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Disulfiram Produces a Non-Carbon Disulfide-Dependent Schwannopathy in the Rat

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Abstract. Disulfiram is a dithiocarbamate drug used for alcohol aversion therapy that produces a distal sensorimotor peripheral neuropathy in certain individuals. Because carbon disulfide, a disulfiram metabolite, produces a peripheral neuropathy clinically similar to disulfiram, it has been postulated that disulfiram neuropathy results from CS_2 release in vivo. The current study evaluated the morphological changes produced by disulfiram and the contribution of CS_2-mediated protein cross-linking to disulfiram-induced neuropathy. Male Sprague–Dawley rats were administered 1% w/w disulfiram in their feed for 2, 4, 5, or 7 wk, and erythrocyte spectrin, hemoglobin, and neurofilament preparations were isolated and the extent of cross-linking assessed by SDS-PAGE, RP-HPLC, and Western blotting, respectively. Spinal cord and peripheral nerve sections were obtained from separate treated animals and assessed by light and electron microscopy. Significant protein cross-linking was only detected in neurofilament preparations obtained after 7 wk of exposure. Morphological changes were observed after 4 wk exposure and consisted of vacuoles within the Schwann cell cytoplasm and segmental demyelination. No neurofilamentous axonal swellings were detected and no significant changes were observed in the CNS. Because disulfiram neuropathy lacks both the morphological changes and intermolecular cross-linking characteristic of CS_2, we conclude that disulfiram neuropathy is not mediated by the axonal toxicant CS_2; instead, disulfiram appears to be a primary Schwann cell toxicant. Recognition of a diethylcarbamoyl adduct on globin and axonal proteins presents a novel putative neurotoxic mechanism for disulfiram.

Key Words: Axonopathy; Carbon disulfide; Demyelination; Disulfiram; Dithiocarbamates; Peripheral neuropathy; Schwann cell.

INTRODUCTION

Disulfiram (bis(diethylthiocarbamoyl)disulfide or Antabuse®) is used in alcohol aversion therapy and in industrial applications including the production of rubber. Coingestion of disulfiram and ethanol produces an unpleasant physiological response, characterized by vomiting and flushing (1–3). This response is believed to result from aldehyde dehydrogenase (ALDH) inhibition by disulfiram (or its metabolites) and subsequent accumulation of high levels of acetaldehyde (1, 4). Inhibition of ALDH has been proposed to occur either by carbamylation (5–7) or by disulfide formation (8).

During the use of disulfiram in alcohol aversion therapy treatment over the past several decades, 2 important side effects have been observed: neurotoxicity and hepatotoxicity (9–11). Although rare, hepatotoxicity is serious and appears to be an idiosyncratic reaction possibly mediated through an immune mechanism (12). Neurotoxicity is more prevalent and is typically the dose-limiting side effect of disulfiram administration. Depending on the dose, neurotoxicity manifests within a few weeks to a few months of initiating disulfiram treatment, and presents as motor and sensory deficits in the distal regions of the limbs, particularly the legs. Patients affected by this peripheral neuropathy complain of loss of sensation and/or painful sensations in the hands and feet, loss of muscle strength, and loss of coordination (11, 13, 14). Disulfiram can also produce behavioral effects including drowsiness, lethargy, sexual dysfunction, headaches, depression, and psychoses (14).

Reports on disulfiram neuropathy date back at least half a century (15) and data on the clinical signs and morphology are available from both human cases and animal studies. Although the nature of some lesions remains controversial, a number of generally accepted characteristics have been established for disulfiram toxicity. The incidence of neuropathy is equivalent in both sexes, and the condition presents as a distal sensorimotor neuropathy. Common findings from morphological studies performed on sural nerve biopsies of patients have included axonal degeneration with concomitant decreased numbers of myelinated axons and changes in myelin thickness and integrity (11, 16–19). Debate exists among the case reports regarding the presence of membranous profiles in Schwann cells and neurofilament accumulations within axons. Studies in the rat have allowed examination of the central nervous system as well and have characterized disulfiram neuropathy in this species as a central-peripheral distal axonopathy exhibiting Wallerian degeneration in the posterior columns of the spinal cord and in peripheral nerves (20, 21). Additional lesions noted in the rat were granular disintegration or proliferation of organelles within the axoplasm, intra-axonal vacuoles, intramyelinic bubbles, and cytoplasmic vacuoles within Schwann cells.

The pathogenic mechanism of disulfiram neuropathy has not been determined. There is considerable support

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in the literature for the hypothesis that disulfiram mediates its neurotoxicity through release of a carbon disulfide (CS_2) metabolite (14, 16, 18, 22). This hypothesis has been advanced based on the clinical similarity between disulfiram and CS_2 neuropathy and the measurement of CS_2 and CS_2 metabolites in the breath and urine, respectively, following administration of disulfiram (23, 24). CS_2 is a well-documented neurotoxicant that produces a central-peripheral distal axonopathy characterized by the formation of axonal swellings, degeneration, and atrophy (25, 26). The axonal swellings contain disorganized masses of neurofilaments and displaced organelles, a lesion unique to the axonopathies of carbon disulfide, γ-diketones, and hereditary giant axonal neuropathy (27). Some investigators have provided data that support the existence of neurofilamentous axonal swellings in sural nerve biopsies of patients presenting with disulfiram neuropathy (16, 18). Others have questioned these findings, however, and have not observed swellings in biopsies from similarly affected patients or nerves obtained from animal models (19–21).

Studies on the mechanism of CS_2-induced neurotoxicity have supported a role for protein cross-linking and have established dose–response relationships for biomarkers of effect based upon protein cross-linking in erythrocyte-associated proteins (28). Using these erythrocyte biomarkers in conjunction with morphological changes and protein cross-linking within the nervous system, it was established that oral administration of *N*,*N*-diethyldithiocarbamate (DEDC), a metabolite of disulfiram, exerts toxicity through in vivo release of CS_2 (29). In the study presented here, rats were administered disulfiram in their feed and erythrocyte and axonal protein cross-linking assessed and morphologic changes evaluated as a function of exposure duration to evaluate the role of CS_2 in disulfiram neurotoxicity. Protein cross-linking was quantified in globin, erythrocyte spectrin and neurofilament proteins using RP-HPLC, SDS-PAGE and Western blotting, respectively. Morphological changes in cervical and lumbar spinal cord, sciatic nerve, and the muscular branch of the posterior tibial nerve were assessed by light and electron microscopy. The results obtained in the current study for disulfiram are compared with those previously reported for CS_2, DEDC, and disulfiram.

MATERIALS AND METHODS

Chemicals

Bis(diethylthiocarbamoyl)disulfide (disulfiram) was obtained from Fluka (Milwaukee, WI). Cacodylic acid, (sodium salt trihydrate), polyvinylpyrrolidone, and monoclonal antibody to the low molecular weight neurofilament subunit protein (NFL) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). Sodium nitroprusside was purchased from VWR Scientific (Atlanta, GA). Glutaraldehyde was obtained from Electron Microscopy Sciences (Ft. Washington, PA). Renaissance oxidizing and Luminol reagents for chemiluminescence detection were purchased from DuPont NEN (Boston, MA). Polyvinylidene difluoride (PVDF) membranes and tris-glycine polyacrylamide gels (4%) were purchased from Novex (San Diego, CA).

Synthesis of d_{10}-DETC-Cys

The d_{10}-diethyldithiocarbamoyl cysteine (DETC-Cys) internal standard was synthesized from di(pentadeuterioethyl)amine (2 mmol; $[(C_2D_5)_2NH]$; 98% enrichment; Isotec, Miamisburg, OH). Roughly 225 μL di(pentadeuterioethyl)amine was transferred to a 25-mL 2-neck flask containing 10 mL ethanol and stirred with 10 N NaOH (250 μL). Carbonyl sulfide (1 mL) was condensed into the flask after fitting with a Dewar condenser filled with dry ice-isopropanol and cooled in the same manner. With the condenser kept cold, the flask was allowed to warm to approximately 20°C and remain at that temperature for 2 h. Iodomethane (300 μL) was added and the stirring was continued for 2 h. Ethanol was removed and the residue was mixed with 20 mL each water and dichloromethane. The organic layer was separated, dried, concentrated to 10 mL, cooled in ice, then oxidized by adding m-chloroperoxy-benzoic acid (mCPBA) (1.5 g) in CH_2Cl_2 and incubating 30 min in ice and 4 h at room temperature. Crude d_{10}-DETC-$MeSO_2$ was purified by flash chromatography (5:1 hexane-ethyl acetate) and stirred with an aqueous solution (10 mL; pH 8) of cysteine (0.24 g, 2 mmol) taken in methanol (5 mL) under Ar for 2 h and concentrated before purification by flash chromatography (silica; 15% water-acetonitrile; MS *m/z* 231 (M^+) and 253.1 [sodium salt]).

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, 225–250 g, obtained from Harlan Sprague–Dawley (Indianapolis, IN), were caged in a room on a diurnal light cycle and given food and water ad libitum. Control rats were given Purina rodent chow; treated rats were given rodent chow formulated with 1% disulfiram w/w (Bioserve, Frenchtown, NJ). Treated animals were given a 1:1 mixture of control and disulfiram-containing food for 1 wk prior to the start of the treatment period in order to allow the animals to acclimate to the food. The amount of food given was recorded daily in order to estimate the total dose. Three exposure groups were used, each initially containing 20 rats each (10 controls, 10 treated); exposure times used were 2, 4, and 5 wk. Body weights of control animals were determined prior to disulfiram exposure and recorded weekly thereafter; body weights of treated animals were measured daily in order to ensure that the animals were consuming sufficient food. Animals were deeply anesthetized with i.p. ketamine and xylazine and euthanized by aortic exsanguination before the collection of tissue for cross-linking.

Evaluation of Clinical Neuropathy

Three criteria were used to assess the extent of the neuropathy produced by disulfiram: 1) the ability of the animals to

replace a hind limb fully extended to the rear by the investigator; 2) the gait of the animal in an open field; and 3) the hind limb grip strength. Hind limb grip strength was assessed as described previously (30) at the beginning and end of each exposure period using a DFIS 10 digital force gauge (John Chantillon & Sons, Greensboro, NC). The compression achieved by each animal on 3 successive trials was recorded and expressed as the average ± SEM.

Erythrocyte Spectrin Isolation and Analysis

Spectrin was purified from blood obtained by aortic exsanguination under deep anesthesia. Spectrin was isolated from erythrocytes as described (29, 31) except erythrocytes were washed with isotonic 5 mM phosphate buffer, pH 7.4 (containing 150 mM NaCl) and lysed in hypotonic 5 mM phosphate buffer, pH 7.4 (no NaCl). Lyophilized spectrin samples were dissolved in 500 μL of water and aliquots diluted 1:1 in Novex reducing sample buffer. The extent of covalent cross-linking was determined by electrophoresis using Novex 4% tris-glycine polyacrylamide gels. Protein bands were visualized by silver staining; monomer and dimer bands were quantified by densitometry using a Bio-Rad GS-700 Imaging densitometer and Molecular Analyst software (Bio-Rad, Hercules, CA). The amount of dimer was expressed as a percent of monomer plus dimer.

Neurofilament Isolation and Electrophoresis

After euthanasia, spinal cords were removed with cold isotonic phosphate buffered saline and used to isolate neurofilaments as previously described (32). Neurofilament preparations were solubilized for electrophoresis 1:1 in buffer (pH 6.8) containing 8 M urea, 1% sodium dodecyl sulfate, 2% dithiothreitol, 2% glycerol, and 0.5 M Tris. Proteins were separated using 6% polyacrylamide and visualized by Western blot procedures using a monoclonal antibody directed towards NFL, goat anti-mouse IgG conjugated to horseradish peroxidase, chemiluminescence detection, and Kodak X-OMAT film. Bands containing NFL epitopes were quantified by densitometry. The quantity of high molecular weight immunoreactive protein was expressed as a percentage of the monomer plus high molecular weight protein.

LC/MS/MS Analysis of Neurofilaments

Two hundred microliters of neurofilament preparations were placed in hydrolysis tubes and lyophilized to dryness. Weights were in the range of 1 to 9 mg (avg. 5.6 ± 0.5 mg, n = 21). Samples were hydrolyzed in 6 N HCL at 110°C for approximately 16 h, then reconstituted with 45 μL solvent and spiked with 2.5 μL d-10 DETC-Cys (5 mM) internal standard. Samples were then filtered using a disposable Centrex MF 0.4 microcentrifuge filter with 0.2-μm pores (Schleicher and Schuell, Keene, NH). DETC-Cys was separated by size-exclusion HPLC on a G2500PW$_{XL}$ column (300 × 7.8 mm ID, 6 μm, TosoHaas, Montgomeryville, PA) using a Waters 2690 liquid chromatograph (Milford, MA) equipped with a diversion valve. The elution conditions were 10 min with solvent A followed by a step to 80% solvent B for 5 min at a flow of 1 mL/min (solvent A = ACN/H_2O/acetic acid/trifluoroacetic acid (TFA) (10:90:0.1:0.05 v/v/v/v); solvent B = ACN/H_2O [40:60 v/v]). Liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis of DETC-Cys with selected reaction monitoring (SRM) detection was performed with a Finnigan TSQ 7000 triple-quadrupole ESI/MS (Finnigan, San Jose, CA). Twenty-five μL of sample was injected on the column and the eluant from 13 to 15.5 min directed into the mass spectrometer. The corona voltage of the atmospheric pressure chemical ionization positive ion source was maintained at 5 kV. The vaporizer temperature was 450°C and the capillary temperature fixed at 250°C; nebulizer pressure was 80 psi and auxiliary gas set to 5 psi. Collision induced dissociation (CID) occurred in Q2, with argon as a collision gas, (2.0 mT) at a collision energy of 17.5 eV (laboratory frame of reference). SRM experiments to detect DETC-Cys were conducted by monitoring the *m/z* 221 → 100 transition (*m/z* 231 → 110 for the internal standard). Normalized responses were obtained by dividing the measured mass spectrometric ratio (*m/z* 221 to *m/z* 231) by the weight of protein analyzed and multiplying by 10,000 to give a dimensionless number.

Globin Isolation and Analysis

Globin was isolated from the hemolysates produced during spectrin isolation as previously described (29). Globin was precipitated in ice-cold 2.5% oxalic acid in acetone, washed in acetone, and dried under a stream of argon. Crude dried globin was solubilized in 0.1% trifluoroacetic acid (TFA) and separated on a Phenomenex Jupiter 5μ column (150 × 460 mm, Torrance, CA) using a Waters 2690 liquid chromatograph and a linear gradient of 20% acetonitrile (0.1% TFA) and 60% acetonitrile (0.08% TFA). The elution of globin peaks was monitored by their UV absorption at 220 nm using a Waters 996 photodiode array detector. Peaks of interest were collected, concentrated and mixed 1:1 with sinapinic acid for analysis by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) on a Voyager Elite 2 mass spectrometer (PerSeptive Biosystems, Foster City, CA).

Preparation of Nerve Tissues for Morphological Assessment

Animals were anesthetized, heparinized, and perfused through the left ventricle of the heart using a multi-speed perfusion pump (Harvard Apparatus Co., Dover, MA). A solution of 0.9% NaCl, 0.2% sodium nitroprusside, and 2.5% polyvinylpyrrolidone was perfused for 2 min, followed by 4% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2 for 12 min. Sections of cervical (C_1–C_2) and lumbar (L_1–L_2) spinal cord, the distal portion of the sciatic nerve, and the muscular branch of the posterior tibial nerve from both hind legs were excised and stored in 4% glutaraldehyde. Thick (1-μm) sections of spinal cord and nerve sections from the right hind limb were prepared as previously described (29) and evaluated by light microscopy on a Zeiss Axiovert 135 digital microscope (Thornwood, NY). Thin (70-nm) sections were prepared from sciatic and posterior tibial nerves and evaluated using a Phillips 300 electron microscope. Teased fiber preparations were made from osmicated segments of the muscular branch of the posterior tibial nerve of the left hind limb, stored in a 3:1 solution of cedarwood oil

TABLE 1
Effect of Disulfiram Consumption on Body Weight[a]

Time (wk)	Control Weight (g)	Exposed Weight (g)
0	288.9 ± 11.07	287.9 ± 9.98
2	345.6 ± 12.57	248.6 ± 23.50[b]
4	366.4 ± 21.20	257.6 ± 16.16[b]
5	355.7 ± 17.90	243.3 ± 24.44[b]
7	374.5 ± 14.97	233.0 ± 21.84[b]

[a] Data are presented as mean ± SEM.
[b] $p < 0.01$ relative to the time-matched control group by Student *t*-test.

and xylene, individual axons were teased apart under a dissecting scope, and evaluated by light microscopy.

Quantification of Morphological Changes

Thick sections of control and exposed nerve and spinal cord sections were randomized and scored blindly by 3 independent observers to determine the incidence and severity of lesions. The severity of axonal degeneration was measured as the total number of degenerating fibers per slide. The severity of demyelinated axons and vacuolated Schwann cells was graded as normal (< 2%), mild (2%–25%), moderate (25%–50%), or severe (> 50%). For each animal, the average score of the 3 observations was determined; average scores from each animal in a treatment group were summed to obtain the total score (either of incidence or severity) for that group.

Histopathological Assessment of Major Organs

Following euthanasia, sections of the spleen, liver, heart, lung, stomach wall, and intestinal wall were excised and placed in 10% buffered formalin. Sections of these organs were subject to routine histopathological examination at Kord Veterinary Diagnostic Laboratory (Nashville, TN) to detect any non-neurological changes that occurred.

Statistical Analysis

F-tests and Student *t*-tests for both equal and unequal variances were performed using Microsoft Excel software. The level of significance was taken to be $p < 0.05$, unless otherwise indicated. Values are expressed as the mean ± SEM.

RESULTS

Animals

During the first 1 to 2 wk of disulfiram administration, animals lost weight and exhibited decreased food consumption relative to control animals. As the animals adjusted to the disulfiram feed, consumption increased and the weight of treated animals stabilized at a level below that of controls (Table 1). At the 2- and 4-wk time points, separate groups of 10 animals (5 treated, 5 controls) were used for morphology and protein collection. Because some animals in the 5-wk exposure group lost excessive weight during weeks 1 and 2, possibly due to respiratory disease (see below), only 3 animals were kept on 1% disulfiram for the entire 5-wk period. These 3 treated animals (plus 3 time-matched controls) were used for morphology at the 5-wk time point. Four animals in the 5-wk group were switched to a 0.25% disulfiram diet for 9 days, after which they were returned to 1% disulfiram for weeks 4 through 7. At 7 wk they were clinically similar in severity to the animals in the 5-wk exposure group, and these animals were used for protein collection (n = 4) along with time matched controls (n = 4). The estimated daily dose of disulfiram (based on the amount of feed used) was 0.9 g/kg/d for the 7-wk exposure group; for all other groups it was 1 g/kg/d. These estimates represent maximum values; the actual consumption may have been considerably less due to the tendency of the rats to chew but not consume the disulfiram feed.

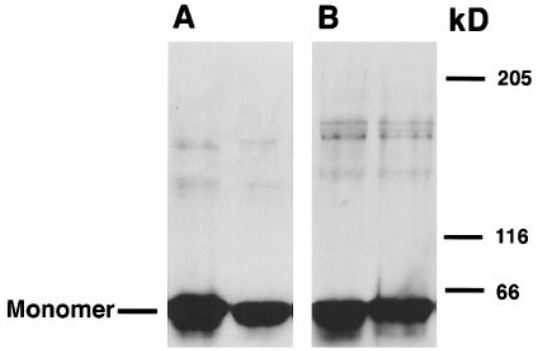

Fig. 1. Covalent cross-linking of NFL by disulfiram. Western blots of spinal cord axonal proteins probed with antibody to NFL of (A) control animals and (B) animals exposed to 1% w/w disulfiram for 7 wk (B). Bars represent the approximate location of molecular weight markers. The NFL monomer is seen just below the 66-kD marker; high molecular weight bands are seen between 116 kD and 205 kD.

No clinical signs of neuropathy were observed in the 2- and 4-wk exposure groups and the hind limb grip strength values for the treated animals were not significantly different from controls. The 5- and 7-wk exposure groups were either slow or unable to replace a limb extended behind them and had an abnormal gait characterized by increased hind leg splay. The most severely affected animals had pronounced paresis in their hind limbs, although they were able to ambulate normally and obtain food and water using their front limbs for locomotion. Hind limb grip strength was significantly decreased in the 5- and 7-wk exposure groups; the average grip strength of the 5-wk exposure group was 533.3 g ± 104.5 for treated animals and 962.2 g ± 109.1 for age-matched controls (n = 3, $p < 0.01$). Hind limb grip strength values for the 7-wk exposure group was 625.8 g ± 98.1 and 977.3 g ± 79.7 for treated and controls, respectively (n = 4, $p < 0.01$). Histopathological examination of major organs showed that some of the animals in the 4-wk group had mild interstitial pneumonia, however, the incidence did not differ between treated and

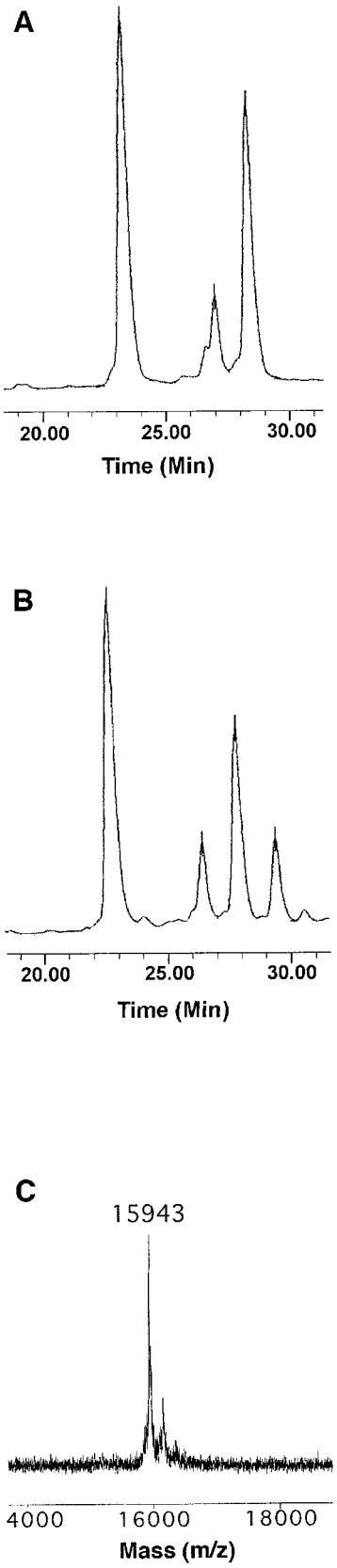

Fig. 2. Formation of modified β-globin by disulfiram. Reverse phase HPLC chromatographs of acid-denatured globins from control rats (A) showing the 2 α-chains eluting as a single peak at 23 min and the 4β-chains eluting in 2 peaks at 27 and 28 min. Globins from rats exposed to 1% w/w disulfiram in the feed for 4 wk (B) showed a peak additional to those present in control samples eluting at 29.5 min. This additional peak was collected and analyzed by MALDI-MS (C) and determined to have a molecular weight of 15943.

control. This respiratory lesion may explain the decrease in body weight of both control and treated animals between weeks 4 and 5 (Table 1). No other significant lesions were identified in any group.

Analysis of Spectrin and Neurofilament Preparations

Silver-stained gels of spectrin preparations from disulfiram-exposed animals did not contain significantly greater amounts of spectrin dimer relative to preparations from control animals at any exposure duration examined (data not shown). Similarly, after 2 and 4 wk of disulfiram exposure, neurofilament preparations did not contain significantly greater amounts of high molecular weight proteins with NFL epitopes relative to controls (data not shown), but neurofilament preparations from animals treated for 7 wk did (Fig. 1). In controls, the background level of high molecular weight bands was 2.7% ± 0.44% of all immunoreactive protein ($n = 4$), while in samples from exposed rats, the level of high molecular weight bands was 8.1% ± 0.88% of all immunoreactive protein ($n = 4$, $p < 0.01$). This level of modification is comparable to that resulting from exposure to CS_2 at 50 ppm for 13 wk (28).

Analysis of Globin Preparations

No CS_2-modified α-globin was detected in preparations from either control or exposed rats at any time point; however, a novel peak, eluting at 29.5 min, was present in preparations from exposed rats at all the time points (Fig. 2B). This peak was not detected in any control animals (Fig. 2A). The area of the new peak was 23.7% ± 1.07% ($n = 5$) of the total β-peak area after 2 wk exposure to disulfiram, and remained at this level at all subsequent time points. The peak at 29.5 min was collected and characterized by MALDI-MS and found to have a molecular weight of 15943 kD (Fig. 2C), corresponding to the β_3 chain plus 100 Daltons.

LC/MS/MS Analysis of Neurofilaments

To assess the formation of *N*,*N*-diethylthiocarbamoyl cysteine (DETC-Cys) in the nervous system, neurofilament protein preparations from control rats ($n = 3$) and DSF exposed rats ($n = 3$) were analyzed for the presence of DETC-Cys by LC/MS/MS. No evidence of DETC-Cys in control neurofilament preparations was obtained as judged by the absence of the diagnostic fragment ion (*m/z* 100) in the ion chromatograms (data not shown). In

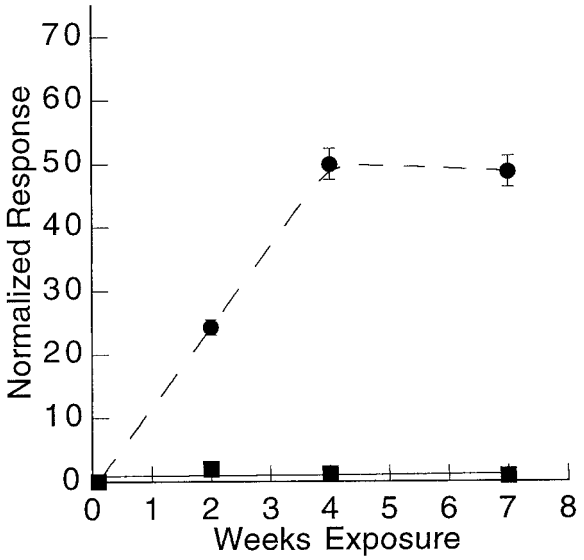

Fig. 3. Normalized LC/MS/MS responses for DETC-Cys as a function of exposure duration for neurofilament preparations obtained from control (squares) and DSF-treated rats (circles). The amount of DETC-Cys increases between 2 and 4 wk and plateaus between 4 and 7 wk. Statistical differences were obtained between control and disulfiram-treated rats at each time point during disulfiram exposure based on paired *t*-tests ($p < 0.05$).

contrast, for disulfiram exposed rats, neurofilament preparations from all exposure groups (2, 4, and 7 wk) contained DETC-Cys (Fig. 3). A time-matched 1-sided *t*-test revealed statistical differences between the normalized responses of controls versus treated rats ($p < 0.05$). Amounts of DETC-Cys were estimated based on regression analysis (ng = 0.0123 + 0.0033 × ratio, R^2 = 0.9995, ratio = response of DETC-Cys ÷ response of d_{10} DETC-Cys internal standard). At 2 wk, the amount of DETC-Cys detected was estimated to be 0.5 ± 0.07 ng/mg of protein. At 4 wk, the level of DETC-Cys was 1.25 ± 0.08 ng/mg and was 1.04 ± 0.04 ng/mg at 7 wk.

Central and Peripheral Nervous System Morphology

Relative to controls (Fig. 4A), no significant changes were observed by light microscopy in the muscular branch of the posterior tibial nerve after 2 wk of disulfiram administration (Fig. 4B). After 4 wk, muscular branch sections contained large vacuoles external to the myelin sheath that compressed the adjacent myelin and axon (Fig. 4C). Some demyelinated axons were also seen, although the extent of vacuolation and demyelination varied between individual animals within this group. Very few normal myelinated axons were found in muscular branch sections from 5-wk exposed animals (Fig. 4D), and many axons appeared to be demyelinated or thinly myelinated, although fewer vacuoles were seen. Structural changes in the distal sciatic nerve were less pronounced and, although not significantly different from controls as a group after 5 wk exposure (Fig. 4E, F), vacuoles in the Schwann cell cytoplasm could be observed in the more severely affected individuals. Teased fiber preparations from the muscular branch of the posterior tibial nerve after 4 wk exposure to disulfiram revealed demyelinated internodes (Fig. 5).

Ultrastructural studies of the muscular branch of the posterior tibial nerve of the 4-wk exposure group disclosed vacuoles within the Schwann cell cytoplasm that were frequently large enough to distort the axon (Fig. 6B, C). Thin sections from animals exposed to disulfiram for 5 wk contained few axons with normal myelin and many large Schwann cells containing myelin whorls and debris-filled vacuoles. Many axons were completely demyelinated (Fig. 6D); others were ensheathed by degenerating myelin (Fig. 6E). Rarely, myelin splitting was also observed (Fig. 6F).

The incidence and severity of degenerated axons, Schwann cell vacuoles, and demyelinated axons is presented in Table 2. No neurofilamentous axonal swellings (characteristic of CS_2 neuropathy) were observed in any of the nerve sections at any time point. Most of the sections examined were indistinguishable from controls in both incidence and severity when compared as a group. The only nerve sections that were significantly different from controls as a group were the muscular branch samples at 5 wk. The incidence of positive observations was 2.7 out of 3 animals for both vacuoles and demyelination (3 observations per animal); the severity of these lesions was given an average score of 0.4 ± 0.2 in controls and 1.8 ± 0.4 in exposed ($n = 3$, $p < 0.01$). Scores for individual animals in the exposed group ranged from 0.7 ± 0.3 to 3. A score of 1.8 corresponds to almost 25% affected axons. At 4 wk, the severity of the exposed group was not significantly different than the controls for any of the lesions. However, the incidence of positive observations for vacuolation and demyelination was 3.3 out of 5 exposed animals in this group, indicating that some of the animals in the group were significantly affected. The 2 most severely affected animals in the 4-wk group had scores of 2.7 ± 0.3 and 2.3 ± 0.7 for vacuolation and 1.7 ± 0.7 and 1.3 ± 0.3 for demyelination.

DISCUSSION

Mechanistic studies on CS_2 have provided evidence for covalent cross-linking of proteins within the axon as the pathogenic mechanism (27, 28) and the series of reactions through which CS_2 covalently cross-links proteins has been delineated in detail (33). Exposure to CS_2, either directly or from oral administration of DEDC results in the formation of intramolecular valine-lysine thiourea cross-links in globin (34) and intermolecular lysine–lysine thiourea cross-links in erythrocyte spectrin (35, 36). In addition, a specific intramolecular cross-link on the major α chain of globin that exhibits a mass consistent

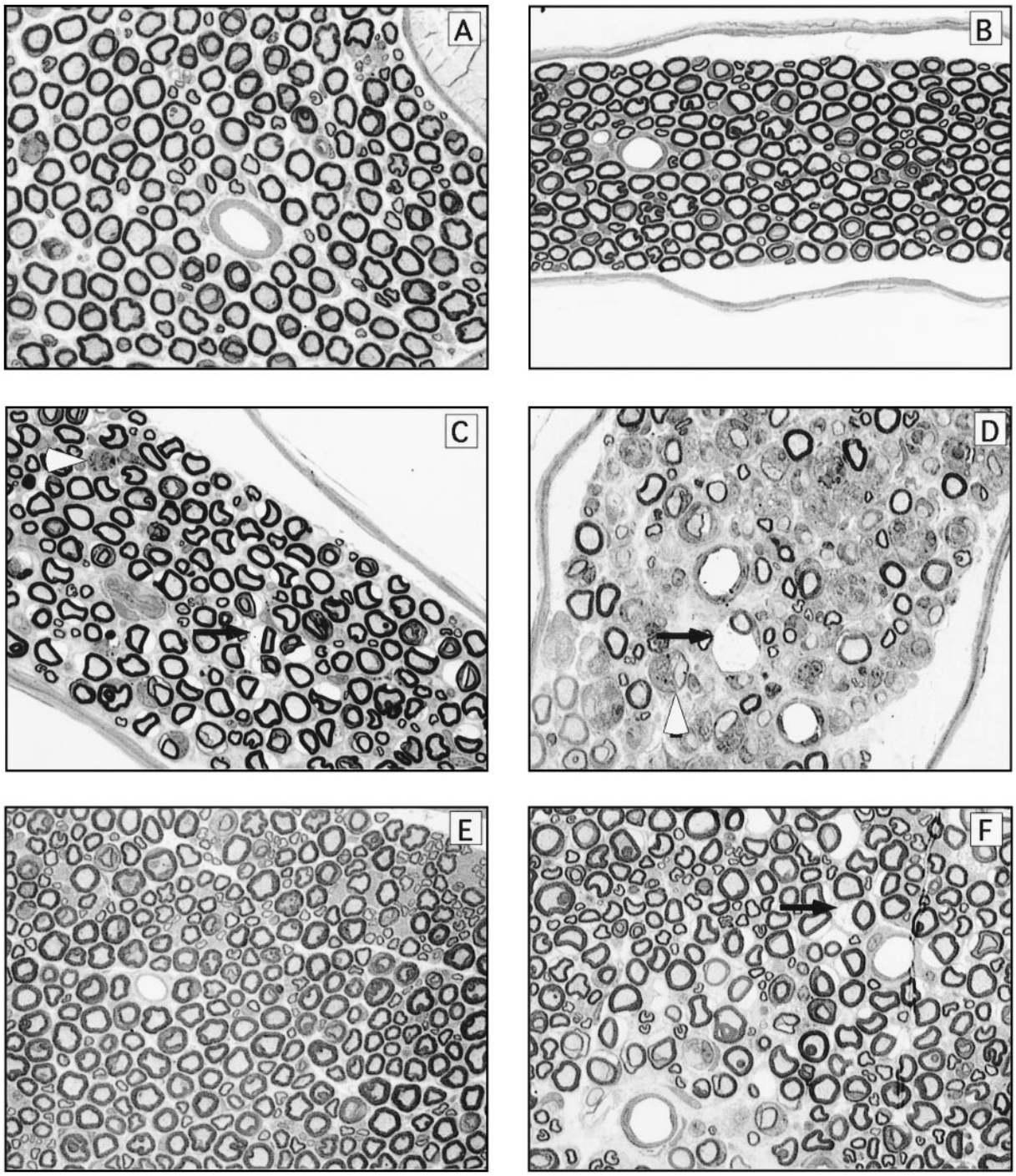

Fig. 4. Cross-sections from peripheral nerves stained with toluidine blue (320×). A: Muscular branch of the posterior tibial nerve from a 2-wk control showing a bimodal distribution of axonal diameters among myelinated axons. B: Sections of the muscular branch of the posterior tibial nerve after 2 wk exposure to disulfiram were indistinguishable from controls. C: After 4 wk exposure to disulfiram, vacuoles were seen external to the myelin sheath, within the cytoplasm of the Schwann cell (arrow). The vacuoles compressed the adjacent myelin sheath. The arrowhead identifies a degenerating axon. D: After 5 wk exposure to disulfiram, many large axons were no longer ensheathed by myelin, but were instead surrounded by Schwann cells containing myelin debris (arrowhead). Occasionally, Schwann cell vacuolation was observed (arrow). E: Cross-section of the distal sciatic nerve from a 5-wk control animal. F: Distal sciatic nerve from an animal exposed to disulfiram for 5 wk demonstrating vacuolated Schwann cells and rare examples of demyelination, but changes were consistently less severe than sections of the more distal muscular branch in the same animal.

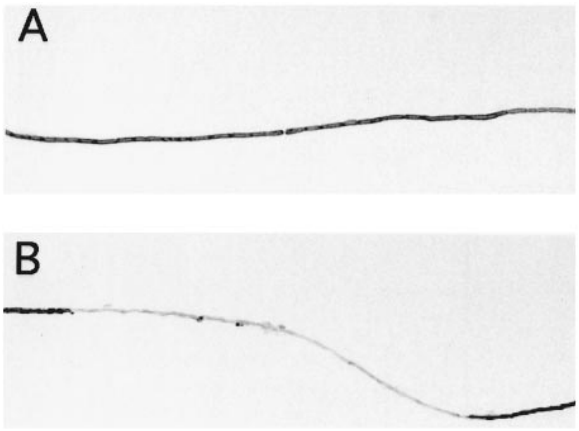

Fig. 5. A: A single teased fiber from the muscular branch of the posterior tibial nerve of a control animal displays constant myelin thickness and a single central node of Ranvier. B: Teased fiber from an animal exposed to 1% w/w disulfiram for 4 wk showing loss of myelin from an entire internode. An abrupt change to normal myelin thickness is seen on both sides of the demyelinated internode at each node of Ranvier.

with the formation of a thiourea or dithiocarbamate ester cross-link has been identified using HPLC and LC/MS (37). These biomarkers have demonstrated a cumulative dose response that correlates to the level of protein cross-linking occurring within the axon, suggesting that cross-linking in peripheral proteins can serve as a surrogate for axonal proteins. Accordingly, a linear relationship between globin cross-linking and neurofilament cross-linking has been reported (37). Equivalent levels of erythrocyte-associated protein cross-linking generated by CS_2 or DEDC were also associated with the same severity and distribution of CS_2-induced morphological changes (29, 34). Similarly, the levels of neurofilament protein cross-linking observed at the onset of axonal swellings produced by either CS_2 or DEDC were equivalent (28, 29). In contrast to the results obtained for CS_2 and DEDC, disulfiram did not elicit detectable levels of protein cross-linking in globin or erythrocyte spectrin. Relative to time-matched controls, significant levels of neurofilament cross-linking were detected in preparations isolated from disulfiram exposed rats, but they were not detected until after the onset of lesions. Also, the levels of neurofilament cross-linking were considerably below those associated with the onset of lesions for either CS_2 (28) or DEDC (29).

Consonant with previous animal studies, the structural changes observed here for disulfiram were more severe distally, and no neurofilamentous axonal swellings were detected in any sections, even in the muscular branch of the posterior tibial nerve (shown to be a sensitive site for this lesion) (25, 26, 38). In contrast to previous studies, there did not appear to be involvement of the spinal cord, and axonal degeneration was a relatively minor component. Moreover, although vacuolization of Schwann cells was noted previously, in the present study it appeared to be the initiating event leading to the development of debris-filled vacuoles, demyelination, and Schwann cell proliferation with a relative sparing of the axon. Also, there was considerable variability in the severity of lesions observed within exposure groups that may have arisen from differences in individual susceptibility or feeding habits or a combination of both.

Although no CS_2-modified major α chain was detected, a new, later eluting globin peak was observed by HPLC following administration of disulfiram. Isolation and analysis of this new peak revealed that its mass corresponded to that of the β_3 globin chain plus 100 Daltons. Such a modification may arise from the addition of an *N*,*N*-diethylcarbamoyl adduct on a cysteinyl residue of the globin chain. This adduct has been produced on glutathione by disulfiram and is believed to result from the reduction of disulfiram to DEDC in blood (39, 40) followed by methylation and oxidation to either an *S*-methyl-*N*,*N*-diethylthiocarbamate sulfoxide or sulfone susceptible to nucleophilic addition (Fig. 7 [Scheme 1]) (41). LC/MS/MS analysis of the protein preparations isolated from the spinal cord provided further insight into the nature of the 100 Dalton protein modification produced by disulfiram by localizing the adduct to cysteine, establishing its generation on proteins other than globin and verifying that it occurred within the nervous system. However, unlike the immunoblotting analysis that is specific for the low molecular weight neurofilament protein in the spinal cord protein preparations, the LC/MS/MS method used does not reveal which of the axonal proteins are modified. Also, considering the absence of lesions within the spinal cord, it is not clear what the biological significance is for the covalent modifications observed on the axonal proteins. Nevertheless, because carbamylation of protein sulfhydryl groups has been demonstrated to disrupt enzyme function, protein carbamylation, presumably within the Schwann cell, presents a novel putative neurotoxic mechanism for disulfiram. Indeed, a similar mechanism, inhibition of squalene epoxidase, has been credited for the Schwann cell toxicity observed for tellurium (42).

The absence of protein cross-linking and neurofilamentous swellings observed here for disulfiram argue against a role for CS_2 and is at odds with the established role of CS_2 in the neurotoxicity resulting from oral administration of DEDC. These differences may arise from the relative acid stability of the 2 compounds. Decomposition of dithiocarbamates to parent amine and CS_2 is promoted by acid (Fig. 7 [Scheme 1]) and is therefore expected to proceed readily within the stomach (43). Bis(thiocarbamoyl) disulfides are more stable in an acidic environment; therefore, they are expected to be absorbed intact to a greater extent following oral administration.

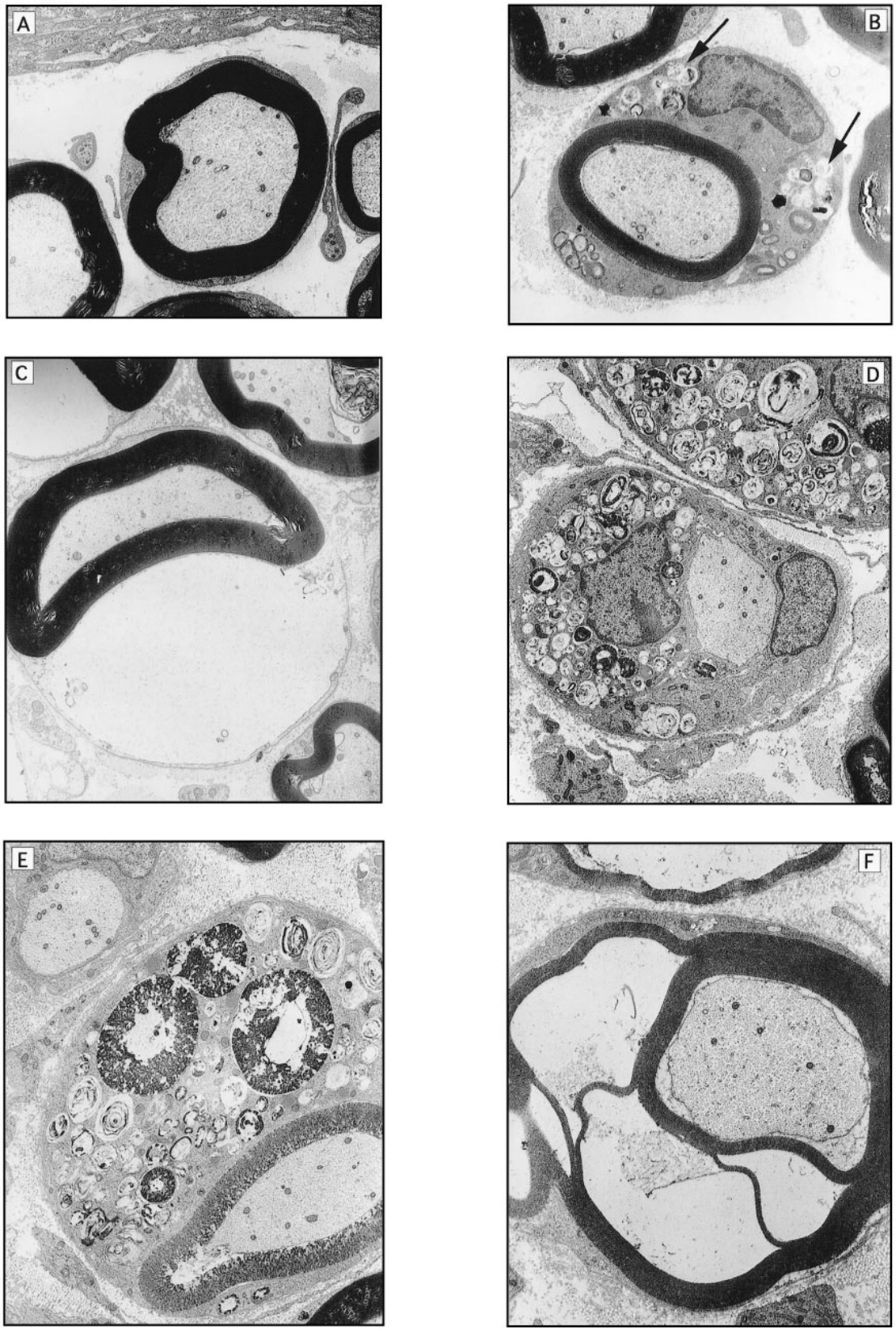

Fig. 6. Ultrastructural changes in the muscular branch of the posterior tibial nerve. A: Myelinated axons from a 4-wk control animal shows normal compact myelin surrounded by a thin rim of Schwann cell cytoplasm. The axoplasm shows the normal distribution of mitochondria, microtubules, and neurofilaments (3470×). B, C: Sections from animals treated with 1% w/w disulfiram for 4 wk. B: Myelin and axoplasm are normal; however, the volume of Schwann cell cytoplasm is greatly increased

TABLE 2
Incidence and Severity of Disulfiram-Induced Lesions[a]

Location	Duration	Treatment	Degenerated axons		Vacuolation		Demyelination	
			Incidence[b]	Severity[c]	Incidence[b]	Severity[d]	Incidence[b]	Severity[d]
Muscular branch	2 weeks	Control	0/5	0	1.3/5	0.3 (0.2)	1.3/5	0.3 (0.2)
		Exposed	0/4	0	0.7/4	0.3 (0.2)	0.7/4	0.3 (0.2)
Muscular branch	4 weeks	Control	0.3/4	0.1 (0.1)	1.3/4	0.5 (0.2)	1.3/4	0.5 (0.2)
		Exposed	0.7/5	0.7 (0.7)	3.3/5	1.3 (0.3)	3.3/5	0.9 (0.2)
Muscular branch	5 weeks	Control	0.7/3	0.4 (0.3)	1/3	0.4 (0.2)	1/3	0.4 (0.2)
		Exposed	0.3/3	2.7 (2.7)	2.7/3	1.8 (0.4)[e]	2.7/3	1.8 (0.4)[e]
Sciatic	5 weeks	Control	1.3/3	0.6 (0.2)	1.3/3	0.4 (0.2)	1.3/3	0.4 (0.2)
		Exposed	0.7/3	0.7 (0.5)	1.7/3	0.7 (0.2)	1.7/3	0.7 (0.2)
Cervical cord	5 weeks	Control	0/3	0	0.3/3	0.1 (0.1)	0.3/3	0.1 (0.1)
		Exposed	1/3	3.3 (1.7)	0.3/3	0.1 (0.1)	0.3/3	0.1 (0.1)
Lumbar cord	5 weeks	Control	0.3/3	0.2 (0.2)	0.3/3	0.1 (0.1)	0.3/3	0.1 (0.1)
		Exposed	0.3/3	1.1 (1.1)	0.3/3	0.1 (0.1)	0.3/3	0.1 (0.1)

[a] Slides were scored blinded by 3 observers.
[b] Number of positive observations/number of animals in the treatment group. (Three observations per animal, so a single observation counts as 0.33).
[c] Values are average number of degenerated axons/section (SEM).
[d] Values are average score (SEM) where 0 = normal (<2% axons affected), 1 = mild (2%–25% axons affected), 2 = moderate (25%–50% axons affected) and 3 = severe (>50% axons affected).
[e] $p < 0.01$.

Thus, if the Schwann cell toxicity is dependent upon either the disulfide linkage or a metabolite other than CS_2 or diethylamine, e.g. *S*-methyl diethylthiocarbamate sulfoxide, disulfiram would be a more potent toxicant than DEDC following oral administration.

Because exposed rats lost weight relative to controls, there may be a concern that the malnourished state of the exposed animals contributed to the neuropathy. Studies on the effects of malnutrition in the developing rat peripheral nervous system have shown that feed restriction from birth results in decreased axonal caliber in peripheral nerves, particularly in the large caliber fibers, and decreased myelin thickness relative to axonal caliber (44, 45). However, reduction in the number of myelinated fibers was not seen and ultrastructural examination of the nerves showed no Schwann cell lesions associated with malnourishment. The rats used in the current study were approximately 9 to 10 wks old when treatment began. Although both axonal caliber and myelin thickness are increasing at a slow rate at this age, the rapid growth and myelination period seen in developing rats is complete (46). Conceivably, the weight loss of exposed animals could have contributed to a reduction in myelin thickness, but it appears unlikely that decreased food intake could have produced the Schwann cell lesions and segmental demyelination documented here.

In the present investigation, administration of disulfiram in the feed to rats produced a peripheral distal axonopathy that targeted Schwann cells, resulting in segmental demyelination with only minor amounts of CS_2-mediated protein cross-linking occurring. These observations are inconsistent with CS_2 being a proximate toxic species of disulfiram. Two important questions that arise from the present study are 1) If neurotoxicity is not mediated through release of CS_2, what is the mechanism of disulfiram-induced injury, and 2) What is the relevance of the data obtained here using the rat to the neuropathy observed in humans? Regarding the first question, the potential bioactivation of disulfiram to an electrophilic species followed by protein carbamylation presents a novel mechanism that warrants further investigation, in particular to determine if these modifications are being generated within Schwann cells. Although it is difficult to address the second question given the current state of

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and contains multiple debris-filled vacuoles (arrows) (3470×). C: The shape of the myelin and its contained axon are distorted by a large fluid-filled vacuole in the cytoplasm of the surrounding Schwann cell. The myelin remains compact and the axoplasm is normal (3470×). D–F: Muscular branch sections from animals treated with disulfiram for 5 weeks. D: The central axon contains a normal complement of axoplasmic structures, but the myelin formerly surrounding this axon has degenerated. Two Schwann cells envelop the axon, and the enlarged cytoplasm contains numerous vacuoles filled with myelin debris (3000×). E: The well-preserved axon in the lower right corner is surrounded by fragmented myelin and additional myelin debris is seen in the Schwann cell cytoplasm vacuoles. A denuded axon is present in the upper left corner surrounded by its Schwann cell (3470×). F: A rare example of intramyelinic edema (3000×).

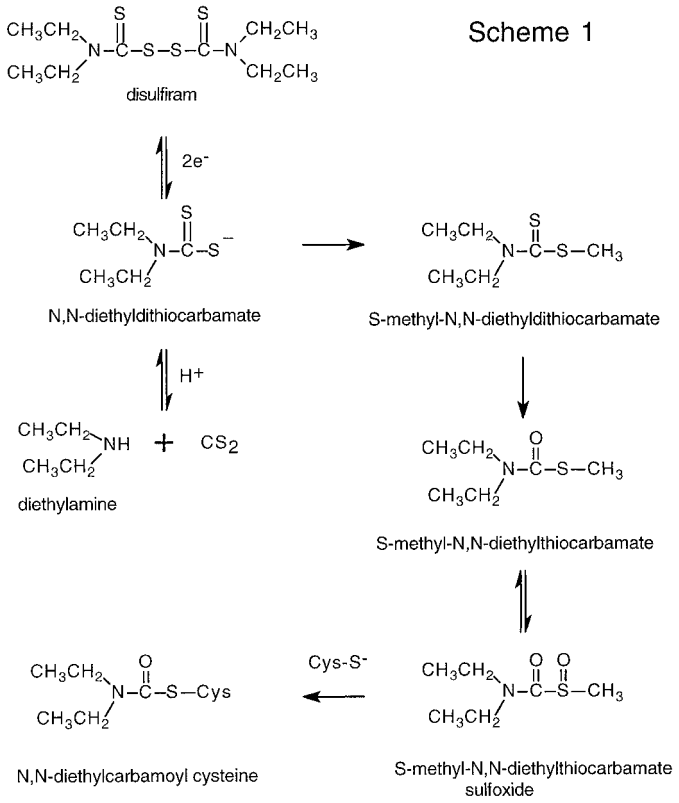

Fig. 7. Decomposition and metabolism of disulfiram and N,N-diethyldithiocarbamate. Oral administration of N,N-diethyldithiocarbamate results in acid promoted decomposition to CS_2 and diethylamine. Disulfiram is more acid stable and can be absorbed intact following oral administration, reduced to N,N-diethyldithiocarbamate in a neutral environment and undergo methylation and oxidation to S-methyl-N,N-diethylthiocarbamate sulfoxide. The electrophilic character of the sulfoxide renders it susceptible to nucleophilic addition by sulfhydryl functions of cysteine residues.

knowledge, the biomarkers developed for CS_2 and disulfiram may help to assess the relative amounts of protein cross-linking and carbamylation occurring in humans administered disulfiram. This information may provide insight into the contributions of these 2 pathways in humans. In addition, the identification of disulfiram-mediated protein modifications on hemoglobin may also provide a method for identifying susceptible individuals and for monitoring compliance of patients on disulfiram therapy.

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