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Comparative Neurotoxicity and Pyrrole-Forming Potential of 2,5-Hexanedione and Perdeuterio-2,5-Hexanedione in the Rat[1,2]

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Comparative Neurotoxicity and Pyrrole-Forming Potential of 2,5-Hexanedione and Perdeuterio-2,5-Hexanedione in the Rat. DECAPRIO, A. P., BRIGGS, R. G., JACKOWSKI, S. J., AND KIM, J. C. S. (1988). *Toxicol. Appl. Pharmacol.* 92, 75–85. 2,5-Hexanedione (2,5-HD), the neurotoxic metabolite of *n*-hexane, reacts with protein amines to form alkylpyrrole adducts. Pyrrolylation of neurofilament protein may be the initiating molecular event in 2,5-HD neuropathy. The present study compares the neurotoxic and pyrrole-forming potentials of 2,5-HD with those of perdeuterio-2,5-HD ([D^{10}]-2,5-HD) in the rat. Due to a requirement for C–H bond breaking in the reaction mechanism, the latter derivative was expected to exhibit a primary isotope effect, thus forming the pyrrole at a slower rate. *In vitro* studies confirmed that [D^{10}]-2,5-HD pyrrolylated protein at only one-third of the initial rate seen with native 2,5-HD. Prolonged incubation resulted in similar pyrrole concentrations with both derivatives. Adult, male Wistar rats were administered daily (5 days/week) ip doses of either 3.5 mmol 2,5-HD or [D^{10}]-2,5-HD/kg/day for 17 days or 2.5 mmol/kg/day for 38 days. At termination, animals administered 2,5-HD and [D^{10}]-2,5-HD exhibited 27 and 8% body weight loss, respectively. Moderate to severe hindlimb paralysis was present in the 2,5-HD groups while only mild effects were seen in [D^{10}]-2,5-HD-dosed rats. Neuropathological changes were prominent in spinal cord sections from 2,5-HD-treated animals, while no effects were present in rats given the deuterated derivative. Pyrrole adduct concentrations in serum and axonal cytoskeletal proteins from 2,5-HD-treated animals were two- to threefold higher than in rats given equimolar doses of [D^{10}]-2,5-HD. Levels of covalent crosslinking of axonal cytoskeletal proteins (assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis) appeared to correlate with pyrrole concentrations. Tissue concentrations of each diketone isomer were not significantly different, indicating similar uptake of native and deuterated 2,5-HD. Mass spectrometry revealed rapid back exchange of the terminal (methyl) but not of the internal (methylene) deuteriums of [D^{10}]-2,5-HD *in vivo*. These findings support an absolute requirement for pyrrole formation in γ-diketone neurotoxicity.

2,5-Hexanedione (2,5-HD) is the neurotoxic γ-diketone metabolite of the industrial solvents *n*-hexane and methyl *n*-butyl ketone (DiVincenzo *et al.*, 1976; Spencer *et al.*, 1980). 2,5-HD administration to experimental animals induces neurofilament (NF)-packed axonal swellings, localized to the distal regions of myelinated nerve fibers (Spencer and Schaumburg, 1977). We have previously demonstrated (DeCaprio *et al.*, 1982) that this derivative reacts with protein amines to form 2,5-dialkylpyrrole residues, a phenomenon which has since been confirmed in a number of *in vitro* and *in vivo* systems (Graham *et al.*, 1982; DeCaprio *et al.*,

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1983; Anthony *et al.*, 1983; DeCaprio and O'Neill, 1985). Formation of pyrrole adducts in axonal cytoskeletal protein, particularly NF protein, has been suggested as the critical initiating event in γ-diketone neuropathy (DeCaprio *et al.*, 1982).

Although other considerations (i.e., structure/activity relationships, *in vivo* covalent protein binding, and the apparent direct axonal action of the γ-diketones) support the pyrrole hypothesis, evidence for an absolute requirement for this reaction is not conclusive. Anthony *et al.* (1983) demonstrated the enhanced neurotoxicity of 3,4-dimethyl-2,5-HD (DMHD) as compared to 2,5-HD in the rat. This derivative also formed pyrroles *in vitro* and *in vivo* at a relatively higher rate, a result which supports the pyrrole hypothesis. However, interpretation of this finding is complicated by the observation that subsequent pyrrole autoxidation was also accelerated. Such autoxidative reactions have been shown to yield chromophores and covalently crosslinked protein polymers in γ-diketone: protein mixtures (DeCaprio *et al.*, 1982; Graham *et al.*, 1982). Covalent NF crosslinking has been suggested to underlie the axonal degeneration seen in γ-diketone neuropathy (Graham *et al.*, 1982). In addition, proximal axonal swellings were produced by DMHD, in contrast to the distal lesions typically encountered with 2,5-HD.

Sayre and co-workers (1986) examined the neurotoxic potential of 3,3-dimethyl-2,5-hexanedione, a γ-diketone which cannot form a pyrrole. This compound was not neurotoxic in the rat, even at high doses. Additional data were reported by Genter *et al.* (1987), who compared *in vitro* rates of pyrrole formation by the diastereoisomers *d,l*- and *meso*-DMHD with their neurotoxic potency in the rat. The *d,l* isomers formed the pyrrole more rapidly and were associated with heightened neurotoxicity. Pyrrole adduct concentrations *in vivo* were not reported in this study.

Recent work has demonstrated the presence of N-substituted imines as intermediates during pyrrole formation under organic (Katritzky *et al.*, 1986) and aqueous (DeCaprio *et al.*, 1987) reaction conditions. Since the rate-determining step in the reaction mechanism appears to involve C–H bond breaking (associated with β-elimination of H_2O), it was hypothesized that this reaction should exhibit a primary isotope effect. Therefore, a perdeuterated γ-diketone should form the pyrrole at a lower rate than the native (protio) isomer. An absolute requirement for pyrrole adduct formation in this neuropathy would thus be reflected by a decreased neurotoxic potential of a perdeuterated γ-diketone.

The present investigation compares the *in vitro* and *in vivo* pyrrole-forming potential of 2,5-HD and perdeuterio-2,5-HD ([D^{10}]-2,5-HD). In addition, the neurotoxic potency and tissue distribution of each isomer is examined following multiple, equimolar doses in rats. Covalent crosslinking of axonal cytoskeletal protein is also assessed. Finally, the relative *in vivo* rates of hydrogen–deuterium exchange at each carbon position in [D^{10}]-2,5-HD are determined. The results provide substantial direct support for a requirement for pyrrole adduct formation in 2,5-hexanedione neuropathy.

METHODS

Chemicals. 2,5-HD was obtained from Eastman Organic Chemicals (Rochester, NY) and was redistilled prior to use. Deuterated reagents were obtained from MSD Isotopes (St. Louis, MO). [D^{10}]-2,5-HD was synthesized from redistilled 2,5-HD by base (NaOD)-catalyzed exchange in D_2O (White *et al.*, 1979). Final purification utilized fractional distillation under N_2 (bp 193–194°C). Isotopic purity as determined by mass spectrometry was >94 atom% D.

In vitro studies. Samples of bovine serum albumin (BSA) (7.5 mg/ml in 200 mM sodium phosphate buffer with 0.1% NaN_3, pH 7.4) were incubated with either 2,5-HD or [D^{10}]-2,5-HD (50:1 molar excess relative to lysine; Peters, 1977) at 37°C under N_2. The buffer used for the [D^{10}]-2,5-HD samples was made up in D_2O rather than H_2O. At timed intervals, aliquots were withdrawn for analysis of pyrrole adduct concentration by a *p*-dimethylaminobenzaldehyde (DMAB)-based colorimetric assay (DeCaprio and O'Neill, 1985). Samples were purged with N_2 following each aliquot withdrawal.

Animals and dosing. Groups of nine adult, male Wistar rats (400–500 g) were administered daily (5 days/

wk) ip injections of either 2,5-HD or [D^{10}]-2,5-HD (3.5 mmol/kg/day for 17 days or 2.5 mmol/kg/day for 38 days). Dosing preparations were made up as 50% solutions in either H_2O or D_2O. An additional group of nine control rats received injections of H_2O only. All animals were observed and graded daily for signs of hindlimb weakness and paralysis (DeCaprio and O'Neill, 1985), and body weights were taken twice weekly.

Biochemical studies. Immediately after termination (by CO_2 asphyxiation), samples of blood, brain stem, and spinal cord were taken from six animals per group. Nervous tissue was processed to yield axonal cytoskeletal protein preparations (Chiu and Norton, 1982), which were analyzed for pyrrole content by DMAB assay. Serum was recovered from blood and also analyzed by DMAB assay. In addition, axonal cytoskeletal protein preparations were separated by SDS–PAGE (Laemmli, 1970). Completed gels were stained with either Coomassie blue R-250 for detection of crosslinked protein or with fast green FCF for densitometric quantitation of high-molecular-weight protein.

Histopathology. For histopathological studies, the remaining three animals per group were deeply anesthetized by ip injection of pentobarbital, followed by heparinization and perfusion via the aorta with 10% phosphate-buffered formalin (pH 7.4) containing 7.5% sucrose. Samples of brain stem, cervical, thoracic, and lumbar spinal cord, terminal sciatic nerve, and testes were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. In addition, thick plastic sections of thoracic spinal cord were prepared and stained with toluidine blue.

Tissue diketone distribution. In a separate study, groups of three rats were administered single, ip doses of 7.5 mmol 2,5-HD or [D^{10}]-2,5-HD/kg (50% solution in H_2O or D_2O, respectively) and then terminated 1, 2, 6, or 18 hr after dosing. Samples of serum, brain stem, spinal cord, and liver were isolated and homogenized with acetone (3 ml/g tissue). Tissue homogenates were centrifuged and the acetone extracts were analyzed for diketone content by gas chromatography as previously described (DeCaprio *et al.*, 1983).

Determination of hydrogen–deuterium exchange rates in [D^{10}]-2,5-HD. Serum samples from rats treated with single doses of [D^{10}]-2,5-HD (see above) were extracted once with methylene chloride (3 ml CH_2Cl_2/ml serum), followed by centrifugation. The methylene chloride layer was then recovered and analyzed by gas chromatography–mass spectrometry as described below. Fresh blood from control rats was also directly spiked with [D^{10}]-2,5-HD and immediately extracted as above to determine the amount of D–H exchange occurring during the extraction process itself. These data were utilized to determine the relative rates of deuterium loss in the molecule as a whole and at each carbon position (i.e., terminal methyl vs internal methylene groups) *in vivo.*

GC/MS analysis. GC separation of CH_2Cl_2-extracted serum samples was carried out by on-column injection

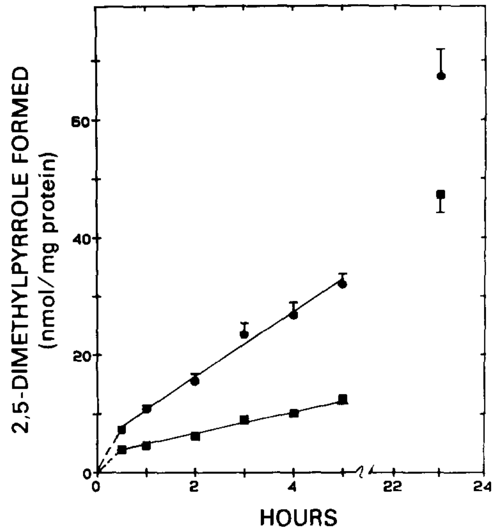

FIG. 1. *In vitro* pyrrole adduct formation in diketone-treated protein. BSA was incubated with either 2,5-HD (●) or with [D^{10}]-2,5-HD (■) as described under Methods, followed by DMAB-based pyrrole assay at timed intervals. Data points represent the mean ± standard deviation of quadruplicate determinations. Where not shown, the error bar lies within the symbol. The best-fit lines were calculated by linear regression analysis using the 0.5-, 1-, 2-, 3-, 4-, and 5-hr time-point data.

onto a 50 m × 0.2-mm fused silica bonded Hewlett–Packard "UltraPerformance" methyl phenyl silicone column with H_2 carrier gas and isothermal (65°C) conditions. MS analysis was performed by direct introduction of the GC eluant into the source of a Kratos MS 25 mass spectrograph at a source temperature of 270°C and 70 eV ionization voltage. Areas under ion chromatograms were obtained for m/z 114 through 124 (molecular ion), 99 through 105 (molecular ion minus methyl), 71 through 78 (molecular ion minus carboxymethyl), and 43 through 45 (carboxymethyl fragment). Total deuterium in each type of fragment was determined by summing the products of the fractions of subspecies and the number of deuteriums in the subspecies. The deuterium content of the terminal methyls was that of the carboxymethyl fragment. The deuterium content of the internal methylenes was calculated as one-half the difference in deuterium content between the fragments lacking one methyl group and the fragment with one methyl group.

RESULTS

Pyrrolylation of BSA in vitro. Figure 1 shows the rate of pyrrole formation in BSA treated with a 50:1 molar excess (relative to

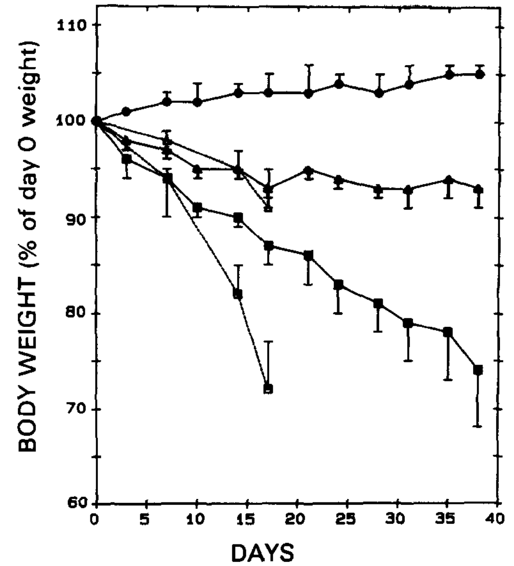

FIG. 2. Body weight changes in diketone-treated rats. Control animals received daily ip injections of H_2O (●). Treated animals were given either 2,5-HD (3.5 mmol/kg/day for 17 days (□); 2.5 mmol/kg/day for 38 days (■)) or [D^{10}]-2,5-HD (same doses and times as for 2,5-HD (△; ▲)). Data points represent the mean ± standard deviation of values from six animals per group.

lysine) of either 2,5-HD or [D^{10}]-2,5-HD for up to 23 hr. These rates were linear between 0.5 and 5 hr of incubation for both isomers (r = 0.995). The rate of pyrrolylation with native 2,5-HD during this time period was calculated by linear regression as 5.6 nmol pyrrole/mg protein/hr, compared to a rate of 1.9 nmol pyrrole/mg protein/hr with the deuterated derivative. Although not determined experimentally, the rate of pyrrolylation during the first 30 min of incubation appeared substantially higher with both isomers. By 23 hr of incubation, the rate of pyrrolylation by 2,5-HD had fallen to 1.3 nmol pyrrole/mg protein/hr while that by [D^{10}]-2,5-HD remained constant at 1.9 nmol pyrrole/mg protein/hr. With prolonged incubation (1 week), absolute pyrrole levels with the deuterated isomer approached those seen with native 2,5-HD (data not shown).

Body weight changes in γ-diketone-treated rats. Figure 2 illustrates the body weight changes observed with multiple, ip doses of 3.5 or 2.5 mmol diketone/kg/day for 17 or 38 days, respectively. Control rats gained approximately 5% additional body weight over 38 days. Animals treated with 2,5-HD exhibited a more severe body weight loss than those treated with equimolar doses of [D^{10}]-2,5-HD. Weight loss was approximately 27% in rats given either 3.5 mmol 2,5-HD/kg/day for 17 days or 2.5 mmol/kg/day for 38 days. In contrast, only 8% of body weight was lost in rats administered identical doses of [D^{10}]-2,5-HD. Animals treated at the 2.5 mmol/kg/day dose level received a total of 70.0 mmol diketone/kg, while those given 3.5 mmol/kg/day received 45.5 mmol diketone/kg.

Clinical and histopathological signs of toxicity. Clinical signs of neuropathy and histopathological alterations resulting from diketone treatment are summarized in Table 1. Rats administered 2,5-HD at either dose progressed to moderate or marked levels of clinical neuropathy by the time of termination. In contrast, animals given equimolar doses of [D^{10}]-2,5-HD exhibited only mild hindlimb weakness at the same time points.

Numerous swollen axons were seen in sections of thoracic and lumbar spinal cord from 2,5-HD-treated rats, primarily in the region of the anterior fasciculi. Thick plastic sections stained with toluidine blue revealed the typical appearance of NF-packed swellings, segregation of other axonal elements, and myelin attenuation. Scattered axonal degeneration was also noted in these preparations. No similar changes were seen in brain stem sections from these animals. Spinal cord sections from [D^{10}]-2,5-HD-treated rats were not distinguishable from those of control. No alterations were seen in preparations of terminal sciatic nerve from any dose group.

Testicular alterations were also prominent in the 2,5-HD-treated rats. These consisted of giant cell formation, arrest of spermatogenesis, and pyknotic changes in Sertoli and germinal cell nuclei. Such changes were noted at both dose levels of 2,5-HD. In contrast, no testicular damage was apparent in control animals or in rats exposed to [D^{10}]-2,5-HD.

TABLE 1

CLINICAL AND PATHOLOGICAL EFFECTS IN γ-DIKETONE-TREATED RATS

Treatment	Dose (mmol/kg/day)	Duration (days)	Clinical signs[a]	Histopathological damage[b]	
				CNS[c]	PNS[d]
Control	—	17	0 ± 0	0	0
2,5-HD	3.5	17	5 ± 1	++	0
[D[10]]-2,5-HD	3.5	17	2 ± 1	0	0
Control	—	38	0 ± 0	0	0
2,5-HD	2.5	38	5 ± 1	++	0
[D[10]]-2,5-HD	2.5	38	2 ± 1	0	0

[a] 0, No observable effects; 2, mild hindlimb weakness; 4, moderate weakness and observable paralysis; 6, marked hindlimb paralysis. Data represent the mean ± standard deviation of values from six animals.

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

[c] Damaged assessed in thoracic spinal cord.

[d] Damaged assessed in terminal branches of sciatic nerve.

Pyrrole formation in serum and axonal cytoskeletal proteins. Pyrrole adduct concentrations in serum and axonal cytoskeletal protein preparations from diketone-treated animals are shown in Table 2. Pyrrole adduct concentrations were significantly (two- to threefold) higher in serum and in axonal cytoskeletal proteins from the 2,5-HD-treated animals as compared to the [D[10]]-2,5-HD-treated rats, with the exception of brain stem samples from the 38-day groups. All values for treated animals were significantly different from corresponding control values. Pyrrole adduct levels were also comparatively higher in the rats treated with 3.5 mmol/kg/day for 17 days than in those given 2.5 mmol/kg/day for 38 days. Relative pyrrole concentrations among CNS tissues from a particular dose group were similar to those measured in serum, a result consistent with facile penetra-

TABLE 2

PYRROLE ADDUCT FORMATION IN PROTEINS FROM γ-DIKETONE-TREATED RATS

Treatment	Dose level (mmol/kg/day)	Duration (days)	Pyrrole concentration (nmol/mg protein)[a]		
			Serum	Brain stem[b]	Spinal cord[b]
Control	—	17	0.2 ± 0.0	0.6 ± 0.1	0.6 ± 0.2
2,5-HD	3.5	17	7.5 ± 1.8	3.7 ± 0.7	4.7 ± 1.2
[D[10]]-2,5-HD	3.5	17	2.8 ± 0.8^c	1.9 ± 0.4^c	2.1 ± 0.5^d
Control	—	38	0.2 ± 0.1	0.6 ± 0.4	0.3 ± 0.2
2,5-HD	2.5	38	3.1 ± 1.0	1.7 ± 0.6	2.2 ± 0.7
[D[10]]-2,5-HD	2.5	38	1.2 ± 0.1^c	1.7 ± 0.8	1.1 ± 0.1^d

[a] Data represent the mean ± standard deviations of duplicate determinations from six animals.

[b] Axonal cytoskeletal protein preparations.

[c] Significantly different from corresponding 2,5-HD value (Student's t test); $p \leq 0.001$.

[d] $p \leq 0.01$.

TABLE 3

COVALENT CROSSLINKING OF AXONAL CYTOSKELETAL PROTEINS FROM γ-DIKETONE-TREATED RATS

Treatment	Dose level (mmol/kg/day)	Duration (days)	Percentage high-molecular-weight protein[a]	
			Brain stem	Spinal cord
Control	—	17	3.7 ± 0.2	3.6 ± 2.4
2,5-HD	3.5	17	6.4 ± 0.9	12.2 ± 1.3
[D[10]]-2,5-HD	3.5	17	4.2 ± 0.7[b]	9.4 ± 0.6[c]
Control	—	38	2.9 ± 1.0	3.8 ± 0.6
2,5-HD	2.5	38	9.9 ± 2.7	11.6 ± 1.8
[D[10]]-2,5-HD	2.5	38	8.3 ± 1.3	7.0 ± 1.7[c]

[a] Covalent crosslinking was assessed by densitometric scanning of Fast Green FCF-stained SDS–PAGE gels of axonal cytoskeletal protein preparations. Percentage high-molecular-weight protein was defined as the percentage of total protein present with an apparent molecular weight >260 kDa. Data represent the mean ± standard deviation of values from five or six animals per group. All data from diketone-treated animals were significantly different (Student's *t* test) from corresponding control values.

[b] Significantly different (Student's *t* test) from corresponding 2,5-HD value; $p \leq 0.001$.

[c] $p \leq 0.01$.

tion of the blood–brain barrier by the diketones.

SDS–PAGE analysis of axonal cytoskeletal proteins. Coomassie blue staining of SDS–PAGE-separated axonal cytoskeletal proteins from treated animals revealed the presence of new, high-molecular-weight protein bands with a pattern identical to that previously reported (DeCaprio and O'Neill, 1985; Carden *et al.*, 1986; Lapadula *et al.*, 1986; DeCaprio, 1987). The same bands were present in preparations from both 2,5-HD- and [D[10]]-2,5-HD-treated rats. The results of densitometric quantitation of high-molecular-weight protein in these gels following Fast Green staining are shown in Table 3. The levels of covalently crosslinked, high-molecular-weight protein were significantly lower in preparations from the [D[10]]-2,5-HD-treated as compared to the 2,5-HD-treated rats, with the exception of brain stem samples from the 38-day dose groups. High-molecular-weight protein values in brain stem samples from the 38-day dose groups tended to be higher than those measured in the corresponding 17-day groups. In contrast, the reverse relationship was seen in spinal cord samples.

Tissue distribution of 2,5-HD and [D[10]]-2,5-HD. The relative distribution of 2,5-HD and [D[10]]-2,5-HD in rat tissues following a single ip dose of 7.5 mmol/kg is shown in Fig. 3. Both diketones were rapidly absorbed into blood and distributed to liver and CNS. Diketone concentrations were slightly higher in serum than in nervous tissue, which were in turn slightly higher than those from liver. The elimination half-life of each diketone in all four tissues examined was approximately 10 hr. No significant differences between tissue concentrations of the diketone isomers were noted at any time point, indicating that deuterium incorporation did not affect tissue uptake.

Deuterium–hydrogen exchange rates in vivo. Results of studies examining the relative rates of exchange of hydrogen for deuterium *in vivo* at each carbon position in [D[10]]-2,5-HD are shown in Fig. 4. Deuterium loss from the whole molecule was approximately 37% during the first hour after dosing, with a total exchange of 47% after 18 hr. A small portion (6%) of this exchange occurred during the extraction procedure itself. Essentially all of the exchange detected in the whole molecule was attributable to loss of deuterium from the terminal methyl groups. In contrast, only a slight (9%) exchange was noted with the internal methylene groups after 18 hr. This low

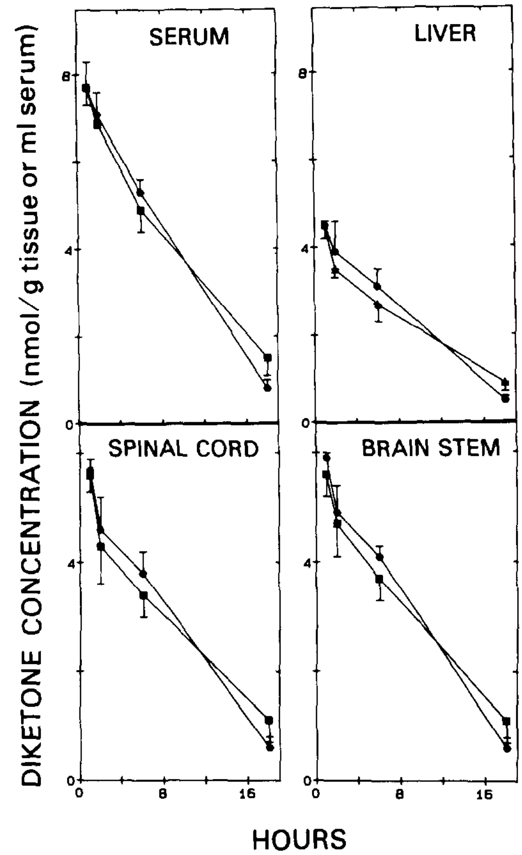

FIG. 3. Relative tissue concentrations of 2,5-HD and [D^{10}]-2,5-HD. Tissue diketone concentrations were determined in serum, liver, spinal cord, and brain stem at various time points following a single ip injection of 7.5 mmol 2,5-HD (●) or [D^{10}]-2,5-HD/kg (■) to rats. Data points represent the mean ± standard deviation of determinations from three animals per group.

rate of exchange indicates the relative stability of the C–D bond in the methylene groups as compared to the terminal methyls under physiological conditions.

DISCUSSION

Substantial indirect evidence is available that pyrrole formation may be the critical initiating event in γ-diketone neuropathy. For example, Spencer and co-workers (1978) first demonstrated that diketone neuropathy is restricted to the γ-isomers. This finding is consistent with the pyrrole hypothesis, since only γ-diketones can form the five-membered pyrrole nucleus (Jones and Bean, 1977). Other workers reported stable, protein-bound radiolabel in rats exposed to [^{14}C]-methyl *n*-butyl ketone, the metabolic precursor of 2,5-HD (DiVincenzo *et al.*, 1977).

The apparent direct toxic action of 2,5-HD upon the axon has also been demonstrated in several *in situ* and *in vitro* systems (Politis *et al.*, 1980; Veronesi *et al.*, 1983; Zagoren *et al.*, 1983). Such direct toxicity would be expected for a mechanism involving covalent binding of γ-diketones with axonal proteins. A number of *in vivo* studies have confirmed the pyrrole-forming reaction in tissue protein, including serum (DeCaprio *et al.*, 1982),

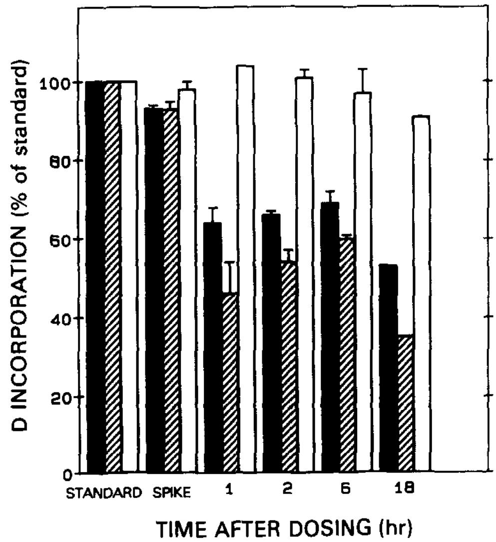

FIG. 4. *In vivo* deuterium–hydrogen exchange in [D^{10}]-2,5-HD. Animals were administered a single ip injection of 7.5 mmol [D^{10}]-2,5-HD, followed by termination and recovery of serum 1, 2, 6, or 18 hr after dosing. Serum diketone was extracted with methylene chloride. Deuterium incorporation in the whole molecule (solid bars), terminal methyl groups (diagonal bars), and internal methylene groups (open bars) was determined by mass spectrometry (see Methods). Data are expressed as percentage of deuterium incorporation at each position relative to pure [D^{10}]-2,5-HD (standard), which contained >94 atom% D. Additional samples (spike) were prepared in which the deuterated derivative was added directly to control rat serum and then extracted. Bars represent the mean ± standard deviation of values from two to three animals per group.

myelin (DeCaprio *et al.*, 1983), red blood cell (Anthony *et al.*, 1983), and axonal cytoskeletal proteins (DeCaprio and O'Neill, 1985) from animals exposed to γ-diketones.

More direct evidence for the critical role of pyrroles in γ-diketone neuropathy has come from recent studies utilizing 2,5-HD isomers substituted at the three and/or four positions. Anthony *et al.* (1983) demonstrated that DMHD was a neurotoxin 20 to 30 times more potent than 2,5-HD in the rat. This derivative was also found to form pyrroles *in vitro* and *in vivo* at a substantially increased rate, although pyrrole levels in axonal cytoskeletal proteins per se were not reported. In general, these results are compatible with the pyrrole hypothesis; however, interpretation of these data may not be straightforward. Unlike the axonal swellings induced by 2,5-HD, those produced by DMHD are localized to the proximal segments of affected axons. This finding is difficult to account for solely on the basis of either an increased rate of pyrrole formation or of secondary autoxidative crosslinking, which also appeared to be accelerated with DMHD.

Sayre and co-workers (1986) provided evidence for the lack of effect in the rat of 3,3-dimethyl-2,5-hexanedione, a γ-diketone which would form an imine that cannot undergo cyclization to the pyrrole. Genter *et al.* (1987) examined the neurotoxicity of a series of *d,l* and *meso* diastereoisomers of several 3,4-disubstituted γ-diketones, including DMHD, 3,4-diethyl-2,5-HD, and 3,4-diisopropyl-2,5-HD. Within the dimethyl-substituted group, the *d,l* isomers were found to be more potent neurotoxins than the corresponding *meso* isomers. The *d,l* isomers also formed pyrroles more rapidly *in vitro*, although *in vivo* pyrrole levels were not reported in these studies. The diethyl- and diisopropyl-substituted derivatives were not neurotoxic and formed pyrroles only very slowly *in vitro*. These findings provide additional support for a requirement for pyrrole formation in this neuropathy.

Until recently, the actual mechanism of the pyrrolylation reaction was uncertain, although several workers had proposed imines as intermediates (Hazlewood *et al.*, 1938; Smith-Broadbent *et al.*, 1968; Graham *et al.*, 1982). Using organic reaction conditions, this mechanism was confirmed by Katritzky and co-workers (1986). They characterized the major rate-determining step in the reaction as the nucleophilic attack of enamine nitrogen (after imine–enamine tautomerization) on the second carbonyl carbon of 2,5-HD, followed by β-elimination of water to form the pyrrole. A secondary rate-limiting step was found to be the initial attack of amine nitrogen on the first carbonyl carbon. We have demonstrated a similar mechanism for pyrrole formation under aqueous reaction conditions (DeCaprio *et al.*, 1987).

In view of this requirement for two C–H bond breaking steps in pyrrole formation, it was hypothesized that the reaction should exhibit a pronounced primary isotope effect. Thus, $[D^{10}]$-2,5-HD should form the pyrrole less readily than native 2,5-HD, and if pyrrole formation is required for γ-diketone neuropathy, the deuterated derivative should be a less potent neurotoxin. In addition, with the exception of its pyrrole-forming potential, $[D^{10}]$-2,5-HD would display physicochemical properties very similar to those of native 2,5-HD. This experimental approach had the advantage of avoiding potential difficulties associated with basing mechanistic conclusions regarding 2,5-HD neuropathy on the chemically distinct 3,4-disubstituted species.

In the present investigation, a primary isotope effect was confirmed by *in vitro* experimentation. $[D^{10}]$-2,5-HD pyrrolylated BSA at only one-third of the initial rate seen with 2,5-HD. However, with prolonged incubation, pyrrole concentrations with both isomers were similar. Thus, the absolute number of lysine ϵ-amine groups in BSA ultimately susceptible to attack by γ-diketone was apparently unaffected by deuterium incorporation. Analysis of the hydrogen–deuterium exchange rates at each carbon position in $[D^{10}]$-2,5-HD by mass spectrometry indicated the relative stability of the internal methylene C–D bonds under physiological conditions. Consequently, it was expected that the magnitude of the isotope effect would not be reduced by back replacement of one or more of the internal deuterium atoms with hydrogen prior to reaction with tissue protein.

Equimolar doses of 2,5-HD were more toxic than [D^{10}]-2,5-HD in the rat, as judged by body weight loss, clinical signs of neurotoxicity, and neuropathological and testicular damage. These findings were correlated with significantly lower pyrrole adduct levels in serum and in brain stem and spinal cord axonal cytoskeletal proteins. That the lower pyrrole concentrations were not simply due to decreased tissue uptake of deuterated as compared to native 2,5-HD was confirmed by determination of tissue diketone levels. These results provide strong additional support for the pyrrole hypothesis in γ-diketone neuropathy and also suggest that pyrrole formation may be important in the mechanism of testicular degeneration encountered with 2,5-HD (Chapin *et al.*, 1983).

The difference in tissue pyrrole content between 2,5-HD and [D^{10}]-2,5-HD-treated rats was more pronounced in those animals given 3.5 mmol/kg/day for 17 days as compared to those administered 2.5 mmol/kg/day for 38 days, despite the higher total dose in the latter groups. In fact, no significant difference in brain stem cytoskeletal protein pyrrole concentrations was detected between the 38-day dosage groups. In view of the *in vitro* findings discussed above, it is likely that pyrrole concentrations in the [D^{10}]-2,5-HD-treated groups had begun to approach those present in the 2,5-HD group after 38 days of exposure. Thus, it might be predicted that prolonged administration of the deuterated isomer should eventually result in frank neurotoxicity and nervous tissue damage. If correct, this hypothesis would be consistent with a required threshold level of pyrrole formation in nerve protein for the induction of neuropathy.

A somewhat surprising result was that pyrrole adduct concentrations in rats administered [D^{10}]-2,5-HD for 17 days were not significantly different from those in animals given 2,5-HD for 38 days, even though only the latter group exhibited neurotoxicity. One explanation for this finding may be that repair and/or degenerative processes within the axon had proceeded to a relatively greater extent in the 38-day treatment group, thus resulting in the removal of axonal protein and consequent decreased pyrrole levels. Some evidence for the existence of clearance mechanisms in the axon capable of removing pyrrolylated protein has been reported (DeCaprio and O'Neill, 1985). In view of the dynamic temporal processes likely to be involved in axonal degeneration, such intergroup (i.e., 17 day vs 38 day) comparisons of absolute pyrrole levels must be made with caution.

Pyrrole formation in axonal cytoskeletal protein now appears to be a necessary step in γ-diketone neuropathy, although it is still unclear whether it alone is sufficient to induce all of the pathological sequelae associated with the syndrome. In addition, the steps linking this reaction with ultimate nerve degeneration are unknown. Several models which involve the disruption of cytoskeletal structure or NF–microtubule (MT) interactions within the axon following NF pyrrolylation have been proposed (DeCaprio and O'Neill, 1985; Sayre *et al.,* 1985). These mechanisms are supported by studies demonstrating rapid NF–MT reorganization following direct, *in situ* exposure of nerve fibers to 2,5-HD (Griffin *et al.*, 1983; Zagoren *et al.*, 1983). In contrast, Graham *et al.* (1982) have postulated that autoxidation and crosslinking of pyrrolylated neurofilaments is also required to induce neuropathy. Such covalent protein crosslinking has been demonstrated in several *in vivo* studies (Anthony *et al.*, 1983; Graham *et al.*, 1984; DeCaprio and O'Neill, 1985; Carden *et al.*, 1986; Lapadula *et al.*, 1986; DeCaprio, 1987).

Although results from the present investigation do not afford unequivocal support for either possibility discussed above, further data related to the mechanism of the autoxidation reactions have been provided. The degree of covalent crosslinking was correlated with pyrrole adduct concentrations in axonal cytoskeletal proteins, suggesting that there was no relative difference in autoxidizability of the pyrrole formed by $[D^{10}]$-2,5-HD as compared to that with native 2,5-HD.

In addition, preliminary *in vitro* studies in our laboratory have indicated similar rates of covalent crosslinking of pyrrolylated BSA synthesized using either isomer. Other workers have demonstrated a minimal exchange of the ring hydrogens and essentially no exchange of the methyl hydrogens in 1,2,5-trimethylpyrrole at neutral pH (Alexander and Butler, 1980). Consequently, deuterium incorporation at each position in the pyrrole adduct should have been equivalent to that in the parent $[D^{10}]$-2,5-HD molecule just prior to reaction. One would therefore expect near 100% D at ring positions three and four and variable incorporation within the 2,5-dimethyl functions.

We have postulated that the autoxidative crosslinking phenomenon is a free-radical-mediated process involving either radical substitution (via H abstraction) or radical addition to the pyrrole ring (DeCaprio, 1986). Both processes have been shown to occur with substituted pyrroles under organic reaction conditions (Conant and Chow, 1933; Bridger and Russell, 1963; Gritter and Chriss, 1964). Since C–H bonds are being broken, the substitution mechanism would be expected to exhibit a primary isotope effect, resulting in a decreased rate of crosslinking. As this was not observed in the present investigation, the results support the alternative free-radical addition mechanism for pyrrole adduct autoxidation *in vivo*.

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