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Alterations in Rat Axonal Cytoskeletal Proteins Induced by *in Vitro* and *in Vivo* 2,5-Hexanedione Exposure[1]

ANTHONY P. DECAPRIO[2] AND ELIZABETH A. O'NEILL

Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201

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Alterations in Rat Axonal Cytoskeletal Proteins Induced by *in Vitro* and *in Vivo* 2,5-Hexanedione Exposure. DECAPRIO, A. P., AND O'NEILL, E. A. (1985). *Toxicol. Appl. Pharmacol.* **78,** 235–247. The neurotoxic γ-diketone 2,5-hexanedione (2,5-HD) reacts *in vitro* and *in vivo* with protein lysine ε-amine moieties to yield 2,5-dimethylpyrrole adducts. It has been hypothesized that pyrrole adduct formation in neurofilament (NF) or other axonal proteins may lead to increased hydrophobicity, secondary autoxidative crosslinking, or the loss of essential lysine amine groups, and that pyrrolylation therefore represents the critical initiating event in γ-diketone neuropathy. The present investigation was designed to evaluate pyrrole levels and other changes in brain stem and spinal cord axonal cytoskeletal proteins from rats receiving 0.5% 2,5-HD in the drinking water for up to 8 weeks and following recovery. Clinical signs of neuropathy were apparent in rats after 5 weeks exposure, while no histopathological effects were seen until 8 weeks. Cessation of dosing resulted in some recovery from clinical neuropathy but virtually no change in histopathologically demonstrable CNS damage. 2,5-Dimethylpyrrole adduct was detected in serum and axonal cytoskeletal proteins from animals in all exposure groups and its formation appeared to reach a plateau in both serum and axonal protein. Assay of total protein lysine vs pyrrole content demonstrated an average conversion of <1% of ε-amine groups into pyrrole adducts in axonal protein after 2 weeks exposure. Gel electrophoresis revealed discrete new protein bands in brain stem and spinal cord axonal protein preparations from treated animals, along with high-molecular-weight, nonmigrating proteinaceous material. Concentration of the nonmigrating material appeared to increase in a time-dependent fashion. A concurrent decrease in the relative amounts of native NF subunit proteins was observed in brain stem but not spinal cord. Reversal of these changes was observed 9 weeks after cessation of dosing, although residual nonmigrating protein and pyrrole adduct were present. *In vitro* incubation of axonal cytoskeletal protein preparations (pH 7.2, 37°C) with 2,5-HD resulted in the formation of high-molecular-weight bands identical to those seen *in vivo*. These findings provide evidence for pyrrole adduct formation and secondary covalent crosslinking in CNS axonal cytoskeletal proteins from 2,5-HD-treated animals.

The widely used industrial and commercial hydrocarbon solvent *n*-hexane undergoes conversion in animals and humans to the neurotoxic metabolite 2,5-hexanedione (2,5-HD) (Couri *et al.*, 1978; Perbellini *et al.*, 1979). This γ-diketone is capable of producing a characteristic distal accumulation of axonal neurofilaments in susceptible central and peripheral myelinated nerve fibers when administered to a variety of animal species (Spencer and Schaumburg, 1975, 1977). Similar neuropathological changes can be induced by direct application of 2,5-HD to exposed nerve fibers (Politis *et al.*, 1980), by intraneural injection of the compound (Griffin *et al.*, 1983; Zagoren *et al.*, 1983), and by its incorporation into the nutrient medium

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[2] To whom correspondence should be addressed.

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of functionally coupled spinal cord, motor nerve, and skeletal muscle tissue culture systems (Veronesi *et al.*, 1983).

The apparent direct axonal toxicity of the γ-diketones suggests that the axonal cytoskeleton is a potentially important site of covalent binding of these compounds. The axonal cytoskeleton is the major proteinaceous component of axoplasm, consisting of a unique system of structural proteins and associated enzymes (including the neurofilament proteins, tubulin, creatine phosphokinase, and neuron-specific enolase) (Lasek and Hoffman, 1976; Brady and Lasek, 1981; Metuzals *et al.*, 1981). These proteins are initially synthesized and organized within the nerve cell body and together comprise the slowly transported components (SCa and SCb) of axoplasm (Black and Lasek, 1980; Grafstein and Forman, 1980; Lasek, 1982). The neurofilament (NF) proteins in particular are highly stable and long-lived, and are believed to undergo turnover almost exclusively within the nerve terminals (Schlaepfer, 1979). It is apparent that such a complex system might be uniquely susceptible to functional damage by covalent binding of xenobiotic chemicals.

We have previously demonstrated the formation of 2,5-dialkylpyrrole adducts as products of the reaction of γ-diketones with protein lysine ε-amine groups *in vitro* and *in vivo* (DeCaprio and Weber, 1980, 1981; DeCaprio *et al.*, 1982, 1983). *In vitro* studies have also indicated that these hydrophobic residues undergo secondary reactions to yield complex oxidized and polymeric products (DeCaprio and Weber, 1980, 1981; DeCaprio *et al.*, 1982; Graham *et al.*, 1982; Anthony *et al.*, 1983). We have hypothesized that pyrrole adduct formation in NF protein might be the critical event leading to pathological NF accumulation in this syndrome, as a result of physicochemical changes induced by the hydrophobic adduct (DeCaprio *et al.*, 1982, 1983).

It has been alternatively proposed that NF accumulation is due to covalent crosslinking of neurofilaments, a scheme which was initially suggested to occur via a "conjugated Schiff base" mechanism (Graham, 1980) which was later abandoned in favor of one involving autoxidative crosslinking of pyrrole adducts (Graham *et al.*, 1982; Anthony *et al.*, 1983). A third hypothesis invokes a disruption in NF–microtubule (MT) interactions as a result of γ-diketone exposure to explain the observed axonal NF accumulation (Griffin *et al.*, 1983). In any case, the net result of these NF alterations is thought to be physical blockade of axonal nutrient flow and eventual nerve degeneration.

Although pyrrole adduct has been detected in whole-brain homogenates from hens administered 2,5-HD (DeCaprio *et al.*, 1983), this phenomenon has not been unequivocally demonstrated in NF or other axonal proteins during exposure to this diketone. The incidence of 2,5-HD-induced crosslinking or other physicochemical changes in axonal protein *in vivo* is similarly obscure, though dimerization of the erythrocyte membrane protein spectrin has been reported in rats after oral exposure to γ-diketones (Anthony *et al.*, 1983). Crosslinking of NF proteins after *in vitro* γ-diketone treatment has also been described (Graham *et al.*, 1984).

The present investigation was designed to evaluate the proposed mechanisms of γ-diketone neuropathy by examining alterations in CNS axonal cytoskeletal proteins from rats during prolonged oral exposure to 2,5-HD and after cessation of exposure. An additional aim was to examine protein changes in axonal cytoskeletal preparations following *in vitro* exposure to the diketone. This report presents evidence for both pyrrole adduct formation and the appearance of high-molecular-weight protein species in axonal cytoskeletal preparations from 2,5-HD-treated animals, and describes similar changes as a result of *in vitro* exposure.

METHODS

Animals and dosing. Adult, male Wistar rats (Griffin Laboratory, New York State Department of Health)

(300 to 340 g) were divided into five groups of 10 animals per group. Groups received 0.5% (v/v) 2,5-HD (Eastman Kodak Co., Rochester, N.Y.) in the drinking water for 1, 2, 5, or 8 weeks before being killed, or for 8 weeks followed by 9 weeks of recovery before being killed. An additional 12 animals received tap water only and served as controls which were killed at the same time as those on the recovery regimen (17 weeks). Animals were housed individually in plastic cages at 22°C and 40 to 60% humidity and were allowed access to standard rat chow and drinking water, *ad libitum*. Body weights and drinking water consumption were monitored weekly and neurotoxic signs were assessed twice weekly using a previously described method (Abou-Donia, 1978).

In a separate experiment, five male Wistar rats were pair-fed with five animals receiving 0.5% 2,5-HD in the drinking water. Water intake in the pair-fed group was also matched to animals receiving 0.5% 2,5-HD, in order to control for the decreased water consumption commonly seen in these animals (Gillies *et al.*, 1980). Body weights and feed and water consumption were monitored daily, and animals were killed at 2 weeks. Animals were killed by CO_2 asphyxiation and blood was collected into nonheparinized tubes by cardiac puncture. Spinal cords were removed using a syringe and ice-cold phosphate-buffered saline as described by deSousa and Horrocks (1979), and brain stems were dissected from the cerebrum and cerebellum. Nervous tissues from all animals in a particular dosing group were pooled and stored at −80°C prior to subcellular fractionation. Samples of thoracic spinal cord and sciatic nerve (tibial branch) from dosed and control animals were fixed in 10% phosphate-buffered Formalin, pH 7.0, for histopathological analysis.

Isolation of the axonal cytoskeleton. Axonal cytoskeletal preparations were obtained from pooled spinal cords and brain stems using the method of Chiu and Norton (1982), which avoids disassembly or denaturation of neurofilaments during isolation. The proteins isolated from these preparations were stable for several months when stored at −80°C.

Analytical methods. Polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulfate (SDS) was performed as previously described (DeCaprio *et al.*, 1982), except that electrophoresis was carried out at 30 mA per gel, constant current. Staining of PAGE-separated proteins for pyrrole adduct and quantitative assay of the adduct in serum and axonal protein preparations utilized the specific colorimetric reagent *p*-dimethylaminobenzaldehyde (DMAB), with 2,5-dimethylpyrrole (Aldrich, Milwaukee, Wisc.) as the standard (DeCaprio *et al.*, 1982, 1983). For the quantitative assay, 1% SDS was used in place of guanidine · HCl as the solubilizing agent. Protein assay was performed using a commercial system (Bio-Rad Inc., Rockville Centre, N.Y.). Pyrrole concentrations were expressed as nanomoles DER (dimethylpyrrole equivalent residues) per milligram protein. Two-dimensional PAGE was performed as described by O'Farrell (1975) and silver staining of completed gels utilized a commercial reagent system (Bio-Rad Inc.). Assay for total protein lysine was performed using the fluorescent reagent *o*-phthalaldehyde (Goodno *et al.*, 1981). Densitometric quantitation of Coomassie blue-stained gels was performed with a Beckman CDS-200 scanning densitometer.

In vitro studies. Frozen brain stems and spinal cords from 10 adult rats (Pel-Freez Biologicals, Rogers, Ark.) were processed as described above to obtain axonal cytoskeletal pellets. The pellets were homogenized in 100 mM sodium phosphate buffer, pH 7.2, containing 100 mM NaCl, 0.1% NaN_3, and 2 mM *trans*-4-(aminomethyl)-cyclohexanecarboxylic acid (AMCA). Protein concentrations were adjusted to 1 mg/ml in the incubation mixtures by dilution with the same buffer. Appropriate amounts of 2,5-HD were added to aliquots of the homogenate to yield diketone:lysine ϵ-amine molar ratios of 100–1000:1. Control samples were incubated with distilled water in place of 2,5-HD. Samples were incubated at 37°C in capped glass tubes for the desired time period, after which unreacted diketone was removed and delipidation was accomplished by treatment with ethanol: ether as described (Chiu and Norton, 1982). Proteins were then extracted using the same method employed for the *in vivo* studies.

RESULTS

Toxicological Effects of 2,5-HD

Results of clinical and neuropathological assessment of rats receiving 0.5% 2,5-HD in the drinking water are summarized in Table 1. All animals exhibited a net body weight gain during the study although the rate of body weight gain was reduced in 2,5-HD-dosed animals. Rats in the recovery group exhibited a near normal rate of body weight gain after being returned to tap water. The average dose of 2,5-HD in treated animals based on drinking water consumption was 300 to 400 mg/kg/day. Animals receiving 2,5-HD displayed a 17% reduction in drinking water consumption and 12% reduction in feed consumption compared to controls. Untreated animals which were pair-fed and pair-watered with 2,5-HD-treated rats for 2 weeks exhibited a similar decrease in body weight gain.

TABLE 1

NEUROPATHY AND BODY WEIGHT CHANGES IN RATS RECEIVING 0.5% 2,5-HD IN THE DRINKING WATER

Weeks of exposure	Number of animals	Body wt at termination (g)[b]	Clinical neuropathy[c]	Histopathological damage[a]	
				PNS[d]	CNS[e]
Control[f]	12	628 ± 20	None	0	0
1	10	355 ± 4 (385 ± 7)	None	0	0
2	10	362 ± 5 (429 ± 8)	None	0	0
5	10	378 ± 10 (496 ± 12)	Mild	0	0
8	10	377 ± 13 (542 ± 14)	Gross/severe	+	++/+++
Recovery[g]	9[h]	499 ± 22 (628 ± 20)	Gross	0	++/+++

[a] 0, No observable damage; +, scattered axonal swellings; ++, numerous axonal swellings, some degeneration; +++, severe axonal swelling and degeneration.

[b] Mean ± SE. Values in parentheses are $\bar{x}$ ± SE for control group at same time point.

[c] Assessed using scale of Abou-Donia (1978).

[d] Damage assessed in terminal branches of sciatic (tibial) nerve.

[e] Damage assessed in thoracic spinal cord.

[f] Terminated at same time as recovery group (17 weeks).

[g] Received 2,5-HD for 8 weeks followed by 9 weeks recovery.

[h] One animal died after 3 weeks exposure.

A time-dependent increase in clinical signs of neuropathy (ataxia and hindlimb paralysis) was observed in treated animals, progressing to moderate to severe levels after 8 weeks exposure. No evidence of neuropathy was apparent after 1 or 2 weeks exposure, and animals on the recovery regimen displayed only slight clinical improvement. Neuropathological damage was only apparent after 8 weeks exposure to 2,5-HD. Swollen and degenerating axons were numerous in the anterior tracts of the thoracic spinal cord from these animals, while peripheral nerve damage was much less marked. Nine weeks of recovery after 8 weeks of 2,5-HD exposure resulted in reversal of peripheral nerve damage but essentially no change in the severity of spinal cord damage.

Isolation of the CNS Axonal Cytoskeleton

The subcellular fractionation method employed was effective in providing samples enriched in axonal cytoskeletal proteins using a minimum of experimental manipulation. This was an important consideration since it was recognized that pyrrolylated species might differ significantly enough in physicochemical properties from native proteins to result in their loss during a more extensive purification procedure.

Six proteins associated with the axonal cytoskeleton (defined as the slow components a and b of axonal transport (Lasek and Hoffman, 1976; Black and Lasek, 1980; Garner and Lasek, 1981) comprised the majority of material in these preparations: the three NF subunit proteins (210, 160, and 70 kDa), clathrin (180 kDa), tubulin (55 kDa), and actin (43 kDa) (Figs. 1 and 2). The 210-kDa NF subunit protein was sometimes visualized as a doublet in spinal cord preparations (Fig. 1, lane 1). Brain stem appeared to contain relatively less clathrin and more tubulin than did spinal cord (Figs. 1 and 2). A substantial amount of nonneural glial fibrillary acidic protein (GFAP, 51 kDa) was found to copurify with these axonal cytoskeletal preparations. Approximately 35 to 40 minor pro-

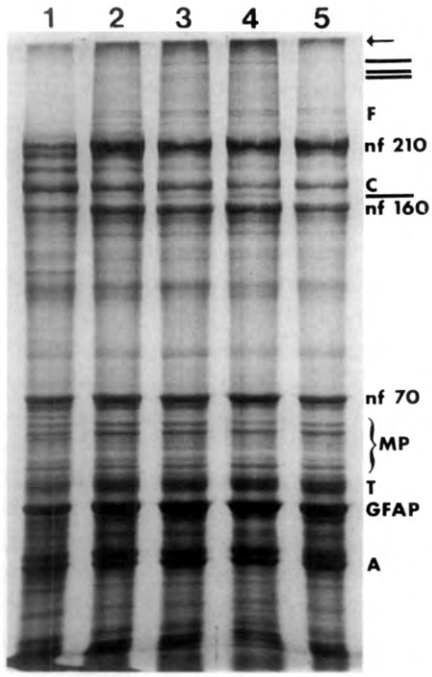

FIG. 1. SDS–PAGE of pooled spinal cord axonal cytoskeletal proteins from 2,5-HD-treated rats. Lanes 1 to 5: Proteins from control, 2, 5, and 8 weeks exposure, and 8 weeks exposure followed by 9 weeks recovery, respectively. Arrow indicates HMW protein which failed to migrate into the gel. Extra discrete protein bands in treated animals are indicated by bars, with apparent molecular weights of 300, 288, 275, and 168 kDa. Note loss of extra bands and reduction in the amount of HMW protein after 9 weeks recovery (lane 5). Identification of major proteins based on published data (Lasek and Hoffman, 1976; Black and Lasek, 1980; Garner and Lasek, 1981): F—fodrin doublet (235/240 kDa); nf—neurofilament subunit proteins (210, 160, and 70 kDa); C—clathrin (180 kDa); MP—microtubule-associated tau proteins (57 to 64 kDa); T—tubulin dimer (55 kDa); GFAP—glial fibrillary acidic protein (51 kDa); and A—actin (45 kDa). Coomassie blue staining of 7.5% acrylamide gel with 5% stacking gel; 100 μg protein each lane.

tein bands were also present, including those presumably corresponding to the fodrin doublet (230/235 kDa) and several microtubule-associated tau proteins (57–64 kDa). Yields of axonal cytoskeletal proteins were approximately 2 mg/g tissue from spinal cord and 6 mg/g tissue from brain stem.

2,5-HD-Induced Changes in CNS Axonal Cytoskeletal Proteins

Several distinct alterations were observed by SDS–PAGE in brain stem and spinal cord axonal protein preparations from animals receiving 2,5-HD (Figs. 1, 2, 3a, b). The first consisted of the appearance of discrete extra protein bands primarily in the region of the gel above 260 kDa. In spinal cord preparations (Fig. 1), these extra protein bands exhibited apparent molecular weights of 168, 275, 288, and 300 kDa, and similar patterns were seen in brain stem preparations (Fig. 2). These molecular weight values should be interpreted with caution, since if the new bands represented polymers of native proteins then an anomalous migration in this SDS–PAGE system may have occurred (Steele and Nielsen, 1978). The new protein bands were visible on heavily loaded gels as early as 1 week following the beginning of exposure to

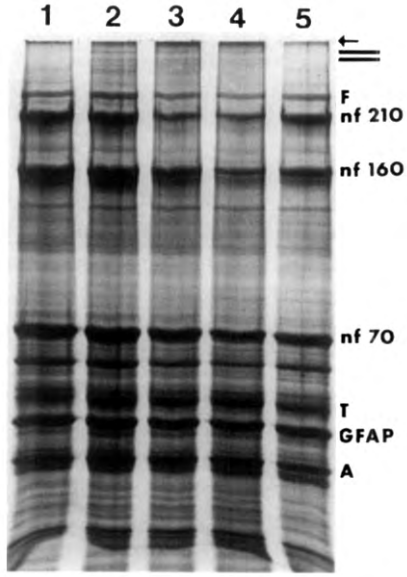

FIG. 2. SDS–PAGE of pooled brain stem axonal cytoskeletal proteins from 2,5-HD-treated rats. Samples and legend are the same as in Fig. 1. In addition to extra protein bands and nonmigrating material note relative reduction in NF-subunit protein concentrations after 5 and 8 weeks of exposure.

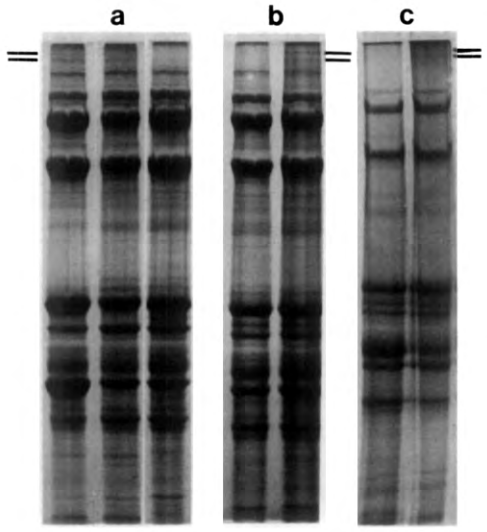

FIG. 3. SDS–PAGE of axonal cytoskeletal proteins. (a) Pooled spinal cord (lane 1) and brain stem (lane 2) proteins from rats receiving 0.5% 2,5-HD in the drinking water for 1 week. Pooled spinal cord protein from control rats (lane 3), 250 μg protein each lane. (b) Pooled spinal cord protein from rats receiving 2,5-HD for 2 weeks (lane 2) and from control rats pair-fed and pair-watered to dosed animals (lane 1), 200 μg protein each lane. (c) Brain stem proteins after *in vitro* incubation for 1 hr (pH 7.2, 37°C). Control proteins (lane 1) and proteins exposed to a 1000-fold molar excess of 2,5-HD to lysine (lane 2), 100 μg protein each lane. Extra discrete protein bands in a, b, and c indicated by bars.

2,5-HD (Fig. 3a). Similar bands could not be detected in axonal protein preparations from rats allowed to recover for 9 weeks after 8 weeks of exposure (Figs. 1 and 2, lane 5) or from control rats pair-fed and watered with dosed animals for 2 weeks (Fig. 3b).

The second type of alteration was the presence of diffusely staining, high-molecular-weight, nonmigrating protein (HMW protein). The concentration of this nonmigrating material appeared to increase progressively as a function of exposure time. This pattern was confirmed by scanning densitometry (Table 2), which demonstrated a twofold increase in brain stem and a threefold increase in spinal cord HMW protein over control values after 8 weeks exposure. Residual HMW protein was still present 9 weeks after cessation of dosing in spinal cord but not brain stem (Table 2). HMW protein was not detected in axonal cytoskeletal preparations from control rats pair-fed and pair-watered with animals receiving 2,5-HD for 2 weeks (Fig. 3b).

The formation of extra protein bands and HMW protein was accompanied by an apparent time-related decrease in the relative concentration of the three NF subunit proteins in brain stem but not spinal cord preparations. The 210-, 160-, and 70-kDa NF subunits were reduced by 44, 35, and 26%, respectively, from control values after 8 weeks exposure (Table 2), although these changes must be interpreted with caution in view of the fact that pooled preparations were used. A return to near control values was seen after cessation of dosing. Relative concentrations of other proteins (e.g., clathrin, tubulin, and GFAP) were unaltered as a result of exposure. The lack of marked changes in spinal cord NF subunit protein concentrations may represent a lack of detection sensitivity rather than a specific tissue difference.

Further evaluation of the high-molecular-weight region was performed using overloaded gels (Fig. 3a). Protein loads of up to 250 μg did not reveal discrete bands above approximately 260 kDa in control axonal cytoskeletal preparations, indicating that the extra bands were not a result of increased synthesis of minor native proteins. The presence of a time-dependent increase in diffuse, high-molecular-weight protein staining density and nonmigrating material was confirmed using 3% acrylamide gels and silver staining (Fig. 4). Clearance of a portion of this protein was apparent after cessation of dosing, although a substantial amount of nonmigrating protein remained. The lack of sharp discrete bands in this PAGE system was most likely due to the relatively low resolving power of the 3% acrylamide gel used.

Staining of PAGE-separated proteins with DMAB was unable to reveal the presence of pyrrole adduct in specific proteins. In addi-

TABLE 2

DENSITOMETRIC QUANTITATION OF BRAIN STEM AND SPINAL CORD AXONAL CYTOSKELETAL PROTEINS FROM RATS RECEIVING 0.5% 2,5-HD IN THE DRINKING WATER[a]

Weeks exposure to 2,5-HD	Protein[b]							
	HMW		NF210		NF160		NF70	
	BS	SC	BS	SC	BS	SC	BS	SC
Control[c]	1.2	2.0	8.6	6.6	9.9	4.7	9.2	7.2
2	1.4	3.6	7.7	7.3	10.1	5.3	9.0	7.1
5	2.7	4.8	5.7	6.0	9.9	4.7	7.8	6.5
8	2.4	6.1	4.7	5.8	6.4	4.7	6.8	7.5
Recovery[d]	1.0	4.3	7.8	6.1	8.9	3.9	8.3	6.2

[a] Obtained by densitometric scanning of Coomassie blue-stained PAGE-separated proteins from pooled brain stem and spinal cord preparations. Values are expressed as percentage of total protein and represent the mean of three determinations for each pooled sample. Standard deviations were ≤0.2 in all cases.

[b] NF subunit proteins abbreviated as in Fig. 1. BS = brain stem, SC = spinal cord.

[c] Sacrificed at same time as recovery group (17 weeks).

[d] Received 2,5-HD for 8 weeks followed by 9 weeks recovery.

tion, two-dimensional PAGE did not demonstrate significant alterations in the native charge of axonal cytoskeletal proteins. This lack of observable effects was attributed (as described later) to insufficient sensitivity of the methods employed in detection of relatively low levels of pyrrole formation (i.e., 1 adduct/molecule protein).

Pyrrole Adduct Formation in 2,5-HD-Treated Animals

Results of the quantitation of pyrrole adduct in serum and axonal cytoskeletal protein preparations are summarized in Table 3. Yields of these proteins were too low to allow the assay of pyrrole adduct in individual animals, thus necessitating the pooling of tissues from all animals in a dosing group prior to subcellular fractionation. Serum pyrrole content appeared to achieve maximum concentrations by 2 weeks exposure, after which little change in relative concentrations was observed. Pyrrole formation in axonal cytoskeletal protein also appeared to reach a plateau, although detailed time-course comparisons could not be made due to the pooled sample method used. Absolute amounts of the adduct were comparable in serum and axonal protein preparations, reflecting a similar distribution of 2,5-HD to neural and nonneural tissue. Cessation of dosing for 9 weeks after 8 weeks exposure resulted in essentially complete loss of detectable pyrrole adduct in serum protein, while residual amounts were present in both brain and spinal cord axonal cytoskeletal proteins.

In Vitro Studies

The experimental protocol for *in vitro* incubation of axonal cytoskeletal preparations with 2,5-HD was designed to promote reassembly of neurofilaments and simulate *in vivo* conditions (i.e., physiological salt concentration, pH, and temperature). The addition of NaN_3 and AMCA allowed incubation to proceed for several days without significant protein degradation.

In vitro incubation of brain stem protein with 2,5-HD at a diketone:lysine ϵ-amine molar ratio of 1000:1 for 1 hr resulted in the

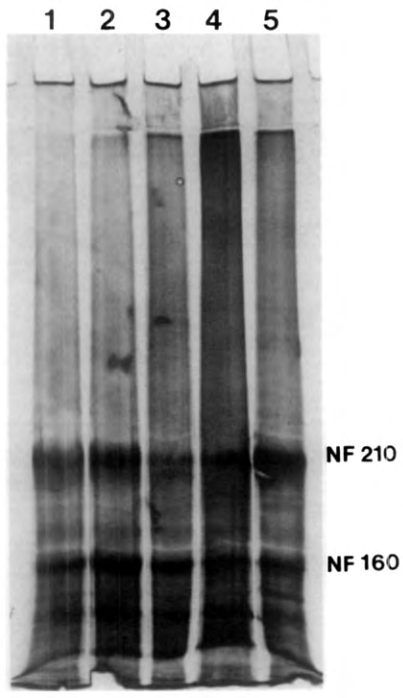

FIG. 4. Silver stain of pooled brain stem axonal cytoskeletal proteins separated on a 3% acrylamide gel, 100 μg protein each lane. Samples and legend are the same as in Fig. 1. An increase in diffusely staining protein is apparent in the region above the NF 210-kDa subunit protein after 5 and 8 weeks diketone exposure (lanes 3 and 4), while a partial return to the control pattern is seen after 9 weeks recovery (lane 5).

formation of extra bands and HMW protein with a pattern identical to that seen after *in vivo* exposure (Fig. 3c). Assay of pyrrole adduct in these samples revealed concentrations approximately 5- to 10-fold higher than those encountered after *in vivo* exposure (Table 4). Continued incubation resulted in a relative increase in the amount of HMW protein and total pyrrole adduct but no change in the pattern of extra protein bands. Incubation without 2,5-HD produced a pattern indistinguishable from protein isolated from control animals.

Estimation of Lysine ϵ-Amine Conversion

Efforts to detect significant differences in total lysine ϵ-amine content between treated and control preparations were unsuccessful due to the relatively low levels of conversion encountered. To obtain some estimate of the average degree of lysine conversion in axonal cytoskeletal preparations, pyrrole adduct concentrations in samples from exposed animals were compared with total lysine concentrations in corresponding control preparations (Table 4). This method assumed a similar molar absorptivity between the 2,5-dimethylpyrrole used as a standard in the colorimetric assay and the ϵ-*N*-(2,5-dimethylpyrrolyl) norleucine adduct formed as a result of lysine conversion. That this was a reasonable assumption is indicated by studies using quantitative amino acid analysis of bovine serum albumin treated *in vitro* with 2,5-HD (DeCaprio *et al.*, 1982; unpublished results).

TABLE 3

PYRROLE ADDUCT LEVELS IN PROTEINS FROM RATS RECEIVING 0.5% 2,5-HD IN THE DRINKING WATER

	Pyrrole adduct levels (nmol DER[a]/mg protein)		
		Axonal cytoskeletal protein[b]	
Weeks exposure to 2,5-HD	Serum protein[c]	Spinal cord	Brain stem
Control[d]	0.04 ± 0.00 (12)	ND[e] (12)	ND (12)
1	1.58 ± 0.10 (10)[f]	2.29 (10)	2.20 (10)
2	3.14 ± 0.20 (9)[f]	3.22 (10)	4.69 (10)
5	3.22 ± 0.27 (10)[f]	4.73 (10)	5.16 (10)
8	2.73 ± 0.15 (9)[f]	—[g]	4.38 (10)
Recovery[h]	0.07 ± 0.00 (9)	0.81 (9)	2.05 (9)

[a] DER = Dimethylpyrrole equivalent residues. Values determined using 2,5-dimethylpyrrole as standard (DeCaprio *et al.*, 1983).

[b] Mean of two determinations on pooled tissues from (*N*) number of animals.

[c] $\bar{x}$ ± SE (*N*).

[d] Sacrificed at same time as recovery group (17 weeks).

[e] No detectable pyrrole adduct.

[f] Significantly different from control, $p \leq 0.001$ by ANOVA and Dunnett's test.

[g] Not determined.

[h] Animals received 2,5-HD for 8 weeks followed by 9 weeks recovery.

TABLE 4

PYRROLE FORMATION AND LYSINE CONVERSION IN AXONAL CYTOSKELETAL PROTEINS EXPOSED *IN VIVO* AND *IN VITRO* TO 2,5-HD

Sample	Pyrrole levels[a] (nmol DER/ mg protein)	Total lysine[b] (nmol/mg protein)	Percentage lysine conversion[c]
In vivo			
Brain stem	4.69 (10)[d]	703.1 (10)[e]	0.7
Spinal cord	3.22 (10)[d]	827.5 (10)[e]	0.4
In vitro			
Spinal cord	35.33[f]	965.4[g]	3.7

[a] DER = Dimethylpyrrole equivalent residues. Values determined using 2,5-dimethylpyrrole as standard. Mean of two determinations on pooled tissues from (*N*) number of animals.

[b] Mean of two determinations on pooled tissues from (*N*) number of animals.

[c] Expressed as (nmol DER/mg protein)/(nmol lysine/ mg protein) × 100.

[d] Animals received 0.5% 2,5-HD in the drinking water for 2 weeks.

[e] Determined from control animals pair-fed and pair-watered to animals receiving 2,5-HD for 2 weeks.

[f] Axonal cytoskeletal protein preparations were obtained from 10 pooled spinal cords and incubated with a 1000: 1 2,5-HD:lysine molar ratio for 4 hr as described under Methods.

[g] Determined from control spinal cord preparations as described under Methods.

The average amount of lysine conversion in axonal cytoskeletal protein preparations from treated animals was <1% after 2 weeks exposure (Table 4). Approximately 4% of lysine ϵ-amine groups were converted to pyrrole adducts during *in vitro* incubation of spinal cord axonal proteins with a 1000-fold molar excess of 2,5-HD to lysine for 4 hr. Total lysine concentrations were similar between *in vivo* and *in vitro* control preparations.

DISCUSSION

An acceptable molecular mechanism of action for the neurotoxic γ-diketones must account for the neuropathological effects, structure–activity relationships, and apparent direct axonal toxicity of these compounds. Although covalent reaction and formation of substituted pyrrole adducts in axonal protein has been suggested as the primary causative factor in this neuropathy (DeCaprio and Weber, 1980; DeCaprio *et al.*, 1982), the events occurring between appearance of the adduct and actual nerve degeneration remain obscure. As discussed below, several mechanisms have been proposed to link these phenomena based upon the potential consequences of pyrrole formation in NF and/or other axonal proteins.

Alkyl pyrrole derivatives are quite susceptible to autoxidation and polymerization (Schofield, 1967; Jones and Bean, 1977), a finding which has been confirmed *in vitro* using various protein preparations (DeCaprio and Weber, 1980, 1981; DeCaprio *et al.*, 1982; Graham *et al.*, 1982, 1984; Anthony *et al.*, 1983). As proposed by Graham *et al.* (1982), autoxidation of pyrrole adducts in NF protein could ultimately result in covalent crosslinking between neurofilaments. This reaction would presumably have an adverse effect upon normal functioning of these cytoskeletal elements which would not be easily reversed.

Alternatively, formation of aromatic pyrrole adducts in NF subunit proteins might result in an increased hydrophobic interaction between these proteins (DeCaprio *et al.*, 1982, 1983). Alterations in the tertiary structure of the NF subunit proteins would also be expected to accompany conversion of cationic lysine ϵ-amine functions into uncharged pyrrole moieties. Such changes might be sufficient to disrupt NF function or transport and cause NF accumulation without (or prior to) actual covalent crosslinking.

As an additional possibility, induction of neuropathy might be due to disruption of the normal relationships between neurofilaments and other cytoskeletal components, particularly microtubules (Griffin *et al.*, 1983). Since NF–MT interactions are believed to be

mediated by specific proteins (Ellisman and Porter, 1980; Leterrier *et al.*, 1982), conversion of lysine moieties on one or more of these proteins into pyrrole adducts might irreversibly alter cytoskeletal organization. This is a particularly exciting possibility in view of the observed rapid segregation of neurofilaments and microtubules after the direct exposure of nerve fibers to 2,5-HD (Griffin *et al.*, 1983; Zagoren *et al.*, 1983). The ultimate result of any one of these schemata would be NF accumulation, reduction of nutrient transport along the axon, and distal axonal degeneration.

The present report describes evidence for both pyrrole adduct formation and covalent crosslinking in axonal cytoskeletal proteins from 2,5-HD-treated rats. These phenomena were observed in brain stem and spinal cord after only 1 week of exposure, indicating the ease with which they can occur *in vivo*. It appears possible that the new discrete protein bands and the HMW protein respectively represent various dimeric and polymeric combinations of NF subunit proteins. Formation of the HMW protein was accompanied by a relative decrease in the amount of native NF subunit proteins in brain stem preparations, suggesting a progressive conversion of monomeric protein with increasing exposure time. This decrease was partially reversed after cessation of exposure. In addition, the HMW protein displayed the strong affinity for silver stain which is characteristic of NF protein (Gambetti *et al.*, 1981). A similar selective vulnerability of NF protein has been reported in rat sciatic nerve exposed *in vitro* to γ-diketones (Graham *et al.*, 1984). It is unlikely that the extra bands represent novel proteins synthesized by the neuron in response to stress induced by decreased feed and water consumption, since pair-fed and pair-watered control animals displayed no similar changes. Characterization of these proteins will ultimately require the use of more specific analytical techniques.

Quantitation of pyrrole adduct revealed that its concentration reached a plateau in serum protein and in brain stem and spinal cord axonal cytoskeletal protein. Absolute serum protein pyrrole concentrations in this study (0.04 to 3.22 nmol/mg) were comparable to those reported for hemolysate protein from rats receiving 1% 2,5-HD in the drinking water (0 to 1.86 nmol/mg) (Anthony *et al.*, 1983). In contrast, significant pyrrole adduct was not detected in "axonal pad" proteins from rats receiving 1% 2,5-HD in the water for 3 weeks (Anthony *et al.*, 1983), compared with demonstrable adduct in axonal cytoskeletal preparations as reported here. This difference may be related to variations in the sensitivities of the pyrrole assays used in these studies. The finding of similar absolute pyrrole adduct concentrations in serum and axonal cytoskeletal protein confirms previous reports of the wide distribution of both diketone (Anthony *et al.*, 1984) and pyrrole adduct (DeCaprio *et al.*, 1983) after 2,5-HD exposure. Thus, the target-organ specificity of this compound cannot be the result of its selective distribution to or uptake by nervous tissue.

An additional important finding in the present investigation is evidence for the partial clearance of adduct and reversal of axonal protein changes after cessation of γ-diketone exposure. The mechanisms behind these phenomena are unclear, although such altered protein could conceivably be removed from axons by glial cell invasion (Spencer and Schaumburg, 1976). In addition, Cavanagh (1982) reported that NF masses that were able to reach the nerve terminal during 2,5-hexanediol intoxication could be cleared by normal NF proteolysis. Such a mechanism might account for a portion of the observed pyrrole adduct clearance in the present study, but it is questionable whether the recovery period (63 days) was long enough to allow for sufficient transport of altered cytoskeletal protein into the nerve terminals for process-

ing. This would be particularly true for axons in which the rate of NF transport had become reduced as a result of γ-diketone exposure (Griffin *et al.*, 1984). Further research is necessary to determine whether an *in situ* process may exist to remove pyrrolylated or crosslinked protein within the axon.

Results from this study indicate that <1% of the total lysine ε-amine functions in axonal cytoskeletal protein were converted to pyrrole adducts as a result of 2,5-HD exposure. If it is assumed that these adducts were equally distributed among the various axonal proteins, then it can be calculated from amino acid composition data (Chiu *et al.*, 1983) that the 70-, 160-, and 210-kDa NF subunit proteins would have contained <0.4, 1.4, and 2.0 adducts/molecule, respectively. Although it is difficult to conceive of significant increases in NF protein hydrophobicity occurring as a result of such low pyrrole adduct concentrations if these estimates are accurate, it is possible that certain axonal proteins are more susceptible to pyrrolylation by 2,5-HD than are others. Such a phenomenon has been demonstrated *in vitro* using purified proteins (DeCaprio *et al.*, 1982). Current studies are examining the degree and specific sites of pyrrolylation of individual axonal proteins after *in vitro* γ-diketone exposure and during 2,5-HD neuropathy.

Based upon our *in vitro* findings which demonstrated an apparent requirement for high diketone concentrations and certain initiating factors, we have previously argued (DeCaprio *et al.*, 1982, 1983) against a proposed mechanism involving NF protein crosslinking (Graham *et al.*, 1982) in this neuropathy. Although it now appears that *in vivo* axonal protein crosslinking does occur in γ-diketone neuropathy, it must be recognized that such covalent crosslinking could be incidental to the actual neurotoxic mechanism. As discussed previously, data from direct-injection studies have demonstrated rapid (within 5 min) reorganization of neurofilaments and microtubules into a pattern similar to that seen after prolonged systemic exposure (Griffin *et al.*, 1983; Zagoren *et al.*, 1983). Significant covalent crosslinking could probably not have occurred during this short time period, suggesting that neurotoxicity might be the result of pyrrole formation itself.

It is possible that conversion of particular lysine amine functions into pyrroles early in the syndrome may produce critical axonal protein alterations which are not clinically manifested until later on. A promising target for such modification is the 210-kDa NF subunit protein, which contains a relatively high proportion of lysine residues in its carboxy-terminal "tail" region (Geisler *et al.*, 1983). This region is believed to participate in NF–MT interaction and may also represent the cross-bridge structures normally seen between neurofilaments (Ellisman and Porter, 1980). Results from the present investigation suggest that the NF 210-kDa subunit protein may be particularly vulnerable to changes induced by 2,5-HD exposure.

Substantial evidence is now available that pyrrole formation in axonal protein is a necessary step in the mechanism of action of the γ-diketones. However, it is not yet possible to determine if it alone is sufficient to induce neuropathy or if secondary oxidation and crosslinking are also required. Anthony *et al.* (1983) reported the enhanced neurotoxic potential of 3,4-dimethyl-2,5-hexanedione (DMHD), a γ-diketone which produced more proximally located axonal swellings than those encountered with 2,5-HD. This derivative exhibited both an increased rate of pyrrole formation and potential for autoxidation and crosslinking as compared to 2,5-HD, suggesting that either process might be critical to neuropathy. Resolution of this question will require the use of γ-diketone derivatives which readily form pyrroles that are less susceptible to autoxidation.

REFERENCES

ABOU-DONIA, M. B. (1978). Role of acid phosphatase in delayed neurotoxicity induced by leptophos in hens. *Biochem. Pharmacol.* **27,** 2055–2058.

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.

ANTHONY, D. C., SZAKALQUIN, G., AND GRAHAM, D. G. (1984). Stable proteins as targets in diketone neuropathy. *J. Neuropathol. Exp. Neurol.* **43,** 305.

BLACK, M. M., AND LASEK, R. J. (1980). Slow components of axonal transport: Two cytoskeletal networks. *J. Cell Biol.* **86,** 616–623.

BRADY, S. T., AND LASEK, R. J. (1981). Nerve-specific enolase and creatine phosphokinase in axonal transport: Soluble proteins and the axoplasmic matrix. *Cell* **23,** 515–523.

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., GOLDMAN, J. E., AND NORTON, W. T. (1983). Biochemistry of neurofilaments. In *Neurofilaments* (C. A. Marotta, ed.), pp. 27–56. Univ. of Minnesota Press, Minneapolis.

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 compositions. *J. Neurochem.* **39,** 1252–1260.

COURI, D., ABDEL-RAHMAN, M. S., AND HETLAND, L. B. (1978). Biotransformation of n-hexane and methyl n-butyl ketone in guinea pigs and mice. *Amer. Ind. Hyg. Assoc. J.* **39,** 295–300.

DECAPRIO, A. P., AND WEBER, P. (1980). *In vitro* studies on the amino group reactivity of a neurotoxic hexacarbon solvent. *Pharmacologist* **22,** 222.

DECAPRIO, A. P., AND WEBER, P. (1981). Conversion of lysine ϵ-amino groups to substituted pyrrole derivatives by 2,5-hexanedione: A possible mechanism of protein binding. *Toxicologist* **1,** 134.

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.

DECAPRIO, A. P., STROMINGER, N. L., AND WEBER, P. (1983). Neurotoxicity and protein binding of 2,5-hexanedione in the hen. *Toxicol. Appl. Pharmacol.* **68,** 297–307.

DESOUSA, B. N., AND HORROCKS, L. P. (1979). Development of rat spinal cord. I. Weight and length with a method for rapid removal. *Dev. Neurosci.* **2,** 115–121.

ELLISMAN, M. H., AND PORTER, K. R. (1980). Microtrabecular structure of the axoplasmic matrix: Visualization of cross-linking structures and their distribution. *J. Cell Biol.* **87,** 464–479.

GAMBETTI, P., AUTILIO-GAMBETTI, L., AND PAPASOZOMENOS, S. C. (1981). Bodian's silver method stains neurofilament polypeptides. *Science (Washington, D.C.)* **213,** 1521–1522.

GARNER, J. A., AND LASEK, R. J. (1981). Clathrin is axonally transported as part of slow component b: The microfilament complex. *J. Cell Biol.* **88,** 172–178.

GEISLER, N., KAUFMANN, E., FISCHER, S., PLESSMAN, U., AND WEBER, K. (1983). Neurofilament architecture combines structural principles of intermediate filaments with carboxy-terminal extensions increasing in size between triplet proteins. *EMBO J.* **2,** 1295–1302.

GILLIES, P. J., NORTON, R. M., AND BUS, J. S. (1980). Effect of 2,5-hexanedione on lipid biosynthesis in sciatic nerve and brain of the rat. *Toxicol. Appl. Pharmacol.* **54,** 210–216.

GOODNO, C. C., SWAISGOOD, H. E., AND CATIGNANI, G. L. (1981). A fluorometric assay for available lysine in proteins. *Anal. Biochem.* **115,** 203–211.

GRAFSTEIN, B., AND FORMAN, D. S. (1980). Intracellular transport in neurons. *Physiol. Rev.* **60,** 1167–1283.

GRAHAM, D. G. (1980). Hexane neuropathy: A proposal for the pathogenesis of a hazard of occupational exposure and inhalant abuse. *Chem.-Biol. Interact.* **32,** 339–346.

GRAHAM, D. G., ANTHONY, D. C., BOEKELHEIDE, K., MASCHMANN, N. A., RICHARDS, R. G., WOLFRAM, J. W., AND SHAW, B. R. (1982). Studies of the molecular pathogenesis of hexane neuropathy. II. Evidence that pyrrole derivatization of lysyl residues leads to protein crosslinking. *Toxicol. Appl. Pharmacol.* **64,** 415–422.

GRAHAM, D. G., SZAKALQUIN, 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., FAHNESTOCK, K. E., PRICE, D. L., AND CORK, L. C. (1983). Cytoskeletal disorganization induced by local application of β,β'-iminodiproprionitrile and 2,5-hexanedione. *Ann. Neurol.* **14,** 55–61.

GRIFFIN, J. W., ANTHONY, D. C., FAHNESTOCK, K. E., HOFFMAN, P. N., AND GRAHAM, D. G. (1984). 3,4-Dimethyl-2,5-hexanedione impairs the axonal transport of neurofilament proteins. *J. Neurosci.* **4,** 1516–1526.

JONES, R. A., AND BEAN, G. P. (1977). *The Chemistry of Pyrroles.* Academic Press, New York.

LASEK, R. J. (1982). Translocation of the neuronal cytoskeleton and axonal locomotion. *Philos. Trans. R. Soc. London Ser. B* **299,** 313–327.

LASEK, R. J., AND HOFFMAN, P. N. (1976). The neuronal cytoskeleton, axonal transport and axonal growth. In *Cell Motility* (R. Golden, T. Pollard, and J. Rosenbaum, eds.), Vol. 3, pp. 1021–1049. Cold Spring Harbor Conf. Cell Proliferation. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

LETERRIER, J. F., LIEM, R. K. H., AND SHELANSKI, M. L. (1982). Interactions between neurofilaments and microtubule-associated proteins: A possible mechanism for intraorganellar bridging. *J. Cell Biol.* **95,** 982–986.

METUZALS, J., MONTPETIT, V., AND CLAPIN, D. F. (1981). Organization of the neurofilamentous network. *Cell. Tissue Res.* **214,** 455–482.

O'FARRELL, P. A. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250,** 4007–4021.

PERBELLINI, L., BRUGNONE, F., PASTORELLO, G., AND GRIGOLINI, L. (1979). Urinary excretion of n-hexane metabolities in rats and humans. *Int. Arch. Occup. Environ. Health* **42,** 349–354.

POLITIS, M. J., PELLIGRINO, R. G., AND SPENCER, P. S. (1980). Ultrastructural studies of the dying-back process. V. Axonal neurofilaments accumulate at sites of 2,5-hexanedione application: Evidence for nerve fiber dysfunction in experimental hexacarbon neuropathy. *J. Neurocytol.* **9,** 505–516.

SCHLAEPFER, W. W. (1979). Nature of mammalian neurofilaments and their breakdown by calcium. in *Progress in Neuropathology,* (H. Zimmerman, ed.). Vol. 4, pp. 101–123. Grune & Stratton, New York.

SCHOFIELD, K. (1967). *Heteroaromatic Nitrogen Compounds: Pyrroles and Pyridines.* Plenum, New York.

SPENCER, P. S., AND SCHAUMBURG, H. H. (1975). Nervous system dying-back disease produced by 2,5-hexanedione. *Trans. Amer. Neurol. Assoc.* **100,** 148–151.

SPENCER, P. S., AND SCHAUMBURG, H. H. (1976). Central–peripheral distal axonopathy—The pathology of dying-back polyneuropathies. In: *Progress in Neuropathology* (H. Zimmermann, ed.). Vol. 3, pp. 253–295. Grune & Stratton, New York.

SPENCER, P. S., AND SCHAUMBURG, H. H. (1977). Ultrastructural studies of the dying-back process. III. The evolution of experimental peripheral giant axonal degeneration. *J. Neuropathol. Exp. Neurol.* **36,** 276–299.

STEELE, J. C. H., AND NIELSEN, T. B. (1978). Evaluation of cross-linked polypeptides in SDS–gel electrophoresis. *Anal. Biochem.* **84,** 218–224.

VERONESI, B., PETERSON, E. R., BORNSTEIN, M. B., AND SPENCER, P. S. (1983). Ultrastructural studies of the dying-back process. VI. Examination of nerve fibers undergoing giant axonal degeneration in organotypic culture. *J. Neuropathol. Exp. Neurol.* **42,** 153–165.

ZAGOREN, J. C., POLITIS, M. J., AND SPENCER, P. S. (1983). Rapid reorganization of the axonal cytoskeleton induced by a gamma-diketone. *Brain Res.* **270,** 162–164.