

AN ABSTRACT OF THE THESIS OF

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Ian J. Tinsley _____

Hydrocarbon solvents are widely used in the production of paints, adhesives, dyes, polymers, plastics, textiles, printing inks, agricultural products and pharmaceuticals. While the neuropathic potential of aliphatic solvents was shown in the 1970s, little is known about the neuropathic potential of aromatic solvents.

The present study examines such solvents, 1,2-diethylbenzene (DEB) and its metabolite 1,2-diacetylbenzene (DAB), to determine (a) the neuropathological evidence for peripheral neuropathy in rodents treated with 1,2-DAB, (b) the neurochemical basis for the neurotoxic properties of this compound, and (c) the structural requirements for nerve fiber damage. The properties of 1,2-DAB and 2,5-hexanedione (HD) are also compared.

A key finding of this thesis is that 1,2-DAB induces a 2,5-HD-like pattern of nerve damage of motor and sensory axons with focal swellings containing neurofilaments. Whereas nerve damage begins distally in 2,5-HD intoxication, with 1,2-DAB treatment axonal swellings begin intraspinally and in the proximal ventral roots of motor nerve fibers.

A second key finding is the reactivity of 1,2-DAB with amino acids, notably lysine, a property that is shared with 2,5-HD. 1,2-DAB and 2,5-HD react with amino acids and proteins to form blue and yellow chromophores, respectively.

Relative to 2,5-HD, 1,2-DAB is three orders of magnitude more reactive in forming high-molecular-weight species.

1,2-DAB treatment of spinal cord slices *in vitro* and intact sciatic nerve *in vivo* showed that neurofilament proteins react more readily than beta-tubulin. The heavy and medium subunits of neurofilament protein were more reactive than the light subunit. The reactivity of these four axonal proteins was in proportion to their lysine content. These data are consistent with selective accumulation of neurofilaments in giant axonal swellings.

In summary, these studies have shown a relationship between the chromogenic and neuropathic properties of two gamma-diketones, one aliphatic (2,5-HD) the other aromatic (1,2-DAB). These studies are relevant to occupational and public health for at least two reasons. First, urinary chromogens generated by neuropathic aliphatic and aromatic hydrocarbons could serve as biological markers of exposure to solvents with neuropathic potential, and second, other chromogenic solvents (such as tetralin) should be considered for neuropathic potential.

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Molecular and Cellular Mechanisms of Aromatic Hydrocarbon Axonopathy.

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Table of Contents

<u>Chapter</u>	<u>Page</u>
1. General Introduction	1
Organic Solvent Neurotoxicity.....	1
Aliphatic Hydrocarbons.....	2
Aromatic Hydrocarbons.....	5
Peripheral Neuropathy.....	9
Axonopathy.....	10
Mechanisms of Aromatic and Aliphatic γ -Diketone Axonopathy.....	11
Hypothesis and Medical Significance.....	13
2. 1,2-Diacetylbenzene, the Neurotoxic Metabolite of a Chromogenic Aromatic Solvent, Induces Proximal Axonopathy.....	15
Abstract.....	16
Introduction.....	17
Materials and Methods.....	19
Animals.....	19
Chemicals.....	19
Animal treatment.....	20
Dosing regimens.....	21
Results.....	22
Animal Observations.....	22
Neuropathology.....	25
Discussion.....	32
Acknowledgements.....	39

Table of Contents (Continued)

<u>Chapter</u>	<u>Page</u>
3. Amino Acid and Protein Targets of 1,2-Diacetylbenzene, a Potent Aromatic γ -Diketone that Induces Proximal Neurofilamentous Axonopathy.....	40
Abstract.....	41
Introduction.....	42
Materials and Methods.....	44
Chemicals.....	44
Animals.....	44
Amino acid and protein chromogenicity.....	45
Protein reactivity and polymer formation.....	45
GAPDH and LDH reactivity.....	46
Neural protein reactivity.....	46
Spinal cord protein reactivity <i>in vitro</i>	47
Sciatic nerve protein reactivity <i>in vivo</i>	47
SDS-PAGE and Western blotting.....	48
Enzyme assays.....	49
Results.....	51
Amino acid reactivity.....	51
Protein reactivity.....	52
GAPDH and LDH activity.....	54
Neural protein reactivity.....	56
Spinal cord protein reactivity <i>in vitro</i>	59
Sciatic nerve protein reactivity <i>in vivo</i>	62
Discussion.....	64
Amino acid and protein reactivity.....	64
Mechanisms of neurotoxicity.....	67
Acknowledgements.....	72

Table of Contents (Continued)

<u>Chapter</u>	<u>Page</u>
4. Conclusion.....	73
Bibliography.....	77

List of Figures

<u>Figure</u>	<u>Page</u>
1.1 Biotransformation of <i>n</i> -hexane and MBK to 2,5-HD in mammalian systems.....	3
1.2 Formula of acetyl ethyl tetramethyl tetralin (AETT) and keto-AETT, the chromogenic putative metabolite of AETT.....	7
1.3 Formula of 1,2-diethylbenzene (DEB) and 1,2-diacetylbenzene, the chromogenic putative metabolite of 1,2-DEB.....	8
2.1 Rats treated with 10 mg/kg/d for ~25 days with 1,2-DAB (A) or saline (B).....	23
2.2 Brains of rats treated for 30 days with saline (A), 1,3-DAB (B), or 1,2-DAB (C).....	24
2.3 Cross sections of lumbar spinal cord (A,C,D) and adjacent ventral spinal root (B).....	26
2.4 Cross sections of lumbar spinal cord	27
2.5 (A) Light micrograph showing a massive focal swelling of the proximal axon segment of a lumbar anterior horn cell. (B) Transmission electron micrograph of a thinly myelinated intraspinal motor axon filled with neurofilaments	28
2.6 Light micrographs of cross sections of lumbar spinal cord and adjacent ventral spinal root.....	29
2.7 Light micrographs of cross sections of tibial nerve branches to the calf musculature.....	30
2.8 Electron micrographs of cross sections of lumbar dorsal roots of rats treated with 10 mg/kg/d 1,2-DAB 5 days/week for 6 weeks.....	31

List of Figures (Continued)

<u>Figure</u>	<u>Page</u>
3.1 <i>In-vitro</i> effect of diacetylbenzene isomers on test proteins bovine serum albumin and ribonuclease analyzed by SDS-PAGE.....	53
3.2 Effect of diacetylbenzene isomers on the protein state and enzyme activity of glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase after treatment <i>in vitro</i> with DAB isomers.....	55
3.3 <i>In-vitro</i> effect of test agents on purified neurofilament triplet proteins and β -tubulin.....	57
3.4 Western blots of rat spinal cord slices incubated with different concentrations of 1,2-DAB, 1,3-DAB or buffer (control).....	60
3.5 Western blots of rat spinal cord slices incubated for varying periods of time with 1,2-DAB, 1,3-DAB or buffer (control).....	61
3.6 Effect of DAB isomers or vehicle (phosphate-buffered saline) on intact rat sciatic nerve bathed <i>in vivo</i> for 5 min at RT.....	62
3.7 Structure and hypothetical reactions of 2,5-HD and 1,2-DAB with amino acids and proteins, forming pyrroles and isoindoles, respectively.....	69
4.1 Sequence of evolution of CNS and PNS nerve fiber pathology.....	75

List of Tables

<u>Table</u>		<u>Page</u>
1.1	List of aromatic hydrocarbons reported to produce blue-colored urine.....	6
3.1	Chromogenic reactivity of selected amino acids with diacetylbenzene isomers.....	52

List of Acronyms

- AETT: acetyl ethyl tetramethyl tetralin
ALS: amyotrophic lateral sclerosis
BSA: bovine serum albumin
CNS: central nervous system
DAB: diacetylbenzene
DEB: diethylbenzene
DMHD: dimethyl hexanedione
GABA: gamma amino butyric acid
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
GSH: glutathione
HD: hexanedione
HRP: horseradish peroxidase
IDPN: β,β' -iminodipropionitrile
MAP: microtubule-associated proteins
MBK: methyl *n*-butyl ketone
NADH: nicotinamide adenine dinucleotide
NF: neurofilament
Nin: ninhydrin
NIOSH: National Institute of Occupational Safety and Health
NMDA: *N*-methyl-*D*-aspartate
PBS-T: phosphate-buffered saline-tween
PEL: Permissible Exposure Limit
PNS: peripheral nervous system
PVDF: polyvinylidene fluoride
RN: ribonuclease
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCE: trichloroethylene
TLV: Threshold Limit Value
TU: tubulin

Molecular and Cellular Mechanisms of Aromatic Hydrocarbon Axonopathy

Chapter 1

General Introduction

Organic solvents are used widely in industry and in everyday life; they are found in everything from paints to cosmetics to drugs to gasoline. Many organic solvents have been reported to be carcinogenic and hazardous for the reproductive system. They also pose a particular threat to the nervous system, which controls vital bodily functions and possesses a low regeneration capacity. Various solvent chemicals and mixtures are reported as chronic neurotoxicants (Spencer and Schaumburg, 1985).

This dissertation examines 1,2-diacetylbenzene (DAB), the putative metabolite of the chromogenic, neurotoxic organic solvent, 1,2-diethylbenzene (DEB). The various studies seek to determine: (a) the neuropathological evidence for peripheral neuropathy in rodents treated with 1,2-DAB, (b) the neurochemical basis for the neurotoxic properties of this compound, and (c) the structural requirements for nerve fiber damage. The properties of the neurotoxic compounds 1,2-DAB and 2,5-hexanedione (HD) are also compared. My hypothesis is that 1,2-DAB affects axons by mechanisms similar to that of aliphatic γ -diketone 2,5-HD, and that this structural similarity provides the link between chromogenicity and neurotoxicity.

ORGANIC SOLVENT NEUROTOXICITY

Organic solvents are used for extracting, dissolving, or suspending materials such as fats, waxes, and resins that are not soluble in water. The National Institute for Occupational Safety and Health (NIOSH) estimates that 9.8 million workers are potentially exposed to organic solvents used in products such as paints, adhesives,

glues, coatings, and degreasing/cleaning agents, as well as in the production of dyes, polymers, plastics, textiles, printing inks, agricultural products, and pharmaceuticals (NIOSH, 1987). Over 1.3 million civilian and military personnel are occupationally exposed to hydrocarbon fuels such as gasoline, jet fuel, diesel fuel, or kerosene (Ritchie et al., 2001). Since organic solvent (chemicals are usually) are mixed together, it is difficult to distinguish the precise mechanism of action of an individual agent. Moreover, it is unknown whether additive or synergistic interactions may result in unpredicted neurotoxicity.

Organic solvents are broadly classified as aliphatic hydrocarbons, halogenated hydrocarbons, and aromatic hydrocarbons. An aromatic compound is an organic compound that contains one or more benzene or equivalent heterocyclic rings. An aliphatic hydrocarbon is any nonaromatic hydrocarbon compound in which the constituent carbon atoms form open chains. Some organic solvents are known carcinogens (benzene, carbon tetrachloride, trichloroethylene, and 1,1,2,2-tetrachloroethane); others are reproductive hazards (2-methoxyethanol, 2-ethoxyethanol, and methyl chloride), or have neurotoxic properties (*n*-hexane, xylene, and tetralin) (NIOSH, 1987).

ALIPHATIC HYDROCARBONS

Aliphatic hydrocarbons and their metabolites are known to pose threats to human health. Motor and sensory neuropathy is induced in humans and animals by repeated exposure to *n*-hexane, methyl *n*-butyl ketone (MBK), which acts via their neurotoxic metabolite, 2,5-HD (Fig. 1.1).

One common aliphatic hydrocarbon solvent is *n*-hexane, once widely used in industrial and commercial processes, including rubber, adhesive, ink, and paint manufacturing, and in the extraction of vegetable oils for human consumption (IPCS, 1991). The first case of peripheral neuropathy following prolonged

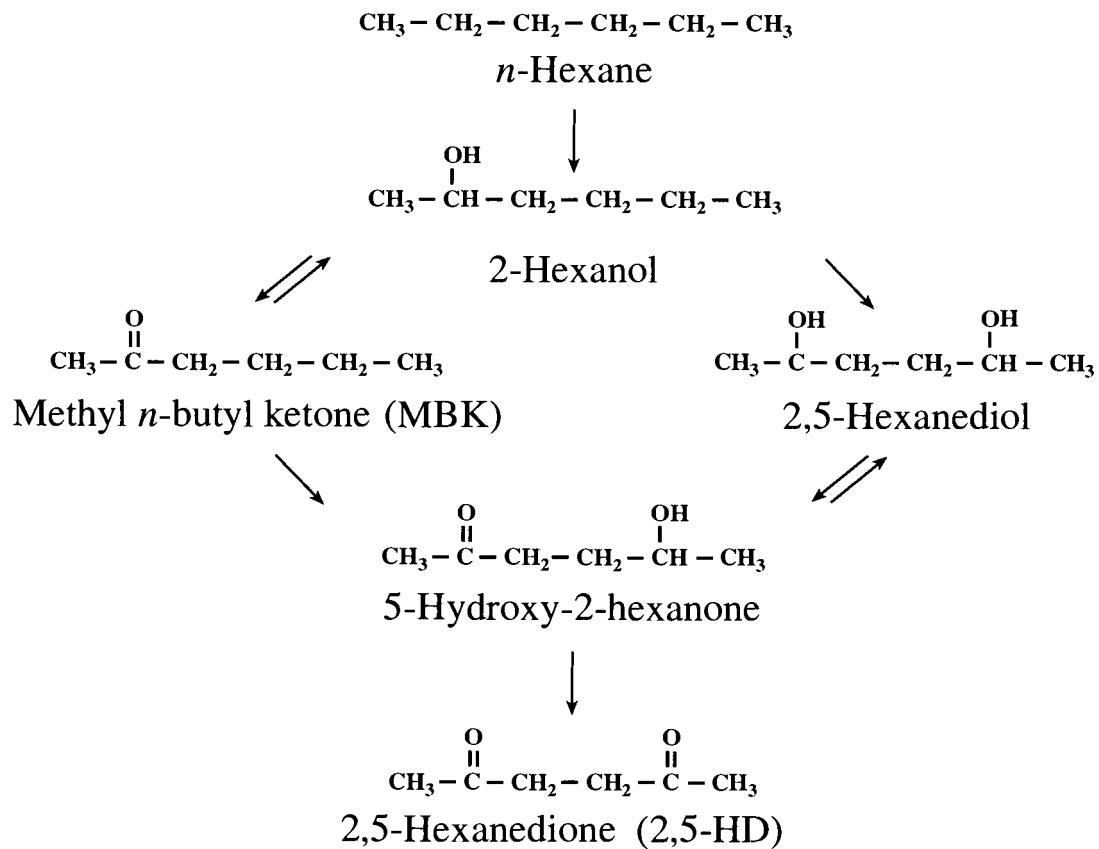


Figure 1.1. Biotransformation of *n*-hexane and MBK to 2,5-HD in mammalian systems.

n-hexane exposure happened in poorly ventilated polyethylene laminating operations (Takeuchi, 1993). Workers presented with an insidious progression of a number of neuropathic signs and symptoms, including acral paresthesia, weakness in the lower limbs, foot-drop, and moderately to severely compromised tendon reflexes. These motor and sensory deficits are symmetrical and usually limited to the feet and hands (Spencer and Schaumburg, 1977b).

The American Conference of Governmental Industrial Hygienists Threshold Limit Value (TLV) for *n*-hexane in occupational exposure is 50 ppm (180mg/m³) in

air, while the U.S. Occupational Safety and Health Administration Permissible Exposure Limit (PEL) is 500ppm (DeCaprio, 2000).

Another aliphatic hydrocarbon, MBK, a neurotoxic metabolite of *n*-hexane, is rarely used today in pure form. However, MBK is still present in some ketone-based solvent mixtures. Additionally, it occurs as a by-product of wood pulping and various petroleum processes; it is also a naturally occurring component of certain foods (Arlien-Soborg, 1992). Human neuropathy due to occupational MBK exposure was first encountered in 1973 at a facility that printed vinyl-coated fabric in Ohio (Billmaier et al., 1974). Eighty-six workers out of 1157 tested subjects in the plant were diagnosed with a toxic peripheral neuropathy. The TLV for MBK is 5ppm (20mg/m³) in air, with a PEL of 100ppm. The U.S. National Institute for Occupational Safety and Health has recommended lowering the TLV for MBK to 1ppm (DeCaprio, 2000).

The neurotoxicity of *n*-hexane and its metabolites has been studied in a number of animal species (Arlien-Soborg, 1992; IPCS, 1991; O'Donoghue, 1985; Spencer et al., 1980b). Exposure to high levels of both *n*-hexane and MBK by inhalation results in typical signs of solvent overexposure, including respiratory depression, narcosis, convulsions, coma, and death (Spencer et al., 1980b). Lower and subchronic exposure induces distal axonopathy in both CNS and PNS nerve fibers. Exposure to a solvent mixture containing *n*-hexane resulted in neurological dysfunction confined to a sensorimotor or motor peripheral neuropathy (Altenkirch et al., 1982). Rats exposed to 1300 ppm MBK exhibited hindlimb foot drop after 3-month exposure, while *n*-hexane-exposed animals displayed an unsteady gait after 6-week exposure, and bilateral hindlimb neuromuscular weakness and paralysis thereafter (De Jesus et al., 1977; Okamoto et al., 1977).

2,5-HD, the γ -diketone metabolite of the aliphatic solvents *n*-hexane and MBK, is responsible for the induction of axonal degeneration. Rats or cats treated with *n*-hexane or 2,5-HD revealed a characteristic pattern of scattered, focal paranodal or internodal axonal swelling, a corresponding thinning of myelin, and axonal degeneration in peripheral nerves. Electron microscopic studies

demonstrated that the axonal swellings consisted of densely packed masses of 10nm neurofilaments with segregation of other axonal organelles (mitochondria, microtubules) into a discrete central zone (Spencer and Schaumburg, 1977a).

Neuropathological examination revealed that the most susceptible sites to *n*-hexane, MBK, 2,5-HD within the CNS appeared to be the distal regions of large myelinated fibers within the dorsal spinocerebellar, gracile, cuneate, ventrolateral, and ventromedial tracts, while the tibial nerve branches to the calf muscle were affected earliest in the PNS (Spencer and Schaumburg, 1977a,b).

Electrophysiological and behavioral deficits are present in experimental animals exposed to *n*-hexane or its metabolite. Monkeys and rats exposed chronically to 100ppm MBK (6h/day, 5 days/week, 10 months) exhibited reduced nerve conduction velocity in sciatic-tibial nerves and decreased amplitude of evoked muscle action potentials (Johnson et al., 1977).

AROMATIC HYDROCARBONS

Several widely used aromatic hydrocarbon solvents reportedly induce blue-green discoloration of tissues and urine in animals (Table 1.1) and humans (Gerarde, 1960; Spencer and Schaumburg, 1980).

Benzene is used extensively as a solvent in the chemical and drug industries, as both a starting and intermediate material in the synthesis of numerous chemicals, and as a constituent of motor fuels. High-level exposure causes CNS depression; chronic, low-level exposure depresses the hematopoietic system and is associated with leukemia (Schaumburg, 2000).

Toluene is used as a solvent for synthetic rubber, paint, and lacquers, as well as a constituent of motor fuels. Chronic inhalation abuse of pure toluene produces irreversible cerebellar, brainstem, and pyramidal-tract dysfunction (Boor and Hurtig, 1977; Grabski, 1961; King et al., 1981; Lazar et al., 1983). Ototoxicity is found in experimental animals exposed to toluene or xylene (McWilliams et al.,

Table 1.1. List of aromatic hydrocarbons reported to produce blue-colored urine

MONOCYCLIC	DICYCLIC
Benzene	Indane
1,2-Xylene	Indene
1,2-Ethyltoluene	Tetralin
1,2-Diethylbenzene	Diphenyl
1,3-Diethylbenzene	Diphenylmethane
1,2-Diisopropylbenzene	1-Methylnaphthalene
Triethylbenzene (mixture)	2-Methylnaphthalene
Diethyldiisopropylbenzene	1-Ethylnaphthalene
	2-Ethylnaphthalene

Data generated from studies of albino rats treated with a single subcutaneous injection of the listed substance (purity not stated) in a dose of *ca.* 5 ml/kg (Gerarde, 1959, 1960). With regard to the diethylbenzene isomers, treatment of male and female rats and hamsters with 1,2-diethylbenzene (1,2-DEB) and 1,3-diethylbenzene (1,3-DEB) caused the excretion of a blue dye in the urine. The sclera, blood, plasma, and tissues were stained a deep blue that persisted for many days. The rabbit did not excrete blue-colored urine but the sclera was stained blue after administration of large doses of 1,2-DEB or 1,3-DEB. The guinea pig did not excrete blue urinary metabolites with these agents. 1,4-DEB was not chromogenic in the rat, hamster, guinea pig, or rabbit (Gerarde, 1960). Later studies using purified compounds found that 1,2-DEB (95%), but neither 1,3-DEB (99%) nor 1,4-DEB (96%), was chromogenic in Sprague-Dawley rats given repeated oral doses of 500 and 750 mg/kg/d, 5 days/week for 10 weeks (Gagnaire et al., 1990).

2000; Pryor and Rebert, 1984). Toluene is a teratogen in humans and experimental animals. In rat studies, high levels of toluene impaired fetal growth and produced a variety of fetal malformations (Pearson et al., 1994).

Tetralin (tetrahydronaphthalene) shows chromogenic and acute neurotoxic properties in animals and humans. Babies, children, and adults are reported to have developed acute neurological disturbances (headache, asthenia, restlessness, stupor) in association with the excretion of green-colored urine after exposure to freshly applied varnishes and waxes containing tetralin or derivatives thereof (Browning, 1965; Longacre, 1987). A woman excreted green-gray urine within approximately

one day of ingesting approximately 250ml of an ectoparasiticide containing 31.5% tetralin (Drayer and Reidenburg, 1979). Tetralin produced hindlimb weakness in guinea pigs treated orally, percutaneously or by inhalation (Smyth et al., 1951).

Musk tetralin (8-acetyl-7-ethyl-1,1,4-tetramethyl-1,2,3,4-tetralin), a white compound once used worldwide in fragrances and as a food additive, also causes blue discoloration of tissue (including the brain), green-colored urine, hyperactivity followed by hindlimb weakness, a progressive neuronal ceroid degeneration, and

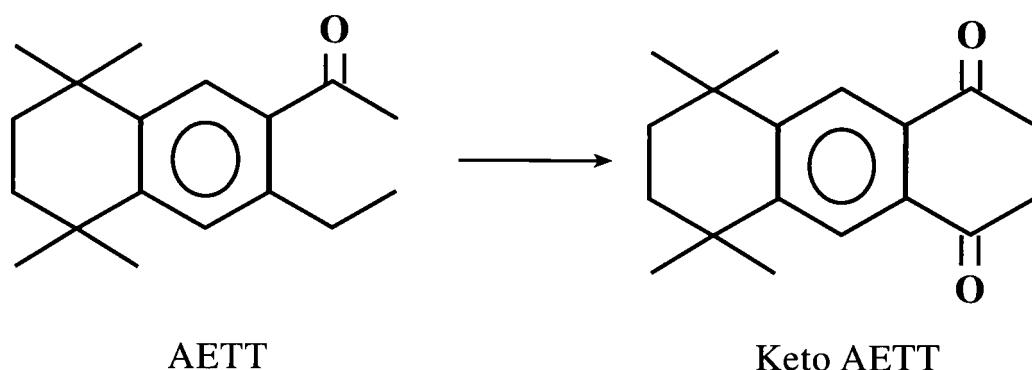


Figure 1.2. Formula of acetyl ethyl tetramethyl tetralin (AETT) and keto-AETT, the chromogenic putative metabolite of AETT.

central and peripheral neurodegeneration (Spencer et al., 1979a). Since methyl substitution of the 7-ethyl group blocked the chromogenic and neurotoxic properties, AETT-induced neurotoxicity may be linked to chromogenicity (Spencer, 1982). The metabolite of AETT (“keto AETT”) is a chromogenic compound that forms a blue color on contact with amino acids, peptides and proteins.

This observation raised the possibility that other chromogenic aromatic hydrocarbons, such as 1,2-DEB, may have neurotoxic properties comparable to those of musk tetralin (<http://www.ohsu.edu/croet/peterbook/pdf/Ch.20.pdf>, Spencer et al., 1980a).

The two compounds addressed in the present are diethylbenzene (DEB) and diacetylbenzene (DAB). DEB is a minor component of aromatic solvents that are widely used in industry and serves as an intermediate for the production of divinylbenzene (Sandmeyer, 1981). It is a colorless, flammable liquid that exists as three isomers: 1,2-(ortho), 1,3-(meta), and 1,4-(para)-DEB. 1,2-DAB is a putative neurotoxic metabolite of 1,2-DEB. By intraperitoneal injection, 1,2-DAB is several-fold more toxic (body weight change) and neurotoxic (peripheral neuropathy) than 1,2-DEB (Gagnaire et al., 1991, 1992a).

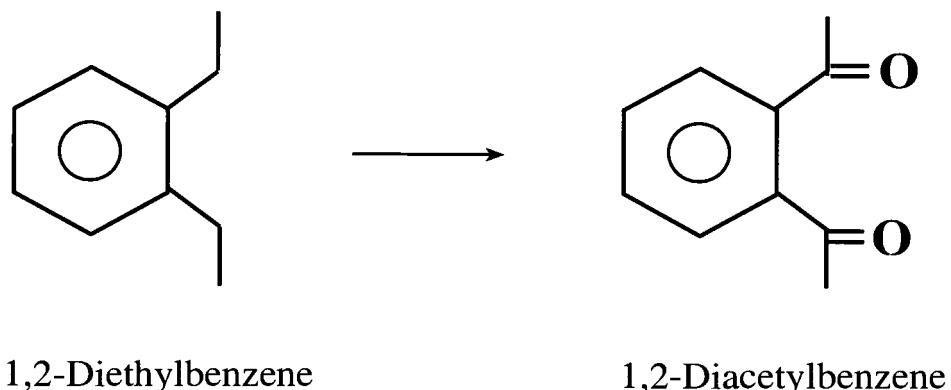


Figure. 1.3. Formula of 1,2-diethylbenzene (DEB) and 1,2-diacetylbenzene, the chromogenic putative metabolite of 1,2-DEB.

Rats treated with 1,2-DEB or 1,2-DAB excrete blue-green urine and develop electrophysiological changes consistent with peripheral neuropathy. By contrast, neither 1,3-DEB nor 1,4-DEB are chromogenic or neurotoxic (Gagnaire et al., 1990). The chromogenic and neurotoxic properties of 1,2-DEB appear to arise from the proposed active metabolite 1,2-DAB (Gagnaire et al., 1991; Payan et al., 1999). In addition, rats treated with 1,2,4-triethylbenzene, but not 1,3,5-triethylbenzene, showed bluish skin with greyish-green urine and decreased motor and sensory conduction velocities. Taken together, these data suggest that two ethyl residues in the ortho position on an aromatic ring (γ -diketone structure) are required

for chromogenicity and neurotoxicity (Gagnaire et al., 1993). While 1,2-DEB and 1,2,4-triethylbenzene cause electrophysiological changes consistent with sensorimotor neuropathy in rodents, but underlying mechanisms and pathogenesis are unclear.

Chromogenicity may thus be a clue to neurotoxicity. Widely used neurotoxic aromatic hydrocarbon solvents (tetraline or tetralin derivatives) reportedly induce blue-green discoloration of tissue and urine in animals and humans. Several reports document the relationship between human exposure to aromatic solvents and the excretion of green-colored urine (Pohl and Rawicz, 1919; Rockemann, 1922; Koelsch, 1926; Lehman and Flury, 1943; Badinand et al., 1947; Browning, 1953; Drayer and Reidenberg, 1979; Grant et al., 1985; Longacre, 1987). The chromophore has been proposed to result from a ninhydrin-like reaction with amino groups in proteins (Spencer et al., 1980a). Systemic treatment of rodents with 2,5-HD results in an orange-reddish discoloration of hair that contains pyrrole substituents (Johnson et al., 1995).

PERIPHERAL NEUROPATHY

Peripheral neuropathy is a disorder of nerves associated with inflammation, decreased blood flow, trauma or exposure to toxic substances (e.g. 2,5-HD or 1,2-DAB). Degeneration of the axon blocks conduction at the point of the degeneration. Demyelination (loss of the myelin sheath that surrounds the axon) greatly decreases the speed of impulse conduction along the nerve.

Damage to sensory nerve fibers results in changes in sensation ranging from pain, decreased sensation, lack of sensation, perception of abnormal sensations, or an inability to determine joint position sense in the area. Sensation changes usually begin in the feet or hands and progress toward the center of the body.

Damage to motor nerve fibers causes muscle weakness, lack of muscle control, difficulty or inability to move a part of the body (paralysis), loss of muscle

function or feeling, muscle atrophy, and muscle twitching (fasciculation)(Kandel et al., 2000).

After prolonged exposure to relatively high levels of certain aliphatic solvents (e.g. *n*-hexane, MBK), humans and animals develop a dose-dependent distal, retrograde degeneration of long and large axons in the peripheral nerve and spinal cord (central-peripheral distal axonopathy). Neurological symptoms initially develop in the extremities (Spencer and Schaumburg, 1975; DeCaprio, 2000).

Rats treated with 1,2-DEB or 1,2-DAB developed blue discoloration of tissue followed by the appearance of electrophysiological deficits (decreased motor and sensory conduction velocities of peripheral nerve fibers in the rat tail) and altered brainstem auditory evoked potentials (Gagnaire et al., 1990, 1991, 1992a,b).

AXONOPATHY

The neuron is the functional unit of the nervous system. It consists of three parts: the cell body, called the soma, and the dendrites and axon. Cytoskeleton proteins in the axon such as microtubules, neurofilaments (NFs), and microfilaments, are responsible for structural integrity and axonal transport. They move anterograde from their site of synthesis in the nerve cell body along the axon to the nerve terminal. Microtubules are 25-28nm in diameter and composed of α - and β -tubulin. NFs are 10 nm in diameter and consist of NF triplet protein NF-H, -M, -L subunits. NF has been proposed as a critical target for a number of agents that induce axonopathy (Graham et al., 1982). Microfilaments, the smallest elements, are 3-5 nm in diameter, and polar polymers of globular actin monomers are wound into a double-stranded helix. The microtubule-associated proteins (MAPs), including tau, form cross bridges between microtubules and other cytoskeletal constituents, as well as cellular organelles (Kandel et al., 2000). While the precise targets of axonal neurotoxins are unknown, plausible candidates also include proteins responsible for maintaining the supramolecular organization of the

cytoskeleton (Monaco et al., 1990), such as plakins; proteins responsible for phosphorylation/dephosphorylation of NF subunits; and the motor proteins kinesin and dynein (Stone et al., 1999).

“Axonopathy” refers to primary degeneration of the axon, such as occurs in 2,5-HD axonopathy. The protein targets of toxic attack in neurofilamentous and other axonopathies have yet to be identified. In this study, it was important to determine first whether neuropathological changes in spinal cord and spinal roots were correlated with the onset of limb weakness in rats treated with DAB isomers. Rats treated with 1,2-DAB, but not 1,3-DAB, developed limb weakness associated with proximal neurofilament (NF)-filled giant axonal swellings comparable to those seen in animals treated with the 3,4-dimethyl- 2,5-HD (DMHD) (Kim et al., 2001).

Several neurotoxic chemicals such as acrylamide, carbon disulfide (CS_2), β,β' -iminopropionitrile (IDPN), 2,5-HD, neurotoxic γ -diketone metabolite of *n*-hexane, or MBK produce axonal swelling filled with 10 nm NFs (Couri and Milks, 1982; Gottfried et al., 1985). These chemicals disrupt microtubule-NF relationships and cause accumulations of NFs in proximal (IDPN) or distal (2,5-HD and CS_2) regions of affected axons (Spencer and Schaumburg, 1975; Tashiro et al., 1994; Stone et al., 1999). Although proteins of all tissues are derivatized and cross-linked after exposure to CS_2 , the toxicologically relevant reactions are those that occur with long-lived proteins, such as the subunits of the neurofilament (Graham et al., 1995).

MECHANISMS OF AROMATIC AND ALIPHATIC γ -DIKETONE AXONOPATHY

Chemicals that cause toxic axonopathy target systems within the axon on which the nerve fiber depends for maintenance of structural and functional integrity. A prominent feature of neurofilamentous axonopathies is the segregation

of microtubules that penetrate amassed NFs in giant axonal swellings. This is seen in the peripheral nerves of rats systemically treated with 2,5-HD or IDPN and can also be reproduced by local application or intraneuronal injection of high concentrations of either agent to intact sciatic nerves in living animals (Zagoren et al., 1983; Griffin et al., 1983). We have observed (Kim et al., 2001) a similar phenomenon after systemic treatment of rats with 1,2-DAB (10-20 mg/g/d i.p.). Western blot analysis of intrafascicular nerve tissue showed that NF-M was more sensitive than β -tubulin to 1,2-DAB-induced cross-linking. Similarly, in spinal cord *in vitro*, NF-H and NF-M were more sensitive than β -tubulin to 1,2-DAB. *In vitro* studies using a native rat NF model system comparably demonstrated that 2,5-HD affects NF-H and NF-M more than NF-L (DeCaprio and Fowke, 1992). Mice heterozygous or homozygous for the NF-H null mutation fail to develop neurofilamentous swellings and segregated microtubules in motor neurons after systemic IDPN treatment. These results indicate that the NF-H subunit is a key mediator of IDPN-induced axonopathy (Zhu et al., 1998).

The γ -diketone structure underlies the neurotoxic property of the aliphatic hydrocarbon 2,5-HD and DMHD neurotoxicity. Of a series of diketones tested for neurotoxicity in rats, only γ -diketones, including 2,5-HD, 2,5-heptanedione, and 3,6-octanedione, were found to induce the classic clinical signs and pathological changes associated with *n*-hexane neuropathy (Spencer et al., 1978). In contrast, α -diketones (2,3-butanedione, 2,3-hexanedione), β -diketones (2,4-hexanedione, 2,4-pentanedione, and 3,5-heptadione), and an ε -diketone (2,7-octanedione) were not neurotoxic.

The γ -diketone neurotoxic agents 2,5-HD and DMHD form pyrrole adducts with axonal and other proteins, particularly NF protein (DeCaprio et al., 1982). Subsequent autooxidation causes cross-linking of NF and development of axonal neuropathy (Anthony et al., 1983a). Heavier molecular weight subunits represent diketone-NF pyrrole adducts that have presumably undergone secondary covalent intermolecular NF-NF cross-linking (Graham et al., 1982). Pyrrole adduct

formation decreased NF solubility within the axoplasm, resulting from formation of hydrophobic pyrrole adducts, which might alter their transport or metabolism (DeCaprio et al., 1983). Progressive proximodistal NF cross-linking is proposed to impair anterograde NF translocation across narrow nodes of Ranvier and thereby promotes accumulation of derivatized NFs with subsequent axon swelling and degeneration.

During treatment with an aliphatic γ -diketone, the extent of longitudinal movement and position of arrest of NF proteins are associated with the protein reactivity and neurotoxic potency of the selected agent. Compared with 2,5-HD, which develops distal giant axonal swellings after chronic treatment, 3-methyl-2,5-hexandione and DMHD cause NF transport arrest at mid-level and proximal locations, respectively (Monaco et al., 1990; Anthony et al., 1983). On the basis of a comparison of the position of arrest of NF proteins, 1,2-DAB thus might have neurotoxic potency similar to that of DMHD.

NF is unlikely to be the crucial lesion, however, because wild-type mice that express NF proteins and transgenic mice that do not, both display similar patterns of distal axonal degeneration in 2,5-HD neuropathy (Stone et al., 2001). Other potential targets of γ -diketones are microtubules, motor proteins such as kinesin or dynein, microtubules, microtubule-associated proteins, and enzymes involved in supplying energy for the transport of materials along the axon (Spencer et al., 1980b; Graham, 1999).

HYPOTHESIS AND MEDICAL SIGNIFICANCE

The studies presented in this dissertation examine the hypothesis that the mechanism of 1,2-DAB axonopathy is similar to that of aliphatic γ -diketone 2,5-HD. To test this hypothesis, the following specific studies were conducted:

1. Examine morphological changes induced by DAB isomers using light and electron microscopy (Chapter 2).

2. Examine reactivity of DAB isomers with amino acids and proteins.

Determine protein adduct formation with 1,2-DAB or 2,5-HD and purified proteins, and enzymes *in vitro*, and proteins present in spinal cord and sciatic nerves treated *in vitro* and *in vivo* (Chapter 3).

My studies lead to conclusion that the structure relationship (γ -diketone) and neurotoxic effects (neurofilamentous axonopathy) of 1,2-DAB and 2,5-HD are shared. Aromatic hydrocarbon 1,2-DAB, compared with 2,5-HD, induced proximal neurofilamentous axonopathy and showed high protein reactivity .

My findings may shed light on medical problems beyond the scope of neurotoxicity. 1,2-DAB-induced proximal axonal swellings filled with 10nm-NFs are the pathological hallmark of amyotrophic lateral sclerosis (ALS). Lewy bodies that contain NF protein are pathological determinants of Parkinson's disease (Hughes et al., 2001). Whether the NF proteins are the cause or consequence of the diseased state remains to be clarified. Recent evidence suggests the involvement of Cu, Zn superoxide dismutase, which leads to accumulation of NF aggregates (Price et al., 1997). Additionally, a recombinant mouse strain that overexpresses NF-H shows abnormal NF accumulations resembling those found in ALS (Julien et al., 1998).

1,2-DAB has been shown to crosslink and polymerize NFs and arrest their movement along the axon. This may explain the appearance of swellings filled with NF in proximal axons. Further studies are required to illuminate the mechanism of NF accumulation and formation of axonal swellings in 1,2-DAB-treated animal.

Chapter 2

1,2-Diacetylbenzene, the Neurotoxic Metabolite of a Chromogenic Aromatic Solvent, Induces Proximal Axonopathy

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ABSTRACT

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Several widely used aromatic hydrocarbon solvents reportedly induce blue-green discoloration of tissues and urine in animals and humans. The chromophore has been proposed to result from a ninhydrin-like reaction with amino groups in proteins. The present study examines the neurotoxic property of 1,2-diacetylbenzene (1,2-DAB), the active metabolite of the chromogenic and neurotoxic aromatic solvent 1,2-diethylbenzene. Rats treated with 1,2-DAB, but not with the non-chromogenic isomer, 1,3-DAB, or with ninhydrin, developed blue discoloration of internal organs, including the brain and spinal cord. Only 1,2-DAB induced limb weakness associated with nerve fiber changes, which were most prominent in spinal cord and spinal roots. Changes began with the formation of proximal, neurofilament-filled axonal swellings of the type seen after treatment with 3,4-dimethyl-2,5-hexanedione, a potent derivative of the active metabolite of the neurotoxic aliphatic hydrocarbon solvents *n*-hexane and methyl *n*-butyl ketone. These compounds are metabolized to a γ -diketone that forms pyrroles with target proteins, such as neurofilament proteins. A comparable mechanism is considered for 1,2-DAB, an aromatic γ -diketone.

Key Words: Diethylbenzene, diacetylbenzene, aromatic solvent, ninhydrin, tetralin, chromophore, neuropathy, axonopathy, green urine.

INTRODUCTION

More than 80 years have elapsed since the first report of a relationship between human exposure to aromatic solvents (tetralin or tetralin derivatives) with acute neurotoxic properties and the excretion of green-colored urine (Pohl and Rawicz, 1919; Rockemann, 1922; Koelsch, 1926; Lehmann and Flury, 1943; Badinand et al., 1947; Browning, 1953; Drayer and Reidenberg, 1979; Grant et al., 1985; Longacre 1987). Over 40 years have passed since green-colored urine was reported in rats treated with a single subcutaneous dose of tetralin, one of a list of reportedly chromogenic aromatic hydrocarbon solvents that includes 1,2-diethylbenzene (1,2-DEB) (Gerarde, 1960, see also Spencer, 2000a,b and Spencer et al., 2001). Over 20 years ago, the chromogenic property of a tetralin derivative (AETT: 1,1,4,4-tetramethyl-6-ethyl-7-acetyl-1,2,3,4-tetrahydronaphthalene), formerly used worldwide in fragrances and as a food additive, was linked to pathological changes in neurons and myelin in the central and peripheral nervous system of rats treated with repeated dermal or oral applications (Spencer et al., 1979a,b; Spencer et al., 1980a). This observation raised the possibility that other chromogenic aromatic hydrocarbons, such as 1,2-DEB, may have comparable neurotoxic properties (Spencer and Schaumburge, 1985).

The first report linking the chromogenic and neurotoxic properties of 1,2-DEB appeared over 10 years ago (Gagnaire et al., 1990). Rats treated orally or by inhalation with 1,2-DEB or its putative metabolite, 1,2-diacetylbenzene (1,2-DAB), developed blue discoloration of tissue followed by the appearance of electrophysiological deficits consistent with sensorimotor neuropathy and altered brainstem auditory evoked potentials (Gagnaire et al., 1990, 1991, 1992a,b). By contrast, the investigators were unable to produce peripheral nerve conduction changes in rats treated orally with either of the non-chromogenic isomers 1,3-diethylbenzene (1,3-DEB) or 1,4-diethylbenzene (1,4-DEB) (Gagnaire et al., 1990). 1,2,4-Triethylbenzene but not 1,3,5-triethylbenzene showed comparable chromogenic and neurotoxic properties, suggesting that the presence of two ethyl

residues in the *ortho* position on an aromatic ring is required for chromogenicity and neurotoxicity (Gagnaire et al., 1993).

The chromogenic and neurotoxic properties of 1,2-DEB seem to arise from the proposed active metabolite 1,2-DAB (Gagnaire et al., 1991; Payan et al., 1999), which reacts with amino acids to form colored pigments (Nan'ya and Maekawa, 1977a; Bayle-Lacoste et al., 1987; M-S Kim et al., 2000b). In contrast to 1,2-DEB, the urinary metabolite 1,2-DAB produces a blue color on contact with human and animal skin (Nan'ya and Maekawa, 1977b; Kim et al., 2000b). By the intraperitoneal route, 1,2-DAB is severalfold more toxic (body weight) and neurotoxic (peripheral neuropathy) than 1,2-DEB (Gagnaire et al., 1991, 1992a). Our preliminary work shows that the structure-activity requirement for chromogenicity and neurotoxicity reported for DEB isomers also holds for DAB isomers (Kim et al., 1999, 2000a). The present study employs light and electron microscopy to confirm the structure-activity requirements for DAB neurotoxicity and to define the neuropathologic process induced by 1,2-DAB. The effects of DAB isomers (1,2-DAB, 1,3-DAB) are compared with those of ninhydrin (2,2-dihydroxy-1,3-indanedione), a compound that reacts with amino acids to form a blue pigment (Ruhemann's purple). The Ruhemann reaction has been postulated to be involved in the genesis of the chromogenicity associated with the diketo derivative of AETT (1,2-diacetyltrimethyltetralin) and 1,2-DAB (Spencer et al., 1980a; Zhan et al., 2001).

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 175-225 g on arrival were used. Animals were acclimated individually in home cages for 5 days prior to treatment. Cages were supplied with soft, absorbent bedding, and animals were given Purina rat chow and water *ad libitum*. Stacked cages were held in a quiet room provided with a 12h/12h light-dark cycle and held at a temperature of 20°C. Animals were randomly selected for treatment with test articles.

Chemicals

1,2-DAB (99%), 1,3-DAB (97%) and ninhydrin (97%) were purchased from Aldrich Chemical Co. (Madison, WI) and dessicated at room temperature (1,2-DAB) or stored at 4°C (1,3-DAB). Purity of all test agents was checked by gas chromatography-mass spectrometry (GC-MS). Each substance was dissolved in 2% HPLC-grade acetone, 0.9% saline added, the solution shaken and (for 1,3-DAB) warmed to achieve maximum solubilization of the test article in target concentrations of approximately 0.06 M or 0.12 M. DAB isomers and ninhydrin were stable when this solution was stored at room temperature for 7 days. All other chemicals were of analytical grade.

Animal treatment

Rats were weighed daily (scoping study), every other day (preliminary study) or twice weekly (formal study) to track body weight changes and adjust the dosage of the test article. Animals were treated in the morning and their behavior and locomotion in an open field examined qualitatively in the afternoon. Video-recordings were made of selected animals in a highly illuminated open-field setting.

Test articles were administered intraperitoneally with a 1-ml disposable plastic syringe equipped with a 27-gauge needle. Injection sites were rotated around the abdomen. Care was taken to minimize leakage. *N*-Butyl benzenesulfonamide, a neurotoxic substance found in some types of plastic syringe (Strong et al., 1991), was not detected by GC-MS in test articles held for up to 1 day in syringes identical to those used for injection. At termination of treatment, rats were individually anesthetized with 3% isofluorane in oxygen, the chest opened and the animal injected intracardially with 200 μ l of heparin (1000 units/ml) solution. Immediately thereafter, the animals were perfused through the ascending aorta with 4% paraformaldehyde (10 sec) followed by 5% glutaraldehyde (1 liter), each in ice-chilled phosphate buffer (pH 7.4).

Tissues were sampled from the central nervous system (CNS), including brain (frontal cortex, midbrain, cerebellum and optic tract), medulla oblongata and lumbar spinal cord. Peripheral nervous system (PNS) tissues included lumbar dorsal and ventral spinal roots, lumbar dorsal root ganglia, sciatic nerve (proximal and distal), tibial nerve branches to the calf muscles, and tibial nerve at the ankle. All tissues were post-fixed with 1% osmium tetroxide in phosphate buffer, dehydrated, and embedded in epoxy resin. One-micrometer-thick tissue sections of all regions were stained with 1% toluidine blue and screened by bright-field microscopy. Thin sections of areas of special interest were stained with 2% uranyl acetate followed by 1% lead citrate and examined by transmission electron microscopy.

Dosing regimens

Scoping studies were carried out to estimate the neurotoxic dosage of 1,2-DAB. Animals were given 9 injections of saline (n=4) or 20 mg/kg/day 1,2-DAB in saline (n=4) administered over 2 weeks. Nine days after treatment began, there was severe weight loss accompanied by tissue and urine discoloration. Changes in neuro-behavioral status were evident in the form of post-injection hyperactivity, aggressive behavior, back-arching and limb weakness. The dosage of test articles was therefore halved in preliminary studies to compare the effects of 1,2-DAB, 1,3-DAB and ninhydrin. Animals were treated for 30 days either with saline (n=4) or with 10 mg/kg/d 5 days/week of each agent in saline (n=4 per group) for a total dose of 220 mg/kg/animal.

Formal studies were carried out to assess the spatial-temporal evolution of neuropathological changes induced by 1,2-DAB. For this purpose, animals were treated in the manner described above with saline for 3 weeks (n=4), 6 weeks (n=4) or 9 weeks (n=4) or saline containing 10 mg/kg/day 1,2-DAB 5 days/week for 3 weeks (total dose: 150 mg/kg, n=4), 6 weeks (300 mg/kg, n=4) or 9 weeks (450 mg/kg, n=4). Daily visual examination was used to monitor general health, skin color, hair texture, posture, and locomotion. Body weights were examined twice weekly and the results used to adjust the dosage of the test article. Animals were randomly selected at each timepoint for neuropathological study.

RESULTS

Animal observations

DAB Treatment. Changes in body weight were related to the daily dosage of 1,2-DAB. Whereas all animals in the scoping study lost body weight (mean, -25%; 20 mg/kg/d for 9 days), all animals gained body weight erratically in the preliminary study (mean, +18%; 10 mg/kg/d for 30 days). Body weight gain in all 1,3-DAB-treated rats (mean, +36%; 10 mg/kg/d for 30 days) was steady and comparable to that in animals treated with saline (mean, +38% in 30 days) in the preliminary study. Whereas all animals treated with saline in the formal study steadily gained body weight, rats treated with 1,2-DAB steadily but more slowly gained body weight for 39 days and lost body weight between day 39 and day 60 of treatment. There were no animal losses. There was a marked difference between the physical appearance of animals treated with 1,2-DAB versus 1,3-DAB, ninhydrin or saline. In the scoping, preliminary and formal studies, all animals treated with 1,2-DAB rapidly developed darkened eyes and a bluish tone to the exposed skin (ears, paws, tail) (Fig. 2.1).

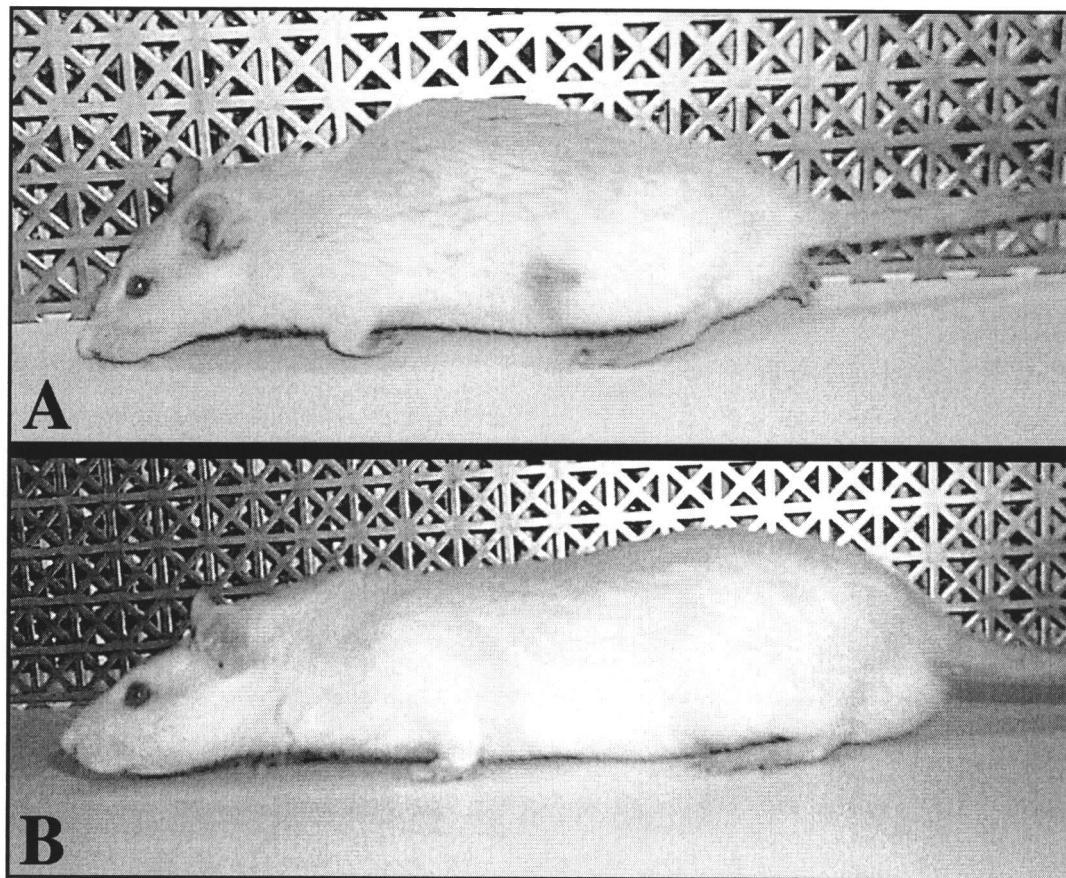


Figure 2.1. Rats treated with 10 mg/kg/d for ~25 days with 1,2-DAB (A) or saline (B). Note the discoloration of the ears, paws and tail with 1,2-DAB treatment.

These animals also developed a reactive bladder and greenish urine, circumoral porphyrin deposition, and late epistaxis. In the absence of aldehyde perfusion, blue discoloration was present throughout body organs and tissues of all 1,2-DAB-treated animals, especially the kidneys and testes, and including the brain, spinal cord and peripheral nerves, and plasma. Skin at injection sites was also densely stained blue. After aldehyde perfusion, the brains of 1,2-DAB-treated animals were discolored relative to the amber coloration of brains removed from animals treated with 1,3-DAB or saline (Fig. 2.2). Intestinal bloating and brittle bones were also noted in 1,2-DAB-treated rats.

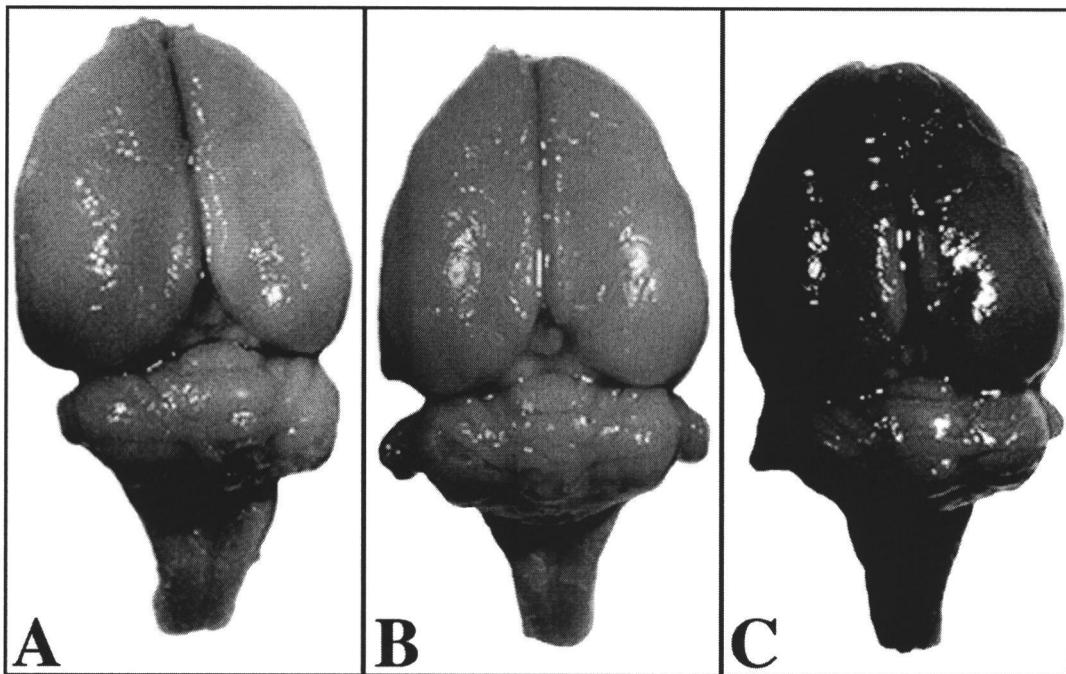


Figure 2.2. Brains of rats treated for 30 days with saline (A), 1,3-DAB (B), or 1,2-DAB (C) (10 mg/kg/d 5 days/week treatment with either DAB isomer).

Most animals treated with 20 mg/kg/d 1,2-DAB showed post-injection and touch-induced hyperactivity, drowsiness, and aggressiveness (including biting) toward the handler. Thereafter, the animals weakened, developed an arched back, distal forelimb weakness, and then hindlimb paresis. Rats treated with 10 mg/kg/d 5 days/week for 30 days showed no clear-cut neurological signs. In the formal study, rats typically showed distal forelimb weakness at 6 weeks, and hindlimb and forelimb splaying at 9 weeks. All animals maintained their ability to eat and drink throughout the course of the experiment.

Ninhydrin Treatment. Three of four animals treated with ninhydrin initially lost body weight, and one animal died after 7 days of progressive body weight loss. All remaining animals subsequently gained body weight (mean of +23% at 30 days). No behavioral or pathological signs of neurodegeneration were evident in

any animal after 30 days of treatment with ninhydrin. These animals showed at the injection site a tendency to hemorrhage (20 days) and a blue discoloration of skin (1 day), but no discoloration of brain, spinal cord, skin, eyes or abdominal organs was seen. At autopsy, the liver was generally speckled and reduced in size, and intestinal bloating and obstruction were evident.

Taken together, these results showed that 1,2-DAB, but neither 1,3-DAB nor ninhydrin, induced blue nervous tissue discoloration associated with the development of progressive limb weakness.

Neuropathology

The quality of tissue fixation was generally of high standard in all regions of the CNS and PNS examined. Focus was placed on the lumbar spinal cord and spinal roots, the site of the earliest and most dramatic nerve fiber changes induced by 1,2-DAB. No such changes were observed in animals treated with 1,3-DAB, saline or ninhydrin.

Scoping Study. All rats developed hind limb weakness and corresponding pathological changes in lumbar motor neurons. Intraspinal motor axons were grossly enlarged and thinly myelinated, a pattern of neuropathology that has been termed *giant axonal swelling* (Fig. 2.3A). Cross sections of axonal swellings revealed densely packed neurofilaments and islands of clustered microtubules, mitochondria, and vesicles (Fig. 2.3B). The soma of spinal neurons occasionally contained abnormal lucent regions (Fig. 2.3C) that corresponded to skeins of neurofilaments running between islands of rough endoplasmic reticulum (Fig. 2.3D). Evidence of abnormal somal lipopigmentation or neuronal degeneration was not found.

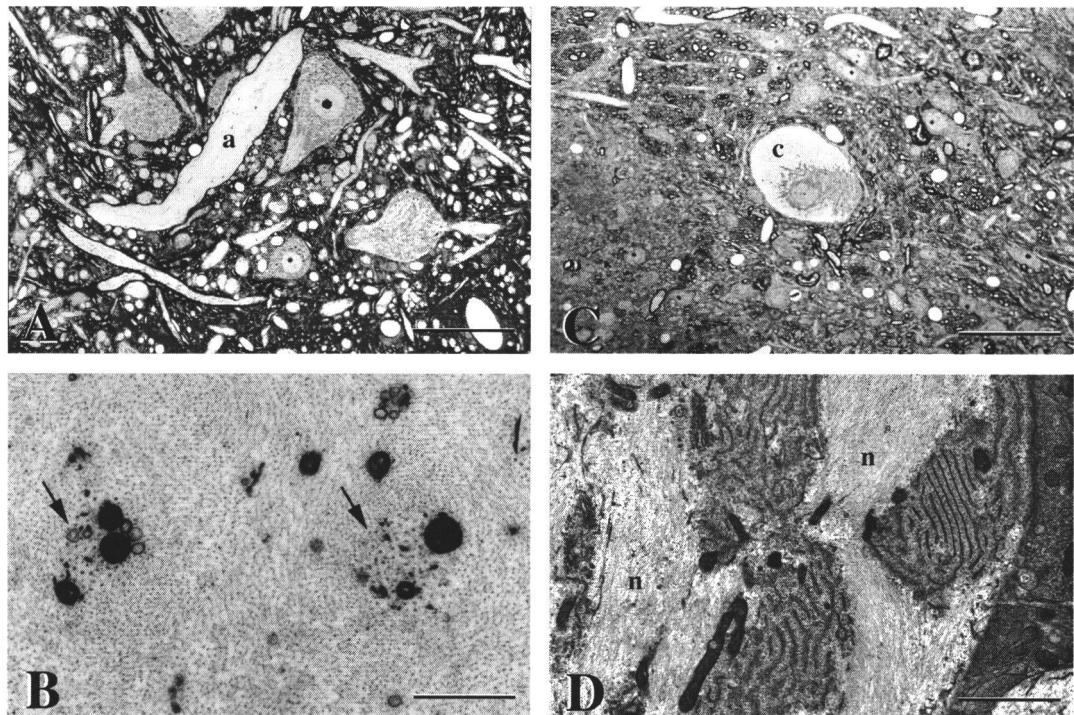


Figure 2.3. Cross sections of lumbar spinal cord (A,C,D) and adjacent ventral spinal root (B). (A) Anterior horn showing several motor neurons and a grossly enlarged intraspinal axon (*a*). (B) Axoplasm containing aggregations (*arrows*) of neurotubules, mitochondria and vesicles, surrounded by densely packed neurofilaments. (C) A distended spinal neuron containing aggregated organelles and abnormal lucent regions of cytoplasm (*c*). (D) Anterior horn cell cytoplasm containing abnormal skeins of neurofilaments (*n*) and islands of ribosome-studded endoplasmic reticulum. Light (A,C) and transmission electron (B,D) micrographs of epoxy-embedded tissue of animals treated with 20 mg/kg/d 1,2-DAB for 2 weeks (9 individual daily injections). Bars represent 38 µm (A), 0.9 µm (B), 38 µm (C) and 2.3 µm (D).

Preliminary Study. Neuropathological changes in these animals were generally less advanced than those found in the scoping study. Scattered intraspinal nerve fibers with giant axonal swelling were visible in the lumbar spinal cord of all animals treated with 1,2-DAB (Fig. 2.4A) but not in corresponding tissues of animals treated with 1,3-DAB (Fig. 2.4B), ninhydrin (Fig. 2.4C), or saline (Fig. 2.4D).

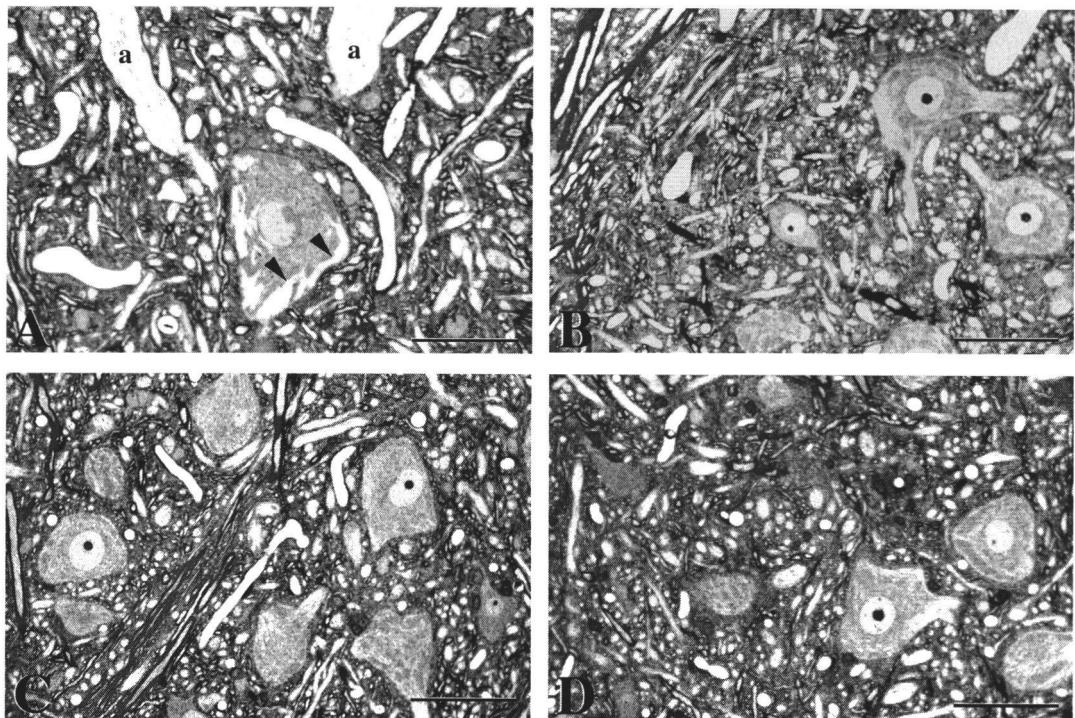


Figure 2.4. Cross sections of lumbar spinal cord. (A) Scattered giant swollen axons (*a*) adjacent to an anterior horn cell containing abnormally lucent regions of cytoplasm (arrows). Animal treated with 10 mg/kg/d 1,2-DAB 5 days/week for 30 days. (B, C) No abnormalities are visible after comparable treatment with 1,3-DAB (B) or ninhydrin (C). (D) Normal tissue from animal treated for a similar period with saline. Light micrographs of epoxy-embedded tissue. Bars represent 38 μm (A), 40 μm (B), 40 μm (C) and 32 μm (D).

Formal Study. CNS and PNS tissues were examined in rats treated for 3 weeks (no apparent limb weakness), 6 weeks (distal forelimb weakness) and 9 weeks (fore- and hindlimb weakness) of 1,2-DAB treatment.

At 3 weeks, intraspinal regions of lumbar motor axons showed a moderate degree of axonal swelling and normal myelination, comparable in degree to that seen in equivalent regions of animals in the preliminary study. Occasional swollen myelinated fibers were seen in cross sections of lumbar dorsal root ganglia. Other

sampled regions of the CNS (medulla oblongata, cerebellum, frontal cortex) and PNS (sciatic nerve, tibial nerve) were within normal limits.

At 6 weeks, the anterior horn of lumbar spinal cord contained massively swollen axons which, on rare occasion, could be seen in continuity with the soma of an anterior horn cell of relatively normal appearance (Fig. 2.5A). Some motor neurons contained zones of clear cytoplasm. Both these somal zones and their associated swollen motor axons contained dense skeins of 10 nm neurofilaments (Fig. 2.5B), together with aggregations of axonal organelles (mitochondria, dense bodies).

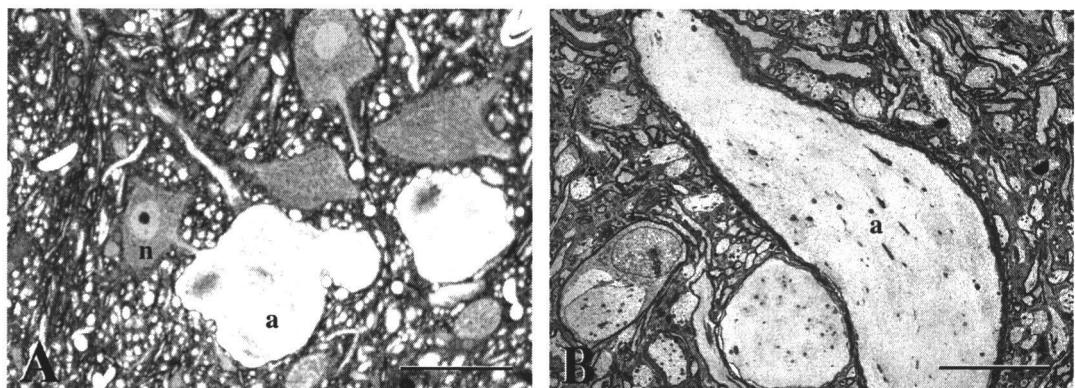


Figure 2.5. (A) Light micrograph showing a massive focal swelling of the proximal axon segment (*a*) of a lumbar anterior horn cell (*n*). (B) Transmission electron micrograph of a thinly myelinated intraspinal motor axon filled with neurofilaments. Tissue from rats treated with 10 mg/kg/day 1,2-DAB 5 days/week for 6 weeks. Bars represent 52 µm (A) and 14.5 µm (B).

In contrast to the lumbar ventral root of animals treated with saline (Fig. 2.6A) or 1,2-DAB for 3 weeks (Fig. 2.6B), pronounced giant axonal swelling was evident in corresponding regions of animals treated for 6 weeks (Fig. 2.6C). Several nerve fibers showed myelin splitting, with the formation of myelin bubbles. Sciatic nerves, including large-diameter myelinated nerve fibers supplying the calf muscles, showed no qualitative evidence of neuropathology.

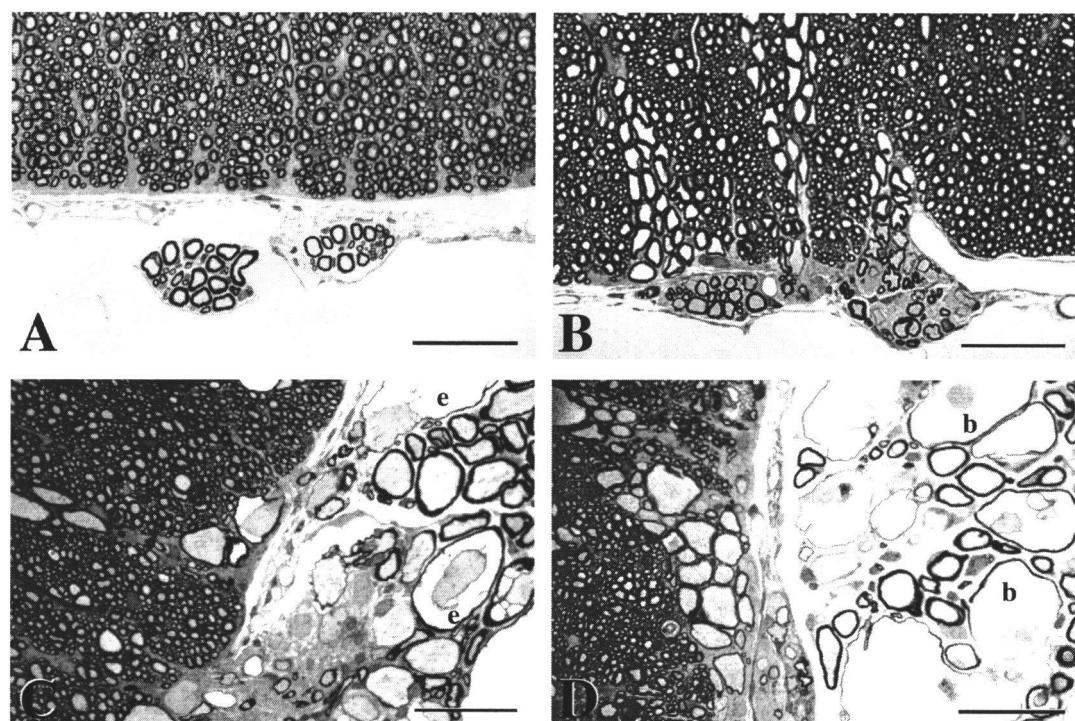


Figure 2.6. Light micrographs of cross sections of lumbar spinal cord and adjacent ventral spinal root. (A) Saline treatment. Spinal cord (upper) and spinal roots (lower) are normal in appearance. (B) 1,2-DAB (10 mg/kg/d, 5 days/week) for 3 weeks. Enlarged axons of some intraspinal myelinated nerve fiber pathways are apparent. (C) Same 1,2-DAB dosage for 6 weeks. Giant axonal swelling and myelin edema (*e*) are evident. (D) Same 1,2-DAB dosage for 9 weeks. Peripheral nerve fibers display myelin bubbles (*b*). Bars represent 38 µm (A), 40 µm (B), 38 µm (C) and 38 µm (D).

At 9 weeks, spinal roots frequently displayed demyelination and myelin bubble formation (Fig. 2.6D). By contrast, relative to the normal structure of cross-sections of tibial nerve branches to the calf muscles of saline-treated rats (Fig. 2.7A), equivalent regions of 1,2-DAB-treated animals occasionally showed myelin bubbles, some possibly shrunken myelinated fibers, and rare remyelinating and degenerating fibers (Fig. 2.7B,C). Electron micrographs of myelin bubbles disclosed an intact, attenuated axon surrounded by an intramyelinic edematous space containing myelin debris (Fig. 2.8A) or phagocytes with ingested myelin debris (Fig. 2.8B).

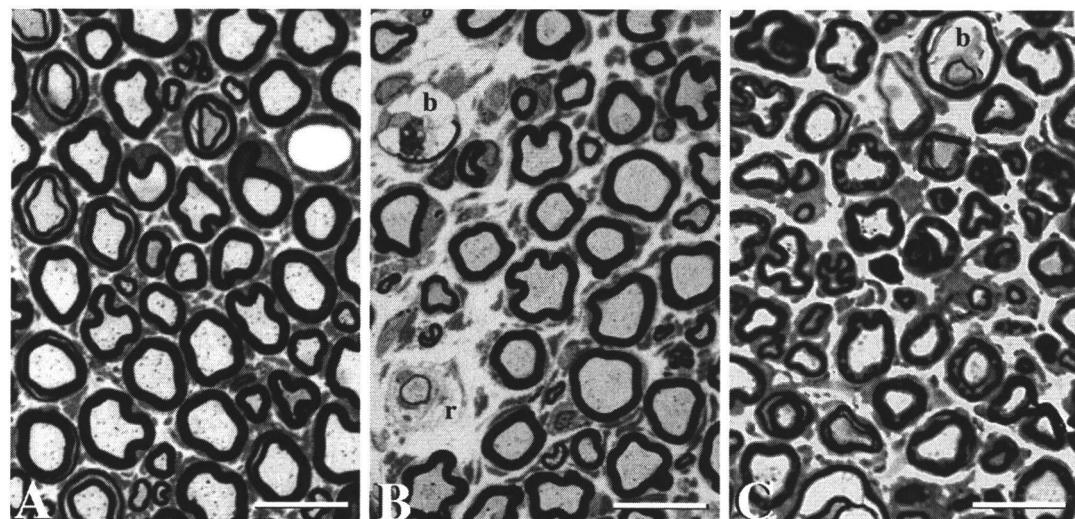


Figure 2.7. Light micrographs of cross sections of tibial nerve branches to the calf musculature. (A) Saline treatment. (B, C) 1,2-DAB treatment (9 weeks). Note the presence of a few scattered myelin bubbles (*b*) among the large majority of normal-appearing and crenated myelinated fibers. One remyelinated fiber (*r*) is present. Bars represent 30 μ m.

Comparable but less advanced pathological changes were seen elsewhere in sampled CNS and PNS tissues of rats treated for 6 weeks (less change) or 9 weeks (more change). As in the lumbar spinal cord and corresponding spinal roots, myelinated fibers of large diameter were most obviously affected in vulnerable

regions of the CNS. Giant axonal swellings were scattered throughout large-diameter fibers in the white matter of the lumbar spinal cord. Spinal gray matter at 9 weeks contained intact anterior horn cells, slight evidence of astrocytosis, and fewer swollen axons than at 6 weeks. In the medulla oblongata, the gracile and cuneate fasciculi/nuclei were spared, but nerve fiber tracts running immediately lateral to intact myelinated fibers in the pyramidal decussation showed progressive

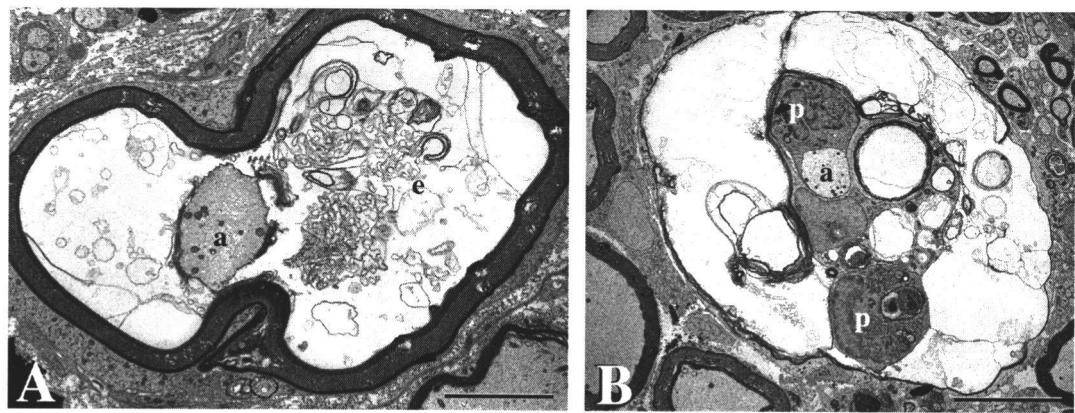


Figure 2.8. Electron micrographs of cross sections of lumbar dorsal roots of rats treated with 10 mg/kg/d 1,2-DAB 5 days/week for 6 weeks. (A) Myelin bubble with shrunken axon (a) associated with an edematous intramyelinic space (e) containing myelin debris. (B) Myelin bubble containing a centrally located axon (a) associated with intramyelinic phagocytes (p) containing myelin debris. Bars represent 4.6 μm (A) and 9.2 μm (B).

abnormalities at the two time points. Scattered giant axonal swelling but no marked lipopigmentation was found in the hypoglossal nucleus. Comparable nerve fiber pathology was evident in the region of the dentate nucleus, but no abnormalities were found in the cerebellar vermis. No convincing changes were seen in motor neurons or axons of the frontal cortex. Giant axonal swelling, demyelination and myelin bubbles were not seen in optic nerves adjacent to the optic chiasm.

DISCUSSION

The present study shows that DAB chromogenicity and neuronal toxicity are properties restricted to the *ortho* isomer, 1,2-DAB. Since 1,3-DAB lacks these properties, the presence (*ortho*) or absence (*meta*) of chromogenicity and neurotoxicity among DAB isomers explains the comparable properties of the corresponding isomers of the aromatic solvent diethylbenzene (DEB) (Gagnaire et al., 1990). Taken together, this provides the first concrete evidence that aromatic hydrocarbon solvents, like as their aliphatic hydrocarbon cousins (*i.e.*, *n*-hexane and methyl *n*-butyl ketone), show structure-dependent axonal neurotoxicity arising from the likely generation of a neurotoxic γ -diketone metabolite (*discussed below*). In both cases, repeated systemic administration induces characteristic pathological changes (giant axonal degeneration) in the CNS and PNS. Additional studies are needed to determine whether pathological changes also develop in other organs. Noteworthy in this regard is the induction of Sertoli cell degeneration in the testes of animals treated with the aliphatic γ -diketone, 2,5-hexanedione (Boekelheide et al., 2000).

The existence of a relationship between the chromogenicity and neuronal toxicity of both DAB and DEB suggests the need to evaluate for neurotoxic properties other reportedly chromogenic aromatic hydrocarbons, notably tetralin (Gerarde, 1960). Tetralin is used as an industrial solvent for a variety of materials, including asphalt, rubber, resins, lacquers, waxes and polishes (Longacre, 1987, Anon., 1997). The U.S. Occupational Health and Safety Administration has not established an occupational exposure limit for tetralin. Tetralin is also a constituent of motor fuels and is used as a substitute for terpentine and as a paint remover and pesticide (Longacre, 1987; Cavender, 1994). The solvent is also a component (31.5%) of Cuprex^R, a non-prescription human pediculocide (Drayer and Reidenberg, 1979). Excessive dermal use of Cuprex to treat a 30-year-old male with generalized pediculosis and extensive areas of excoriation was associated with headache, nausea, conjunctivitis, cough, dry mouth and excretion of green urine

(Grant et al., 1985). Tetralin-based waxes and varnishes have been held responsible for acute CNS effects (restlessness, headache, stupor) in infants and children (Longacre, 1987, Spencer, 2000a). Comparable acute neurotoxicity (drowsiness, stimulus-induced hyperactivity, occasional biting of handler, tremulousness) was noted in rats treated with AETT (Spencer et al., 1979a) and, in the present study, in animals receiving 1,2-DAB. Biting behavior has also been reported in association with an aminotetralin (Costall et al., 1980). Although the mechanism underlying the acute neurotoxic actions of these compounds is unknown, it is perhaps noteworthy that phenylaminotetralins may be agonists at a novel sigma-like site that has neuromodulatory activity that results in increases of brain catecholamine synthesis via activation of tyrosine (Booth et al., 1993). Sigma receptors are found in motor and limbic areas in the brains of humans, non-human primates, and rodents (Gonzalez and Werling, 1997). 5-Methoxy-2-[*N*-(2-benzamidoethyl)-*N*-n-propylamino]tetralin has a high affinity for the dopamine D2A receptor, the dopamine D3 receptor and the serotonin 5-HT1A receptor (Homan et al., 1998).

The acute neurotoxic responses to 1,2-DAB are incidental to the principal observation of this study, namely that repeated parenteral treatment induces neuronal, axonal and myelin pathology associated with progressive limb weakness preceded and accompanied by abnormal body weight gain. Examination of the temporal evolution of the neuropathological process demonstrates that the accumulation of neurofilaments in intraspinal motor axons and sometimes in anterior horn cells precedes the appearance of intramyelinic edema and (secondary) phagocyte-mediated demyelination in spinal roots. Comparable changes are seen in lumbar dorsal spinal roots and in distal peripheral (tibial) nerves, but the latter appear *much later* in the pathological process. Thus, distal forelimb weakness and hindlimb paresis seem to arise predominantly from radiculopathy (ventral root pathology) rather than peripheral neuropathy. Whether distal nerve fibers eventually undergo atrophy or degeneration, or both, would require animal treatment regimens that result in a degree of clinical deficit that may be difficult to justify on ethical grounds.

The myelin changes induced by 1,2-DAB are indistinguishable from those seen in rats treated with AETT, a compound that (with prolonged treatment) induced a spectacular pattern of CNS and PNS vacuolar demyelination reminiscent of *status spongiosus* (Spencer et al., 1979a,b). Based on the present observations, it seems likely that 1,2-DAB and AETT-induced demyelination are equivalent, originate *secondary* to axonal degeneration, and are followed in the region of nerve fiber damage by axonal attenuation and remyelination. This would account for the observation that AETT does not interfere with Schwann cell behavior during wallerian degeneration, regeneration after crush injury, or remyelination in the perineurial window model of primary demyelination (Sterman and Spencer, 1981). Prolonged AETT treatment was also associated with a marked neuronal cytoplasmic lipopigmentation (ceroid type) that was prominent in the pyramidal cells of the entorhinal cortex, hypoglossal nuclei in the brainstem, anterior horn cells in the lumbar spinal cord, and the spinal ganglia. The reason for the absence of comparable neuronal and glial lipopigmentation in rats treated with 1,2-DAB is unknown; possibly, the explanation may be the shorter period of animal treatment with 1,2-DAB vs. AETT.

From 1960, published reports indicated *n*-hexane was able to induce peripheral neuropathy in humans who were overexposed to this aliphatic hydrocarbon solvent in the setting of either inhalant abuse or occupational exposure (Spencer et al., 1980b). Controlled studies with rats reproduced *n*-hexane neuropathy (Schaumburg and Spencer, 1976, 1978) and showed the neuropathological substrate to be a central-peripheral distal axonopathy hallmark by focal axonal swellings containing excessive numbers of maloriented but otherwise normal-appearing 10 nm neurofilaments (Spencer and Schaumburg, 1977a,b). By contrast, three hexane isomers of *n*-hexane were found together to lack the ability to induce peripheral neuropathy in subchronically treated rats (Egan et al., 1980). The explanation for the special structural relationship between *n*-hexane and axonal neuropathy awaited experimental studies stimulated by an industrial outbreak of a peripheral neuropathy attributed to methyl ethyl ketone

acting to potentiate the neurotoxic properties of methyl *n*-butyl ketone (2-hexanone) (Mendell et al., 1974). Both *n*-hexane and its metabolite 2-hexanone were shown to be biotransformed to 2,5-HD, the serum levels of which accounted for the ascending neurotoxic potency of the three compounds (Krasavage et al., 1980). Unlike 2,5-HD and 2,5-hexanediol, neither 2,4-hexanedione nor 2,3-hexanediol was able to induce experimental neuropathy (Spencer et al., 1978). However, other γ -diketone compounds, such as 2,5-heptanedione, induced peripheral neuropathy (O'Donoghue et al., 1984).

The neurotoxic potency of the 6-carbon γ -diketone 2,5-HD was stepwise increased by the addition of methyl groups, such that the potency of 3,4-dimethyl-2,5-HD > 3-methyl-2,5-HD > 2,5-HD (Anthony et al., 1983a,b; Monaco et al., 1990). These compounds were proposed to induce neurotoxicity by reaction with amino groups of proteins to form pyrroles that spontaneously oxidized to form pyrrolated polymers (Anthony et al., 1983a; DeCaprio et al., 1992). It is unclear whether the second step is required for neurotoxicity (DeCaprio, 2000), but new probes have been developed to examine this question (Xu et al., 2001). By contrast, the non- γ -diketones (which failed to induce peripheral neuropathy in rats) were non-reactive with amino groups. Since pyrrole formation is apparently required for the induction of γ -diketone axonopathy, and the rate of pyrrole formation correlates directly with the methylation status and corresponding potency of the γ -diketone species, it seems likely that this reaction is responsible for the induction of giant axonal neuropathy (DeCaprio, 2000).

Neurofilaments are hypothesized to accumulate in axonal swellings during γ -diketone treatment because the anterogradely transported neurofilament proteins undergo progressive pyrrolation and cross-linking (Graham et al., 1985; Pyle et al., 1992). In the case of 2,5-HD, the reactions leading to protein cross-linking are apparently relatively slow processes that allow neurofilament protein to reach *distal* axons before further anterograde transport of the altered proteins fails. The highly potent γ -diketone, 3,4-dimethyl-2,5-HD, reacts much more rapidly with amino

groups to form methylpyrrole dimers leading to more rapid cross-linking of proteins, thereby causing transported proteins to be rapidly modified and neurofilament peptides to accumulate in *proximal* giant axonal swellings in dorsal and ventral roots (Anthony et al., 1983b). Subsequent experiments with 3-acetyl-2,5-HD, which forms pyrroles that are not susceptible to auto-oxidative cross-linking, failed to cross-link tissue protein and did not appear to be neurotoxic (Genter St. Clair et al., 1988). Although this evidence supports the proposal that the cross-linking of neurofilament protein is the crucial step in the induction of axonal neuropathy, pyrrole adduct levels at the proposed neurofilament target sites have not been compared in 2,5-HD-treated and 3-acetyl-2,5-HD-treated animals (DeCaprio, 2000). Just as it is logical to propose that anterogradely transported neurofilament proteins serve as the critical target of γ -diketones, a protein-based neurofilament-transport mechanism that moves anterograde in concert with neurofilaments is also a plausible hypothetical target for these agents.

The molecular mechanisms underlying the chromogenic and neurotoxic properties of aromatic hydrocarbons such as 1,2-DAB and AETT are under active investigation. Both 1,2-DAB and the keto derivative of AETT have chromogenic behaviors, but the protein adducts responsible for these reactions have yet to be identified. One possibility for the active chromogen is Ruhemann's purple (Spencer et al., 1980a; Zhan et al., 2001), the pigment formed in the familiar reaction of ninhydrin with amino acids. The formation of colored adducts with neurofilament peptides or the proteins required for their axonal transport might then account for the neurotoxicity of 1,2-DAB. However, animals treated with ninhydrin failed to develop global chromogenic changes and behavioral or pathologic evidence of neurotoxicity, even though there was evidence of systemic toxicity in the form of body weight changes and liver toxicity. Taken together, these observations appear to be inconsistent with a ninhydrin-like mechanism for the induction of 1,2-DAB axonopathy.

Formation of protein complexes analogous to the pyrroles formed by 2,5-HD is perhaps a more likely mechanism for 1,2-DAB neurotoxicity, and there are

various routes that can lead to protein cross-linking. However, unreacted (unoxidized) pyrroles, whether as monomer or dimer and without special substitutions, do not show absorption in the visible range (Zhan et al., 2001). Several other structures have been proposed that could account for the chromogenic behavior: These include conjugated pyrroles or other structures derived from pyrrole dimers that are highly colored, for example, indole and isoindole derivatives (e.g. structures 2-5 in Nan'ya and Maekawa, 1975; Nan'ya and Maekawa, 1977a; Nan'ya et.al., 1982), structures resembling indigo-based dyes (Streitwieser and Heathcock, 1981; Roberts and Caserio, 1965) or structures based on condensed tetra-pyrroles (Bourhis et.al., 1972). Resolution of the question of relating chromogenicity to neurotoxicity is important not only to understand molecular mechanisms of neurotoxicity but also to assess whether chromogen in urine can serve as a useful biomarker of exposure to 1,2-DAB and other neurotoxic aromatic hydrocarbons (Spencer et al., 2001a,b).

The potential health significance of these observations merits consideration. While 1,2-DAB appears to be a potent neurotoxic substance, its use may be restricted to that of a laboratory indicator for amino acids (Roth, 1971; Ebrahim and Dakshinamurti, 1986; Bayle-Lacoste et al., 1987). The parent neurotoxic substance, 1,2-DEB, is used as a specialty paint solvent (Welsh, 1980) and is present in small concentrations in the jet fuels JP4 and JP8 (Hughes et al., 1982-83), together with a number of other reportedly chromogenic aromatic hydrocarbons. 1,2-DEB comprises approximately 7% of DEB (Gagnaire et al., 1990), which is used as a heat-transfer fluid and in the production of vinyl benzene (styrene) (Payan et al., 1999, Saellenfait et al., 1999). DEB is a constituent of organic solvent mixtures (Czeski and Kostrzewski, 1995; Gagnaire et al., 1990).

It is noteworthy that the neurotoxic potency of 1,2-DEB in the rat is 5 times greater than that of *n*-hexane (Gagnaire et al., 1990), a neuropathy-producing substance that has been assigned a NIOSH time-weighted recommended exposure limit for workroom air of 50 ppm. Formal studies should be conducted to compare the neurotoxic potency of these compounds and their active neurotoxic metabolites

(2,5-HD and 1,2-DAB). Judged solely from the proximal distribution of giant axonal swellings induced by 1,2-DAB, a neuropathological pattern seen with 3,4-dimethyl-2,5-HD (which is 20-30 times more potent than 2,5-HD) (Anthony et al., 1983a), 1,2-DAB is likely to have a neurotoxic potency significantly higher than that of 2,5-HD. This relationship will be important for the establishment of exposure limits for 1,2-DEB and DEB mixtures.

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Chapter 3

Amino Acid and Protein Targets of 1,2-Diacetylbenzene, a Potent Aromatic γ -Diketone that Induces Proximal Neurofilamentous Axonopathy

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ABSTRACT

Amino acid and protein targets of 1,2-diacetylbenzene, a potent aromatic γ -diketone that induces proximal neurofilamentous axonopathy. Kim, M-S., Spencer, P.S., and Sabri, M.I. (XXX) *Toxicol. Appl. Pharmacol.* XXX, XXX-XXX.

The γ -diketone analogs 1,2-diacetylbenzene (1,2-DAB) and 2,5-hexanedione (2,5-HD), but not the δ -diketone 1,3-diacetylbenzene (1,3-DAB) or the β -diketone 2,4-hexanedione (2,4-HD), induce neuropathological changes in the rodent central and peripheral nervous system. The molecular targets of the neurotoxic aromatic and aliphatic γ -diketones, and of their non-neurotoxic structural analogs and ninhydrin, are examined by assessing their differential reactivity with neural and non-neural amino acids and proteins *in vitro* and *in vivo*. Whereas 1,2-DAB is chromogenic and forms polymers with amino acids (notably lysine) and proteins (especially lysine-rich proteins), 1,3-DAB lacks these properties. Ninhydrin forms a chromophore without evidence of protein polymerization. 1,2-DAB preferentially targets neurofilament relative to microtubule protein *in vitro* and *in vivo*. Based on protein reactivity, 1,2-DAB is three orders of magnitude more potent than 2,5-HD. Lysine-rich neurofilament protein subunits NF-H and NF-M are relatively more susceptible than lysine-poor NF-L and β -tubulin to 1,2-DAB. These observations correlate with the development of proximal (1,2-DAB) and distal (2,5-HD) neurofilament-filled axonal swellings and segregated intact microtubules observed during systemic treatment with aromatic and aliphatic γ -diketones.

KEY WORDS. γ -diketone, diacetylbenzene, aromatic solvent, neurofilament, axonopathy, chromophore, protein adducts

INTRODUCTION

1,2-Diacetylbenzene (DAB) is the putative active metabolite of 1,2-diethylbenzene (1,2-DEB), a minor component of aromatic solvents used in industry. 1,2-DAB reacts with amino acids and proteins to form a blue pigment or chromophore (Gagnaire et al., 1991). Rats treated orally or by inhalation with 1,2-DEB or 1,2-DAB develop bluish discoloration of tissue and greenish urine. The chemical structure(s) of the chromophore formed by reaction of 1,2-DAB with amino acids and primary amines has been examined (Nan'ya and Maekawa, 1975, 1977; Nan'ya et al., 1982). Continued treatment with 1,2-DEB or 1,2-DAB causes limb weakness accompanied by electrophysiological deficits consistent with sensorimotor neuropathy and altered brainstem auditory evoked potentials (Gagnaire et al., 1990, 1991, 1992a,b).

We have reported that the chromogenic and neurotoxic properties of 1,2-DAB (a γ -diketone) are absent in its structural analog 1,3-DAB (a δ -diketone) (Kim et al., 2001). The *aromatic* γ -diketone induced a pattern of neuropathology comparable to that seen with the high-potency *aliphatic* γ -diketone, 3,4-dimethyl-2,5-hexanedione (DMHD) (Anthony et al., 1983b). Animals treated with daily intraperitoneal injections of 1,2-DAB developed neurofilament accumulations in anterior horn cells and in extreme proximal regions of motor and sensory nerve fibers in the anterior spinal roots and dorsal root ganglia, respectively . Ultrastructural examination of cross sections of 1,2-DAB-induced (and DMHD-induced) axonal swellings revealed densely packed neurofilaments and islands of segregated microtubules, mitochondria, and vesicles. Beginning pathological changes in lumbar spinal roots of 1,2-DAB-treated rats occurred in the absence of overt nerve fiber atrophy or degeneration in sciatic and tibial nerves (Kim et al., 2001).

Neurofilaments are composed of cytoskeletal triplet proteins that move anterogradely from their site of synthesis in the nerve cell body along the axon to the nerve terminal (Hoffman and Lasek, 1975). Neurofilament (NF) transport is

blocked by systemic treatment with aliphatic γ -diketones, which cross-link NF (and other) proteins to form polymers (reviewed by DeCaprio, 2000). Low potency 2,5-hexanedione (2,5-HD) and high potency DMHD react with proteins slowly and rapidly, respectively, and, in turn, arrest NF transport distally (nerve) and proximally (spinal roots), respectively (Anthony et al., 1983a,b). Formation of pyrrole adducts with ϵ -amino groups in lysine residues of protein, particularly NF protein, has been suggested as the critical step in the genesis of aliphatic γ -diketone axonopathy (Graham et al., 1982; DeCaprio et al., 1982). The diketone-NF pyrrole adducts are believed to undergo secondary covalent intermolecular NF-NF cross-linking (Graham et al., 1982). The cross-linked NFs are unable to move anterogradely along the axon and consequently accumulate in focal axonal swellings visible by light microscopy (Anthony et al., 1983b). Rats treated with 2,5-HD, which is less reactive than DMHD, showed preferential reduction of the heavy NF protein (NF-H) (Carden et al., 1986), or all three NF protein subunits (Lapadula et al., 1986), and accumulation of higher-molecular-weight products immunoreactive with NF-specific antibodies. 3-Acetyl-2,5-HD, which forms pyrroles that are not susceptible to auto-oxidative cross-linking, failed to cross-link tissue protein and did not cause neurofilamentous axonal swelling (Genter St. Clair et al., 1988). These findings suggest that polymerization of target proteins is a required step for axonal neurotoxicity.

The present studies compare the neural targets of aromatic and aliphatic γ -diketones by examining their differential reactivity with neural and non-neural amino acids and proteins *in vitro* and *in vivo*, and the effects of 1,2-DAB on protein function. The properties of these compounds are compared with that of ninhydrin, a chromogenic amino acid reagent that fails to induce neurofilamentous axonopathy in systemically treated rats (Kim et al., 2001). The studies suggest that aromatic and aliphatic γ -diketones have common neural targets.

MATERIALS AND METHODS

Chemicals

1,2-DAB (99% pure), 1,3-DAB (97%), and ninhydrin (97%) were purchased from Aldrich Chemical Co. (Madison, WI) and dessicated at RT (1,2-DAB) or stored at 4°C (1,3-DAB). 2,5-HD (94% pure) and 2,4-HD (94%) were purchased from Eastman Chemical Co., Rochester, N.Y. Chemical purity was confirmed by gas chromatography-mass spectrometry (GC-MS). Purified amino acids (99+%), reduced glutathione (GSH), non-neural proteins (crystalline) and enzymes (lyophilized) were obtained from Sigma Chemical Co. (St. Louis, MO) and neural proteins (1mg/ml solutions) from Cytoskeleton Co., Denver, CO. Proteins included: bovine serum albumin (BSA), crystalline bovine pancreatic ribonuclease (RN), bovine spinal cord NF proteins (mixture of NF-H, NF-M and NF-L subunits), and bovine brain tubulin. Enzymes included rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH). All other chemicals used were of analytical grade.

Animals

Male Sprague-Dawley rats, weighing 175-225 g on arrival, were housed at 20°C on a 12h/12h light-dark cycle. Purina rat chow and water were supplied *ad libitum*. Animals were acclimated individually in home cages for 5 days. Eleven rats were used to examine the action of DAB isomers on peripheral nerve *in vivo*, and the spinal cord was removed from an additional 20 animals to study the protein reactivity of these agents *in vitro*. These studies are described below.

Amino acid and protein chromogenicity

We examined the differential reaction *in vitro* of neurotoxic and non-neurotoxic DAB isomers with 22 protein and non-protein (ornithine, γ -aminobutyric acid) amino acids. Test articles were dissolved in 0.1M phosphate buffer (pH 7.4). Equal volumes of each test article and either 1,2-DAB or 1,3-DAB (1mM) were incubated at 37°C for 15, 30 or 60 min. Absorbance of the resulting purple/blue color was determined between 350 and 650 nm in a scanning spectrophotometer (Perkin Elmer Lambda Bio UV/VIS), with a peak absorbance at 540 nm. The intensity of the color formed from the reaction in triplicate of 1,2-DAB or 1,3-DAB with lysine, glycine, ornithine, γ -aminobutyric acid (GABA) or cysteine (negative control) was measured after incubation at 37°C for 15 min, 30 min or 60 min.

We used L-lysine (the most reactive amino acid) and BSA (a representative protein) to compare the chromogenic reaction of DAB isomers. BSA (0.5mg/ml) and L-lysine (0.5mg/ml) were individually reacted with 1mg/ml 1,2-DAB or 1,3-DAB for 1h at 37°C in 0.1M phosphate buffer, pH 7.4. The peak pattern of the chromogen formed in each reaction was determined as described above.

Protein reactivity and polymer formation

We examined the ability of neurotoxic and non-neurotoxic aliphatic and aromatic diketones to form polymers with test proteins, namely BSA (MW~67,000) and RN (MW ~13,000). In addition, BSA was studied in the presence and absence of GSH. Polymer formation with diketones was also compared with that of ninhydrin, which also forms a chromophore with amino acids and proteins but lacks systemic neurotoxicity (Kim et al., 2001). The following incubations were performed: (a) BSA (16 mg/ml in 0.1M phosphate buffer, pH 7.4) was incubated

for 30 min. at 37°C with 1,2-DAB (1,2,3,4 or 5mM) or 1,3-DAB (5mM); (b) an equivalent concentration of RN was incubated for 5, 10 or 15 min. at 37°C with 25mM 1,2-DAB or 1,3-DAB in phosphate buffer (0.1M, pH 7.4). Under comparable conditions, BSA and RN were individually incubated in an equivalent volume of buffer (control); (c) BSA was incubated for 30 min. with 2,5-HD (2M), 2,4-HD (2M), 1,2-DAB (2mM), 1,3-DAB (2mM), or ninhydrin (0.5 and 2mM) in 0.1M phosphate buffer pH 7.4 at 37°C. In addition, BSA in the presence or absence of GSH (5 or 10mM) was incubated with 1,2-DAB (5mM) in 0.1M phosphate buffer pH 7.4 for 30 min. at 37°C. Aliquots of reacted protein (10 μ g) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).

GAPDH and LDH reactivity

We compared the protein reactivity and enzyme activity of GAPDH and LDH after their individual treatment *in vitro* with DAB isomers. Lyophilized GAPDH (100 μ g/20 μ l of 0.1M pyrophosphate buffer, pH 8.5) or LDH (100 μ g/20 μ l of 0.1M phosphate buffer, pH 7.2) was incubated for 30 min. at 37°C with 1, 2 or 5mM 1,2-DAB or 1,3-DAB in the same buffer. Controls were incubated under identical conditions in the respective buffer alone. Aliquots of reacted GAPDH or LDH solution were examined spectrophotometrically for enzyme activity using the method of Sabri (1983) as described below. Additional aliquots (6 μ g protein) were used for SDS-PAGE.

Neural protein reactivity

We examined the differential protein reactivity of DAB isomers and ninhydrin with purified tubulin and NF proteins containing all three subunits. Each purified protein (6 μ g) was treated for 30 min at 37°C (pH 7.4) with 1, 2 or 5mM

1,2-DAB, 1,3-DAB, or ninhydrin in 0.1M phosphate buffer (pH 7.4) (3-5 trials per compound). In controls, DAB isomers were replaced by an equivalent volume of buffer. The relative reactivity of NF proteins with DAB isomers was compared in a single trial with that of 2,5-HD or 2,4-HD (1mM-2.0M). Samples of reacted protein were subjected to SDS-PAGE.

Spinal cord protein reactivity in vitro

We determined the DAB reactivity of neural cytoskeletal elements in spinal cord tissue, the locus of early pathological changes in animals treated with 1,2-DAB. Rats were decapitated by guillotine and the spinal cord expelled with ice-cold 0.1M phosphate-buffered saline by the method of deSousa and Horrocks (1979). The lower half of the spinal cord tissue was stored at -80°C.

Thawed spinal cord tissue was cut into 2-mm lengths on a chilled dissecting board. Tissue was incubated with individual DAB isomers in 0.1M phosphate buffer (pH 7.4, 37°C) for different times (15, 30, 60min) and at varying concentrations (0.1, 0.5, 1, 2, 5, 10mM). Incubated tissue was twice rinsed with ice-cold 0.1M phosphate buffer and homogenized on ice for 10 sec. with 8M urea containing complete protease-inhibitor cocktail (one tablet/50ml buffer) (Roche Molecular Biochemicals, Indianapolis, IN). The homogenate was centrifuged at 10,000 x g for 5 min at 4°C and the supernatant collected. Protein concentration in the supernatant was determined using the Coomassie-plus-200 protein reagent (Pierce Co., Rockford, IL) according to the manufacturer's instructions. Aliquots (6µg total protein) were subjected to SDS-PAGE.

Sciatic nerve protein reactivity in vivo

We examined the reactivity of DAB isomers in living peripheral nerve tissue. High DAB concentrations for short periods were used to minimize the

treatment period and to maximize the ability to detect and identify susceptible neural proteins. Animals were placed on a warming pad and deeply anesthetized by inhalation of halothane or isofluorane. The thigh of the anesthetized animal was shaved bilaterally and the exposed skin cleaned with isopropanol. The sciatic nerve was surgically exposed in the thigh, connective tissue surrounding the nerve gently removed, and a 1-cm piece of Parafilm slipped under the nerve to form a fluid trap. Exposed nerves (~1 cm) were each continuously bathed for 5 min with a total of ~1ml of the test article (n=22). Test agents included 1,2-DAB (0, 2, 5, 25 or 50 mM) or 1,3-DAB (25 mM) in isotonic saline + 2% acetone at RT. Treated nerves were excised from animals, which were then immediately terminated by anesthetic overdose. Excised tissue was placed on an ice-cold dissecting board, the epineurium and endoneurial blood vessels removed, and the remaining intrafascicular tissue (comprising nerve fibers and endoneurial connective tissue) stored at -80°C until use. Methods of homogenization, centrifugation, protein determination and SDS PAGE were those employed for spinal tissue.

SDS-PAGE and Western blotting

Six micrograms of reacted protein and low molecular weight- or high molecular weight protein markers (Bio-Rad, Richmond, CA) were separated by SDS-PAGE (a 4-15% gradient polyacrylamide gel for spinal cord tissue and 4-20% for sciatic nerve tissue) using optimal separation conditions (80V for 15min plus 100V for 1-2 h) and a Mini Protein II Gel Apparatus (Bio-Rad, Richmond, CA). Gels were stained with 0.2% Coomassie blue for ~1h to visualize native protein and diketone polymers formed therefrom. Visualization of NF proteins in neural tissue used an immunoblotting technique employing monoclonal mouse antibodies to β -tubulin, NF-H (200kD, clone RT97), NF-M (160kD, clone NN18) and NF-L (70kD) subunits (Chemicon International Inc., Temecula, CA). For this purpose, electrophoresed proteins were transferred to polyvinylidene fluoride (PVDF) membranes using Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Richmond,

CA), according to manufacturer's instructions. PVDF membranes were stained with Ponceau-S solution (Fluka, Milwaukee, WI) to check for successful transfer of separated proteins. PVDF membranes were washed 3X with PBS-T (0.1M phosphate-buffered saline plus 0.1% Tween-20, pH 7.4) and blocked for 1h with 5% non-fat milk in PBS-T or blocking agent (3% casein in PBS-T, Amplified Opti-4CN Detection Kit, Bio-Rad, Richmond, CA). Membranes were incubated with constant shaking for 1h at RT with the appropriate dilution (3000-10,000 X) of primary monoclonal antibody in 1% BSA in PBS-T. Incubated membranes were washed twice with PBS-T before applying secondary antibody conjugated with either (a) horseradish peroxidase (HRP) for detection on X-ray film (Eastman Chemical Co., Rochester, N.Y.) using a chemiluminescence substrate (Pierce, Rockford, IL) or (b) biotinylated goat anti-mouse IgG for colorimetric detection after treatment with streptavidin-HRP amplifying reagent (Bio-Rad, Richmond, CA).

Enzyme assays

The glycolytic enzymes GAPDH [E.C. 1.2.1.12] and LDH [E.C. 1.1.1.27] were used as convenient testbeds to assess whether changes in protein integrity correlated with alterations in protein function as measured by enzyme activity. The enzyme activities of GAPDH and LDH were assayed by the method of Sabri (1983).

For the GAPDH assay, 20 μ l of 5mg/ml (100 μ g) of lyophilized enzyme dissolved in 0.1M pyrophosphate buffer (pH 8.5) was incubated for 30 min. at 37°C with 20 μ l of 1,2-DAB or 1,3-DAB (final concentration 1, 2 or 5mM) in the same buffer. Control enzyme samples were identically incubated with buffer alone. The reaction was stopped by cooling with ice and the solution immediately diluted 10X with buffer. Enzyme activity determined spectrophotometrically at 340 nm by the rate of reduced nicotinamide adenine dinucleotide (NADH) formed per min

from the reduction of NAD in the presence of substrate (glyceraldehyde-3-phosphate), glycolysis uncoupler (sodium arsenate) and sulfhydryl agent (dithiothreitol).

For the LDH assay, 100 μ g of lyophilized enzyme dissolved in 0.1M phosphate buffer (pH 7.2) was incubated at 37°C for 30 min. with 20 μ l of 1,2-DAB or 1,3-DAB (final concentration 1-5mM) in the same buffer. Control enzyme samples were identically incubated with buffer alone. Incubated samples were placed cooled on ice and diluted 100X with ice-cold buffer prior to LDH assay. LDH activity was assayed spectrophotometrically by measuring the rate of NADH decrease at 340nm in the presence of pyruvate.

RESULTS

Amino acid reactivity

1,2-DAB, but not 1,3-DAB, formed a blue pigment (chromophore) with selected amino acids (Table). Spectrophotometric analysis of the chromophore revealed three peaks; the peak at 540 nm was the most consistent and most prominent at pH 7.0. Under these conditions, the rate and amount of chromophore formation was directly dependent on the amino acid, the concentration of 1,2-DAB and the incubation temperature. Of the 22 amino acids tested, 1,2-DAB reacted most rapidly with L-lysine and other primary amines. Pigment color with L-lysine varied with hydrogen ion concentration and ranged between pink (pH 2.4), purple-blue (pH 5-7) and brown (pH 11.0). Chromophore density varied with time of incubation: for example, L-lysine and ornithine showed a similar intensity of chromophore at 60 min, but not at 15 min, when L-lysine intensity was two-fold that of ornithine. Glycine and GABA reacted similarly at all time points tested. The reactivity of other amino acids with 1,2-DAB ($n=1/\text{amino acid}$) was as follows: L-histidine > L-asparagine > L-methionine = L-glutamine > L-arginine = L-phenylalanine > L-aspartic acid = L-leucine, L-serine, L-alanine, L-glutamic acid, L-tryptophan, L-cysteine > L-threonine = L-isoleucine, L-tyrosine, L-valine and L-proline. As expected, no chromophore was detected with 1,3-DAB.

Table 3.1. Chromogenic reactivity of selected amino acids with diacetylbenzene (DAB) isomers.

Amino Acid	1,2-DAB			1,3-DAB
	15min	30min	60min	60min
L-Lysine	2.04±0.02	3.22±0.02	3.51±0.02	0.00
Ornithine	0.99±0.02	2.80±0.01	3.56±0.01	0.00
Glycine	0.95±0.01	2.21±0.02	3.32±0.04	0.00
GABA	0.86±0.03	1.93±0.03	3.02±0.02	0.00
L-Cysteine	0.00	0.00	0.02	0.00

Values represent mean absorbance \pm standard deviation at 540nm. In triplicate, 0.5ml of amino acid solution (0.1M, pH 7.0) was incubated with 0.5 ml of 1,2- or 1,3-DAB (1mM) at 37°C for the stated times. L-Lysine, glycine, γ -aminobutyric acid (GABA) and ornithine, a non-protein amino acid and higher homologue of L-lysine, were the most reactive of 22 tested amino acids. L-Cysteine was among the least reactive of amino acids tested.

Protein reactivity

BSA and RN served as test articles to assess the protein reactivity of DAB isomers. Spectrophotometric analysis of the chromophore formed between 1,2-DAB and BSA revealed a lysine-like pattern of peaks with the most prominent peak at 540 nm at pH 7.4. Treatment with 1,2-DAB, but not 1,3-DAB, resulted in reduced staining intensity of test protein bands and appearance of protein adducts of higher molecular weight (dimers, trimers etc.) (Fig.3.1).

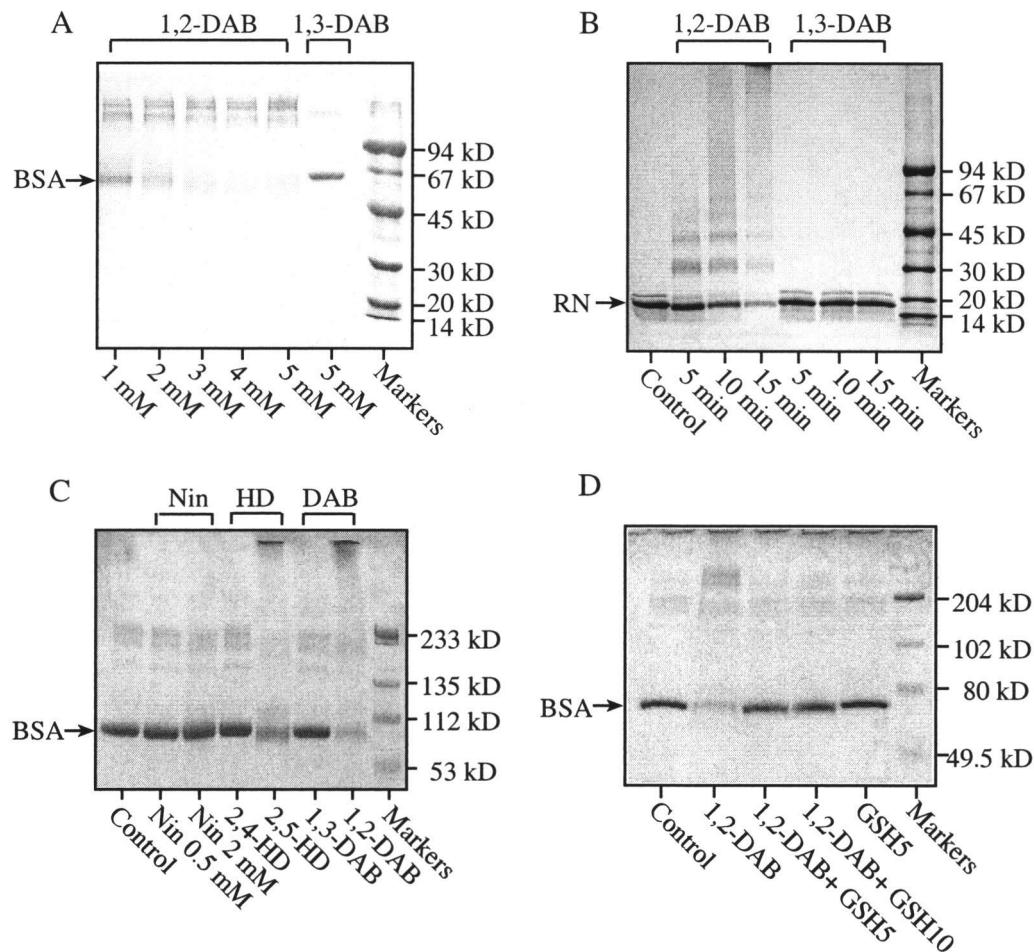


Figure 3.1. *In-vitro* effect of diacetylbenzene (DAB) isomers on test proteins bovine serum albumin (BSA) and ribonuclease (RN) analyzed by SDS-PAGE. (A) BSA (16mg/ml) was incubated for 30 min at 37°C with 1,2-DAB (1-5mM) or 1,3-DAB (5mM). 1,2-DAB, but not 1,3-DAB, reduced the native protein and polymerized BSA in a concentration-dependent manner. (B) RN (16mg/ml) was incubated with buffer or 25mM 1,2-DAB or 1,3-DAB for 5-15min at 37°C. 1,2-DAB, but not 1,3-DAB, caused a temporal reduction of native protein and increase of 30kD and 45kD dimers and trimers, respectively. (C) BSA (16mg/ml) was incubated at 37°C for 16h with DAB isomers, hexanedione (HD) isomers, or ninhydrin at the stated concentrations. Insoluble protein adducts formed with 2,5-HD and 1,2-DAB remained on the top of the gel, with the native BSA band proportionately decreasing in staining intensity. No adducts were seen with ninhydrin (Nin), 2,4-HD or 1,3-DAB. (D) In the presence of glutathione (5 and 10mM) (GSH5, GSH10), 1,2-DAB did not decrease the intensity of BSA (10mg/ml) or trigger the formation of BSA-related polymers.

These changes were prominent after 30 min. of BSA incubation with 1mM 1,2-DAB and concentration-dependent up to 5mM (Fig. 3.1A). There was a gradual reduction of the intensity of RN bands (Fig. 3.1B) and the appearance of protein adducts of high molecular weight in 1,2-DAB-treated samples (Fig. 3.1A,B). Native protein reduction and corresponding polymer formation was more pronounced with 1,2-DAB (2mM) than 2,5-HD (2M); no clearcut changes were evident with equimolar concentrations of ninhydrin or of the corresponding non-chromogenic and non-neurotoxic isomers 1,3-DAB and 2,4-HD, respectively (Fig. 3.1C). When BSA was incubated with 1,2-DAB in the presence of GSH, protein reactivity failed to occur as judged by the absence of chromophore formation, preservation of native protein, and absence of high molecular weight species (Fig. 3.1D).

GAPDH and LDH activity

1,2-DAB, but not 1,3-DAB, caused the formation of high molecular weight species and inhibited the enzyme activity of test proteins (GAPDH, LDH) in a concentration-dependent manner (Fig. 3.2). The staining intensity of high molecular weight species increased for both GAPDH (Fig. 3.2A) and LDH (Fig. 3.2B) over the range of 1-5mM. Incubation of these proteins with 1,2-DAB for 30 min. resulted in a mean loss of enzyme activity of >50% (1mM),~85% (2mM) and ~100% (5 mM) for GAPDH (Fig. 3.2C) and ~20%, ~30% and ~90%, respectively, for LDH (Fig. 3.2D).

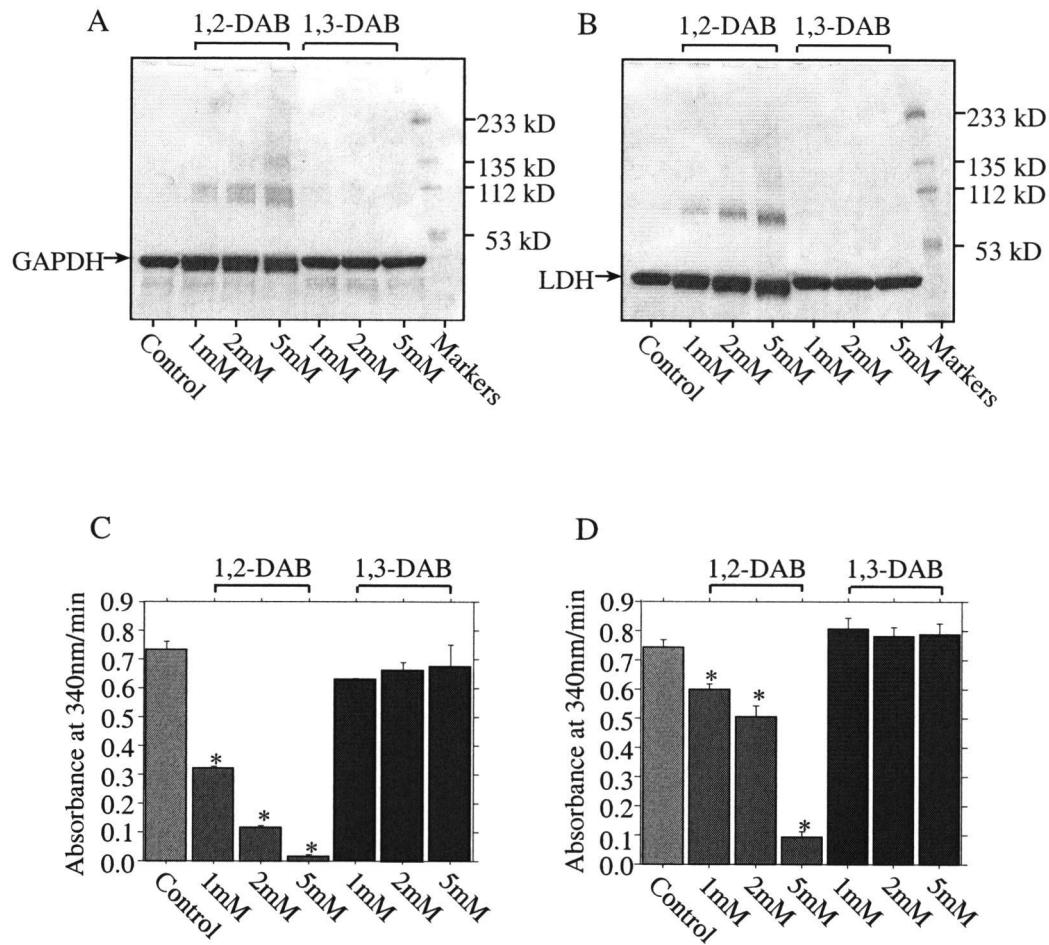
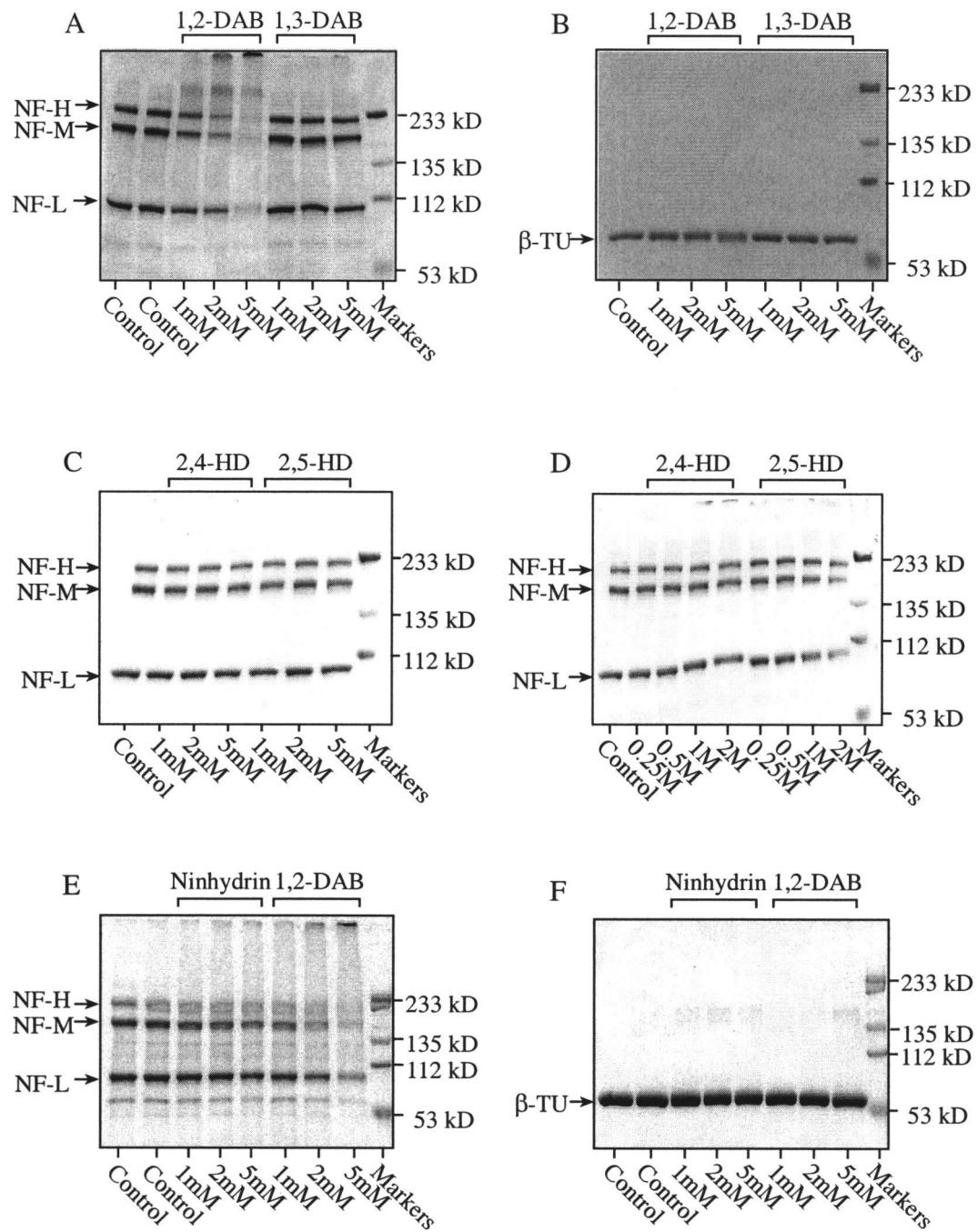


Figure 3.2. (A,B) Effect of diacetylbenzene (DAB) isomers on the protein state (SDS-PAGE) and enzyme activities of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH) after treatment *in vitro* with the test agents. One hundred microgram of GAPDH or LDH in 20 μ l buffer were incubated for 30 min at 37°C with 1-5mM 1,2-DAB or 1,3-DAB, or an equivalent volume of buffer (control). 1,2-DAB, but not 1,3-DAB, produced high molecular weight derivatives of GAPDH (A) and LDH (B) in a concentration-dependent manner. (C,D) Enzyme activity of these proteins decreased (GAPDH > LDH) with increasing 1,2-DAB concentration. * $p < 0.0001$. 1,3-DAB produced no significant changes in enzyme activity.

Neural protein reactivity

1,2-DAB, but not 1,3-DAB, reacted with purified NF proteins (NF-H, NF-M, NF-L) in a concentration-dependent manner (Fig. 3.3). At 1-5 mM 1,2-DAB, a qualitative inspection of band densities revealed greater reductions in NF-H and NF-M than in NF-L, with corresponding increases of high molecular weight proteins (Fig. 3.3A). By contrast, under the same conditions, 1-5mM 2,5-HD had no detectable effect (Fig. 3.3C), and changes were only seen at 2M 2,5-HD (Fig. 3.3D). No reactivity was seen with 2,4-HD (Fig. 3.3C,D). Reductions of NF subunit staining intensity were evident in the presence of ninhydrin; however, protein bands of high molecular weight were not detectable (Fig. 3.3E). No changes were evident when purified β -tubulin was incubated with 1 or 2 mM 1,2-DAB; there was some reduction in the density of the protein band at 5 mM 1,2-DAB relative to 1,3-DAB (Fig. 3.3B). Ninhydrin produced little or no detectable change in β -tubulin staining (Fig. 3.3F).

Figure 3.3. *In-vitro* effect of ninhydrin, diacetylbenzene (DAB) isomers or hexanedione (HD) isomers on purified neurofilament (NF) triplet proteins and β -tubulin. Six micrograms of each protein were treated with the test article or buffer (control) for 30 min at 37°C and resolved by SDS-PAGE as described in Methods. (A) Treatment of NF with 1,2-DAB (1-5mM), but not with 1,3-DAB or buffer (control), resulted in a concentration-dependent decrease in intensity of native triplet proteins (NF-H and NF-M > NF-L), with corresponding formation of increasing amounts of high molecular weight proteins. (B) Treatment of β -tubulin with 1,2-DAB, 1,3-DAB or buffer was without significant effect. (C,D) Treatment of NF with 2M 2,5-HD, but not with lower concentrations of 2,5-HD or with 2,4-HD (1mM-2M), or with buffer (control), resulted in a marginal decrease in intensity of native triplet proteins. (E) Ninhydrin and 1,2-DAB reduced native NF protein in a concentration-dependent manner, but high molecular weight species (top of gel) were seen only with the higher concentrations of 1,2-DAB. (F) Ninhydrin and 1,2-DAB were largely unreactive with β -tubulin.



Spinal cord protein reactivity in vitro

1,2-DAB, but not 1,3-DAB, altered NF subunit proteins in a manner that was both concentration -dependent (Fig. 3.4) and time-dependent (Fig. 3.5). Incubation of spinal tissue with increasing 1,2-DAB concentrations for 30 min. resulted in corresponding reductions of band intensities of NF-H, NF-M and NF-L (Fig. 3.4A-C). Similarly, incubation of tissue with 2mM 1,2-DAB for 15-60 min. produced corresponding increases in high molecular weight proteins that were recognized by monoclonal antibodies to individual NF subunits (Fig. 3.5A-C). β -Tubulin failed to show reactivity and form polymers with 1,2-DAB when spinal tissue was incubated up to 60 min. with 2mM 1,2-DAB (Fig. 3.4D, 5D). However, high molecular weight protein immunoreactive to anti- β -tubulin was evident after treatment with 10mM 1,2-DAB for 30 min. (Fig. 3.4D).

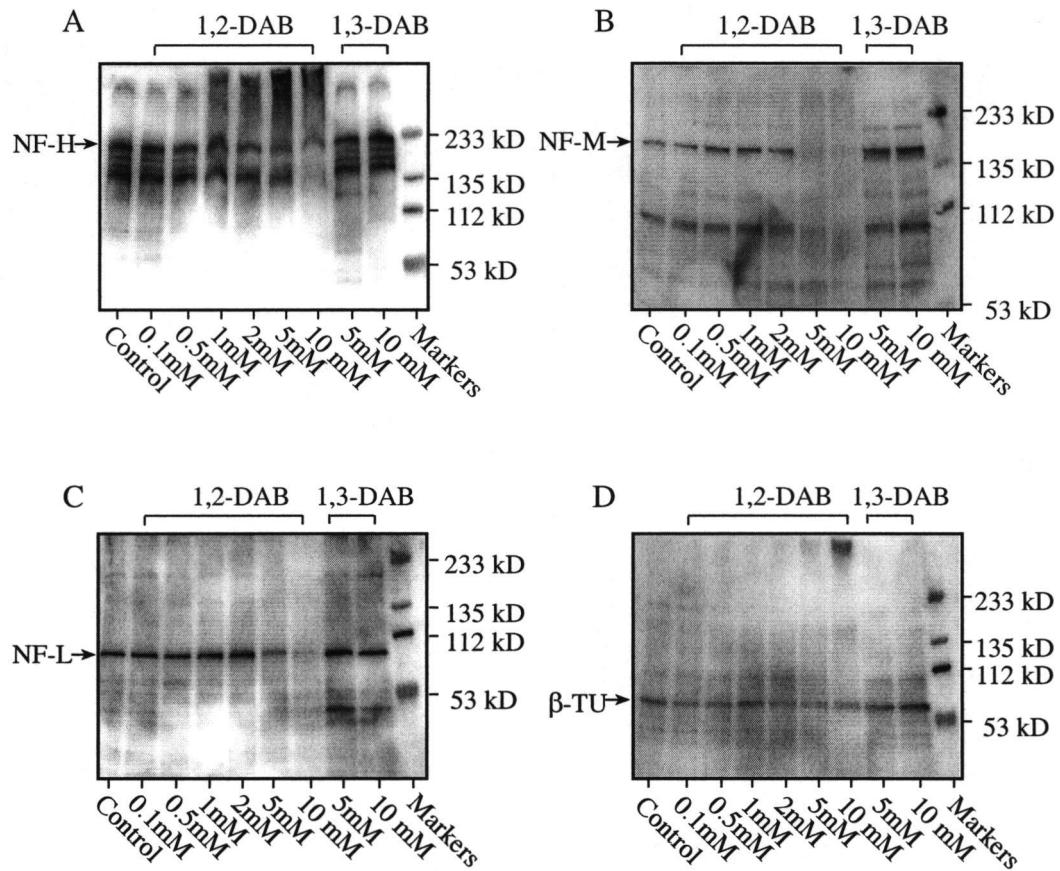


Figure 3.4. Western blots of rat spinal cord slices incubated with different concentrations (0.1–10 mM) of diacetylbenzene (DAB) isomers or buffer (control) for 30 min. at 37°C. Six micrograms of protein from each sample were resolved by SDS-PAGE. Proteins were transferred to PVDF membranes as described in Methods. Blots were probed with (A) anti-neurofilament H (NF-H) monoclonal antibody; (B) anti-neurofilament M (NF-M) monoclonal antibody; (C) anti-neurofilament L (NF-L) monoclonal antibody, and (D) anti-β-tubulin (β-TU) monoclonal antibody. (A) Native protein was reduced and high molecular weight proteins were formed selectively when NF-H was treated with 1,2-DAB but not 1,3-DAB. (B,C) Immunostaining for NF-M and NF-L was reduced by 5 or 10 mM 1,2-DAB but not by equivalent concentrations of 1,3-DAB. (D) Immunostaining for β-tubulin appeared unaltered up to 5 mM 1,2-DAB and 10 mM 1,3-DAB. Evidence of high molecular weight derivatives of β-tubulin was seen with 10 mM 1,2-DAB.

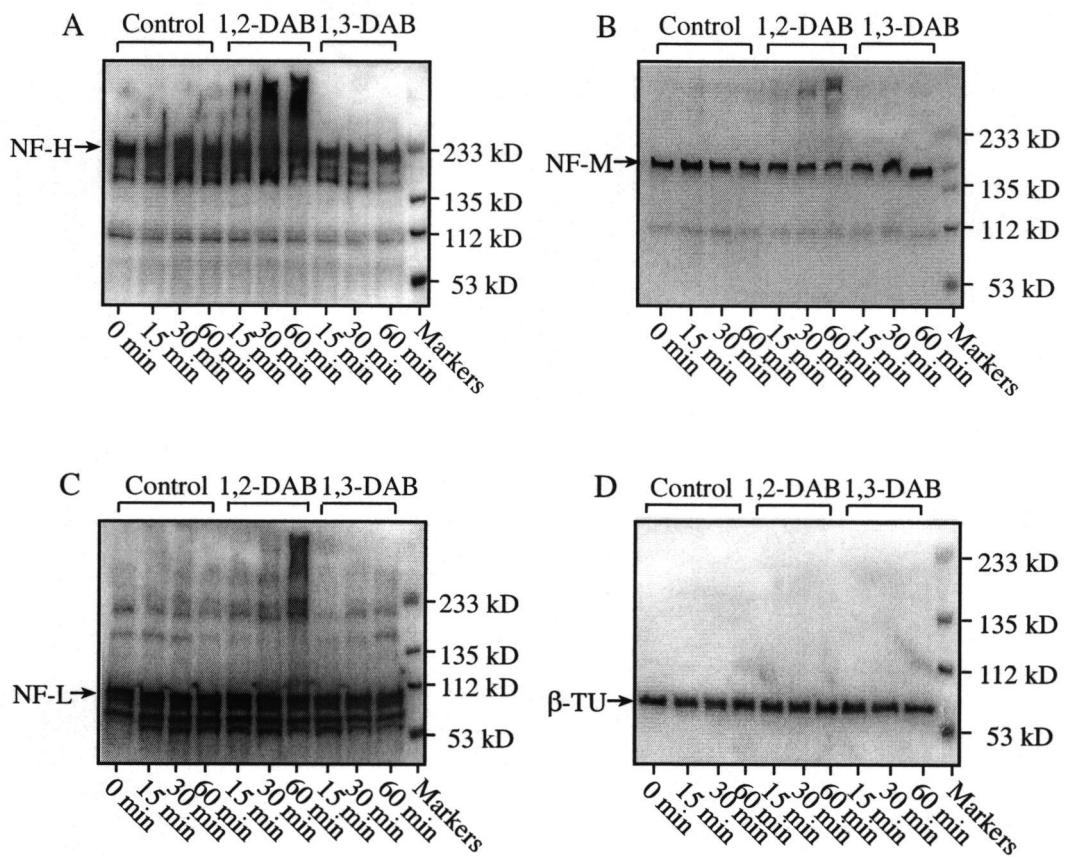


Figure 3.5. Western blots of rat spinal cord slices incubated for varying period of time (0-60 min) with 2mM diacetylbenzene (DAB) isomers or buffer (control) at 37°C. SDS-PAGE and immunostaining are as described in figure 4 legend. (A-C) All NF subunit blots showed a temporal increase of NF polymers of high molecular weight after treatment with 1,2-DAB but not 1,3-DAB or buffer. (D) No polymers of β -tubulin (β -TU) were seen either 1,2-DAB or 1,3-DAB.

Sciatic nerve protein reactivity in vivo.

Bathing intact sciatic nerve in 1,2-DAB, but not in 1,3-DAB, selectively and differentially changed the pattern of cytoskeletal proteins recognized by monoclonal antibodies specific for NF proteins (Fig. 3.6A,B,D), with no apparent

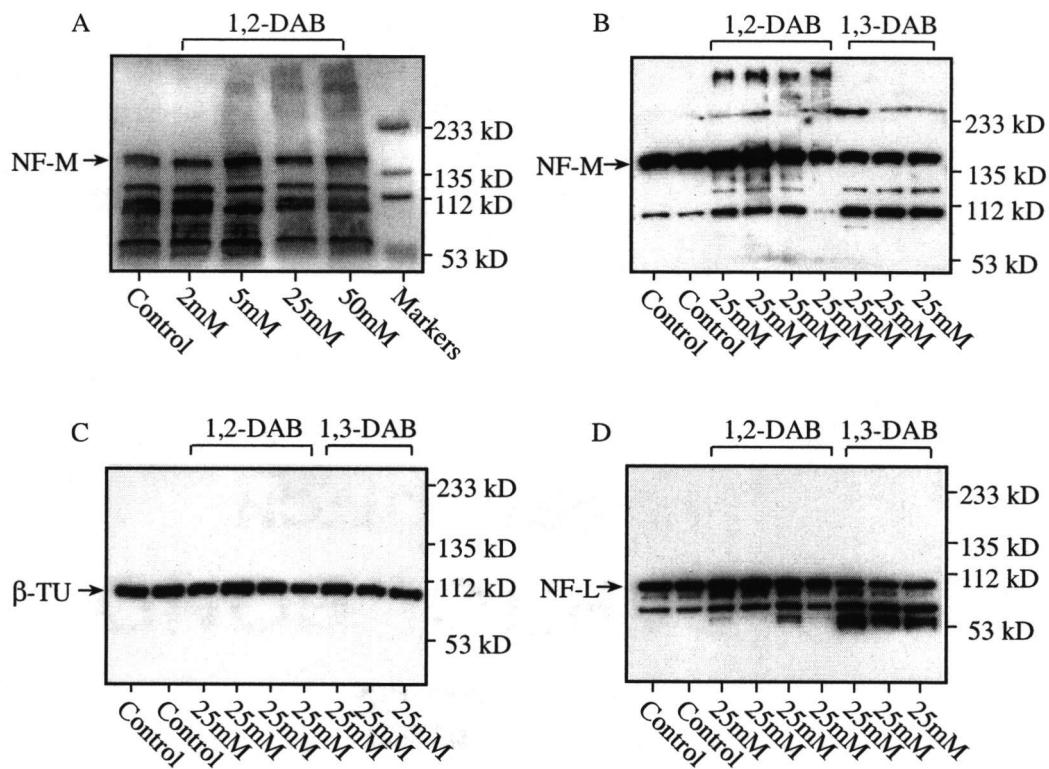


Figure 3.6. Effect of diacetylbenzene (DAB) isomers (2-50mM) or vehicle (phosphate-buffered saline) on intact rat sciatic nerve bathed *in vivo* for 5 min at RT. Western blot of 6 µg intrafascicular tissue proteins separated on 4-20% SDS-PAGE and immunopropbed with anti-neurofilament (NF) peptide (NF-M, NF-L) or anti-β-tubulin (β-TU) monoclonal antibodies. (A) 1,2-DAB treatment induced a concentration-dependent increase of immunostaining corresponding to high molecular weight derivatives of NF-M. (B) Immunostaining with anti-NF-M showed polymer formation with 1,2-DAB but not with 1,3-DAB. (C, D) Polymers of β-tubulin and NF-L were not seen after treatment with either 1,2-DAB or 1,3-DAB.

changes in β -tubulin (Fig. 3.6C). Formation of high molecular weight species of NF-M was evident in sciatic nerve tissue treated with 1,2-DAB, but not 1,3-DAB (Fig 3.6 A,B).

DISCUSSION

The present findings reveal a robust relationship among DAB structure, amino acid reactivity, targeting of neural (NF) and non-neural (GAPDH, LDH) protein structure and function, respectively, and chromophore formation and neurotoxicity (axonopathy) potential. Whereas the neurotoxic aromatic γ -diketone, 1,2-DAB, is a chromogenic amino acid and protein reagent, the non-neurotoxic γ -diketone, 1,3-DAB, lacks both properties. 1,2-DAB targets NF (relative to microtubule) proteins *in vitro* and *in vivo* at markedly lower concentrations than the corresponding neurotoxic aliphatic γ -diketone 2,5-HD. Neurofilament protein subunits NF-H and NF-M are more susceptible than NF-L and β -tubulin to 1,2-DAB. These observations correlate with the proximal (1,2-DAB) and distal (2,5-HD) induction of NF-filled axonal swellings and segregated intact microtubules during systemic treatment with these aromatic and aliphatic γ -diketones, respectively. These phenomena do not occur after comparable exposure to their corresponding non-pyrrole-forming, non-neurotoxic isomers (1,3-DAB, 2,4-HD) or to ninhydrin (Spencer et al., 1978; DeCaprio et al., 1982; Kim et al., 2001).

Amino acid and protein reactivity

1,2-DAB reacts with various amino acids and proteins, including those present in human and animal skin, to form colored pigments (Nan'ya et al., 1977; Kim et al., 2001). Among the amino acids tested here, those containing a primary amino group -- such as lysine and glycine --- react most strongly with 1,2-DAB to form a purple-blue chromophore at pH 7.0. Basic amino acids, such as arginine and histidine, are much less reactive, while the thioamino acid cysteine, is essentially unreactive with 1,2-DAB under these conditions. While prolonged incubation of cysteine with 1,2-DAB produces a chromophore closely resembling the pigment formed between 1,2-DAB and human skin, the latter reaction is near-instantaneous and therefore difficult to attribute to cysteine, as proposed by Nan'ya and Maekawa

(1977b). Lysine more than any other amino acid is a likely candidate for rapid reaction with 1,2-DAB because of the presence of its exposed (and therefore reactive) ϵ -amino group. Similarly, 2,5-HD is reported to target the ϵ -amino group of lysine to form pyrroles, the first step in the formation of cross-linked proteins (Graham et al., 1982; DeCaprio et al., 1982). Just as ornithine forms insoluble polymers with 2,5-HD (Yamamoto et al., 1994), we find that reactive amino acids (lysine) and proteins (BSA) form a polymer (solid or rubbery, respectively, after prolonged incubation) that is insoluble in water, common organic solvents and SDS. The formation of polymers by reaction of 2,5-HD with proteins *in vitro* is blocked by GSH, which is thought to undergo cross-linking and thereby prevent pyrrole oxidation and polymerization (Zhu et al., 1995). GSH also blocks the formation of chromophore and polymers formed between BSA and 1,2-DAB. Since lysine forms a blue pigment *in vitro* both in the presence of oxygen and in its absence (N_2 atmosphere, up to 24h, RT, pH 7.0), we are uncertain whether the chromophore corresponds to the proposed 1,2-DAB-related isoindole monomer (see below) or a polymerized derivative.

The chemical structure of the chromophore formed between 1,2-DAB and amino acids/proteins has yet to be established. However, this is unlikely to be related to the bluish chromophore (Ruheman's purple) formed between ninhydrin and amino acids because ninhydrin (a) fails to form high molecular weight protein adducts with BSA, NF, β -tubulin), and (b) does not induce behavioral and pathological indices of neurotoxicity in systemically treated animals (Kim et al., 2001). The blue chromophore formed by the aromatic γ -diketone neurotoxin 1,2-DAB compares with the orange-reddish, pyrrole-related pigment associated with the aliphatic γ -diketone 2,5-HD (Johnson et al., 1995). Nan'ya and associates have proposed a number of structures for the 1,2-DAB-derived chromogenic polymer, including the pyrrole-related isoindole structure shown in figure 7 (Nan'ya and Maekawa, 1975, 1977a,b; Nan'ya et al., 1982). Our preliminary GC-MS studies show that lysine and 2,5-HD (0.1M) or 1,2-DAB (0.1M), under conditions of high pH (~ 11), form identical elution peaks (21.73 > 21.07 > 22.06/min) and

fragmentation patterns corresponding to the pyrrole moiety of both chromophores. Similarly, the urinary chromophore collected from rats treated with 1,2-DAB (20 mg/kg/d i.p. for 8-9 days) demonstrates the presence of pyrrole by GC-MS (M-S Kim and J. Muniz et al., unpublished data). Others have shown that 2,5-HD forms pyrrole adducts with BSA, ovalbumin, RN and NF protein (Graham et al., 1982; DeCaprio et al., 1982; Anthony et al., 1983a; Yan et al., 1996). These pyrrolated proteins are proposed to undergo further oxidative reaction *in vitro* to form secondary, cross-linked derivatives (dimers and higher polymers). Based on lysine (chromogenicity) and protein reactivity (polymer formation), the present findings show that 1,2-DAB is at least three orders of magnitude more reactive than 2,5-HD. Given that the rate of γ -diketone-induced pyrrole formation and polymerization are related to neurotoxic potency for axonopathy, as shown for 2,5-HD (low reactivity/potency) versus DMHD (20-30X greater reactivity/potency than 2,5-HD) (Anthony et al., 1983a), it is likely that the neurotoxic potency of 1,2-DAB far exceeds that of 2,5-HD. It is noteworthy that whereas axonal neurofilaments accumulate in distal axons of animals treated with 2,5-HD (Spencer and Schaumburg, 1977a,b), both 1,2-DAB and DMHD cause proximal accumulations, presumably because these NF proteins are rapidly cross-linked and polymerized and cannot be transported further along the axon (DeCaprio et al., 1982; Graham, 1995). Formal toxicology studies are needed to compare the protein reactivity and neurotoxic potency of 2,5-HD and 1,2-DAB; these appear to be the active neurotoxic metabolites of the organic solvents *n*-hexane and 1,2-diethylbenzene, which have a five-fold difference in neurotoxic potency (Gagnaire et al., 1990).

The protein reactivity of 1,2-DAB and formation of high molecular weight adducts appear to correlate with the lysine content of the proteins studied here (*see* ProtParam database at <http://www.expasy.ch/>). Both *in vitro* and *in vivo*, rat NF-H (13-16% lysine) and NF-M (11-12%) formed polymers more readily than either NF-L (6-7%) or tubulin (3-4%). Other potential protein targets involved in anterograde axonal transport include the rat microtubule-associated protein tau (7-10% lysine), the rat motor proteins kinesin (chain A, 8%; chain B, 12%) and dynein

(8%), and the rabbit glycolytic enzymes GAPDH (8%) and LDH (7-8%). While GAPDH and LDH are similarly reactive with 1,2-DAB *in vitro*, the enzyme inhibition at equimolar concentrations (1 mM, 30 min.) of GAPDH is greater (~50%) than that of LDH (~20%). These observations show that 1,2-DAB may differentially impact protein structure and function. Presumably, changes in protein function (e.g. GAPDH and LDH enzyme activity) relate to 1,2-DAB-reactive lysine moieties at the active site.

While the reaction of 1,2-DAB with proteins is non-specific, the present studies demonstrate important differences in reactivity and functional consequences. The test proteins GAPDH and LDH are both chromogenically reactive *in vitro*, but enzyme inhibition at equimolar concentrations (1 mM) was one-half and one-fifth, respectively.

Mechanisms of neurotoxicity

The molecular mechanisms underlying the neurobehavioral effects of 1,2-DAB, such as hyperactivity, drowsiness, are unknown. 1,2-DAB might react with free amino acids that subserve neurotransmitter function in the central nervous system (CNS). The CNS neurotransmitters glycine and GABA are highly reactive with 1,2-DAB as judged by the rate and amount of chromophore formation *in vitro*. Free GABA is the dominant brain inhibitory neurotransmitter. Free glycine, which serves as an important *inhibitory* neurotransmitter in the brainstem and spinal cord, binds to strychnine-sensitive receptors that regulate motor neuron activity (Hunter et al., 1989). Very low levels of glycine are also required to activate ion channels associated with the neurotransmitter receptor sensitive to *N*-methyl-D-aspartate (NMDA), one of several glutamate receptors that mediate CNS *excitation* (Kandel et al., 2000). Reduction in the level of free glycine through isoindole formation with 1,2-DAB might therefore be expected to impact NMDA-receptor function (decreased excitation state) before glycinergic and GABA receptor function

(increased excitation through reduced inhibition). This provides a possible explanation for the observation that 1,2-DAB-treated rats become both drowsy and hyperreactive to stimuli.

Neuropathological changes in spinal cord and spinal roots correlate with the onset of limb weakness in 1,2-DAB-treated rats (Kim et al., 2001). Focal NF-filled axonal swellings in the initial segments of motor nerve fibers are very early features. Comparable neuropathological changes are found in rats treated with DMHD or β,β' -iminodipropionitrile (IDPN) as a result of the arrest of slow anterograde NF transport (Griffin et al., 1978; Sickles, 1989). The swollen axon disrupts the integrity of the myelin sheath, which is required for maintenance of normal impulse conduction. Since peripheral nerves distal to the sites of axonal swelling are relatively normal at this time (no prominent atrophy or wallerian-like degeneration), the beginning neuropathological picture during 1,2-DAB treatment is more consistent with a radiculopathy than a neuropathy. However, treatment with 1,2-DAB or 1,2-DEB does result in a decrease of both motor and sensory nerve conduction velocities of peripheral nerve fibers in the rat tail (Gagnaire et al., 1990, 1991). DMHD, another potent γ -diketone that rapidly cross-links target proteins (notably neurofilaments), causes giant axonal swellings to form in proximal axons (Anthony et al., 1983a). Cross-linking of NF proteins and their anterograde transport arrest likely account for the formation of intraspinal and spinal root giant axonal swellings and the onset of limb weakness in DMHD (Anthony et al, 1983b) and in 1,2-DAB neurotoxicity (Fig 3.7).

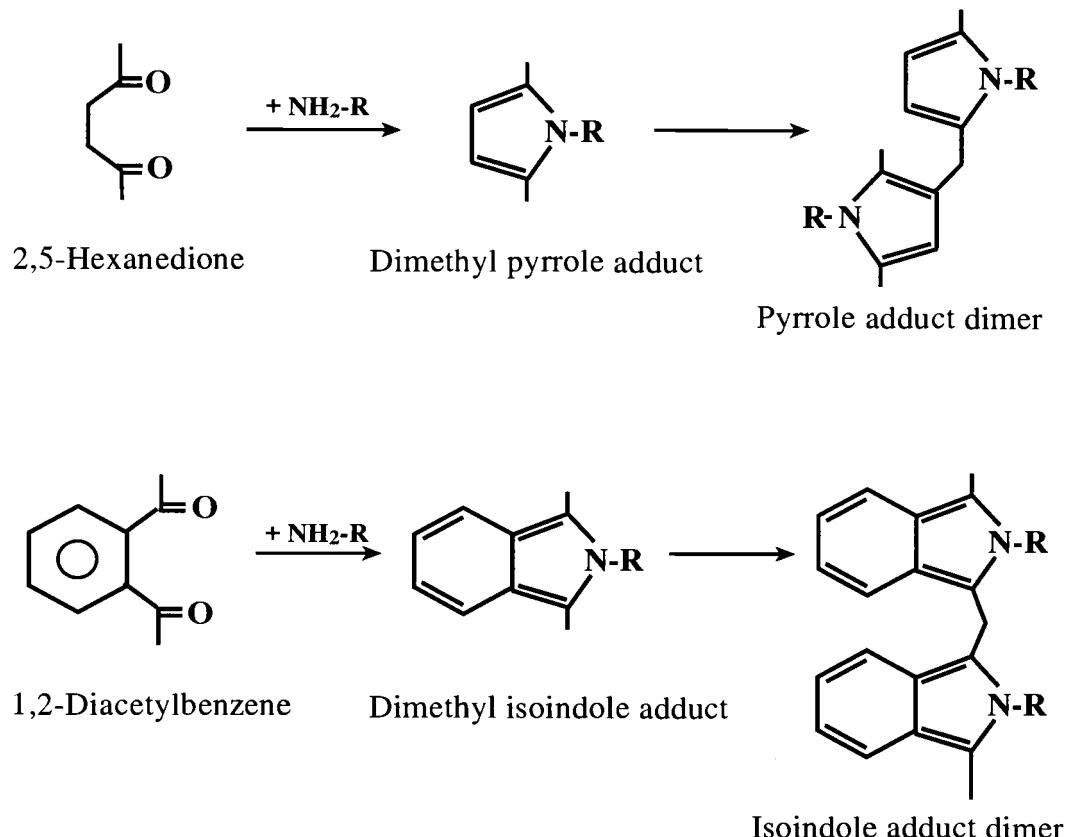


Figure 3.7. Structure and hypothetical reactions of 2,5-HD and 1,2-DAB with amino acids and proteins (R-NH_2), forming pyrrole (DeCaprio et al., 1982) and isoindole adducts, respectively. Chromogenicity is related to the first or second step of polymer formation: at pH ~7, 2,5-HD forms a yellow chromophore and 1,2-DAB a purple-blue chromophore.

A prominent feature of proximal neurofilamentous axonopathies is the segregation of microtubules that penetrate amassed neurofilaments in giant axonal swellings. This is seen in the peripheral nerves of rats systemically treated with 2,5-HD or IDPN and can also be reproduced by local application or intraneuronal injection of high concentrations of either agent to intact sciatic nerves in living animals (Zagoren et al., 1983; Griffin et al., 1983). We have observed (*data not shown*) a similar phenomenon after systemic treatment of rats with 1,2-DAB (10–20 mg/g/d i.p.). Western blot analysis of intrafascicular nerve tissue showed that NF-M was more sensitive than β -tubulin to 1,2-DAB-induced cross-linking.

Similarly, in spinal cord *in vitro*, NF-H and NF-M were more sensitive than β -tubulin to 1,2-DAB. Mice heterozygous or homozygous for the NF-H null mutation fail to develop neurofilamentous swellings and segregated microtubules in motor neurons after systemic IDPN treatment. These results indicate that the NF-H subunit is a key mediator of IDPN-induced axonopathy (Zhu et al., 1998). Whether IDPN (like 1,2-DAB) is related to lysine reactivity in this lysine-rich NF subunit is unknown. While these data suggest that specific NF subunits are preferentially targeted by both compounds, application of a high concentration of 1,2-DAB (0.2M, a saturated solution) or 2,5-HD (8M) to intact sciatic nerve for 5 min to 2h induced microtubule aggregation only in the latter (*data not shown*). The explanation for this unexpected negative result is not apparent.

It is important to emphasize that neuropathological changes (proximal axonal swellings and microtubule segregation) in 1,2-DAB-induced radiculopathy are seen in the absence of overt nerve fiber atrophy and axonal degeneration in the more distally located peripheral nerves. These two phenomena are likely to be unrelated to the mechanisms underlying the apparent arrest of slow anterograde NF transport. Study of animals treated with 2,5-HD or IDPN demonstrate that prominent nerve fiber atrophy occurs distal to NF-filled swellings in which fast anterograde axonal transport is maintained (Griffin et al., 1978; Spencer and Griffin, 1982). The recent suggestion of LoPachin (2000) that nerve fiber atrophy, not giant axonal swelling, is a more prominent hallmark of aliphatic γ -diketone axonopathy is based on studies of animals treated with large doses of 2,5-HD in which proximal regions (spinal roots) were not examined for the presence or absence of NF-filled swellings. Single teased nerve fibers reveal that distal, wallerian-like degeneration also occurs below focal nerve fiber swellings in 2,5-HD axonopathy (Spencer and Schaumburg, 1977a). However, it is clear from more recent studies that the trigger for wallerian degeneration is unrelated to NFs. This is evident from the presence of 2,5-HD-induced peripheral nerve degeneration in both wild-type and transgenic mice lacking genes coding for NF triplet proteins (Stone et al., 1999, 2001). The precise trigger for the onset of distal axonal

degeneration in γ -diketone axonopathy is unknown: possibilities include (a) chemical modification of motor or other proteins involved in axonal transport or (b) the accumulation of axonal organelles, release of their calcium-rich contents into the axoplasm, and activation of calcium-dependent calpains that destroy local axon integrity protein (Spencer et al., 2001a,b). While the mechanism underlying axonal degeneration is an important question to resolve, the 1,2-DAB radiculopathy model shows that proximal giant axonal swelling (not axonal atrophy or degeneration) correlates with the onset of marked limb dysfunction.

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Chapter 4

Conclusion

These studies with 1,2-diacetylbenzene (1,2-DAB) have increased knowledge on the neurotoxicity of aromatic hydrocarbons as follows:

- (1) A specific aromatic hydrocarbon structure (1,2-DAB) has been shown to cause neurotoxicity.
- (2) Molecular and cellular targets of 1,2-DAB have been identified.
- (3) 1,2-DAB has been shown to induce neurofilamentous axonopathy.
- (4) Aliphatic γ -diketone 2,5-HD and aromatic γ -diketone 1,2-DAB share similar mechanisms of neurotoxicity.

Several widely used aromatic hydrocarbon solvents reportedly induce blue-green discoloration of tissue and urine in animals and humans. The chromophore is proposed to result from an isoindole reaction with amino groups in proteins (Nan'ya and Maekawa, 1977a,b; Kim et al., 2001).

1,2-DAB, the putative metabolite of 1,2-DEB, behaves as a neurotoxic substance. Animals treated with 1,2-DAB developed limb weakness associated with proximal giant axonal swellings filled with 10nm neurofilaments. Neurofilaments accumulated in the neuronal soma, intraspinal regions, proximal ventral roots of peripheral motor axons and dorsal roots (Kim et al., 1999). Continued treatment of rats with 1,2-DAB results in primary axonal degeneration followed by secondary vacuolar demyelination of spinal roots indistinguishable from the neuropathology associated with chronic treatment with musk tetralin (Spencer et al., 1979a,b, 1980a).

The chromogenicity of 1,2-DAB was related to neurotoxicity but these properties were apparently not induced not related to a ninhydrin reaction. Ninhydrin-treated rats failed to develop blue discoloration of internal organs and showed no neurological symptoms. Ninhydrin did not form higher molecular weight bands with neuronal and non-neuronal proteins.

The blue chromogen induced by treatment with 1,2-DAB can be used as a possible urinary biomarker of exposure to aromatic solvents with neurotoxic potential. Biomarkers can alert one to avoid exposure to specific substances, as well as offering an early warning to avoid further exposure.

The structural relationships (γ -diketone) and neurotoxic effects (neurofilamentous axonopathy) shared by 1,2-DAB, DMHD, 3-methyl-2,5-hexanedione, and 2,5-HD suggest the possibility of similar molecular mechanisms. 1,2-DAB, compared with 2,5-HD, showed high protein reactivity and neurotoxic potency. Much lower concentrations of 1,2-DAB versus 2,5-HD were required to reduce native protein and form polymers with BSA, ribonuclease or NF proteins.

Neuronal proteins were differentially reactive with 1,2-DAB. In the western blot of the spinal cord treated with 1,2-DAB *in vitro*, NF-H and NF-M were more sensitive than β -tubulin. Similarly, sciatic nerve tissue treated with 1,2-DAB showed that NF-M was more sensitive than β -tubulin.

The common amino acid, protein reactivity and comparable neurofilamentous pathology associated with 1,2-DAB and 2,5-HD, suggests that the neurotoxic properties of these two gamma diketones develop by common mechanisms. In particular, other compounds, such as IDPN induce comparable patterns of axonal degeneration. The potent 3,4-dimethylated derivative of 2,5-HD (DMHD) to produce a similar spatial-temporal pattern of nerve damage (Fig. 4.1).

In summary, this study has identified the molecular and cellular targets of 1,2-DAB in the central and peripheral nervous system of rats. The results reported in this thesis are expected to enhance our understanding of the toxic properties of other chromogenic aromatic hydrocarbons, such as tetralin and hydroxyquinone

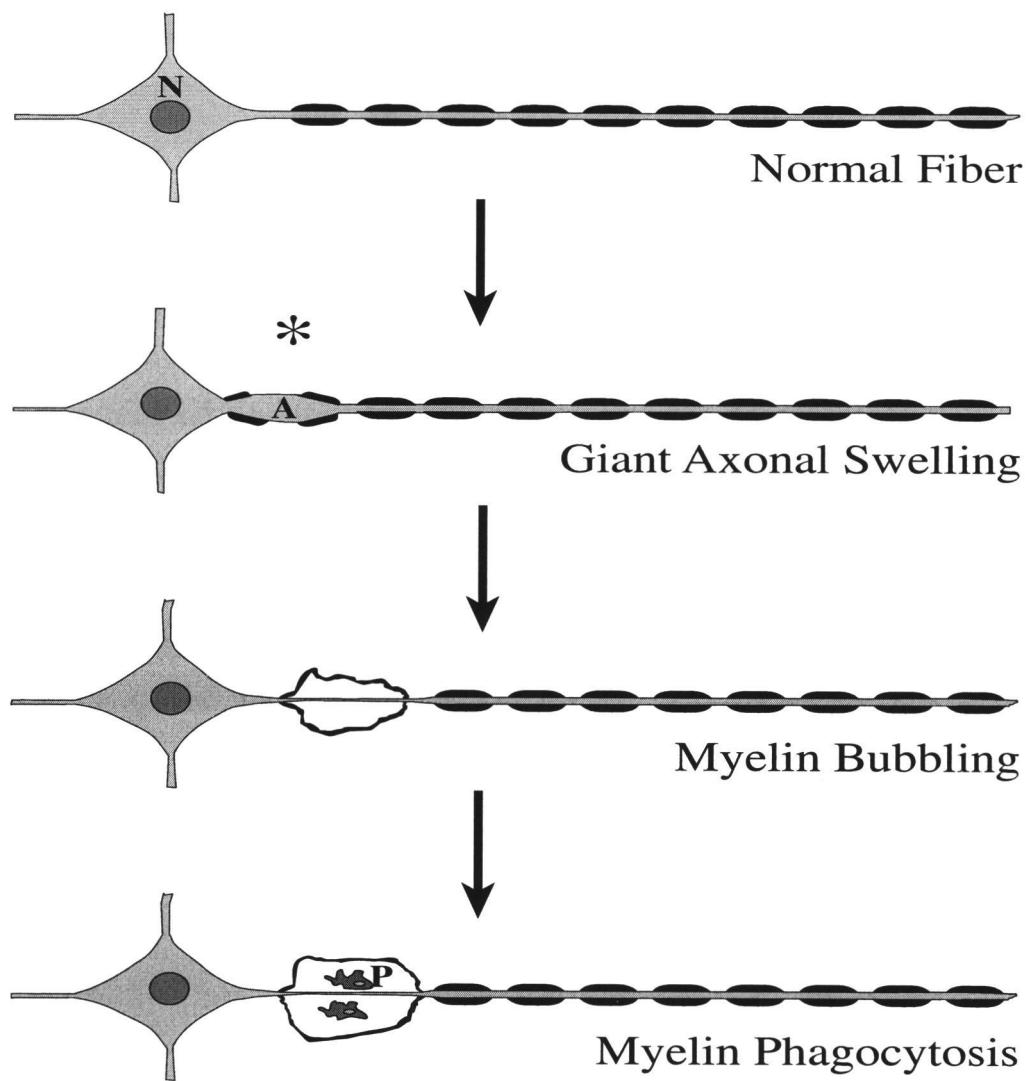


Figure 4.1. Sequence of CNS and PNS nerve fiber pathology: Repeated systemic treatment with 1,2-diacetylbenzene induces the formation of proximal giant axonal swellings (*) containing excessive members of neurofilament. The affected region undergoes myelin splitting and phagocytosis. Subsequently the axon undergoes local segmental demyelination and remyelination (not shown)

Since 1,2-DAB targets a broad spectrum of proteins, it is likely that several other targets contribute to the neuropathologic response to this compound. Since these targets may be selected in part on the basis of their lysine contents, future studies ought to seek to determine, on this basis of this property, other molecular targets by 1,2-DAB. Other functions that might be relevant are the longevity of proteins and their need for transport run great distances with cells such as neurons. Specific protein targets could also be examined using microarray and proteomic techniques to determine 1,2-DAB *vs.* 1,3-DAB-induced change in transcription and translation.

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