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Isotope Effects on the Metabolism and Pulmonary Toxicity of Butylated Hydroxytoluene in Mice by Deuteration of the 4-Methyl Group[1]

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Isotope Effects on the Metabolism and Pulmonary Toxicity of Butylated Hydroxytoluene in Mice by Deuteration of the 4-Methyl Group. MIZUTANI, T., YAMAMOTO, K., AND TAJIMA, K. (1983). *Toxicol. Appl. Pharmacol.* **69,** 283–290. A comparative test in mice for pulmonary toxicity between butylated hydroxytoluene (2,6-di-*tert*.-butyl-4-methylphenol, BHT) and 2,6-di-*tert*.-butyl-4-[α,α,α-$^2H_3$]methylphenol (BHT-d_3) showed a significantly lower toxic potency of the latter. The rate of *in vitro* BHT metabolism to 2,6-di-*tert*.-butyl-4-methylene-2,5-cyclohexadienone (BHT-QM) was slowed by deuterating BHT in the 4-methyl group. On the other hand, the rate of *in vitro* metabolism to 2,6-di-*tert*.-butyl-4-hydroxy-4-methyl-2,5-cyclohexadienone (BHT-OH) was increased with the deuteration. A similar isotope effect of the deuterium substitution on the *in vivo* metabolic rates of BHT was observed. These observations support the concept that the lung damage caused by BHT is mediated by BHT-QM. The pulmonary toxicity of 2-*tert*.-butyl-4-ethylphenol (4-EP) and their deuterated analogs was also compared. 2-*tert*.-Butyl-4-[1,1-$^2H_2$]ethylphenol (4-EP-d_2) showed a significantly lower toxic potency than 4-EP, whereas 2-*tert*.-butyl-4-[2,2,2-$^2H_3$]ethylphenol (4-EP-d_3) showed a toxic potency comparable to that of 4-EP. This result is consistent with the hypothesis that a quinone methide metabolite is responsible for the onset of lung damage produced by 4-EP as well as BHT.

Butylated hydroxytoluene (2,6-di-*tert*.-butyl-4-methylphenol, BHT) is a widely used antioxidant. BHT has been shown to cause lung damage in mice (Marino and Mitchell, 1972; Saheb and Witschi, 1975), which is characterized by early necrosis of type I alveolar cells and subsequent proliferation of type II alveolar cells (Adamson *et al.*, 1977).

Malkinson (1979) reported that BHT-induced lung damage can be prevented by exposing mice to cedar terpenes and that immature mice are nonresponsive to BHT. Kehrer and Witschi (1980) demonstrated the prevention of BHT-induced lung damage by the coadministration of drug metabolism inhibitors SKF-525A or piperonyl butoxide. BHT given to mice becomes covalently bound to lung tissue and this covalent binding can be prevented by the administration of SKF-525A (Kehrer and Witschi, 1980). These findings suggest that a reactive metabolite of BHT is responsible for the onset of lung damage in mice.

Based upon a structure–activity study with BHT analogs, we have recently suggested that a quinone methide, 2,6-di-*tert*.-butyl-4-methylene-2,5-cyclohexadienone (BHT-QM), or closely related metabolites may play a role in producing lung damage in mice dosed with BHT (Mizutani *et al.*, 1982). The present work was undertaken to add positive support for this suggestion and deals with the isotope ef-

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fects on the metabolism and pulmonary toxicity of BHT in mice by deuteration of the 4-methyl group.

METHODS

Chemicals

Chemicals were purchased as follows: lithium aluminum deuteride ($LiAlD_4$, 98% isotopic purity) from E. Merck A. G., Darmstadt, West Germany; sodium borodeuteride ($NaBD_4$, 97.4% isotopic purity) from Commissariat al' Energie Atomique, France; BHT from Nakarai Chemicals, Ltd., Kyoto, Japan; *p*-bromothiophenol from Aldrich Chemical Company, Milwaukee, Wisconsin; NADP from Kohjin Company Ltd., Tokyo, Japan; glucose 6-phosphate from Sigma Chemical Company, St. Louis, Missouri. All other reagents were of the highest purity available.

2,6-Di-*tert.*-butyl-4-hydroxy-4-methyl-2,5-cyclohexadienone (BHT-OH) (Kharasch and Joshi, 1957) and 2-*tert.*-butyl-4-ethylphenol (4-EP) (Mizutani *et al.*, 1982) were synthesized according to the described methods.

Syntheses

2,6-Di-tert.-butyl-4-[α,α,α-$^2H_3$]methylphenol (BHT-d_3). 3,5-Di-*tert.*-butyl-4-hydroxybenzoic acid (Yohe *et al.*, 1956) was converted to the methyl ester, mp; 166–167°C. The ester (0.03 mol, 7.9 g) dissolved in ether (100 ml) was added to a solution of $LiAlD_4$ (0.09 mol, 3.8 g) in ether (200 ml), and the mixture was refluxed under nitrogen for 12 hr. The excess reagent was decomposed with ethyl acetate and 10% sodium hydroxide, and the resulting precipitate was filtered off. The organic layer was washed with water, dried, and freed from the solvent. Column chromatography on silica gel gave BHT-d_3, mp 68–69°C; MS *m/e* 223 (M^+, 32%), 208 (100), 180 (5), 148 (11), isotopic purity 98%; NMR ($CDCl_3$) δ 7.05 (s, 2 H), 5.04 (s, 1 H), 1.44 (s, 18 H).

2,6-Di-tert.-butyl-4-hydroxy-4-[α,α,α-$^2H_3$]methyl-2,5-cyclohexadienone (BHT-OH-d_3). According to the procedure of Kharasch and Joshi (1957), BHT-d_3 was oxygenated to BHT-OH-d_3, mp 110–112°C; MS *m/e* 239 (M^+, 21%), 221 (21), 183 (100), 168 (97), isotopic purity 97%; NMR ($CDCl_3$) δ 6.66 (s, 2 H), 1.92 (s, 1H), 1.21 (s, 18 H).

2,6-Di-tert.-butyl-4-(4'-bromophenylthio[α,α-$^2H_2$]-methyl)phenol (BHT-SPhBr-d_2). 3,5-Di-*tert.*-butyl-4-hydroxybenzoic acid (Yohe *et al.*, 1956) was converted to the acid chloride, which was hydrogenated with $NaBD_4$ in dioxane to 3,5-di-*tert.*-butyl-4-hydroxy[α,α-$^2H_2$]benzyl alcohol, mp 142–143°C. Treatment of the alcohol with thionyl chloride yielded 3,5-di-*tert.*-butyl-4-hydroxy[α,α-$^2H_2$]benzyl chloride. A solution of the chloride (2 mmol, 500 mg) in acetone (2 ml) was added to a mixture of *p*-bromothiophenol (2 mmol, 380 mg) and potassium carbonate (200 mg) in acetone (8 ml), and the mixture was stirred at room temperature for 2 hr. The mixture was poured into water and extracted with benzene. The organic layer was washed with 5% sodium hydroxide and then with water, dried, and freed from the solvent. Column chromatography on silica gel gave BHT-SPhBr-d_2, mp 95–96°C; MS *m/e* 408 (M^+, 1.5%), 221 (100), 205 (7); NMR ($CDCl_3$) δ 7.48, 7.25 (q, J = 8 Hz, 4 H), 7.11 (s, 2 H), 5.23 (s, 1 H), 1.40 (s, 18 H), isotopic purity 96%.

2,6-Di-tert.-butyl-4-(4'-bromophenylthiomethyl)-phenol (BHT-SPhBr). This compound was prepared by the same method as described above for BHT-SPhBr-d_2 with 3,5-di-*tert.*-butyl-4-hydroxybenzyl chloride (Chasar and Westfahl, 1977) and *p*-bromothiophenol as starting materials. BHT-SPhBr, mp 93–95°C; MS *m/e* 406 (M^+, 1.2%), 219 (100), 203 (6); NMR ($CDCl_3$) δ 7.48, 7.25 (q, J = 8 Hz, 4 H), 7.11 (s, 2 H), 5.22 (s, 1 H), 4.08 (s, 2 H), 1.40 (s, 18 H).

2-tert.-Butyl-4-[1,1-$^2H_2$]ethylphenol (4-EP-d_2). According to the procedure of Brown and White (1957), 4-hydroxyacetophenone was hydrogenated with $LiAlD_4$ and aluminum chloride to 4-[1,1-$^2H_2$]ethylphenol, bp 64–66°C/4 mm Hg. The ethylphenol was alkylated with *tert.*-butyl chloride as described previously (Mizutani *et al.*, 1982). 4-EP-d_2, bp 110–113°C/14 mm Hg; MS *m/e* 180 (M^+, 32%), 165 (100), 137 (27), isotopic purity 95%; NMR ($CDCl_3$) δ 7.15 (d, J = 2 Hz, 1 H), 6.97 (d of d, J = 2, 8 Hz, 1 H), 6.61 (d, J = 8 Hz, 1 H), 4.72 (s, 1 H), 1.41 (s, 9 H), 1.20 (s, 3 H).

2-tert.-Butyl-4-[2,2,2-$^2H_3$]ethylphenol (4-EP-d_3). 4-Hydroxyphenylacetic acid was converted to methyl 4-methoxyphenylacetate, which was hydrogenated with $LiAlD_4$ to 4-methoxy[α,α-$^2H_2$]phenethyl alcohol, bp 125–126°C/10 mm Hg. Treatment of the alcohol with phosphorous tribromide in carbon tetrachloride yielded 4-methoxy[α,α-$^2H_2$]phenethyl bromide, bp 114–117°C/11 mm Hg. The bromide was hydrogenated with $LiAlD_4$ to 4-methoxy[2,2,2-$^2H_3$]ethylbenzene by refluxing for 10 hr in tetrahydrofuran. Demethylation with boron tribromide in dichloromethane yielded 4-[2,2,2-$^2H_3$]ethylphenol, which was treated with *tert.*-butyl chloride as descibed above. 4-EP-d_3, bp 112–114°C/14 mm Hg; MS *m/e* 181 (M^+, 32%), 166 (100), 138 (35), isotopic purity 97%; NMR ($CDCl_3$) δ 7.13 (d, J = 2 Hz, 1 H), 6.97 (d of d, J = 2, 8 Hz, 1 H), 6.63 (d, J = 8 Hz, 1 H), 4.71 (s, 1 H), 2.58 (s, 2 H), 1.41 (s, 9 H).

Animals

Male ddY mice (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan), 8 weeks of age, were used.

Mice were housed in plastic cages on a wood chip bedding (White Flake, Charles River Japan, Inc., Kanagawa,

Japan) and received a standard laboratory chow (Funabashi F-2, Funabashi Farms, Chiba, Japan) and water *ad libitum*. The animal room was controlled for temperature (23 ± 2°C) and light cycle (12 hr).

Lung Toxicity

BHT, BHT-d_3, 4-EP, 4-EP-d_2, and 4-EP-d_3 were each dissolved in olive oil and administered ip to mice. Control animals were treated with the vehicle alone.

Lung damage was assessed by determining wet and dry lung weights 4 days after the administration.

In Vitro Metabolism

The livers of mice were homogenized in 3 vol of 0.15 M KCl, and the homogenate was centrifuged for 20 min at 9000 *g*. Each incubation mixture contained 2 ml of the 9000 *g* supernatant fraction, 0.5 mM NADP, 5 mM glucose 6-phosphate, and 2 mM $MgCl_2$ in a total volume of 5 ml of 0.1 M phosphate buffer (pH 7.4). Either BHT or BHT-d_3 (2.5 μmol in 50 μl ethanol) was added to the incubation mixture, and the mixture was shaken at 37°C for 10 min in air.

Measurement of BHT-OH. The incubation mixture was poured into 3 ml of acetone and the mixture was centrifuged. The supernatant solution was extracted with hexane and the extract was subjected to gas chromatography with an electron-capture detector (GC–ECD).

Measurement of BHT-QM. The incubation mixture was poured into 3 ml of acetone containing 25 μmol of *p*-bromothiophenol. After standing for 30 min at room temperature, the mixture was centrifuged and the supernatant solution was extracted with hexane. The extract was subjected to GC-ECD analysis after purification by thin-layer chromatography.

In Vivo Metabolism

Measurement of BHT-OH. BHT and BHT-d_3 were each administered ip to mice at a dose of 1500 mg/kg. The lungs and livers were excised after 4 hr and homogenized in 0.1 M phosphate buffer (4 ml/g of tissue). The homogenate was extracted with benzene and the extract was analyzed by GC–ECD.

Measurement of BHT-QM. An equimolar mixture (3000 mg/kg) of BHT and BHT-d_3 was administered ip to mice. The lungs and livers were excised after 4 hr and homogenized in 0.1 M phosphate buffer (4 ml/g of tissue). A solution of 5 mM *p*-bromothiophenol in dioxane (1 ml/g of tissue) was added to the homogenate and the mixture was shaken for 20 min at room temperature. The reaction mixture was extracted with benzene. The extract was dried and evaporated to dryness under a stream of nitrogen. The residue dissolved in hexane was chromatographed on a silica gel dry column (Wakogel C-200, Wako Pure Chemical Industries, Ltd., Osaka, Japan; 10-cm × 8-mm i.d.). Elution with hexane–benzene (4:1) gave fractions 1 (8 ml) and 2 (10 ml). Fraction 2 was analyzed by selected ion monitoring to determine the ratio of the abundance of BHT-SPhBr and BHT-SPhBr-d_2.

Instrumental Analyses

GC–ECD was performed on a Shimadzu GC-3AE gas chromatograph fitted with a 2-m × 3-mm-i.d. glass column packed with Chromosorb W containing 2% OV-1. The analyses of BHT-OH and BHT-SPhBr were conducted at 100 and 175°C, respectively.

Mass spectra were obtained by using a JEOL JMS-D 100 GC–MS spectrometer equipped with a JEOL MS-PD-01 multiple ion detector. The ionizing energy was 22 eV. The isotopic purities of BHT-d_3, 4-EP-d_2, and 4-EP-d_3 were determined from the parent ions in the electron-impact mass spectra. To determine the ratio of the abundance of BHT-SPhBr and BHT-SPhBr-d_2, the selected ion monitor was focused on the ions *m/e* 219 for BHT-SPhBr and *m/e* 221 for BHT-SPhBr-d_2, and the peak height ratio was measured.

NMR spectra were measured in $CDCl_3$ with tetramethylsilane as an internal standard on a JEOL JMN MH-100 100 MHz spectrometer. The isotopic purity of BHT-SPhBr-d_2 was determined by measuring the residual methylene resonance at δ 4.08.

RESULTS

Isotope Effect on Pulmonary Toxicity of BHT

The effects of 1.39, 1.67, and 2.00 mmol/kg BHT or BHT-d_3 on lung/body weight ratio are shown in Fig. 1A. Both compounds caused dose-related increases in lung/body weight ratio. At each dose, however, BHT-d_3 produced a significantly lower increase in lung/body weight ratio than did BHT.

Similarly, BHT and BHT-d_3 caused dose-related increases in dry lung weight, and the increase caused by BHT-d_3 was consistently lower than that caused by BHT (Fig. 1B). The differences were statistically significant except at the highest dose level, 2.00 mmol/kg.

The effects of BHT and BHT-d_3 on the percentage change in body weight of mice during the experiment (4 days) are shown in Fig. 1C. A dose-dependent reduction in body weight

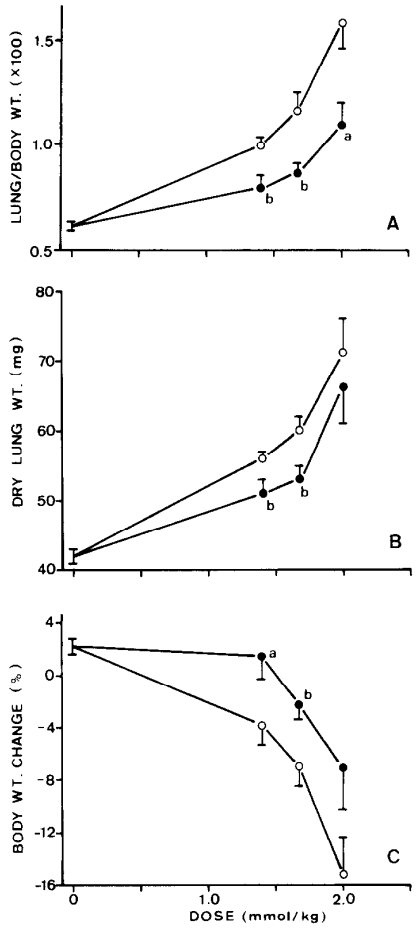

FIG. 1. Effects of different doses of BHT (○) and BHT-d_3 (●) on (A) lung/body weight ratio, (B) dry lung weight, and (C) body weight change during the experiment. Mice were injected ip with an olive oil solution of each agent at doses of 1.39, 1.67, and 2.00 mmol/kg. Control mice were given the vehicle alone. Mice were killed 4 days after injection, and the lung and body weights were determined. Each point represents mean ± SE of 8 to 18 animals. (a) and (b) Indicate values are significantly different from corresponding BHT values ($P < 0.01$ and $P < 0.05$, respectively).

was found for both BHT and BHT-d_3. At each dose, mice receiving BHT lost more weight than did mice treated with BHT-d_3, and the differences were statistically significant except at the highest dose level.

Isotope Effect on in Vitro Metabolism of BHT

A procedure was developed to determine BHT-OH and BHT-QM formed by *in vitro* or *in vivo* metabolism of BHT. ECD exhibited a sensitive response toward BHT-OH possibly because of its conjugated structure. Therefore, BHT-OH at nanogram levels could be detected directly by GC–ECD. BHT-QM, which is known to be an unstable metabolite (Tajima *et al.*, 1981), was trapped with *p*-bromothiophenol and analyzed as BHT-SPhBr by GC–ECD. There was no isotope effect on this trapping reaction. Although the trapping reaction resulted in a more than 80% recovery, the data obtained through this method should be considered as semiquantitative, because it appears rather likely that the resulting BHT-QM also reacts with cellular nucleophiles, such as glutathione and the cysteine components of proteins. BHT-OH and BHT-SPhBr (derivatized from BHT-QM) were identified from their mass spectral fragmentation patterns and by comparison of their GC retention times with those of authentic samples. Typical gas chromatograms of BHT-OH and BHT-SPhBr formed in the *in vitro* experiments are shown in Fig. 2.

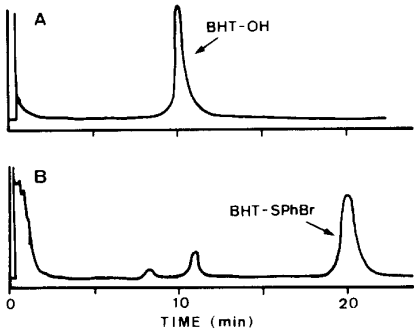

FIG. 2. Typical GC–ECD chromatograms of *in vitro* metabolites, (A) BHT-OH and (B) BHT-QM (after being converted to BHT-SPhBr). Analyses were performed on a 2-m × 3-mm-i.d. glass column of 2% OV-1 operated at (A) 100°C with an inlet pressure of 1.0 kg/cm^2 of nitrogen and (B) 175°C with an inlet pressure of 1.4 kg/cm^2 of nitrogen.

By the above method, the rates of *in vitro* metabolism of BHT and BHT-d_3 were compared (Table 1). Deuteration of BHT in the 4-methyl group resulted in a significant reduction (approximately 40%) in the rate of metabolism to BHT-QM. On the contrary, the rate of metabolism to BHT-OH was significantly increased (approximately 70%) with the deuterium substitution.

Isotope Effect on in Vivo Metabolism of BHT

An equimolar mixture of BHT and BHT-d_3 was administered to mice, and the ratio of deuterated to undeuterated BHT-QM levels in the lung and liver was determined by mass spectrometry with selected ion monitoring. In the lung the amount of BHT-QM per gram of tissue was approximately two times greater than that in the liver 4 hr after dosing. As shown in Table 2, the ratios of deuterated to undeuterated BHT-QM in the lung and liver were 0.66 and 0.85, respectively, indicating that BHT-d_3 was metabolized *in vivo* to BHT-QM at a lower rate than BHT. Although the isotope effect seen in the liver was somewhat small, the effect of the deuteration observed in the lung was comparable to that in the *in vitro* study.

TABLE 1

RELATIVE RATES OF *IN VITRO* METABOLISM OF BHT AND BHT-d_3[a]

Substrate	Metabolite formed (nmol/g liver/min)	
	BHT-QM[b]	BHT-OH
BHT (A)	1.50 ± 0.19	0.91 ± 0.06
BHT-d_3 (B)	0.89 ± 0.18[c]	1.53 ± 0.25[c]
Ratio (B/A)	0.59	1.68

[a] Values represent means ± SE of five determinations.
[b] BHT-QM was determined after being converted to BHT-SPhBr.
[c] Significantly different from BHT values ($P < 0.05$).

TABLE 2

RATIO OF DEUTERATED TO UNDEUTERATED BHT-QM FORMED IN MICE 4 hr AFTER ip ADMINISTRATION OF 3000 mg/kg EQUIMOLAR MIXTURE OF BHT AND BHT-d_3[a]

Tissue	BHT-QM (d_2/d_0)[b]
Lung	0.66 ± 0.03
Liver	0.85 ± 0.01

[a] Values represent means ± SE of four determinations.
[b] BHT-QM was determined after being converted to BHT-SPhBr.

A similar attempt to determine the relative levels of deuterated and undeuterated BHT-OH was unsuccessful because the mass spectrum of control tissue samples gave interfering background peaks. Therefore, *in vivo* BHT-OH (BHT-OH-d_3) levels were determined by GC–ECD after the separate administration of BHT and BHT-d_3. A comparison of the tissue levels of BHT-OH and BHT-OH-d_3 after the administration of BHT or BHT-d_3 is presented in Table 3. In both the lung and liver, BHT-d_3 resulted in a significantly higher level of BHT-OH than did BHT in agreement with the *in vitro* results.

Isotope Effect on Pulmonary Toxicity of 4-EP

The effects of 3.41 mmol/kg 4-EP or its deuterated analogs on lung/body weight ratio are shown in Fig. 3A. 4-EP and 4-EP-d_3 resulted in significant increases in lung/body weight ratio to 161 and 172%, respectively, of the control. 4-EP-d_2, on the contrary, did not cause any significant change in lung/body weight ratio.

With the effects on dry lung weight, a similar pattern was observed (Fig. 3B), that is, 4-EP and 4-EP-d_3 resulted in about the same magnitude of increases (approximately 145% of control); 4-EP-d_2, however, caused a significant, but only small, increase (113%). The change in dry lung weight caused by 4-EP-d_2 was significantly lower than that by either 4-EP or 4-EP-d_3.

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