TOXICOLOGY AND APPLIED PHARMACOLOGY **88,** 370–382 (1987)

2,5-Hexanedione Alters Microtubule Assembly

I. Testicular Atrophy, Not Nervous System Toxicity, Correlates with Enhanced Tubulin Polymerization[1]

KIM BOEKELHEIDE

Department of Pathology and Laboratory Medicine, Brown University, Providence, Rhode Island 02912

Received January 31, 1986; accepted November 4, 1986

2,5-Hexanedione Alters Microtubule Assembly. I. Testicular Atrophy, Not Nervous System Toxicity, Correlates with Enhanced Tubulin Polymerization. BOEKELHEIDE, K. (1987). *Toxicol. Appl. Pharmacol.* **88,** 370–382. Charles River CD rats (200 g) were divided into three groups receiving either 1% 2,5-hexanedione (2,5-HD) or 0.035% 3,4-dimethyl-2,5-hexanedione (DMHD) in the drinking water or water alone (control) for 4 weeks. The two treated groups experienced similar nervous system dysfunction and systemic toxicity. Testicular toxicity, as evidenced by histological changes and decreased testis weight, was present only in 2,5-HD-treated rats. Tubulin was purified from brain and testis and assembly properties were determined. Purified brain and testis tubulin derived from the 2,5-HD-intoxicated rats displayed altered assembly with a shortened nucleation phase and more rapid rate of elongation. Brain tubulin from DMHD-intoxicated rats displayed assembly behavior similar to controls, while testis tubulin from DMHD-intoxicated rats displayed assembly behavior intermediate between the control and 2,5-HD tubulin preparations. The presence of γ-diketone-induced assembly alterations following *in vivo* intoxication was accompanied by the formation of a high-molecular-weight protein identified as crosslinked tubulin. From these data, we conclude that microtubule assembly alterations are not etiologic in the development of nervous system dysfunction following intoxication but may represent the biochemical mechanism of 2,5-HD-induced testicular atrophy.

n-Hexane is an environmental toxicant which, following chronic exposure, produces peripheral polyneuropathy and testicular atrophy (Krasavage *et al.*, 1980). Microsomal oxidation of *n*-hexane yields the ultimate active metabolite 2,5-hexanedione (2,5-HD).[2] Histopathological examination of the peripheral nervous system reveals characteristic massive accumulations of neurofilaments (Spencer and Schaumburg, 1975, 1977) while the testicular atrophy is first manifested by degenerative changes within Sertoli cells (Chapin *et al.*, 1982, 1983). Reaction of 2,5-HD with tissue nucleophiles, particularly protein lysyl amines, leads to formation of cyclic covalent adducts called pyrroles (DeCaprio *et al.*, 1982). The resulting alterations in charge and solubility of derivatized neuro-

[1] This project was supported by Grant R01 OH 02191 from the National Institute for Occupational Safety and Health of the Centers for Disease Control and a Pharmaceutical Manufacturers Association Foundation Faculty Development Award in Toxicologic Pathology. The author thanks Julia Eveleth for excellent technical assistance.

[2] Abbreviations used: DMHD, 3,4-dimethyl-2,5-hexanedione; DMP, 2,5-dimethylpyrrole; 2,5-HD, 2,5-hexanedione; GTP, guanosine 5′-triphosphate; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; tV_{max}, time required to reach maximal velocity of assembly; V_{max}, maximal velocity of assembly.

0041-008X/87 $3.00

filaments (DeCaprio *et al.*, 1983), pyrryl-dependent crosslinking between adjacent neurofilaments (Graham *et al.*, 1982a,b, 1984), and disruption of cytoskeletal interconnections (Griffin *et al.*, 1983) have been proposed as pathogenetic mechanisms in the development of the neurofilamentous neuropathy (DeCaprio and O'Neill, 1985).

3,4-Dimethyl-2,5-hexanedione (DMHD) is a congener of 2,5-HD with several interesting chemical and toxicologic differences from the parent γ-diketone (Anthony *et al.*, 1983a,b). DMHD is approximately 20–30 times as potent a nervous system toxicant as 2,5-HD. In addition, with rats intoxicated to the same degree of nervous system dysfunction, 2,5-HD-treated animals were found to have an accompanying testicular atrophy while DMHD-treated animals did not (Anthony *et al.*, 1983a). Chemically, DMHD cyclizes more rapidly with nucleophiles and demonstrates increased reactivity of the resulting pyrryl intermediate.

Microtubules play an important structural and functional role in both the nervous system and testis as demonstrated by the effect of microtubule poisons such as colchicine. In the nervous system, axonal transport is disrupted following exposure to colchicine (Fink *et al.*, 1973; Komiya and Kurokawa, 1980). In the testis, colchicine alters both Sertoli cells and germ cells leading to hypospermia and testicular atrophy (Handel, 1979; Russell *et al.*, 1981; Vogl *et al.*, 1983b). Because of the importance of microtubules in the nervous system and testis, the potential effects of 2,5-hexanedione upon this cytoskeletal system have been studied. The proportion of tubulin which polymerized into microtubules was not altered by chronic 2,5-HD intoxication (Nachtman and Couri, 1979). The extent and kinetics of tubulin polymerization *in vitro* were not changed significantly by addition, without preincubation, of high concentrations of *n*-hexane, methyl *n*-butyl ketone, or 2,5-HD to the assembly buffer (Selkoe *et al.*, 1978). We have recently reported alterations in microtubule assembly kinetics in 2,5-HD-intoxicated rats (Boekelheide, 1985, 1986). Microtubule assembly occurred both earlier and more rapidly in the crude brain extracts and in the temperature-dependent cycled brain tubulin preparations from treated rats.

In the following article, assembly and structural alterations in purified brain tubulin following *in vitro* incubation with 2,5-HD are investigated in detail (Boekelheide, 1987). In this paper, an intoxication protocol is described whereby 2,5-HD and DMHD produced similar systemic and nervous system toxicity but differential testicular effects. This selective intoxication protocol was used to study brain and testis crude supernatant properties and the assembly of tubulin purified from the brains and testes of treated and control rats. The following hypothesis is proposed: 2,5-HD intoxication alters microtubules in Sertoli cells resulting in testicular atrophy.

MATERIALS AND METHODS

Intoxication protocol. Charles River CD rats (200 g) were divided into three groups. The animals were housed at a constant temperature (70 ± 2°F) in 35–70% humidity with an alternating 12-hr light–dark cycle. All animals received food (Pro-Lab Rat, Mouse and Hamster Chow No. 3000) *ad libitum*. Rats treated with 2,5-HD (>98% pure, Eastman Kodak Co., Rochester, NY) received the toxicant as a 1% solution in the drinking water. Rats treated with DMHD (>99% pure, the kind gift of Dr. Szakal-Quin, Duke University Medical Center, Durham, NC) received the toxicant as a 0.035% solution in the drinking water. Control animals received drinking water *ad libitum*. After 4 weeks of treatment, the animals were assessed for clinical neurotoxicity. On a smooth surface, the hindlimbs were extended back parallel to the tail and released. The time required for the rat to reposition the hindlimbs under the body was measured. The rats were then weighed and anesthetized with pentobarbital, and the brain and testes from each animal were removed and weighed individually.

Crude supernatants. Brains and testes from pairs of rats were combined and processed by three strokes of a glass/Teflon homogenizer at 1500 rpm on ice with 0.1 M 2-(N-morpholino)ethane sulfonic acid, 1 mM ethylene glycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid, 0.5 mM $MgCl_2$, 4 M glycerol, pH 6.75 (1 ml/g

tissue). Ultracentrifugation at 4°C for 45 min at 100,000*g* gave the crude supernatants.

Protein content was determined in duplicate by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The pyrrole content of the 2,5-hexanedione crude supernatants was determined with Ehrlich's reagent. Duplicate 50-μl aliquots of control and 2,5-hexanedione brain and testis crude supernatants were incubated with 40 μg chymotrypsin at 37°C for 30 min. Dimethyl sulfoxide (400 μl) was added by mixing followed by 1 ml of Ehrlich's reagent (12 mg/ml *p*-dimethylaminobenzaldehyde in 50% aqueous methanol with 1% HCl). The samples were incubated for 1 hr at 37°C, cooled, and the absorbance at 530 nm was determined. A standard curve was constructed by using 50-μl aliquots of 15 mg/ml bovine serum albumin in the supernatant buffer with added 2,5-dimethylpyrrole. Therefore, the pyrrole content of the 2,5-hexanedione crude supernatants is expressed in equivalents of 2,5-dimethylpyrrole above the background level produced by control. The amount of tubulin in the crude supernatants was determined by DEAE-filter binding (Borisy, 1972) using [*ring C-methoxyl*-^{3}H]colchicine (Amersham Corp., Arlington Heights, IL). Crude supernatant (50 μl) was incubated for 1 hr at 37°C with 50 μM colchicine and then bound to a fourply stack made from DEAE cellulose paper (Bio-Rad, Rockville Centre, NY). A standard curve of binding efficiency was constructed using DEAE-purified three times-cycled bovine brain tubulin. The binding efficiency (44%) was linear over a wide range of tubulin concentrations (0.29–2.08 mg/ml) and was not altered by *in vitro* reaction with 2,5-hexanedione.

Light and electron microscopy. A portion of each right testicle was fixed in 10% neutral buffered formalin. One-millimeter-thick cross sections were washed, dehydrated, and embedded in glycol methacrylate. Sections 2-μm thick were cut and stained with periodic acid-Schiff's reagent and hematoxylin. Negative staining was performed with 1% uranyl acetate (Boekelheide, 1987).

Tubulin purification and assembly. A temperature-dependent cycle of microtubule assembly and disassembly effects purification by differential centrifugation of the tubulin polymer following warm incubation and subsequent solubilization of the polymer in the cold and recentrifugation. Rat brain and testis tubulins were purified by DEAE-Sephacel binding and elution followed by temperature-dependent cycles of assembly and disassembly with minor modifications (Boekelheide, 1987). An additional DEAE-Sephacel washing step performed in duplicate with 0.1 M NaCl in 1 M sodium glutamate, pH 6.6, was included prior to tubulin elution with 0.4 M NaCl in 1 M sodium glutamate, pH 6.6. Rat brain tubulin from the *in vivo* experimental groups was analyzed for assembly properties after two cycles of temperature-dependent assembly and disassembly. The rat testis tubulin from the *in vivo* experimental groups was analyzed for assembly properties after three cycles of temperature-dependent assembly and disassembly. Microtubule assembly was measured as the change in optical density at 350 nm for samples adjusted to similar protein concentrations (Boekelheide, 1987).

Electrophoretic procedures. SDS–PAGE analyses with silver staining, densitometric scanning, and immunoblotting were performed as described in the companion article (Boekelheide, 1987). The nitrocellulose protein blot was stained with a mixture of mouse monoclonal antibodies recognizing epitopes of chick brain α-tubulin (Amersham Corp.) and sea urchin β-tubulin (the kind gift of Dr. Richard McIntosh, University of Colorado, Boulder, CO).

Statistical methods. For statistical purposes, the two testis weights from each animal were averaged to provide an average testis weight per animal. Data were analyzed for mean and standard error and compared by the two-tailed Student *t* test with significance assigned at $p < 0.05$.

RESULTS

Intoxication with 2,5-HD and DMHD

The intoxication protocol was devised to induce similar systemic and nervous system toxicity in the two treatment groups. Exposure to 1% 2,5-HD or 0.035% DMHD in the drinking water for 4 weeks produced this result. The weights of the 2,5-HD and DMHD animals did not differ significantly from each other at any time, while both were significantly different (at least $p < 0.001$) from the untreated control group at all time points after initiation of intoxication (Fig. 1). Both the 2,5-HD- and DMHD-treated rats showed a range of nervous system impairments including moderate and severe degrees of hindlimb weakness (Table 1).

The testicular effects of 2,5-HD and DMHD intoxication were assessed by comparing testicular weights and histology. The average testis weights of the 2,5-HD-treated rats (1.38 ± 0.03, mean ± SE, $n = 17$) differed significantly from control rats (1.72 ± 0.06, $n = 8$, $p < 0.001$) and from DMHD rats (1.57 ± 0.07, $n = 8$, $p < 0.005$), while the average testis weights of the DMHD-treated rats were not significantly different from controls. Histologically, the DMHD testes appeared sim-

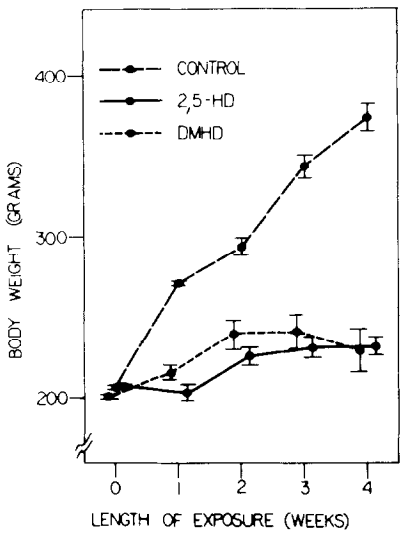

FIG. 1. The effect of intoxication upon body weight. Control rats ($n = 8$) and rats intoxicated with 1% 2,5-HD ($n = 17$) or 0.035% DMHD ($n = 8$) in the drinking water were weighed each week during 4 weeks of treatment and the means ± SE for body weight were determined.

ilar to controls while the testes from 2,5-HD animals tended to have larger and more numerous membrane-bound vacuoles basally located within Sertoli cells, occasional giant cells, and chromatin margination in spermatids (Fig. 2). These histologic features of 2,5-HD-induced testicular injury have been previously well described by Chapin *et al.* (1983).

Properties of Brain and Testis Crude Supernatants

Brain and testis crude supernatants obtained after 4 weeks of treatment were analyzed for protein concentration, pyrrole content, and the concentration of tubulin (Table 2). The protein concentration was significantly increased in the 2,5-HD brain and testis crude supernatants and in the the DMHD testis crude supernatants, though the magnitude of this increase was small (10–23%). Pyrrole content was determined by the Ehrlich's reaction using DMP as a standard. In 2,5-HD crude supernatants, there was an excess of 1.5–1.8 nmol of DMP equivalents per milligram protein when compared with control. The proportion of tubulin in the supernatants was determined by binding [^{3}H]colchicine. Treatment did not alter the tubulin content of crude supernatants (brain, average of 12%; testis, average of 2.5%).

Assembly of Brain Tubulin Derived from Intoxicated Rats

A series of preliminary experiments established that (1) microtubule assembly properties were altered by 2,5-HD intoxication and (2) the alterations in assembly could be explained by a modification of tubulin alone without dependence upon the presence of cytosolic proteins or microtubule-associated proteins. Polymerization of tubulin was recorded in the high-speed supernatant of the crude homogenate (Margolis and Rauch, 1981). In this state, the myriad of cytosolic proteins which might influence tubulin assembly were present. A cycle of assembly/disassembly (Shelanski *et al.*, 1973) gave primar-

TABLE 1

CLINICAL EVALUATION OF NEUROTOXICITY IN CONTROL, 3,4-DIMETHYL-2,5-HEXANEDIONE (DMHD)-, AND 2,5-HEXANEDIONE (2,5-HD)-TREATED RATS

	Clinically normal (No. rats)	Moderate neurotoxicity (No. rats)	Severe neurotoxicity (No. rats)
Control	8	—	—
DMHD	1	5	2
2,5-HD	9	6	2

Note. Control rats and rats treated with 1% 2,5-HD or 0.035% DMHD in the drinking water were tested after 4 weeks of treatment. Neurological evaluation entailed placement of the rat on a smooth surface, extension of the hindlimbs, and measurement of the time required to reposition the limbs next to the body. The retraction time was measured in triplicate and averaged. Clinical categories were assigned according to the average retraction time as follows: clinically normal, less than 1 sec; moderate neurotoxicity, 1–10 sec; severe neurotoxicity, greater than 10 sec.

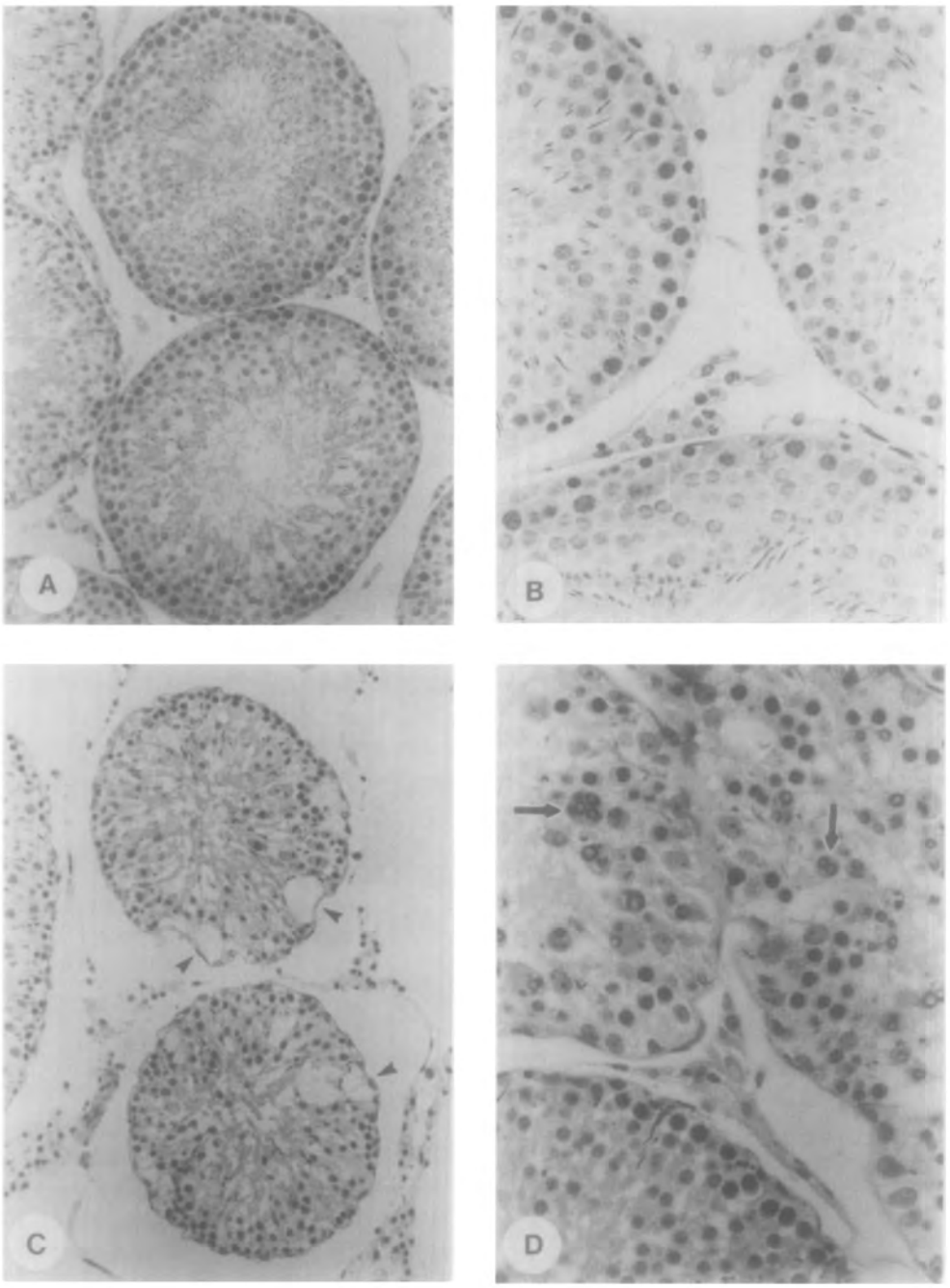

FIG. 2. Only 2,5-HD intoxication altered testicular morphology. Control rats and rats intoxicated with 1% 2,5-HD or 0.035% DMHD in the drinking water were sacrificed after 4 weeks of treatment. Glycol methacrylate-embedded sections were stained with periodic acid-Schiff's reagent and hematoxylin. The morphology of the DMHD-treated rat testes ((A) ×150; (B) ×300) was similar to control. The 2,5-HD-treated rat testes showed larger and more numerous basally located membrane-bround Sertoli cell vacuoles (arrowheads, (C) ×150) and occasional seminiferous tubules containing spermatids with condensed marginated chromatin, some of which were multinucleated (arrows, (D) ×300).

TABLE 2

CRUDE BRAIN AND TESTIS SUPERNATANT PROPERTIES FROM CONTROL, 3,4-DIMETHYL-2,5-HEXANEDIONE (DMHD)- AND 2,5-HEXANEDIONE (2,5-HD)-TREATED RATS

	Protein concentration (mg/ml)		Pyrrole (nmol DMP equivalents/ mg protein)		Tubulin (% total protein)	
	Brain	Testis	Brain	Testis	Brain	Testis
Control ($n = 4$)	15.64 ± 0.49	15.43 ± 0.30	—	—	12.64 ± 0.31	2.57 ± 0.32
DMHD ($n = 4$)	16.30 ± 0.72	18.99 ± 0.33[a]	—	—	11.14 ± 1.76	1.91 ± 0.40
2,5-HD ($n = 8$)	17.28 ± 0.20[b]	18.44 ± 0.57[c]	1.83 ± 0.25	1.56 ± 0.10	12.07 ± 1.24	2.91 ± 0.25

Note. Values shown are the means ± SE. For each group, brains and testes from pairs of rats were homogenized and processed together. Protein concentration was determined by the method of Lowry *et al.* (1951), pyrrole content was determined by the Ehrlich's reaction, and tubulin was determined by [^{3}H]colchicine binding.

[a] $p < 0.001$ when compared to control by two-tailed t test.

[b] $p < 0.005$ when compared to control by two-tailed t test.

[c] $p < 0.01$ when compared to control by two-tailed t test.

ily microtubule-derived material with the predominant components being tubulin and specific microtubule assembly-enhancing proteins. Binding and elution from DEAE-Sephacel (Hamel and Lin, 1981) followed by a cycle of temperature-dependent assembly/disassembly provided purified tubulin freed of contaminating proteins. Only 2,5-HD treatment induced alterations in the kinetics of tubulin assembly, alterations which were present at every stage of purification. The changes consisted of (1) earlier initiation of assembly and therefore a shorter time required to reach maximal velocity of assembly (tV_{max}) and (2) a greater maximal rate of assembly (V_{max}). The polymerization of DMHD-treated rat brain tubulin was always similar to that of control.

Detailed studies of purified tubulin polymerization were conducted to evaluate the effect of stringent assembly conditions as well as the statistical significance of the assembly alterations induced by 2,5-HD intoxication. Crude homogenates of brains from control rats and rats intoxicated for 4 weeks with drinking water containing 1% 2,5-HD or 0.035% DMHD were bound and eluted from DEAE-Sephacel followed by cycles of temperature-dependent assembly and disassembly to provide purified rat brain tubulin. A high-molecular-weight protein contaminant which comigrated with *in vitro* crosslinked tubulin was identified by SDS–PAGE analysis. This high-molecular-weight band was prominent in purified 2,5-HD rat brain tubulin and represented approximately 1% of the total protein (Fig. 3). The nature of the high-molecular-weight protein identified by SDS–PAGE analysis in purified tubulin derived from 2,5-HD-treated rat brain was further investigated by immunoblotting. Samples of purified tubulin from 2,5-HD, DMHD, and control rat brain were subjected to SDS–PAGE followed by electrophoretic transfer to nitrocellulose. The high-molecular-weight protein present in brain tubulin purified from 2,5-HD-intoxicated rats was stained by reaction with antitubulin antibodies (Fig. 4). This specific immunological staining verified the formation of a covalently crosslinked tubulin following *in vivo* intoxication with 2,5-HD.

The assembly of purified brain tubulin from 2,5-HD-treated rats was significantly different from both control and DMHD brain tubulin preparations (Table 3). The 2,5-HD-treated brain tubulin assembled both earlier and more rapidly. Control and

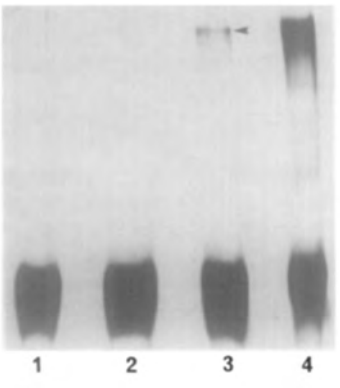

FIG. 3. Silver-stained 7.5% polyacrylamide minigel of purified rat brain tubulin. Aliquots of purified tubulin (2 μg) from control (lane 1), DMHD (lane 2), and 2,5-HD (lane 3) rat brain preparations were coelectrophoresed with bovine brain tubulin (lane 4) crosslinked by 2,5-HD *in vitro* (Boekelheide, 1987). The common band present in all samples represented denatured α- and β-tubulin subunits. A high-molecular-weight protein comigrated with *in vitro* crosslinked tubulin and was present only in purified tubulin derived from 2,5-HD-intoxicated rats (arrowhead).

DMHD brain tubulin preparations did not differ in their assembly characteristics. All three groups assembled to a similar extent as determined by the final maximal absorbance of assembly. The unique behavior of 2,5-HD-treated brain tubulin was emphasized by analysis under stringent assembly conditions. Addition of 2.5 mM calcium prior to assembly or decreasing the assembly temperature to 30°C markedly delayed assembly of control brain tubulin preparations while only moderately altering 2,5-HD brain tubulin assembly (Fig. 5).

Assembly of Testis Tubulin Derived from Intoxicated Rats

The relatively low tubulin concentration in testis complicated tubulin purification from this target organ of toxicity. Approximately 1 mg of purified tubulin was obtained from each group of 15 rats following the 4-week intoxication period. SDS–PAGE analysis of the purified testis tubulin revealed a high-molecular-weight protein contaminant present in both treated and control samples which comigrated with *in vitro* crosslinked tubulin (Fig. 6). This common high-molecular-weight band was visualized only after extensive silver-stain development and likely represented an irreducible protein dimerization product. A second high-molecular-weight band which migrated at a slightly higher apparent molecular weight was seen only in the samples from γ-diketone-intoxicated rats. This second band was most prominent in 2,5-HD testis tubulin and detectable in DMHD testis tubulin. These high-molecular-weight components were visualized by the silver-stain technique, but were not seen following electrotransfer and immunologic staining with antitubulin antibodies. In our hands, the silver-stain technique has been consistently more sensitive in detecting crosslinked tubulin than the immunoblot technique, likely due to the inefficiency of transfer of high-molecular-weight proteins.

All three groups differed in the assembly of purified testis tubulin (Table 4). The γ-dike-

FIG. 4. Intoxication with 2,5-HD induced a crosslinked tubulin. Purified rat brain tubulin (40 μg per lane) was isolated from control rats (lane 1) and rats intoxicated with 0.035% DMHD (lane 2) or 1% 2,5-HD (lane 3) in the drinking water for 4 weeks. Following SDS–PAGE on a 7.5% gel, the samples were electrophoretically transferred to nitrocellulose and stained with a mixture of monoclonal antibodies to α- and β-tubulin. A crosslinked tubulin was identified in brain tubulin purified from 2,5-HD intoxicated rats (arrow) which comigrated with crosslinked tubulin induced by *in vitro* 2,5-HD derivatization.

TABLE 3

ASSEMBLY PROPERTIES OF PURIFIED BRAIN TUBULIN FROM CONTROL, 3,4-DIMETHYL-2,5-HEXANEDIONE (DMHD)-, AND 2,5-HEXANEDIONE (2,5-HD)-TREATED RATS

	tV_{max} (min)	V_{max} (Δ OD_{350}/min)	Final OD_{350}
Control	7.8 ± 0.73	0.024 ± 0.004	0.252 ± 0.014
DMHD	8.0 ± 0.54	0.021 ± 0.002	0.243 ± 0.009
2,5-HD	2.9 ± 0.24[a]	0.043 ± 0.005[b,c]	0.230 ± 0.014

Note. Values shown are the means ± SE. Purified tubulin was prepared by DEAE-Sephacel binding and elution of brain crude supernatants from control, DMHD, and 2,5-HD experimental groups followed by cycles of temperature-dependent assembly and disassembly. Assembly was measured as the change in optical density at 350 nm. Control, DMHD, and 2,5-HD preparations were assembled in quadruplicate at 37°C in 1 M sodium glutamate, pH 6.6, at 0.40 mg/ml. The time required to reach maximal velocity of assembly (tV_{max}) is a measure of the nucleation phase of assembly. The maximal velocity of assembly (V_{max}) is a measure of the rate of microtubule elongation. The final optical density at 350 nm (final OD_{350}) is a measure of the ultimate extent of microtubule assembly and is dependent upon the optical scattering properties of the assembled polymer.

[a] $p < 0.001$ when compared with control or DMHD.

[b] $p < 0.05$ when compared with control.

[c] $p < 0.01$ when compared with DMHD.

tone-treated testis tubulins assembled both earlier and more rapidly than control. DMHD testis tubulin demonstrated assembly behavior intermediate between that of 2,5-HD and control testis tubulin.

Purified rat testis and brain tubulins differed in their assembly parameters (compare Tables 3 and 4). Despite the lower tubulin concentration, testis assemblies displayed more rapid nucleation and elongation and a similar final optical density. Differences in the morphology of the final assembly product may partly or fully explain these differences in assembly behavior. Rat testis tubulin, examined by negative-stain electron microscopy, tended to form wide, open protofilamentous sheets which aggregated into bundles while rat brain tubulin tended to form narrow, open and closed tubules (Fig. 7). The optical scattering volume of an assembled tubulin solution is a function of the width and length of the assembly product (Detrich *et al.*, 1985).

DISCUSSION

The environmental toxicant *n*-hexane and its oxidized metabolites are known industrial health hazards as well as injurious components of solvents inhaled for their euphoric effect (Spencer *et al.*, 1978). The biologically active γ-diketone metabolite 2,5-HD has been studied intensively and represents a prototypal agent for a class of nervous system and testicular toxicants (Graham *et al.*, 1982a).

The available knowledge of the chemical interaction between 2,5-HD and tissue proteins has allowed for the design and testing of several reasonable hypotheses of biochemical mechanisms of its nervous system toxicity (DeCaprio *et al.*, 1983; DeCaprio and O'Neill, 1985; Graham *et al.*, 1982b; Griffin *et al.*, 1983; Sabri, 1984). Because of its dramatic nature, the chronic polyneuropathy has received the primary investigatory attention. In this study, an intoxication protocol is described which controls for the systemic and nervous system toxic effects of γ-diketones while producing differential testicular toxicity, allowing for the investigation of fundamental biochemical alterations associated with toxic testicular injury.

Several properties of brain and testis crude supernatants from treated and control animals were examined. The protein concentra-

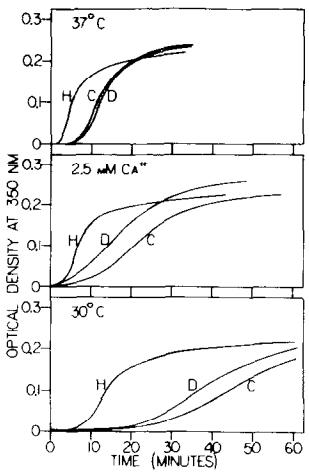

FIG. 5. Assembly of control (C), 2,5-HD (H)-, and DMHD (D)-purified rat brain tubulin following *in vivo* intoxication. Microtubule assembly was observed as the increased scattering of 350-nm light at a tubulin concentration of 0.40 mg/ml in 1 M sodium glutamate, pH 6.6, and 0.25 mM GTP. As shown in the top panel, 2,5-HD tubulin demonstrated earlier and more rapid assembly compared to both control and DMHD tubulin under standard conditions at 37°C. As shown in the middle panel, addition of 2.5 mM calcium to the assembly at 37°C altered DMHD and control assembly with little effect upon 2,5-HD assembly. As shown in the bottom panel, assembly at 30°C in the standard buffer markedly delayed and slowed DMHD and control microtubule polymerization while only moderately postponing 2,5-HD tubulin assembly.

tion was higher in treated supernatants, likely reflecting a state of dehydration. The tubulin content expressed as a percentage of total protein in brain and testis crude supernatants was not altered by 2,5-HD or DMHD treatment. A previous study of rats treated for 2 months with 0.5% 2,5-HD in the drinking water indicated that 80% of control total protein and 50% of control tubulin levels remained in brain homogenates (Couri and Nachtman, 1979). These differences in results may be explained by variable buffers and homogenization procedures, as well as the differences in treatment protocols.

The pyrrole content of 2,5-HD crude brain and testis supernatants was 1.5–1.8 nmol DMP equivalents per milligram protein. Assuming a similar Ehrlich's reactivity for the 2,5-HD pyrrole adduct and DMP and a 4.7% lysine content by weight of whole rat protein (Block, 1935), about 0.5% of protein lysyl ϵ-amines have been derivatized by a 4-week treatment of rats with 1% 2,5-HD in the drinking water. This is similar to literature reports of the extent of pyrryl derivatization following 2,5-HD intoxication (Anthony *et al.*, 1983b; DeCaprio *et al.*, 1983; DeCaprio and O'Neill, 1985).

The selective intoxication protocol induced alterations in brain microtubule assembly only in rats treated with 1% 2,5-HD in the drinking water for 4 weeks. The microtubule assembly alterations consisted of a shortened nucleation phase and a more rapid maximal rate of assembly. These assembly alterations were observed in brain crude supernatants, in the presence of microtubule associated proteins following a cycle of assembly and disassembly, and in electrophoretically pure tubulin following DEAE-Sephacel binding and elution. Repetitive assembly assays of purified rat brain tubulin demonstrated highly statistically significant assembly alterations in the 2,5-HD group than in control or DMHD groups and no significant differences between the control and DMHD groups.

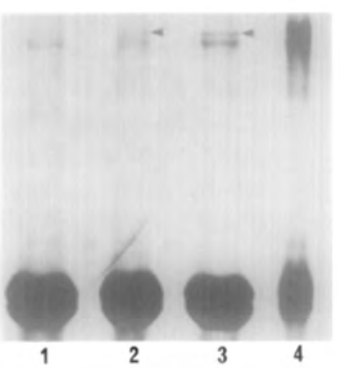

FIG. 6. Silver-stained 7.5% polyacrylamide gel of purified rat testis tubulin. Aliquots of purified tubulin (70 μg) from control (lane 1), DMHD (lane 2), and 2,5-HD (lane 3) rat testis preparations were coelectrophoresed with *in vitro* crosslinked bovine brain tubulin (lane 4, 4 μg). A band unique to the γ-diketone-intoxicated testis tubulins (arrowheads) migrated slightly above a common high-molecular-weight band. Both of these bands comigrated with *in vitro* crosslinked tubulin.

TABLE 4

ASSEMBLY PROPERTIES OF PURIFIED TESTIS TUBULIN FROM CONTROL, 3,4-DIMETHYL-2,5-HEXANEDIONE (DMHD)-, AND 2,5-HEXANEDIONE (2,5-HD)-TREATED RATS

	tV_{max} (min)	V_{max} (ΔOD_{350}/min)	Final OD_{350}
Control	3.9 ± 0.16	0.041 ± 0.001	0.227 ± 0.005
DMHD	$3.0 \pm 0.08^{a,b}$	$0.054 \pm 0.001^{c,d}$	0.231 ± 0.003^{b}
2,5-HD	2.5 ± 0.11^{c}	0.060 ± 0.002^{c}	0.242 ± 0.001^{e}

Note. Values shown are the means ± SE. Purified tubulin was prepared by DEAE-Sephacel binding and elution of testis crude supernatants from control, DMHD, and 2,5-HD experimental groups followed by cycles of temperature dependent assembly and disassembly. Assembly was measured as the change in optical density at 350 nm. Control, DMHD, and 2,5-HD preparations were assembled in quadruplicate at 37°C in 1 M sodium glutamate, pH 6.6, at 0.34 mg/ml. The time required to reach maximal velocity of assembly (tV_{max}) is a measure of the nucleation phase of assembly. The maximal velocity of assembly (V_{max}) is a measure of the rate of microtubule elongation. The final optical density at 350 nm (final OD_{350}) is a measure of the ultimate extent of microtubule assembly and is dependent upon the optical scattering properties of the assembled polymer.

[a] $p < 0.01$ when compared with control.
[b] $p < 0.02$ when compared with 2,5-HD.
[c] $p < 0.001$ when compared with control.
[d] $p < 0.05$ when compared with 2,5-HD.
[e] $p < 0.05$ when compared with control.

Electrophoretic transfer to nitrocellulose following SDS–PAGE and reaction with specific antitubulin monoclonal antibodies were used to verify that the high-molecular-weight band uniquely present in purified brain tubulin from 2,5-HD-intoxicated rats was crosslinked tubulin. The common presence of nervous system dysfunction and the contrasting effect upon brain microtubule assembly following 2,5-HD and DMHD intoxication argues against a role for tubulin modification in γ-diketone-induced peripheral neuropathy.

This is the first report of the purification and assembly of testis tubulin. "Testis tubulin" is clearly a heterogeneous tubulin mixture derived from structural, endocrine, and germ cells. The multiplicity of cellular sources within the testis may result in dilution of a tubulin assembly alteration or structural modification specific to one cell type when testis tubulin is examined in aggregate. However, the low tubulin content of the testis renders purification of assembly competent tubulin difficult and the purification of assembly competent tubulin from a specific cell type within the testis impractical.

Despite these limitations, the selective intoxication protocol used in this study induced assembly behavior in γ-diketone-treated testis tubulin which differed significantly from that of control. Purified testis tubulin from γ-diketone-intoxicated animals assembled earlier and more rapidly than control testis tubulin and these assembly alterations were associated with the presence of a high-molecular-weight band. A common high-molecular-weight band was present in both treated and control testis samples, likely representing nonspecifically crosslinked tubulin. A unique band seen only in γ-diketone testis tubulin migrated at a slightly higher apparent molecular weight and likely represents tubulin crosslinked during intoxication.

2,5-HD caused testicular weight loss and histological changes, the most extreme alterations in microtubule assembly, and the most crosslinked tubulin. DMHD induced no apparent testicular injury, an intermediate level of microtubule assembly alterations, and

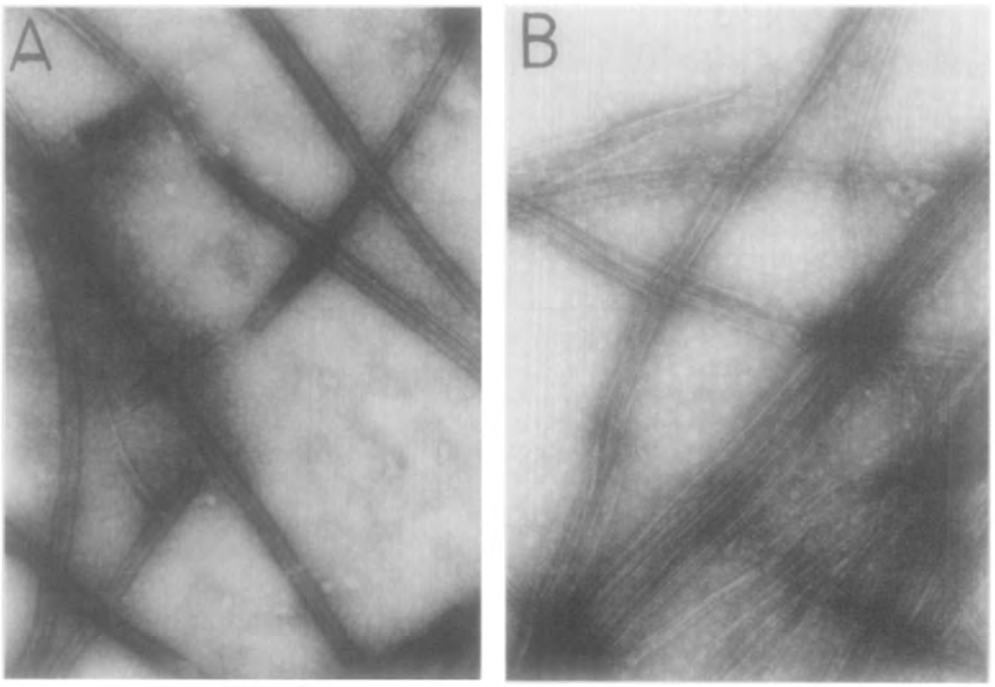

FIG. 7. Polymer morphology of purified rat brain tubulin and purified rat testis tubulin. Brain tubulin (0.40 mg/ml) and testis tubulin (0.34 mg/ml) purified from 2,5-HD-intoxicated rats were negatively stained after assembly to steady-state at 37°C in 1 M sodium glutamate, pH 6.6, with 0.25 mM GTP. Closed microtubules predominated as the polymeric form of assembled purified 2,5-HD brain tubulin ((A) magnification ×63,000). Purified 2,5-HD testis tubulin formed predominantly open polymeric sheets which tended to aggregate into bundles ((B) same final magnification as A, ×63,000).

small amounts of crosslinked tubulin. Therefore, the presence of testicular injury correlated with both the extent of altered assembly of purified testis tubulin and the amount of crosslinked tubulin.

The γ-diketone-induced modification of tubulin appears to involve the process of microtubule nucleation: the presence of additional nucleating centers would explain the earlier onset and the increased rate of microtubule formation. A result of an alteration in the nucleation event for microtubule formation is a change in average microtubule length. An increased concentration of nucleating centers would lead to an increased number of shorter microtubules. We have documented a decrease to one-third of control microtubule length following *in vitro* 2,5-HD derivatization of bovine brain tubulin (Boekelheide, 1987).

The nurturing, transport, and structural roles of the Sertoli cell are uniquely dependent upon microtubule integrity (Vogl *et al.*, 1983a). Given the sensitivity of the testis to microtubule poisons and the importance of microtubules to Sertoli cell function, the following hypothesis is proposed: (1) intoxication with 2,5-HD alters microtubule assembly kinetics, (2) altered assembly produces changes in the number and length of Sertoli cell microtubules which compromises Sertoli cell function, and (3) a malfunctioning, nonsupportive Sertoli cell disrupts germ cell maturation resulting in testicular atrophy.

This study has examined only a single time point and a single dose of 2,5-HD and DMHD in an attempt to correlate testicular effects with microtubule alterations. The absence of DMHD-induced testicular injury in the presence of significant microtubule alter-

ations may be explained by a threshold requirement for induction of injury. Future studies will examine the relationship between the evolution of the microtubule abnormality and testicular injury during intoxication and recovery.

REFERENCES

ANTHONY, D. C., BOEKELHEIDE, K. AND GRAHAM, D. G. (1983a). The effect of 3,4-dimethyl substitution on the neurotoxicity of 2,5-hexanedione. I. Accelerated clinical neuropathy is accompanied by more proximal axonal swellings. *Toxicol. Appl. Pharmacol.* **71**, 362–371.

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

BLOCK, R. J. (1935). On the nature and origin of proteins. *Yale J. Biol. Med.* **7**, 235–252.

BOEKELHEIDE, K. (1985). 2,5-Hexanedione induces microtubule dysfunction and testicular atrophy. *J. Androl.* **6**, 58-P.

BOEKELHEIDE, K. (1986). Rat microtubule assembly properties are altered following *in vitro* and *in vivo* modification by 2,5-hexanedione. *The Toxicologist* **6**, 190.

BOEKELHEIDE, K. (1987). 2,5-Hexanedione alters microtubule assembly. II. Enhanced polymerization of crosslinked tubulin. *Toxicol. Appl. Pharmacol.* **88**, 383–396.

BORISY, G. G. (1972). A rapid method for quantitative determination of microtubule protein using DEAE-cellulose filters. *Anal. Biochem.* **50**, 373–385.

CHAPIN, R. E., MORGAN, K. T., AND BUS, J. S. (1983). The morphogenesis of testicular degeneration induced in rats by orally administered 2,5-hexanedione. *Exp. Mol. Pathol.* **38**, 149–169.

CHAPIN, R. E., NORTON, R. M., POPP, J. A., AND BUS, J. S. (1982). The effects of 2,5-hexanedione on reproductive hormones and testicular enzyme activities in the F-344 rat. *Toxicol. Appl. Pharmacol.* **62**, 262–272.

COURI, D., AND NACHTMAN, J. P. (1979). Biochemical and biophysical studies of 2,5-hexanedione neuropathy. *Neurotoxicology* **1**, 269–283.

DECAPRIO, A. P., OLAJOS, E. J., AND WEBER, P. (1982). Covalent binding of a neurotoxic *n*-hexane metabolite: Conversion of primary amines to substituted pyrrole adducts by 2,5-hexanedione. *Toxicol. Appl. Pharmacol.* **65**, 440–450.

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

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

DETRICH, H. W., JORDAN, M. A., WILSON, L., AND WILLIAMS, R. C. (1985). Mechanism of microtubule assembly. Changes in polymer structure and organization during assembly of sea urchin egg tubulin. *J. Biol. Chem.* **260**, 9479–9490.

FINK, B. R., BYERS, M. R., AND MIDDAUGH, M. E. (1973). Dynamics of colchicine effects on rapid axonal transport and axonal morphology. *Brain Res.* **56**, 299–311.

GRAHAM, D. G., ANTHONY, D. C., AND BOEKELHEIDE, K. (1982a). *In vitro* and *in vivo* studies of the molecular pathogenesis of *n*-hexane neuropathy. *Neurobehav. Toxicol. Teratol.* **4**, 629–634.

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

GRAHAM, D. G., SZAKAL-QUIN, G., PRIEST, J. W., AND ANTHONY, D. C. (1984). *In vitro* evidence that covalent cross linking of neurofilaments occurs in γ-diketone neuropathy. *Proc. Natl. Acad. Sci. USA* **81**, 4979–4982.

GRIFFIN, J. W., FAHNESTOCK, K. E., PRICE, D. L., AND CORK, L. C. (1983). Cytoskeletal disorganization induced by local application of β,β′-iminodipropionitrile and 2,5-hexanedione. *Ann. Neurol.* **14**, 55–61.

HAMEL, E., AND LIN, C. M. (1981). Glutamate-induced polymerization of tubulin: Characteristics of the reaction and application to the large-scale purification of tubulin. *Arch. Biochem. Biophys.* **209**, 29–40.

HANDEL, M. A. (1979). Effects of colchicine on spermiogenesis in the mouse. *J. Embryol. Exp. Morph.* **51**, 73–83.

KOMIYA, Y., AND KUROKAWA, M. (1980). Preferential blockade of the tubulin transport by colchicine. *Brain Res.* **190**, 505–516.

KRASAVAGE, W. J., O'DONOGHUE, J. L., DIVINCENZO, G. D., AND TERHAAR, C. J. (1980). The relative neurotoxicity of methyl *n*-butyl ketone, *n*-hexane and their metabolites. *Toxicol. Appl. Pharmacol.* **52**, 433–441.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.

MARGOLIS, R. L., AND RAUCH, C. T. (1981). Characterization of rat brain crude extract microtubule assembly: Correlation of cold stability with the phosphorylation state of a microtubule-associated 64K protein. *Biochemistry* **20**, 4451–4458.

NACHTMAN, J. P., AND COURI, D. (1979). Studies on hexane 2,5-dione neurotoxicity: No effect on microtubular properties. *Toxicol. Appl. Pharmacol.* **48**, 54. [Abstract]

RUSSELL, L. D., MALONE, J. P., AND MACCURDY, D. S. (1981). Effect of the microtubule disrupting agents, colchicine and vinblastine, on seminiferous tubule structure in the rat. *Tissue Cell* **13**, 349–367.

SABRI, M. I. (1984). *In vitro* effect of *n*-hexane and its metabolites on selected enzymes in glycolysis, pentose phosphate pathway and citric acid cycle. *Brain Res.* **297**, 145–150.

SELKOE, D. J., LUCKENBILL-EDDS, L., AND SHELANSKI, M. L. (1978). Effects of neurotoxic industrial solvents on cultured neuroblastoma cells: Methyl *n*-butyl ketone, *n*-hexane and derivatives. *J. Neuropathol. Exp. Neurol.* **37**, 768–789.

SHELANSKI, M. L., GASKIN, F., AND CANTOR, C. R. (1973). Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. USA* **70**, 765–768.

SPENCER, P. S., BISCHOFF, M. C., AND SCHAUMBURG, H. H. (1978). On the specific molecular configuration of neurotoxic aliphatic hexacarbon compounds causing central–peripheral distal axonopathy. *Toxicol. Appl. Pharmacol.* **44**, 17–28.

SPENCER, P. S., AND SCHAUMBURG, H. H. (1975). Experimental neuropathy produced by 2,5-hexanedione—a major metabolite of the neurotoxic industrial solvent methyl *n*-butyl ketone. *J. Neurol. Neurosurg. Psychiatry* **38**, 771–775.

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

VOGL, A. W., LIN, Y. C., DYM, M., AND FAWCETT, D. W. (1983a). Sertoli cells of the golden-mantled ground squirrel (*Spermophilus lateralis*): A model system for the study of shape change. *Amer. J. Anat.* **168**, 83–98.

VOGL, A. W., LINCK, R. W., AND DYM, M. (1983b). Colchicine-induced changes in the cytoskeleton of the golden-mantled ground squirrel (*Spermophilus lateralis*) Sertoli cells. *Amer. J. Anat.* **168**, 99–108.