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# *In vitro* evidence that covalent crosslinking of neurofilaments occurs in γ-diketone neuropathy

(hexanes/cytoplasmic filaments/neurotoxins/axonal transport/pyrroles)

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ABSTRACT **We have postulated that the toxic neuropathies associated with neurofilament-filled axonal swellings have a common pathogenesis, the covalent crosslinking of neurofilaments during anterograde transport. The newly described γ-diketone, 3,4-dimethyl-2,5-hexanedione (DMHD), is a more potent analogue of the toxic metabolite of *n*-hexane, 2,5-hexanedione. The axonal swellings observed in DMHD toxicity are in the proximal axon, as seen in intoxication with β,β′-iminodipropionitrile, rather than in the distal axon, where neurofilamentous swellings are observed in *n*-hexane, carbon disulfide, and acrylamide neurotoxicity. In these studies, $^{14}$C-labeled DMHD and 2-butanone were synthesized and allowed to react with peripheral nerve. Only $^{14}$C-labeled DMHD resulted in stable radiolabeled protein polymers, which were retained by nitrocellulose filters with pore sizes as large as 12 μm. More specific evidence for covalent crosslinking of neurofilaments was obtained when DMHD was allowed to react with peripheral nerve in which the neurofilaments had been pulse-labeled with L-[$^{35}$S]methionine.**

The common solvent, *n*-hexane, has resulted in sensorimotor neuropathies in industry (1) and after inhalation for its euphoric effects (2). In humans, as well as in experimental animals, the initial alteration is an accumulation of 10-nm neurofilaments in the distal, preterminal axon (3). This is accompanied by huge swellings of the axon, most often proximal to nodes of Ranvier (4). Marked distortions of nodal and paranodal structure are associated with the masses of neurofilaments (5, 6). In large myelinated axons, the neurofilament-filled axonal swellings are followed by Wallerian-like degeneration of the distal axon (4). A similar sequence of events is observed after exposure to carbon disulfide (7) or acrylamide (8), although with the latter toxicant neurofilament-filled axonal swellings are a less common event. An inherited neuropathy in children, giant axonal neuropathy, is also characterized by neurofilamentous swellings of the distal axon (9).

β,β′-Iminodipropionitrile is a compound that has produced a unique clinical and pathological picture in which the neurofilament-filled axonal swellings occur in the proximal rather than the distal axon (10–12). This proximal axonopathy has been classified separately from the distal axonopathies caused by *n*-hexane, carbon disulfide, or acrylamide. Similar proximal swellings filled with neurofilaments are seen early in amyotrophic lateral sclerosis (13).

The genesis of the neurofilament accumulations in these disorders has been a point of great controversy. Some have postulated that neurofilaments accumulate because of a defect in axonal transport, perhaps secondary to a defect in energy metabolism (14). Cavanagh and Bennetts have suggested that the defect may lie in the catabolism of neurofilaments (15). Studies in this laboratory have led to the proposal that the accumulation of neurofilaments is the primary event in this group of neuropathies. In *n*-hexane neuropathy, we have proposed that 2,5-hexanedione (2,5-HD), the γ-diketone resulting from metabolism of *n*-hexane, reacts with lysyl amino groups to form first an imine, followed by cyclization to a pyrrole, and that autoxidation of pyrrole rings leads to covalent crosslinking between proteins (16). We have postulated that the neurofilament, by virtue of its unique stability in the axon, is particularly vulnerable to this series of reactions (16, 17). Further, the greater incidence of Wallerian-like degeneration in large, as compared with small, myelinated axons appears to be related to the greater constrictions of axonal diameters that are found at nodes of Ranvier in large axons (5, 16, 18). This association suggests that these constrictions impede the proximo-distal transport of the crosslinked neurofilament aggregates, leading to swellings of the axon proximal and Wallerian-like degeneration distal to the points of occlusion (5, 16).

Recent studies have shown that an analogue of 2,5-HD, 3,4-dimethyl-2,5-hexanedione (DMHD) is much more potent as a neurotoxicant than 2,5-HD (16, 17, 19, 20). We had predicted that 3,4-dimethyl substitution of 2,5-HD would enhance the rate of cyclization to pyrroles and that the resulting increased electron density of the tetramethylpyrrole ring would lead to accelerated autoxidation and crosslinking (21). Indeed, the rate of protein crosslinking by DMHD exceeds the rate by 2,5-HD by a factor of 40 (21). Further, DMHD results in neurofilamentous swellings of the proximal axon, reproducing the axonal pathology and defects in slow axonal transport that are observed with β,β′-iminodipropionitrile (17, 20, 22). In addition, smaller doses of DMHD result in swellings of the more distal axon (19). Thus, we have proposed the neurofilamentous axonopathy caused by DMHD as a heretofore unavailable missing link between the proximal axonopathy from β,β′-iminodipropionitrile and the distal axonopathies caused by *n*-hexane, carbon disulfide, and acrylamide (17, 19). This observation is the basis for a unifying hypothesis for the neurofilament axonopathies—namely, that the accumulation of neurofilaments occurs as the result of covalent crosslinking and that the rate of crosslinking determines the proximo-distal location of the neurofilament-filled axonal swellings (17, 19).

In this report, we present evidence that covalent crosslinking of neurofilaments results when DMHD is incubated *in vitro* with peripheral nerve.

## MATERIALS AND METHODS

Synthesis of [$^{14}$C]DMHD (3,4-dimethyl-2,5-[2,5-$^{14}C_2$]hexanedione) began with the generation of 2-[$^{14}$C]butanone (ethyl methyl [$^{14}$C]ketone) from the reaction of [1-$^{14}$C]acetyl chloride (ICN) with diethylzinc (Aldrich) in toluene. The resulting 2-[$^{14}$C]butanone was >99% pure by gas chromatogra-

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Abbreviations: 2,5-HD, 2,5-hexanedione; DMHD, 3,4-dimethyl-2,5-hexanedione; SCa, slow component "a" of axonal transport; SCb, slow component "b" of axonal transport.

phy and had a specific activity of 30 μCi/mmol (1 Ci = 37 GBq). DMHD was synthesized by the dehydrodimerization (23) of 2-butanone with $PbO_2$. [$^{14}$C]DMHD was purified by chromatography on silica gel eluted with acetone/*n*-hexane, 1:3 (vol/vol). Sixty percent of the radioactivity was isolated as DMHD (>99% pure by gas chromatography). It was further identified by its $^1$H NMR spectrum (IBM NR-80 FT spectrometer) and by mass spectroscopy (Hewlett–Packard 5992A GC/MS system; $M_r$ 142.1) and had a specific activity of 60 μCi/mmol.

Rat spinal cord neurofilament preparations (24) were allowed to react *in vitro* with DMHD, followed by polyacrylamide gel electrophoresis with 1% $NaDodSO_4$/4% acrylamide gels (25).

To determine whether DMHD resulted in neurofilament crosslinking in intact axons, sciatic nerve along with its roots and major branches was removed from two rats and desheathed, and duplicate 50-mg aliquots were incubated for 8 and 24 hr at 37°C with 20 mM 2-[$^{14}$C]butanone or 20 mM [$^{14}$C]DMHD in Dulbecco's phosphate-buffered saline in a volume of 1 ml. In addition, 1 mM EDTA was added to retard proteolysis. The nerve pellets were washed by centrifugation in phosphate-buffered saline/EDTA, sonicated for 30 sec at 60 W in 1-ml volumes of 1% $NaDodSO_4$, and boiled for 10 min. After cooling, proteins were precipitated with the addition of 9 ml of acetone and, after 15 min at −20°C, collected by centrifugation at 20,000 × *g* for 10 min. Delipidation was effected by washing with two 10-ml quantities of acetone/ether, 1:1 (vol/vol), followed by one 10-ml wash with ether. After drying, the resulting pellets were solubilized in 8 M urea/5% 2-mercaptoethanol/1% $NaDodSO_4$ with sonication and boiling. In order to determine whether high molecular weight species had resulted from the incubation of nerve with [$^{14}$C]DMHD, aliquots were filtered through nitrocellulose filters of pore size 0.025, 0.2, and 12.0 μm (Schleicher & Schuell). Each filter was washed with 5 ml of water.

Crosslinking of neurofilament proteins was investigated more directly by allowing DMHD to react with portions of sciatic nerve in which these proteins were specifically labeled. Since neurofilament proteins travel in slow component "a" of axonal transport [SCa of Hoffman and Lasek (26)], it is possible to label this component largely to the exclusion of other axonal proteins or myelin and connective tissue proteins. By using the technique described by Griffin *et al.* (27), 380- to 540-g Sprague–Dawley (Charles River Breeding Laboratories) rats were anesthetized with chloral hydrate, and the lumbar spinal cord was exposed through laminectomy. High specific activity L-[$^{35}$S]methionine (800–1200 Ci/mmol; Amersham) was injected into the ventral horns of spinal cord segments giving origin to L4 and L5 nerve roots. Each rat received a total of 1 mCi of L-[$^{35}$S]-methionine in three 1-μl injections on each side. After 56–71 days, the SCa peak in each nerve was identified by solubilizing the first 2 mm of each 10-mm segment of nerve in NCS solubilizer (Amersham), beginning at the junction of L4 and L5 roots with the spinal cord. The peak of SCa was present in segments representing a migration rate of 0.5–1.5 mm/day, whereas the peak of the smaller slow component "b" of axonal transport (SCb) had migrated ≈2–3 mm/day. Segments containing SCa were desheathed, then exposed to 20 mM DMHD or 40 mM acetone for 24 hr at 37°C in 100 mM sodium phosphate buffer, pH 7.4/1 mM EDTA. The segments were then homogenized and delipidated, and the resulting protein pellet was solubilized in 8 M urea/1% $NaDodSO_4$/5% 2-mercaptoethanol as described above.

Nonspecific radiolabeling of nerve proteins without crosslinking was performed by reacting desheathed nerve with $^{125}$I-labeled Bolton–Hunter reagent (New England Nuclear) at room temperature. The nerve was then homogenized, its lipids were extracted, and then the proteins were solubilized in urea/$NaDodSO_4$/2-mercaptoethanol.

## RESULTS

When rat neurofilament preparations were treated with DMHD at 37°C, there was a progressive loss of neurofilament bands by Coomassie blue staining of polyacrylamide gels, with the production of large molecular weight species that failed to enter the stacking gel. This pointed out two pitfalls in the analysis of neurofilament crosslinking by $NaDodSO_4$/polyacrylamide gel electrophoresis. First, a polydisperse population of large polymers is difficult to define by polyacrylamide gel electrophoresis; the same would be true for gel filtration or sucrose density gradients. Second, large polymers are revealed mainly by the disappearance of protein bands from the separating gel. A similar observation has been made by Selkoe *et al.* in their analysis of neurofibrillary masses in Alzheimer disease (28) or when neurofilaments were crosslinked by transglutaminase (29). Our subsequent approach was to allow DMHD to react with intact peripheral nerve, using either radiolabeled DMHD or radiolabeled neurofilaments, and to employ nitrocellulose filters to estimate the range of molecular sizes resulting from polymerization.

A major clue in our pursuit of the molecular events underlying γ-diketone neuropathies has been the chromophore that develops in the reaction of γ-diketones with proteins and model amines (16). The dimethylpyrrole that results from the reaction between 2,5-HD and lysyl amino groups of proteins autoxidizes to an orange chromophore, the intensity of which is proportional to the number of lysyl residues that have been derivatized (16). The orange chromophore represents a poorly defined collection of oxidative products, which are potent electrophiles and the ultimate crosslinking species. In these experiments, nerve pellets incubated with 2-[$^{14}$C]butanone remained white, while those incubated with [$^{14}$C]DMHD were noted to become progressively pink, turning a deep rose pink after 24 hr (Fig. 1). This color is characteristic of the oxidation product of pure tetramethylpyrroles

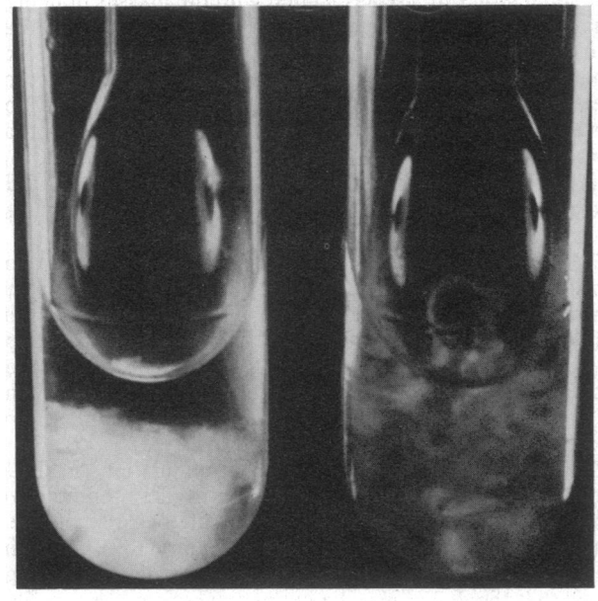


FIG. 1. Reaction of peripheral nerve with 2-butanone (MEK) and DMHD. After 24 hr of reaction with 20 mM 2-butanone, the desheathed nerve remained white, whereas that which reacted with 20 mM DMHD is dark pink.

and also can be seen when DMHD is incubated *in vitro* with proteins or model amines. We have observed that brain, spinal cord, spinal nerve roots, and seminal vesicles turn pink during chronic intoxication of rats with DMHD.

The incorporation of [$^{14}$C]DMHD into the nerve protein pellet was roughly linear for the 24-hr period, with 493.5 ± 44.6 nmol bound per mg of protein (mean ± SEM) after 24 hr (Fig. 2*A*). By contrast, the amount of 2-[$^{14}$C]butanone bound after 24 hr was 0.2 nmol/mg of protein. Of the bound [$^{14}$C]DMHD, 31% was retained by 0.025-μm filters both at 8 and 24 hr (Fig. 2*B*). Between 8 and 24 hr, a greater amount of the [$^{14}$C]DMHD was retained by filters with pore size 0.2 and 12 μm. The percentage of bound label retained on 12-μm filters at 24 hr was 11.3 ± 0.0%, compared with 1.5 ± 1.1% at 8 hr. Thus, with time an increasing quantity of the bound [$^{14}$C]DMHD was incorporated into larger polymers, while the proportion of nerve proteins that were derivatized but not polymerized remained constant. This observation may be explained by the fact that larger proteins with a greater quantity of lysyl residues available for pyrrole derivatization would polymerize to a greater extent than would smaller proteins. Within the axon the neurofilaments exist as stable, insoluble filaments of massive molecular weight (26) and, thereby, are more vulnerable to becoming crosslinked.

A more direct measurement of neurofilament crosslinking was provided by the reaction of unlabeled DMHD with nerve containing $^{35}$S-labeled SCa. The proportion of SCa retained by nitrocellulose filters is given in Fig. 3. The quantity of SCa trapped by 0.025-μm filters increased from 12.2 ± 2.5% in acetone-treated nerve to 63.7 ± 9.7% in DMHD-treated nerve, whereas with the 12.0-μm filter, the amount retained increased from 1.9 ± 0.9% to 32.4 ± 10.1%, with intermediate levels retained by 0.2-μm filters. Thus, although only 31%, 18%, and 11% of label was retained by the 0.025-, 0.2-, and 12.0-μm filters, respectively, when [$^{14}$C]DMHD was reacted with all the proteins of the nerve, the proportion of labeled SCa retained was 2–3 times as great in each case, suggesting that neurofilaments are more vulnerable than are other nerve proteins to covalent crosslinking by DMHD.

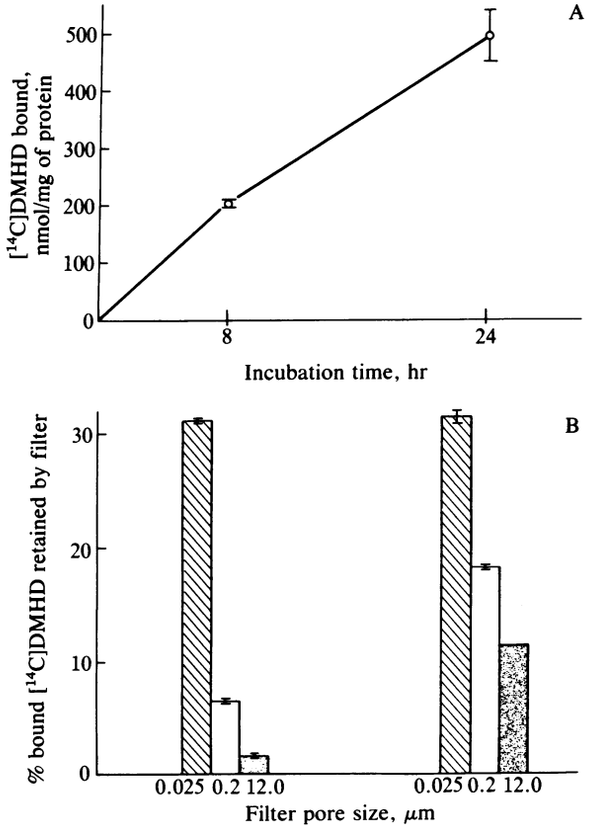


FIG. 2. (*A*) Covalent binding of [$^{14}$C]DMHD to nerve proteins. The amount of [$^{14}$C]DMHD bound (nmol/mg of nerve protein) as a function of time. (*B*) Proportion of bound [$^{14}$C]DMHD retained by nitrocellulose filters at 8 hr (*Left*) and 24 hr (*Right*). The percentage of total radioactivity retained by filters with pore sizes 0.025, 0.2, and 12 μm is presented for each of the two time points in *A*.

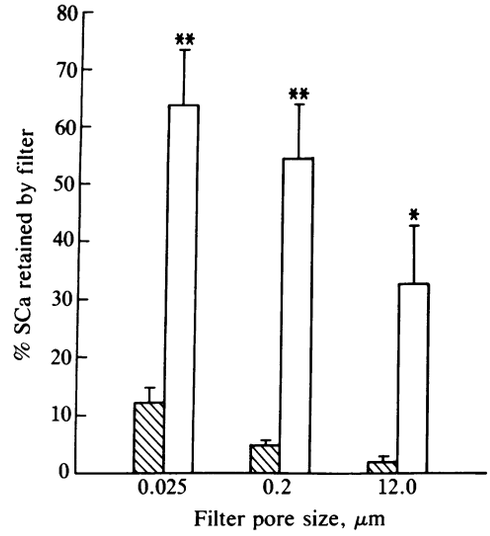


FIG. 3. Retention of $^{35}$S-labeled SCa by nitrocellulose filters after reaction with acetone or DMHD. The percent of SCa retained by filters with pore sizes of 0.025, 0.2, and 12 μm is presented for SCa-containing nerve segments exposed to acetone (crosshatched bars) or DMHD (clear bars). **, $P < 0.01$; *, $P < 0.05$ (Student's *t* test).

The fact that the amount of radiolabeled protein retained by nitrocellulose filters varied greatly between pore sizes is inconsistent with nonspecific adsorption of derivatized protein to nitrocellulose. An additional control for the concern that retention of proteins by nitrocellulose filters might represent nonspecific adsorption was provided by $^{125}$I-labeled Bolton–Hunter reagent. This compound is the *N*-hydroxysuccinimide ester of iodinated *p*-hydroxyphenylpropionic acid. Lysyl residues are acylated to form a stable secondary amide, which is labed with $^{125}$I. Reaction of nerve with Bolton–Hunter reagent would lead to derivatization of any protein within the nerve, probably including a few neurofilament proteins, since this highly reactive reagent would most likely react with endoneurial or myelin proteins before it could reach the axon. Desheathed rat peripheral nerve showed no additional derivatization after 1 hr at 19°C. After delipidation and solubilization in urea/NaDodSO$_4$/mercaptoethanol as outlined above, the retention of radiolabeled protein by nitrocellulose filters was found to parallel that of control SCa that had been pulse-labeled with [$^{35}$S]methionine (Table 1). The concurrence of these two control conditions lends support to the concept that enhanced retention of

Table 1. Nitrocellulose filter retention of radiolabeled nerve proteins

| Nitrocellulose filter pore size, μm | Control SCa retained, % | Bolton–Hunter-labeled nerve protein retained, % |
|---|---|---|
| 12 | 1.93 ± 0.88 | 1.38 ± 0.25 |
| 0.2 | 4.82 ± 0.59 | 5.48 ± 1.44 |
| 0.025 | 12.18 ± 2.52 | 11.45 ± 4.36 |

radiolabeled, denatured, and reduced nerve protein after reaction with DMHD reflects protein crosslinking. The conclusion that can be reached from experiments with prelabeled SCa is that there was extensive covalent crosslinking of neurofilaments.

## DISCUSSION

These studies show that, with either radiolabeled diketone or radiolabeled target, masses of labeled protein result that are stable to boiling in urea, $NaDodSO_4$, and 2-mercaptoethanol (28, 29) and are retained by nitrocellulose filters with pore sizes as large as 12 $\mu$m. Thus, nerve proteins, particularly neurofilaments, have been covalently crosslinked *in vitro*. Derivatization of purified proteins in solution with 2,5-HD or DMHD results in both intramolecular and intermolecular crosslinking (16, 21), so that both types of crosslinking would be expected to occur *in vivo*. Intramolecular crosslinking could retard transport by interfering with the interaction between neurofilaments and other axonal proteins. In concept, intramolecular crosslinking could impair eventual protease digestion. Intermolecular crosslinking of neurofilaments might be between neurofilaments and also could involve other axonal proteins as well as "bridging" molecules such as polyamines. Indeed, it is reasonable to postulate that all of these reactions occur within the axon to lead to the aggregation of neurofilaments. It remains to be shown, however, whether covalent crosslinking underlies neurofilament aggregation *in vivo*.

The concentration of DMHD used in these experiments is in the range of 100 times greater than tissue levels we have observed in chronic DMHD intoxication (unpublished observations). Thus, the extensive degree of crosslinking observed in these experiments is not obtained *in vivo*. Two features of the anatomy of the axon suggest, however, that a much lesser degree of neurofilament crosslinking may be biologically significant. The neurofilament is an extremely stable protein, unlike microtubules, which are in equilibrium with tubulin dimers, and microfilaments, which reversibly dissociate to actin monomers. The neurofilament appears to be a continuous molecule from neuronal perikaryon to synapse (26). Considering that its rate of progression down the axon is only 1 mm/day (26), stable covalent crosslinks could bind neurofilaments to each other and to other axoplasmic components over an extended period of exposure. Repeated exposures to a crosslinking toxicant could result in masses of neurofilaments unable to progress down the axon in a normal fashion. The second pertinent anatomical feature is that axonal diameter is significantly reduced at nodes of Ranvier (18). Thus, an ever-growing mass of neurofilaments would have even greater difficulty moving through nodes. The studies of Jones and Cavanagh (5, 6) lend strong support to this concept. During chronic intoxication of rats with 2,5-hexanediol, the neurofilament masses behaved as cohesive aggregates which both remained intact during anterograde transport and greatly distorted the nodal and paranodal anatomy during their passage through the nodes of Ranvier.

Thus, an obstruction to the distal transport of neurofilaments in the face of continued transport to the site of obstruction could explain the large paranodal accumulations. The observation that distal degeneration is most prevalent among large myelinated axons, where the percentage of constriction of axonal diameter at nodes of Ranvier is greatest, supports a causal relationship between neurofilament-filled axonal swellings and degeneration of the distal axon (5, 16). However, the observation that little axonal degeneration is seen distal to large swellings in ($\beta$,$\beta'$-iminodipropionitrile) intoxication (12) suggests that additional factors participate in the evolution of distal axonal degeneration in $\gamma$-diketone neuropathy.

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