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Cross-Linking of Neurofilament Proteins of Rat Spinal Cord In Vivo After Administration of 2,5-Hexanedione

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Abstract: The aliphatic hexacarbons *n*-hexane, methyl-*n*-butyl ketone, and 2,5-hexanedione are known to produce a peripheral neuropathy that involves an accumulation of 10-nm neurofilaments above the nodes of Ranvier in the spinal cord and peripheral nerve. In this study, rats were treated with 0.5% 2,5-hexanedione in drinking water for 180 days, and their spinal cord neurofilaments were isolated after development of the neuropathy. Visualization by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a significant reduction in content of the neurofilament triplet proteins in treated animals and the presence of bands migrating at 138K and 260K that were not present in control animals. Analysis of the lanes using immunoblotting procedures and anti-70K, anti-160K, and anti-210K neurofilament antibodies revealed many cross-linked peptides. The 138K band cross-reacted with the anti-160K neurofilament antibody. This suggests that the 138K band is an intramolecular cross-link of the 160K neurofilament subunit. In addition to this peptide, there were numerous high-molecular-weight peptides immunoreactive with all three neurofilament protein antibodies. In addition to cross-linking, there was also a diminished amount of immunoreactive breakdown product of all three neurofilament proteins. This report demonstrates direct evidence of 2,5-hexanedione-induced cross-linking of neurofilament proteins in vivo, which maybe responsible for the accumulation of neurofilament proteins pathognomic of this neuropathy. **Key Words:** Neuropathy—Neurofilament proteins—Cross-linking—2,5-Hexanedione—Spinal cord. **Lapadula D. M. et al.** Cross-linking of neurofilament proteins of rat spinal cord in vivo after administration of 2,5-hexanedione. *J. Neurochem.* **46,** 1843–1850 (1986).

Several types of neurotoxicants are known to produce a central-peripheral axonopathy that results in the accumulation of neurofilaments (Spencer and Schaumburg, 1980). These toxicants include β,β-iminodiproprionitrile (Griffin et al., 1978), carbon disulfide (Juntunen et al., 1974), and aliphatic hexacarbons (Spencer and Schaumberg, 1975; Spencer et al., 1975). Methyl *n*-butyl ketone and *n*-hexane are hexacarbons that have been implicated in the production of neuropathies in factory workers and in glue sniffers (Mendell et al., 1974; Allen et al., 1975), with the apparent neurotoxic metabolite being 2,5-hexanedione (Kramer et al., 1974; Abdel-Rahman et al., 1976; DiVincenzo et al., 1976; Abou-Donia et al., 1982). Although the production of the neuropathy is apparently related to the accumulation of neurofilaments, the mechanism of how the increase occurs is unknown.

Several theories have been proposed to explain the mechanisms involved in the production of aliphatic hexacarbon-induced neuropathy. Spencer and collaborators have proposed that 2,5-hexanedione inhibits energy production by reacting with sulfhydryl groups on phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase, resulting in an inhibition of energy-dependent transport of proteins (Sabri et al., 1979; Spencer et al., 1979). Subsequently, Graham and Abou-Donia (1980) demonstrated that inhibition of the glycolytic enzyme (glyceraldehyde-3-phosphate dehydrogenase) by 2,5-hexanedione required concentrations in the range of 10–50 m*M* and may involve the formation

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Abbreviations used: GFAP, glial fibrillary acidic protein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

of a Schiff base on ε-amino groups of lysine residues. Because the concentration range necessary for inhibition of glycolytic enzymes in vitro was extremely high, a direct effect on neurofilaments was postulated. They proposed that the neurofilaments were cross-linked by 2,5-hexanedione (Graham, 1980; Graham and Abou-Donia, 1980). However, Graham et al. (1982, 1984) were unable to demonstrate in vivo formation of cross-links among neurofilament proteins. The formation of a pyrrole adduct on bovine serum albumin and various blood and nervous system proteins was found by DeCaprio et al. (1982). De Caprio and O'Neill (1985) have found high-molecular-weight proteins in cytoskeletal preparations from animals treated with 2,5-hexanedione. They have postulated that the hydrophobic interaction of the pyrrole adduct on various nervous system proteins accounted for the accumulation of neurofilaments. The theory of Spencer and co-workers was subsequently modified to include the formation of a Schiff base or pyrrole adduct on ε-lysine groups of glycolytic enzymes and thus inhibiting them (Spencer and Griffin, 1982). Another theory accounts for the accumulation of neurofilaments by proposing the inhibition of a Ca^{2+}-activated protease that normally breaks down neurofilaments (Cavanagh, 1982), although no evidence was presented. Recently, Monaco et al. (1985) have proposed that an acceleration of neurofilament transport may be responsible for the accumulation of neurofilaments.

In this article, we have examined the effect of 2,5-hexanedione administration on the protein composition of neurofilament preparations isolated from the spinal cord of control and 2,5-hexanedione-treated rats. Direct evidence of in vivo cross-linking of neurofilament proteins in 2,5-hexanedione-treated animals is presented.

MATERIALS AND METHODS

Chemicals

2,5-Hexanedione (98%) was obtained from Eastman Kodak Co. (Rochester, NY, U.S.A.). Acrylamide, sodium dodecyl sulfate (SDS), and urea were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Triton X-100 (reagent grade) was obtained from Research Products International Corp. (Elk Grove Village, IL, U.S.A.). Aminomethylcyclohexanecarboxylic acid and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *Staphylococcus aureus* protease V8 was obtained from Miles Laboratories (Elkhart, IN, U.S.A.). Sepharose CL-6B was obtained from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). Dithiothreitol was obtained from Bachem Inc. (Torrance, CA, U.S.A.). Monoclonal antibodies to the neurofilament triplet proteins (anti-70K, anti-160K, and anti-210K) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). ^{125}I-Protein A was obtained from New England Nuclear (Newark, DE, U.S.A.).

Animals

Male Sprague-Dawley rats (body weight, 200–225 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN, U.S.A.). Twenty-three rats were treated with 0.5% 2,5-hexanedione in their drinking water for 180 days. Seventeen age-matched rats served as controls. All rats were given food and water ad libitum and maintained on a 12-h light/dark cycle in a temperature (23–25°)- and humidity (40–60%)-controlled room. Body weights were monitored weekly.

Neurofilament preparation

Neurofilaments were prepared according to the method of Chiu and Norton (1982). In brief, spinal cords from either control or treated animals were removed by injection of ice-cold saline. Spinal cords were not pooled to obtain 2 g of tissue, but instead spinal cords from individual animals (0.7–0.9 g) were homogenized in 25 ml of buffer T, which consists of 50 m*M* Tris-HCl (pH 6.8), 2 m*M* EDTA, 2 m*M* phenylmethylsulfonyl fluoride, and 0.5% (vol/vol) Triton X-100 (Chiu and Norton, 1982) in a glass-Teflon homogenizer. The homogenate was centrifuged at 13,000 *g* for 15 min at 4°C, the supernatants were discarded, and the pellet was rehomogenized in the above buffer made up to 0.9 *M* sucrose and recentrifuged as before. The pellet was saved at −70°C in 2 ml of phosphate-buffer (0.1 *M* NaH_2PO_4, pH 7.4). After thawing, an aliquot of the cytoskeleton in phosphate-buffer was saved for SDS-polyacrylamide gel electrophoresis (PAGE), and the rest was processed for gel filtration chromatography (Chiu and Norton, 1982). Individual neurofilament bands were harvested from a pool of appropriate aliquots. Samples were concentrated by use of a protein concentrator.

SDS-PAGE

Cytoskeletal proteins as well as column fractions were monitored by SDS-PAGE. Electrophoresis was performed by the procedure of Laemmli (1970) with 4% stacking and 5–10% or 5–8% resolving gels. All samples were prepared with a final concentration of 1% SDS, 1% β-mercaptoethanol, 10% glycerol, 0.015% bromophenol blue, and 0.125 *M* Tris-HCl (pH 6.8), followed by heating for 5 min in a 95°C water bath. Molecular weight standards consisted of ovalbumin (45K), tubulin (53–55K), bovine serum albumin (66.2K), phosphorylase B (92.5K), β-galactosidase (116K), and myosin (205K). Tubulin was prepared by the two-cycle polymerization method of Shelanski et al. (1973). All other markers were purchased from Bio-Rad. Samples were subjected to electrophoresis under conditions of constant power (7.5 W/slab) until the tracking dye reached the bottom of the gel. Coomassie Blue-stained gels were scanned with laser densitometer hooked up to a recording integrator (LKB Instruments, Inc., Bromma, Sweden).

Limited proteolysis of proteins

The method of Cleveland et al. (1977) was used to generate peptide maps of cytoskeletal proteins using 0.02 μg of protease. The gel was stained with Coomassie Brilliant Blue or by the silver staining procedure of Wray et al. (1981).

Electrophoretic elution of proteins

Electrophoretic elution of proteins was performed by the method of Hunkapillar et al. (1983). Electrophoretic

elution was carried out using a soaking buffer of 2% SDS in 0.4 *M* NH_4HCO_3 and 0.1% dithiothreitol. The soaking buffer was overlaid with elution buffer (0.1% SDS in 0.05 *M* NH_4HCO_3) and eluted for 16 h. The elution buffer was replaced with dialysis buffer (0.02% SDS in 0.01 *M* NH_4HCO_3), and elution-dialysis was continued for another 24 h. The concentrated, eluted proteins were collected and subjected to peptide mapping.

Blotting to nitrocellulose paper and antibody detection

The cytoskeletal proteins were prepared as previously described and electrophoresed on 5–7% gradient slab gels. The proteins in the gels were subsequently transferred to nitrocellulose paper (pore size 0.2 μm; Schleicher and Schuell, Keene, NH, U.S.A.) according to the method of Towbin et al. (1979) as modified by O'Callaghan and Miller (1985). After transfer, the nitrocellulose paper was incubated for 1 h in a blocking solution of 0.5% gelatin in Tris-buffered saline (200 m*M* NaCl and 50 m*M* Tris-HCl, pH 7.4). The nitrocellulose paper was then incubated with the primary antibody (diluted 1:250) in blocking solution with 0.1% Triton X-100. The nitrocellulose paper was washed for 30 min in Tris-buffered saline followed by incubation with the secondary antibody (rabbit anti-mouse IgG, diluted 1:500; Dako, Santa Barbara, CA, U.S.A.) in the blocking solution with 0.1% Triton X-100 for 1 h. The paper was washed, incubated with blocking solution, and then incubated for 1 h with ^{125}I-protein A in blocking solution with 0.1% Triton X-100. The nitrocellulose paper was washed overnight with Tris-buffered saline with 0.1% Triton X-100. It was then air-dried and exposed to x-ray film.

RESULTS

Gel electrophoresis of crude neurofilament fractions isolated from control and 2,5-hexanedione-exposed animals differed significantly. Neurofilament fractions from 2,5-hexanedione-exposed animals showed a substantial reduction in the amount of protein migrating in the 70K, 160K, and 210K bands, relative to control fractions (Table 1), and contained bands migrating at 58K, 138K, and 260K that were not present in control fractions (Fig. 1; 260K protein is not visible here because of its low amount). The loss of protein in the 70K, 160K, and 210K bands in fractions from 2,5-hexanedione-exposed animals was not an artifact of purification, because the major band migrating at 51K, corresponding to glial fibrillary acidic protein (GFAP; Chiu and Norton, 1982), was constant across all lanes from treated and untreated animals. The presence of GFAP served as an internal control for protein extraction efficiency and protein loading in each lane during electrophoresis.

The loss of protein from the three major neurofilament bands and the appearance of new bands in fractions isolated from 2,5-hexanedione-exposed animals suggest that the 138K and 260K bands may arise by chemical cross-linking of neurofilament proteins. The 138K protein was partially purified by gel filtration chromatography (Chiu and Norton, 1982); however, contamination from the 160K band

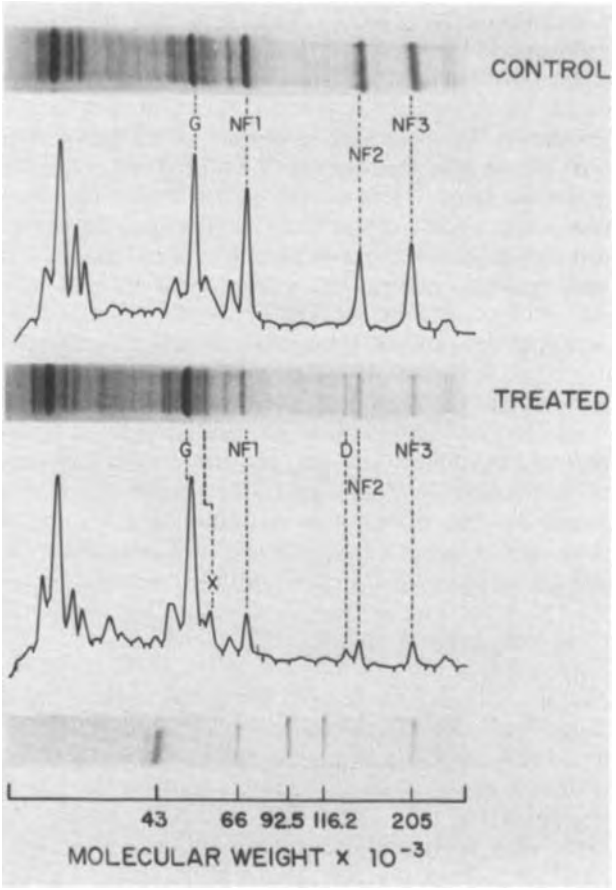

FIG. 1. SDS-PAGE of treated and control neurofilament preparations. Proteins were resolved on 10-well 0.1% SDS-polyacrylamide vertical slab gels (10 × 16 × 0.15 cm; Bio-Rad Laboratories). Forty micrograms of protein was loaded on each lane of a 5–10% gradient resolving gel with a 4% stacking gel: NF1, NF2, and NF3, neurofilament triplet proteins (M_r, 70K, 160K, and 210K, respectively); D, the 138K peptide; X, the extra protein band at the 58K position; G, GFAP (which is identical in control and 2,5-hexanedione-treated animals). Scans were obtained using an LKB laser densitometer.

TABLE 1. *Comparison of neurofilament proteins of 2,5-hexanedione (2,5-HD)-exposed and control animals*

	Control	2,5-HD	p value
70K (NF1)	100 ± 7	56 ± 6	0.01
160K (NF2)	100 ± 14	45 ± 3	0.01
210K (NF3)	100 ± 10	32 ± 3	0.01
51K (GFAP)	100 ± 9	96 ± 6	NS

Forty micrograms of protein from five individual treated or control animals were loaded per well. All lanes were scanned twice using an LKB laser densitometer with a recording intergrator. Data are mean ± SEM values. NF1, NF2, and NF3, neurofilament triplet proteins. All numbers are ± SE.

Statistics were performed using a Student's two-tailed *t* test.

was difficult to remove. To obtain electrophoretically pure 138K protein, the method of Hunkapillar et al. (1983) was used, in which proteins are purified by electrophoretic elution from polyacrylamide gel slices. We were able to obtain ~0.15 mg of protein using this technique. Results from peptide mapping (Fig. 2) revealed some homology between the 138K protein and the 70K neurofilament protein. Because the presence of cross-links in the 138K peptide will result in the appearance of peptides not present in the 70K or 160K neurofilament proteins, complete homology would not be expected, and more detailed studies to confirm the identity of the 138K band were undertaken.

Blotting to nitrocellulose revealed several high-molecular-weight species showing immunoreactivity to the 70K, 160K, and 210K antibodies (Figs. 3 and 4). There was also immunoreactivity of all three antibodies to material that did not enter the gels. Reactivity to the 70K antibody revealed several high-molecular-weight species; however, no band was present in the 130K–160K region. The 138K peptide cross-reacted with the 160K neurofilament antibody. There also appeared to be at least three well-defined bands that reacted with the anti-160K antibody at higher molecular weights, as well as a smearing of immunoreactivity at the top of the resolving gel. The 210K antibody revealed at least one well-defined higher-molecular-weight band as well as material that did not enter the resolving gel. At higher protein loads, immunoreactive material that did not enter the stacking gel was also evident. Another finding that is shown by the immunoblots was a diminished amount of immunoreactive breakdown product, which was most evident in the 70K neurofilament protein. Control samples had a significant amount breakdown product; however, these products were virtually nonexistent in preparations from treated animals.

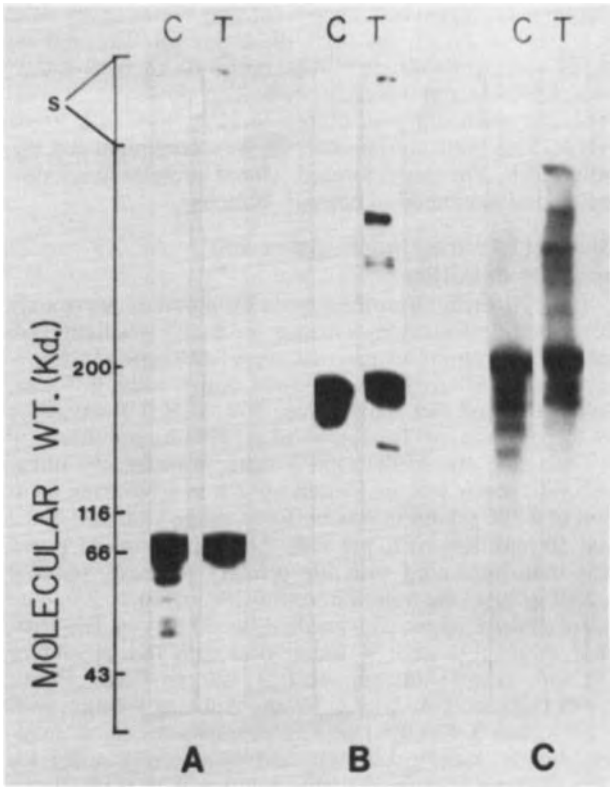

FIG. 3. Immunoblots of cytoskeletal proteins from control (C) and treated (T) animals to anti-70K **(A)**, anti-160K **(B)**, and anti-210K **(C)** antibodies. Immunoreactive material that did not enter the stacking gel (S) is evident in all three immunoblots from treated animals as well as higher-molecular-weight proteins. The diminished content of immunoreactive breakdown products is evident in immunoblots from treated animals. X-ray film was exposed for 2 h at −70°C.

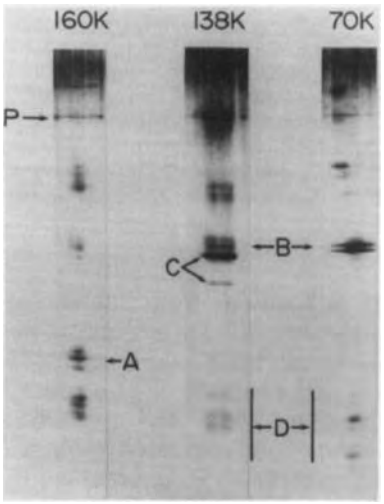

FIG. 2. Peptide maps of the 70K, 138K, and 160K proteins. The presence of sequence homology of the 70K and 138K proteins are demonstrated, as well as the differences between all three proteins: A, an area unique to the 160K protein not found in either the 70K or 138K digests; B, a doublet found in the digests of both the 138K and 70K proteins; D, a second region of homology between the 70K and 138K protein digests; C, a major peptide generated in the 138K digest not present in either the 70K or 160K protein; P, the protease band as determined from blank lanes with the protease alone. The gels were stained by the method of Wray et al. (1981).

DISCUSSION

Chemical cross-linking of neurofilament proteins has been suggested to be the primary mechanism through which aliphatic hexacarbons exert their neuropathic effect (Graham, 1980; Graham and Abou-Donia, 1980). Although direct evidence of in vivo cross-linking of neurofilament proteins could not be demonstrated in γ-diketone-treated animals by Graham et al. (1984), evidence has been presented on in vitro cross-linking (Graham et al., 1984). In addition, DeCaprio and O'Neill (1985) have demonstrated the appearance of high-molec-

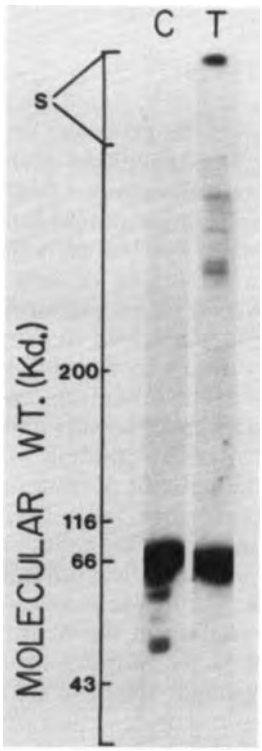

FIG. 4. Immunoblot of anti-70K antibody to cytoskeletal protein from control (C) or treated (T) animals. X-ray film exposure was for 6 h to demonstrate higher-molecular-weight cross-links.

ular-weight proteins in cytoskeletal preparations from 2,5-hexanedione-treated animals.

The accumulation of 10-nm filaments is pathognomic of neurotoxicity produced by 2,5-hexanedione and the related hexacarbons *n*-hexane, 2,5-hexanediol, and methyl-*n*-butyl ketone (Spencer and Schaumburg, 1975, 1980; Spencer et al., 1975). The accumulation of neurofilaments above the nodes of Ranvier in peripheral nerve results in the formation of giant axonal swellings. The Wallerian-type degeneration that ensues is secondary to the formation of these swellings. It appears that the blockage caused by the neurofilaments is directly responsible for the axonal damage that occurs.

SDS-PAGE analysis revealed a diminished amount of neurofilament triplet proteins. This decrease could presumably be due to a number of potential sources. If the neurofilaments are being cross-linked, there would most likely be a loss of neurofilament protein on gel electrophoresis. A second possibility is that the reduction in content of the neurofilament proteins reflects an overall decrease in the amount of neurofilament protein present in the spinal cords, not due to cross-linking but merely due to a loss of protein in the distal part of any axons undergoing Wallerian-type degeneration. Although the second possibility could not be entirely ruled out, similar cytoskeletal preparations isolated from hens with organophosphorus compound-induced delayed neurotoxicity, which produces a distal axonopathy with a Wallerian-type degeneration in the spinal cord (Abou-Donia, 1981), did not demonstrate any alteration in cytoskeletal protein content (authors' unpublished data). In addition, if there were fewer cytoskeletal proteins present, there would have been an increase in the amount of other proteins loaded onto the lanes. The internal control of GFAP is important in this respect. Chiu and Norton (1981, 1982) have demonstrated that GFAP is a major component in this preparation. Thus, if there were a decrease in the amount of neurofilament protein loaded onto the gels, there would be a concomitant increase in GFAP content. Furthermore, in a nervous system undergoing Wallerian type degeneration, an increase in GFAP level due to a reactive gliosis would be expected (Latov et al., 1979; O'Callaghan and Miller, 1985). Because no alteration in the GFAP content was observed, either one of two explanations is possible. The GFAP may also be cross-linked and therefore decreased in content, and at the same time a reactive gliosis is occurring that would increase the GFAP level in the preparation. The net result would be an absence of change on SDS-PAGE analysis. The other alternative is that Wallerian-type degeneration in these animals is minimal, with little or no change in GFAP content occurring, in which case the GFAP content would indicate that neurofilament proteins are present in the gels in an altered state, i.e., cross-linked, and are not migrating with their normal electrophoretic mobilities.

Similar patterns of protein staining have been seen by Selkoe et al. (1982*a*,*b*) in neurofilament proteins from patients with Alzheimer's disease or in neurofilaments cross-linked by transglutaminase. These investigations, as well as the work of Graham et al. (1982, 1984) on in vitro cross-linking, suggest that diminished protein staining is indicative of either in vitro or in vivo protein-protein cross-linking.

To confirm the hypothesis that the neurofilaments are present in an altered state, immunoblots of the three neurofilament proteins were obtained. The immunoblots demonstrated unequivocally that there was a significant amount of cross-linked neurofilament protein. Not only are there new bands migrating at higher molecular weights that are reactive with all three antibodies, but there was also a significant amount of immunoreactive product that did not enter the gels. However, one cannot estimate the amount of cross-linked product present in the immunoblots, because it is entirely possible that even more cross-linked products are present but were not revealed due to blockage of the epitope by the cross-linking. From these immunoblots, it is apparent that the 58K protein was not of neurofilament origin and may be vimentin, which would be produced in response to a gliosis (Latov et al., 1979; Chiu and Norton, 1981).

Studies of the polymerization of purified neurofilament proteins in vitro have demonstrated that all three neurofilament proteins are capable of assembling into filaments in vitro (Geisler et al., 1984, 1985; Minami et al., 1984). However, other investigators have proposed that although all three neurofilament proteins are capable of forming filaments, the 70K subunit forms the backbone of the neurofilament whereas the 160K and 210K subunits are accessory proteins (Heimann et al., 1985). In either case, a perturbation such as cross-linking may alter the interaction between the neurofilament proteins.

One interesting finding from this study is the identity of a 138K protein observed in the gels from treated animals. Peptide map analysis of this peptide revealed an apparent similarity to the 70K neurofilament protein; however, antibodies to the neurofilament proteins clearly demonstrate that this band was derived from the 160K neurofilament protein. Although the 160K neurofilament subunit is known to undergo proteolysis in its transport down the axon, producing several smaller fragments (Nixon et al., 1982, 1983), the 138K protein was apparently not one of the fragments. Nixon et al. (1982, 1983) found that as the 160K (NF2) protein is transported down the nerve, it undergoes proteolysis; however, its peptide mapping pattern was not altered. In all gels, at least two proteins of M_r between 150K and 160K were seen in both control and treated animals. These bands may be the proteolytic products seen by Nixon et al. (1982). However, the 138K protein was distinctly found only in treated animals, with no corresponding protein band in control animals. The uniqueness of the band indicates that it is probably an intramolecular cross-linked product of the 160K neurofilament protein. Such intramolecular cross-linked proteins would appear electrophoretically at a lower molecular weight. Intramolecular cross-linking by 2,5-hexanedione had previously been observed in vitro (Graham et al., 1982). Because neurofilament proteins have previously been shown to have remarkably similar, although not identical, peptide maps (Chiu and Norton, 1981), the similarity of the peptide mapping patterns of the 138K peptide and the 70K neurofilament subunit is not totally surprising. The absence of any protein band in the 120K–170K range to cross-react with the anti-70K neurofilament antibody suggests that it is unlikely that there is 70K-70K protein cross-linking.

The extra protein bands found in this study were the products of protein cross-linking; however, the mechanism by which this occurs is unknown. There have been two postulated mechanisms of cross-linking by 2,5-hexanedione. The first, presented by Graham and Abou-Donia (1980), proposed protein cross-linking by the formation of a conjugated Schiff base on the ε-amino groups of lysine. In this theory, it is only necessary for two lysine groups to come into proximity to one another, with one of them already bound to 2,5-hexanedione. The other theory, presented by Graham et al. (1982, 1984), used the formation of pyrrole, which was found by DeCaprio et al. (1982), to explain the proposed cross-linking. In this theory, the 2,5-hexanedione derivative of the lysyl residue of neurofilaments, *N*-(2,5-dimethylpyrrole)norlysine, undergoes autoxidation and cross-links with another pyrrole derivative molecule, resulting in the cross-linking of two neurofilament proteins. This would be an extremely rare event and would most likely not lead to formation of enough cross-linked product to be visible on SDS-PAGE. However, these mechanisms are not mutually exclusive, and both mechanisms may be acting in this case.

Another finding in this report is a reduction of the amount of immunoreactive breakdown product present in the untreated animals. Three possible causes of this reduction are (a) binding of the hexacarbon to neurofilament proteins blocks the site of action of proteases responsible for the normal degradation of the neurofilaments, (b) the hexacarbon directly inhibits the action of the protease (Cavanagh, 1982), or (c) the binding of the hexacarbon to the degradation product blocks the epitope. The blocking of the epitope is unlikely to occur, because the anti-70K antibody reacts with the 70K subunit protein from treated animals. The present studies do not ascertain which of these is occurring. However, this finding raises some interesting questions as to the mechanism of the neuropathy. The mechanism of 2,5-hexanedione-induced neuropathy may not only result from protein-protein cross-linking, but may also be due to a prevention of action of the proteases responsible for the breakdown of the neurofilament proteins. At protein loads of twice the control level, there was still a decreased amount of immunoreactive breakdown product in the treated animals. Therefore, not only is the hexacarbon cross-linking the neurofilament proteins, but it is also inhibiting the breakdown of the non-cross-linked protein.

In summary, we have found evidence for in vivo cross-linking of neurofilaments to occur in 2,5-hexanedione-treated rats. Although more work needs to be done to confirm the nature of the cross-links, i.e., cross-linking between different neurofilament subunits (e.g., 70K to 160K), as well as the molecular mechanism behind the cross-linking, the evidence presented supports the theory that neurofilament cross-linking is occurring in hexacarbon-treated animals. Further studies are underway to determine the relative contribution of cross-linking and prevention of breakdown of neurofilament proteins in the pathogenesis induced by 2,5-hexanedione.

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REFERENCES

Abdel-Rahman M. S., Hetland L. B., and Couri D. (1976) Toxicity and metabolism of methyl *n*-butyl ketone. *Am. Ind. Hyg. Assoc.* **37**, 95–102.

Abou-Donia M. B. (1981) Organophosphorus ester induced delayed neurotoxicity. *Ann. Rev. Pharmacol.* **21**, 511–548.

Abou-Donia M. B., Makkawy H. M., and Graham D. G. (1982) The relative neurotoxicities of *n*-hexane, methyl *n*-butyl ketone, 2,5-hexanediol, and 2,5-hexanedione following oral administration or intraperitoneal administration in hens. *Toxicol. Appl. Pharmacol.* **62**, 369–389.

Allen N., Mendell J. R., Billmaier D. J., Fontaine R. E., and O'Neill J. (1975) Toxic polyneuropathy due to methyl *n*-butyl ketone. An industrial outbreak. *Arch. Neurol.* **32**, 209–218.

Anthony D. C., Boekelheide K., Anderson C. W., and Graham D. G. (1983) The effect of 3,4-dimethyl substitution on the neurotoxicity of 2,5-hexanedione. II. Dimethyl substitution accelerates pyrrole formation and protein crosslinking. *Toxicol. Appl. Pharmacol.* **71**, 372–382.

Cavanagh J. B. (1982) The pattern of recovery of axons in the nervous system of rats following 2,5-hexanediol intoxication: a question of rheology. *Neuropathol. Appl. Neurobiol.* **8**, 19–34.

Chiu F.-C. and Norton W. T. (1981) The cytoskeleton of primary astrocytes in culture contains actin, glial fibrillary acidic protein, and the fibroblast-type protein, vimentin. *J. Neurochem.* **37**, 147–155.

Chiu F.-C. and Norton W. T. (1982) Bulk preparation of CNS cytoskeleton and the separation of individual neurofilament proteins by gel filtration: dye binding characteristics and amino acid composition. *J. Neurochem.* **39**, 1252–1260.

Cleveland D. W., Fischer S. G., Kirshner M. W., and Laemmli U. K. (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252**, 1102–1106.

DeCaprio A. P. and O'Neill E. A. (1985) Alterations in rat cytoskeletal proteins induced by *in vitro* and *in vivo* 2,5-hexanedione exposure. *Toxicol. Appl. Pharmacol.* **78**, 235–247.

DeCaprio A. P., Olajos E. J., and Weber P. (1982) Covalent binding of a neurotoxic *n*-hexane metabolite: conversion of primary amines to substituted pyrrole adducts by 2,5-hexanedione. *Toxicol. Appl. Pharmacol.* **65**, 440–450.

DiVincenzo G. D., Kaplan C. J., and Dedinas J. (1976) Characterization of the metabolites of methyl *n*-butyl ketone in guinea pig serun and their clearance. *Toxicol. Appl. Pharmacol.* **36**, 511–522.

Geisler N., Fischer S., Vanderchkove J., Plessmenn U., and Weber K. (1984) Hybrid character of a large neurofilament protein (NF-M); intermediate filament type sequence followed by a long and acidic carboxy-terminal extension. *EMBO J.* **3**, 2701–2706.

Geisler N., Fischer S., Vanderchkove J., Van Damme J., Plessmann U., and Weber K. (1985) Protein-chemical characterization of NF-N, the largest mammalian neurofilament component; intermediate filament-type sequences followed by a unique carboxy-terminal extension. *EMBO J.* **4**, 57–63.

Graham D. G. (1980) Hexane neuropathy: a proposal for pathogenesis of a hazard of occupational exposure and inhalane abuse. *Chem.-Biol. Interact.* **32**, 339–345.

Graham D. G. and Abou-Donia M. B. (1980) Studies on the molecular pathogenesis of hexane neuropathy. I. Evaluation of the inhibition of glyceraldehyde-3-phosphate dehydrogenase by 2,5-hexanedione. *J. Toxicol. Environ. Health* **6**, 623–631.

Graham D. G., Anthony D. C., Boekelheide K., Maschmann N. A., Richards R. G., Wolfram J. W., and Shaw B. R. (1982) Studies on the molecular pathogenesis of hexane neuropathy. II. Evidence that pyrrole derivitization of lysyl residues leads to protein crosslinking. *Toxicol. Appl. Pharmacol.* **64**, 415–422.

Graham D. G., Szakai-Quin G., Priest J. W., and Anthony D. C. (1984) *In vitro* evidence that covalent crosslinking of neurofilaments occurs in γ-diketone neuropathy. *Proc. Natl. Acad. Sci. USA* **81**, 4979–4982.

Griffin J. W., Hoffman P. N., Clark A. W., Carroll P. T., and Price D. L. (1978) Slow axonal transport of neurofilament proteins: impairment by β,β′-iminodiproprionitrile administration. *Science* **202**, 633–635.

Heimann R., Shelanski M. L., and Liem R. K. (1985) Microtubule-associated proteins bind specifically to the 70-KDa neurofilament protein. *J. Biol. Chem.* **260**, 12160–12166.

Hunkapillar M. W., Lujan E., Ostrander F., and Hood L. E. (1983) Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. *Methods Enzymol.* **91**, 227–236.

Juntunen J., Haltia M., and Linnoila I. (1974) Histochemically demonstrable non-specific cholinesterase as an indicator of peripheral nerve lesion in carbon disulphide-induced polyneuropathy. *Acta Neuropathol. (Berl.)* **29**, 361–366.

Krammer A., Staudinger H., and Ullrich V. (1974) Effect of *n*-hexane inhalation on the monooxygenase system in mice liver microsomes. *Chem-Biol. Interact.* **8**, 11–18.

Laemmli U. K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Latov N., Nilaver G., Zimmerman E. A., Johnson W. G., Silverman A.-J., Defindini R., and Cote L. (1979) Fibrillary astrocytes proliferate in responses to brain injury. *Dev. Biol.* **72**, 381–384.

Mendell J. R., Saida K., Ganansia M. F., Jackson D. B., Weiss H. S., Gardier R. W., Chrisman C., Allen N., Couri D., O'Neil J., Marks B., and Hetland L. (1974) Toxic polyneuropathy produced by methyl *n*-butyl ketone. *Science* **185**, 787–789.

Minami Y., Endo S., and Sakai H. (1984) Participation of 200K or 150K subunit of neurofilament in construction of the filament core with 70K subunit and promotion of tubulin polymerization by incorporated 200K subunit. *J. Biochem.* **96**, 1481–1490.

Monaco S., Autilio-Gambetti L., Zabel D., and Gambetti P. (1985) Giant axonal neuropathy: acceleration of neurofilament transport in optic axons. *Proc. Natl. Acad. Sci. USA* **82**, 920–924.

Nixon R. A., Brown B. A., and Marotta C. A. (1982) Posttranslational modification of a neurofilament protein during axoplasmic transport: implications for regional specialization of CNS axons. *J. Cell Biol.* **94**, 150–158.

Nixon R. A., Brown B. A., and Marotta C. A. (1983) Limited proteolytic modification of a neurofilament protein involves a proteinase activated by endogenous levels of calcium. *Brain Res.* **275**, 384–388.

O'Callaghan J. P. and Miller D. B. (1985) Cerebellar hypoplasia in the Gunn rat is associated with quantitative changes in neurotypic and gliotypic proteins. *J. Pharmacol. Exp. Ther.* **234**, 522–533.

Sabri M. I., Moore C. L., and Spencer P. S. (1979) Studies on the biochemical basis of distal axonopathies. I. Inhibition of glycolysis of neurotoxic hexacarbon compound. *J. Neurochem.* **32**, 683–689.

Selkoe D. J., Abraham C., and Ihara Y. (1982*a*) Brain transglutaminase: *in vitro* crosslinking of human neurofilament proteins into insoluble polymers. *Proc. Natl. Acad. Sci. USA* **79**, 6070–6074.

Selkoe D. J., Ihara Y., and Salazar F. J. (1982*b*) Alzheimer's disease: insolubility of partially purified paired helical filaments in sodium dodecyl sulfate and urea. *Science* **215**, 1243–1245.

Shelanski M. L., Gaskin F., and Cantor C. R. (1973) Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. USA* **70**, 765–768.

Spencer P. S. and Griffin J. W. (1982) Disruption of axoplasmic transport by neurotoxic agents. The 2,5-hexanedione model, in *Axoplasmic Transport in Physiology and Pathology* (Weiss D. G. and Gorio, A., eds), pp. 92–103. Springer-Verlag, Berlin.

Spencer P. S. and Schaumberg H. H. (1975) Ultrastructural studies of the dying back process. *J. Neuropathol. Exp. Neurol.* **36**, 276–299.

Spencer P. S. and Schaumberg H. H. (1980) *Experimental and Clinical Neurotoxicology*. Williams and Wilkins, Baltimore.

Spencer P. S., Schaumburg H. H., Raleigh R. L., and Terhaar C. J. (1975) Nervous system degeneration produced by industrial solvent methyl *n*-butyl ketone. *Arch. Neurol.* **32**, 219–222.

Spencer P. S., Sabri M. I., Schaumburg H. H., and Moore C. L. (1979) Does a defect of energy metabolism in the nerve fiber underlie axonal degenerations in polyneuropathies: *Ann. Neurol.* **5**, 501–507.

Towbin H., Staehelin T., and Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.

Wray W., Boulikas T., Wray V. P., and Hancock R. (1981) Silver staining in polyacrylamide gels. *Anal. Biochem.* **118**, 197–203.