Communications

Aliphatic Diketones: Influence of Dicarbonyl Spacing on Amine Reactivity and Toxicity

Sir: The hexacarbons *n*-hexane and methyl *n*-butyl ketone produce a well-defined syndrome of nervous system and testicular dysfunction in the rat following their metabolism to the γ-diketone, 2,5-hexanedione (*1*). The synthesis and study of multiple congeners has helped define the chemistry of γ-diketone toxicity. That 2,5-hexanedione first acts by forming pyrroles with protein lysyl ε-amines is supported by (1) the increased neurotoxicity and pyrrole forming ability of *dl*-3,4-dimethyl-2,5-hexanedione, which favors the synclinal (gauche) conformation, compared with 2,5-hexanedione or *meso*-3,4-dimethyl-2,5-hexanedione, both of which favor an antiperiplanar conformation (*2*), (2) the lack of neurotoxicity with 3,3-dimethyl-2,5-hexanedione, a γ-diketone incapable of pyrrole formation (*3*), and (3) the decreased neurotoxicity and decreased pyrrole forming ability of perdeuterio-2,5-hexanedione when compared with 2,5-hexanedione (*4*).

An alteration in neurofilament solubility resulting from pyrrole derivatization has been proposed as the mechanism of γ-diketone-induced neurotoxicity (*5*, *6*). We have argued that an additional cross-linking reaction involving the pyrrole ring is required for the expression of toxicity. Evidence supporting the requirement for an additional reaction of the pyrrole ring includes (1) the greater neurotoxicity of 2,5-hexanedione when accompanied by exposure to hyperbaric oxygen, a potential mediator of pyrrole oxidation and further reaction (*7*), (2) the enhanced neurotoxicity of *meso*-3,4-dimethyl-2,5-hexanedione which forms a more reactive pyrrole than 2,5-hexanedione in the presence of equivalent rates of cyclization,[1] and (3) the correlation between testicular injury and altered microtubule assembly which occurs as a consequence of tubulin cross-linking (*8–10*).

To further examine the roles of amine derivatization and cross-linking in toxicity, we have explored the chemical reactivity and in vivo effects of the δ-diketone 2,6-heptanedione and the ε-diketone 2,7-octanedione. The spacing between the two carbonyl functions of δ-diketones allows for cyclization with amines to form dihydropyridine derivatives (*11*). The chemistry of dihydropyridines is complex, and numerous further reactions can occur with these labile intermediates (*12*). Oxidation of a single dihydropyridine may occur to produce a stable pyridinium moiety while reactions between dihydropyridines or complex condensations of δ-diketones with amines may result in cross-linking. Such cross-linking products, specifically a 3-(2-piperidyl)pyridinium derivative and a complex quaternary pyridinium system, have been identified in the reaction of the δ-dialdehyde glutaraldehyde with model amines and proteins (*13*, *14*).

The relative amine reactivities of 2,5-hexanedione,[2] 2,6-heptanedione, and 2,7-octanedione were determined by incubation with ovalbumin. 2,6-Heptanedione derivatized ovalbumin lysyl ε-amines more rapidly than 2,5-hexanedione (Figure 1). In contrast, the ε-diketone, 2,7-octanedione did not decrease the free amine concentration of ovalbumin during incubation, being essentially incapable of forming long-lived adducts with amines under biologic conditions since this would require the development of a seven-membered ring.

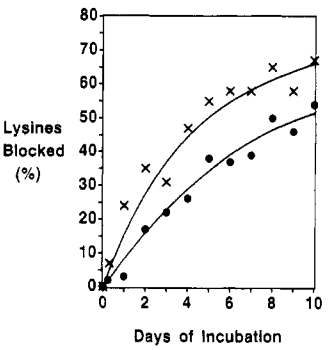

Figure 1. Comparative derivatizing abilities of 2,6-heptanedione (×) and 2,5-hexanedione (●). Ovalbumin (2 mmol/L in lysyl residues) and 200 mmol of diketone/L were reacted in 100 mmol of triethanolamine/L, pH 9.0, at 37 °C. After various times, aliquots were withdrawn and dialyzed. Protein concentration was determined by the Biuret method (*17*), and the free lysyl ε-amine content was determined by reaction with trinitrobenzenesulfonic acid (*18*). Only 80% of ovalbumin lysyl amines are available for reaction (*19*).

To compare their relative cross-linking abilities, the diketones were incubated with ovalbumin and [^{3}H]lysine (Figure 2[3]). The amount of covalently bound label was determined following trichloroacetic acid precipitation of the protein. 2,5-Hexanedione was the most active cross-linking agent with 2,6-heptanedione demonstrating 10–20% of the 2,5-hexanedione activity and no cross-linking by 2,7-octanedione.

NMR spectroscopy was performed on polylysine following incubation with 2,6-heptanedione in the presence of sodium dithionite. The NMR spectrum (Figure 3) supported 2,6-dimethylpiperidine as the structure of the reduced, covalently bound 2,6-heptanedione adduct (*20*, *21*), as predicted by the known imine-reducing ability of sodium dithionite (*22*) and the known cyclizing potential of δ-amino ketones[4] (*11*). Integration of the peaks showed

[1] The additional electron-releasing methyl groups destabilize the pyrrole ring as evidenced by the greater ease of oxidation determined by cyclic voltammetry (*2*).

[2] 2,5-Hexanedione was obtained commercially (Aldrich Chemical Co., Milwaukee, WI) and distilled prior to use, while 2,6-heptanedione and 2,7-octanedione were synthesized by established methods (*15*, *16*) with structural identity and purity confirmed by GC–MS, NMR, and mp [2,6-heptanedione, mp 30-31 °C (lit. mp 33–34 °C); 2,7-octanedione mp 41 °C (lit. mp 44 °C)].

[3] All multiple determinations reported in the text and in figures are expressed as the mean ± standard error. Statistical determinations use the two-tailed Student's *t* test with significance assigned to $p < 0.05$.

[4] The NMR distinguishes between the potential derivatives, namely, an open chain secondary diamine resulting from ketimine reduction and a cyclic piperidine. Since the reducing agent used, sodium dithionite, is essentially incapable of reducing ketones to alcohols under the described reaction conditions (*23*), the only open chain adduct to consider is the diamine (the monoamine adduct would cyclize). The symmetric diamine adduct would not demonstrate the complex methyl resonance shown following acidification; however, such a shift of methyl resonances following acidification is characteristic for piperidines (*20*, *21*).

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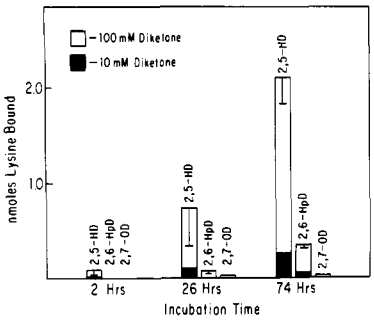

Figure 2. Comparative cross-linking abilities of the three diketones. Ovalbumin (3.72 mmol/L in lysyl residues) and 37.2 μmol of L-[4,5-^{3}H]lysine/L (New England Nuclear, Boston, MA; 30 000 cpm/nmol) were reacted with either 10 or 100 mmol of diketone/L at 37 °C in 160 mmol of sodium phosphate/L, pH 7.4. A background control was provided by a reaction mixture containing either 20 or 200 mmol of acetone/L. Reaction aliquots of 1 mL were withdrawn at various times, and 5 mL of 10% trichloroacetic acid was added. The precipitated protein was collected on a glass fiber filter, washed with 5% trichloroacetic acid and ethanol, dried, and then digested with 1 mL of NCS Tissue Solubilizer (Amersham, Arlington Heights, IL) overnight. Counts were obtained with a Beckman LS-150 liquid scintillation system (scintillation cocktail: 5.0 g of PPO/L and 0.2 g of POPOP/L in toluene). Cross-linking activity was determined in duplicate and is reported as nmol of lysine bound to 0.18 μmol of ovalbumin.

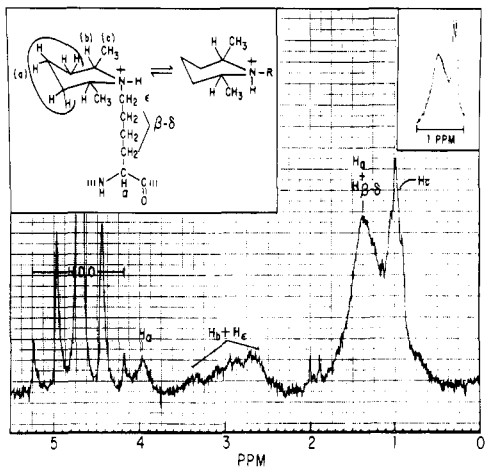

Figure 3. NMR spectrum of 2,6-heptanedione-derivatized polylysine. Polylysine (15 mmol/L in lysyl residues, average molecular weight of 190 kDa, Sigma Chemical Co., St. Louis, MO) in 100 mmol of sodium phosphate/L pH 8.0, with added 200 mmol of 2,6-heptanedione/L and 50 mmol of sodium dithionite/L was incubated at 37 °C for 7 days followed by exhaustive dialysis and lyophilization. The derivatized polylysine was dissolved in 8 mol of urea/L in D_2O, and NMR using an IBM NR-80 FT spectrometer demonstrated the presence of a doublet (methyl resonance) at 1.0 ppm (right inset). The main NMR spectrum shown was obtained after the addition of thionyl chloride. The complexity of the methyl resonance following addition of thionyl chloride can be explained by the presence of relatively long-lived equatorial and axial protonated species which have slightly different chemical shifts (left inset). No attempt is made to distinguish between the cis and trans isomers which likely add further complexity to the resonance pattern. Peak assignments were made by extrapolation from spectra of unreacted polylysine and 2,6-dimethylpiperidine.

that approximately 75% of the ε-amines of polylysine were derivatized. NMR spectra obtained with polylysine reacted with 2,6-heptanedione without added reducing agent were limited by the low solubility of the resulting polymeric material. Low resolution precluded definitive identification of the adducts formed under nonreducing conditions; however, the presence of a peak at 6.9 ppm and the downfield shift of the methyl groups were most consistent with a cyclic aromatic derivative, most likely the 2,6-dimethylpyridinium species.

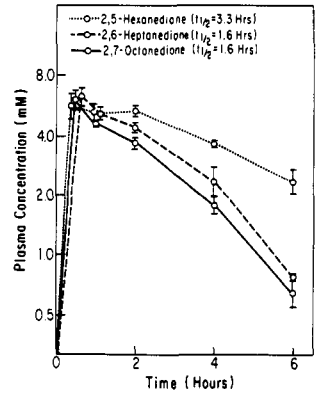

Figure 4. Plasma concentration of diketone (log scale) was determined in two Sprague–Dawley rats (250–300 g) following intraperitoneal injection (4 mmol/kg). Tail vein blood was withdrawn and 1 μL of plasma was injected directly into a Varian series 1400 gas chromatograph with a Chromosorb 101 column set to an injector temperature of 140 °C, column temperature of 120–130 °C, and detector temperature of 170 °C. Peak areas were quantitated by the cut and weigh method. Peak plasma concentrations reached approximately 6 mmol/L. The half-lives ($t_{1/2}$) were calculated by determining the best linear fit to the 2-, 4-, and 6-h time points.

Plasma concentrations of the diketones were determined in Charles River CD rats after intraperitoneal injection (Figure 4). Absorption of the compounds occurred rapidly, yielding similar peak plasma concentrations. However, the biologic half-lives varied, with a $t_{1/2}$ of 3.3 h for 2,5-hexanedione and a $t_{1/2}$ of 1.6 h for 2,6-heptanedione and 2,7-octanedione.

To assess in vivo toxicity, rats received daily intraperitoneal injections of 4 mmol of 2,6-heptanedione/kg. Only three of seven treated rats survived the entire injection period with necrotizing pneumonia as the predominant cause of premature death. After 11 weeks (total dose 308 mmol/kg), treated rats exhibited generalized wasting but no clinically detectable neuropathy (body weights: treated, 289.7 ± 12.9 g; control 416.3 ± 11.7 g; $p < 0.001$). Following perfusion fixation, Epon embedding, and teased fiber preparation (*24*), examination of the muscular branch of the tibial nerve revealed no treatment-related light microscopic alterations. The testis weights of rats injected with 2,6-heptanedione did not differ significantly from controls (testis weight: treated, 1.15 ± 0.05 g; control: 1.62 ± 0.11 g).

In a second intoxication experiment, 2,6-heptanedione was administered in the drinking water as a 1% solution for 7 weeks followed by a 10-week period of 2% 2,6-heptanedione exposure, achieving an average daily dose of 11.3 mmol/kg (total dose 1,342 mmol/kg). No mortality or clinical neurotoxicity was experienced. When sacrificed at 17 weeks, body weights were similar to control (treated, 509.4 ± 8.7 g, $n = 6$; control, 560.2 ± 22.5, $n = 6$). Histopathologic examination of peripheral nerve cross sections and teased fiber preparations showed no treatment-related light microscopic effects. The testis weights were normal (treated, 1.97 ± 0.04 g; control 2.09 ± 0.05 g).

Intraperitoneal injection of 2,7-octanedione (4 mmol/kg/day for 11 weeks) produced a severe local inflammatory reaction with intraperitoneal abscess formation and fibrosis. Since only one of six rats survived the entire

treatment period, this compound was not adequately tested; however, no clinical signs of neurotoxicity were observed and evaluation of the survivor revealed unremarkable light microscopy of peripheral nerve cross sections and normal testis weight.

Diketones are metabolic end products arising from hepatic microsomal ω-1 oxidation of aliphatic compounds (*1*, *25–28*). The chain length of the parent hydrocarbon determines the spacing between the carbonyl functions of the diketone metabolite, and this spacing strongly influences toxicity (*29*, *30*). In this study, we compared the in vitro reactivities and in vivo toxicities of three aliphatic diketones. 2,6-Heptanedione formed covalent derivatives with amines in vitro; in addition, protein cross-linking occurred on incubation with this δ-diketone. NMR spectra of 2,6-heptanedione-derivatized polylysine supported the formation of cyclic adducts. Compared to 2,5-hexanedione, 2,6-heptanedione more readily cyclized with amines but was less effective at forming cross-links between amines. The ϵ-diketone 2,7-octanedione produced neither derivatives nor cross-links with amines. Following intraperitoneal injection, the biologic half-life of 2,5-hexanedione was approximately twice that of 2,6-heptanedione and 2,7-octanedione. Subchronic exposure to 2,6-heptanedione produced neither neurotoxicity nor testicular atrophy, confirming a previous report of 2,6-heptanedione administered by gavage at 3.9–7.8 mmol/kg/day 5 days/week for 13 weeks (*31*, *32*).

The neurotoxic potency of the γ-diketones is clearly dependent upon their rate of pyrrole formation, identifying cyclization as an essential step in the development of an injury. The lack of neurotoxicity or testicular atrophy with δ-diketone exposure indicates a requirement for a cross-linking reaction in the development of organ-specific injury: 2,6-heptanedione more easily forms cyclic derivatives with amines when compared with 2,5-hexanedione, but its rate of subsequent cross-linking is comparatively low.

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Registry No. 2,5-hexanedione, 110-13-4; 2,6-heptanedione, 13505-34-5; 2,7-octanedione, 1626-09-1; lysine, 56-87-1; polylysine, 25104-18-1; polylysine, SRU, 38000-06-5.

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