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THE TOXICOLOGICAL IMPLICATIONS OF THE INTERACTION OF BUTYLATED HYDROXYTOLUENE WITH OTHER ANTIOXIDANTS AND PHENOLIC CHEMICALS

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Abstract—Butylated hydroxyanisole (BHA) enhanced both the *in vitro* peroxidase-catalysed covalent binding of butylated hydroxytoluene (BHT) to microsomal protein and the formation of BHT-quinone methide. Eugenol, methylparaben, vanillin, guaiacol, ferulic acid and several other phenolic compounds commonly used in food and cosmetic products also enhanced the metabolic activation of BHT. BHA was the most effective compound tested. Microsomes from lung, bladder, kidney medulla and small intestine of various animal species, including man, were also able to support this interaction of BHA and BHT using either hydrogen peroxide or arachidonic acid as the substrate. These *in vitro* observations were extended to an *in vivo* mouse lung model. Subcutaneous injections of BHA significantly enhanced the lung/body weight ratio of mice given intraperitoneal injections of subthreshold doses of BHT. The toxicological implications of the interactions of BHT with other antioxidants and phenolic chemicals and their potential relevance to human risk are discussed.

Introduction

Like many xenobiotic compounds, the toxic effects of BHT are thought to be caused by metabolites rather than the parent compound. From a toxicological standpoint, one of the most interesting metabolites of BHT is BHT-quinone methide (2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone). BHT-quinone methide is a relatively recently described hepatic metabolite of BHT in rats (Tajima, Yamamoto & Mizutani, 1981; Takahashi & Hiraga, 1979). The formation of BHT-quinone methide has also been observed *in vitro* in mouse liver microsomes and *in vivo* in mouse liver and lung (Mizutani, Yamamoto & Tajima, 1983). BHT-quinone methide has been suggested to be the ultimate toxic metabolite of BHT responsible for causing lung damage in mice (Mizutani, Ishida, Yamamoto & Tajima, 1982; Mizutani *et al.* 1983) It has also been shown to interfere with vitamin K-dependent protein carboxylation (Takahashi & Hiraga, 1981) and thus may play a role in BHT-induced haemorrhagic death in rats (Takahashi & Hiraga, 1978).

BHT-quinone methide is a reactive compound capable of reacting with various cellular nucleophiles, including glutathione (Tajima, Yamamoto & Mizutani, 1983 & 1985). Administration of high doses of BHT has been shown to deplete levels of glutathione in rat liver and mouse lung (Mizutani, Nomura, Yamamoto & Tajima, 1984; Nakagawa, Tayama, Nakao & Hiraga, 1984). Depletion of glutathione levels with diethyl maleate or buthionine sulphoximine enhanced the toxicity of BHT to mouse lung, whereas dietary administration of cysteine protected mice from BHT-induced lung toxicity (Mizutani *et al.* 1984; Nakagawa, Suga & Hiraga, 1984).

Because of the high reactivity of quinone methides (Turner, 1964), metabolic reactions that result in the formation of these compounds in mammalian systems are of significant interest. BHT-quinone methide is thought to arise from the biotransformation of BHT by a cytochrome *P*-450-related enzyme (Kehrer & Witschi, 1981; Tajima *et al.* 1985). We have recently demonstrated that peroxidase enzymes can catalyse the *in vitro* formation of BHT-quinone methide (Thompson, Cha & Trush, 1986). This report describes the *in vitro* formation of BHT-quinone methide by peroxidase enzymes, the influence of various phenolic compounds on this reaction, and the possible *in vivo* significance of these peroxidase reactions.

Experimental

Materials. (Ring U-^{14}C) BHT (20 mCi/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). Arachidonic acid was obtained from Nu Chek Prep (Elysian, MN). BHT, BHA and other test compounds were obtained from Sigma Chemical Co. (St Louis, MO) or from Aldrich Chemical Co. (Milwaukee, WI). Horseradish peroxidase (type II) was obtained from Sigma and prostaglandin H synthase was prepared from ram seminal vesicles obtained from Dr L. Marnett, Wayne State University. Microsomes from ram seminal vesicles and other tissues were prepared as 25% homogenates in 0.15 M-KCl adjusted to pH 7.8 with dibasic potassium phosphate. The homogenates were centrifuged at 9000 **g** for 20 min, filtered through cheesecloth and then centrifuged at 100,000 **g** for 1 hr. The final microsomal pellets were resuspended in 0.15 M-buffer at

Abbreviations: BDMP = 2-*tert*-butyl-4,6-dimethylphenol; BHA = butylated hydroxyanisole; BHT = butylated hydroxytoluene; BMP = 2-*tert*-butyl-4-methylphenol.

protein concentrations of approximately 10 mg/ml and were then rapidly frozen in a methanol/dry-ice bath and stored at −80°C.

Covalent binding of BHT metabolite(s) to microsomal protein. Reactions were initiated by the addition of 330 μM-arachidonic acid or 0.9 mM-hydrogen peroxide and were allowed to proceed for 10 min at 37°C. Reactions contained 1 mg microsomal protein, 100 μM-BHT (0.25 μCi/tube), and 100 μM-BHA (when specified) in a total of 1 ml 0.1 M-phosphate buffer, pH 7.5. Incubations with ram seminal vesicle microsomes were carried out at room temperature with 200 μM-BHT (0.25 μCi/tube) and 100 μM-BHA (when specified) in 1 ml 0.1 M-Tris buffer, pH 8.0. Reactions were stopped with 4 ml methanol, and the protein pellets were extracted repeatedly with 2 ml methanol or methanol–ether (3:1) until no further radioactivity could be extracted (generally 12–15 washes). Pellets were dissolved in 1 ml 1 N-NaOH, and the radioactivity of an aliquot was counted in 10 ml of scintillation fluid. Protein was determined using the method of Lowry, Rosebrough, Farr & Randall (1951).

In vivo *mouse lung toxicity.* Male CD-1 mice (4–5 wk old) were given subcutaneous injections of BHA 30 min prior to intraperitoneal injections of BHT. Corn oil was used as the vehicle (≤0.2 ml/mouse ip, <0.1 ml/mouse sc). Animals were allowed food (Purina 5001) and water *ad lib.* throughout the experiment, were housed on Alpha-Dry bedding, and kept on a 12-hr light/dark cycle. Lung (wet) and body weights were recorded on day 4 after injection. Toxicity was measured as an increase in lung/body weight ratio (%).

In vitro *measurement of BHT-quinone methide and stilbenequinone*

Reactions contained 15 U horseradish peroxidase (0.1 mg), 0.9 mM-hydrogen peroxide, 100 μM-BHA and 200 μM-BHT (or analogues) in 1 ml 0.01 M-phosphate buffer, pH 7.0. The rate of formation of BHT-quinone methide was measured spectrophotometrically at 300 nm using an extinction coefficient of 27,000/M/cm (Becker, 1965). BHT-quinone methide has an absorption maximum of 285 nm (Bauer & Coppinger, 1963; Becker, 1965; Filar & Winstein, 1960) in organic solvents such as isooctane. We offer the following observations as proof that the product from our reaction (with an absorption maximum at 300 nm) was really BHT-quinone methide: (1) when the product formed in aqueous solution was extracted with hexane, the absorbance shifted back to 285 nm; (2) the product in aqueous solution had similar spectral properties to BHT-quinone methide synthesized by the method of Becker (1965); (3) upon standing, the product formed 1,2-bis-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethane and 3,5,3′,5′-tetra-*tert*-butylstilbene-4,4′-quinone (stilbenequinone), two compounds known to be formed by the dimerization of BHT-quinone methide; (4) deuterated BHT, synthesized by the method of Mizutani *et al.* (1983), in which the three hydrogens on the C-4 methyl group were replaced by deuterium, dramatically slowed the rate of formation of this product; (5) glutathione blocked the formation of the product in this reaction and caused the rapid disappearance of previously formed product immediately upon addition to the reaction mixture, leading to the formation of a BHT-glutathione conjugate.

Similarly, we observed a spectral shift in the absorbance maximum for stilbenequinone from 445 nm (hexane) to 460 nm in aqueous solution. The rate of formation of stilbenequinone was measured at 460 nm using an extinction coefficient of 72,000/M/cm (Kharasch & Joshi, 1957). Authentic stilbenequinone was synthesized (Cook, Nash & Flanagan, 1955) and compared (UV–visible spectra, NMR) with the reaction product at 460 nm, to confirm the identity of this spectral peak.

Results and Discussion

Prostaglandin H synthase and horseradish peroxidase have often been used as model enzyme systems for the study of the peroxidative activation of xenobiotic chemicals (Marnett & Eling, 1983; Subrahmanyam & O'Brien, 1985). The ability of BHA to enhance the metabolism of BHT to a reactive species in these two model enzyme systems is shown in Table 1. Three endpoints were measured: covalent binding of BHT to microsomal protein and the rates of formation of BHT-quinone methide and stilbenequinone. In the presence of BHA the prostaglandin H synthase-catalysed covalent binding of BHT was greatly enhanced (3.9-fold). Similarly, the horseradish peroxidase-catalysed formation of both BHT-quinone methide and stilbenequinone were greatly enhanced in the presence of BHA. In the absence of BHA, no BHT-quinone methide or stilbenequinone were detected. Prostaglandin H synthase also catalysed the formation of BHT-quinone methide in the presence of BHA, but no stilbenequinone was formed in either the presence or absence of BHA (not shown). The lack of formation of stilbenequinone might be due to the presence of microsomal protein which would bind to the BHT-quinone methide before it could dimerize to form the stilbenequinone.

Synergistic effects from various combinations of antioxidants have long been noted (Kurechi and Kato, 1981 & 1982; Kurechi, Kikugawa & Kato, 1980; Kurechi, Kikugawa, Kato & Numasato, 1980). For example, enhanced protective effects in food products can be achieved by using a combination of antioxidants, with the added benefit of reducing the

Table 1. Effect of BHA on the *in vitro* peroxidative metabolism of BHT

Reactants	Covalent binding (nmol BHT-bound/ mg protein/10 min)	BHT-quinone methide formation (nmol formed/min/15 U HRPase)	Stilbenequinone formation (nmol formed/min/ 15 U HRPase)
BHT	1.7 ± 0.3	ND	ND
BHT + BHA	27.6 ± 1.9	43.5 ± 2.2	7.7 ± 0.2

HRPase = Horseradish peroxidase ND = Not detectable

absolute amount of any one antioxidant present in the food product itself. The effectiveness of certain individual antioxidants and some combinations of antioxidants has been attributed to the ability of the antioxidant to regenerate itself (Chen & Shaw, 1974; Kurechi *et al.* 1980). In a study on the synergistic effect of a combination of BHA and BHT on hydrogen donation to the stable free radical 2,2-diphenyl-1-picrylhydrazyl, Kurechi *et al.* (1980) attributed the mechanism of increased hydrogen donation to the regeneration of the parent BHA molecule. This regeneration of BHA was accomplished, however, at the expense of BHT, which was converted into BHT-quinone methide. The oxidation and regeneration of BHA at the expense of BHT might also explain our results in peroxidase systems. The crucial point is the direct interaction of an oxidized metabolite of BHA with BHT. If true, this would represent a novel mechanism of activation for BHT in biological systems.

We investigated the ability of a number of other phenolic compounds to stimulate the metabolic activation of BHT. This was assessed by measuring each compound's ability to enhance or inhibit the covalent binding of BHT. Compounds that stimulated BHT binding are shown in Table 2. Although all the simple phenolic compounds tested were able to stimulate BHT binding, the most effective compound was BHA. The compounds selected for this experiment were chosen because of their presence in or addition to food and cosmetic products or medicinal preparations. In many instances one or more of these compounds is present in the same product with BHT.

The abilities of peroxidase enzymes from various mammalian tissues to catalyse the interaction of BHA with BHT are presented in Table 3. Using arachidonic acid as a substrate, BHA stimulated the covalent binding of BHT in microsomes from sheep, guinea-pig, dog and human lung, as well as dog bladder and rabbit kidney medulla. Using hydrogen peroxide as a substrate, microsomes from rat, mouse and human lung, as well as rat intestine, catalysed a similar enhancement of BHT binding by BHA. These results indicate that peroxidase enzymes from a variety of mammalian tissues, including human lung, can peroxidatively metabolize BHT to a reactive intermediate, which covalently binds to protein, and demonstrate that this binding can be enhanced by BHA, presumably through the increased formation of BHT-quinone methide. In a related experiment, guaiacol stimulated the formation of BHT-quinone methide from BHT using myeloperoxidase isolated from human neutrophils (not shown).

In order to assess whether the interaction of phenolic compounds with BHT to form BHT-quinone methide might have any *in vivo* significance, we measured the effect of various doses of BHA on BHT-induced mouse lung toxicity (Fig. 1). BHT causes the destruction of type I alveolar cells (Marino & Mitchell, 1972; Witschi & Saheb, 1974) and pulmonary endothelial cells (Smith, 1983). This toxic response is generally reversible (in 6–10 days) unless a second stress impedes the proliferation and repair process by the type II pneumocytes. For example, if

Table 2. Ability of various phenolic chemicals to enhance peroxidase-catalysed covalent binding of BHT to protein

Compound	Use	Relative enhancement*
BHA	Antioxidant	+++
Methylparaben	Preservative in beverages, foods and cosmetics	++
Eugenol	In perfumes, manufacture of vanillin, dental analgesic	++
Vanillin	Flavouring agent	++
Diethylstilboestrol	Oestrogen	++
Phenol	Disinfectant, antimicrobial agent	++
Guaiacol	Expectorant	+
Acetaminophen	Analgesic, antipyretic	+
Ferulic acid	Natural plant constituent	+
Oestradiol	Oestrogen	+

*(+) represents 1–2-fold enhancement of BHT covalent binding; (++) represents 2–3-fold enhancement; (+++) represents >3-fold enhancement.

Table 3. Peroxidase-dependent covalent binding of BHT to protein by microsomes from various animal tissues

Tissue	Covalent binding (nmol BHT bound/mg protein/10 min*) in incubations containing	
	BHT	BHT + BHA
	Arachidonic acid-dependent	
Sheep lung	0.18 ± 0.02	1.63 ± 0.06
Guinea-pig lung	0.15 ± 0.02	0.37 ± 0.12
Dog lung	ND	0.68 ± 0.08
Human lung	0.10 ± 0.01	0.35 ± 0.05
Dog bladder	0.44 ± 0.05	7.90 ± 0.65
Rabbit kidney medulla	ND	0.69 ± 0.23
	Hydrogen peroxide-dependent	
Rat lung	0.06 ± 0.02	0.32 ± 0.04
Mouse lung	0.13 ± 0.01	0.80 ± 0.03
Human lung	1.03 ± 0.01	6.14 ± 0.03
Rat intestine	4.33 ± 0.51	27.66 ± 4.15

ND = Not detectable

*Except for hydrogen peroxide-dependent binding by human lung (30-min incubation).

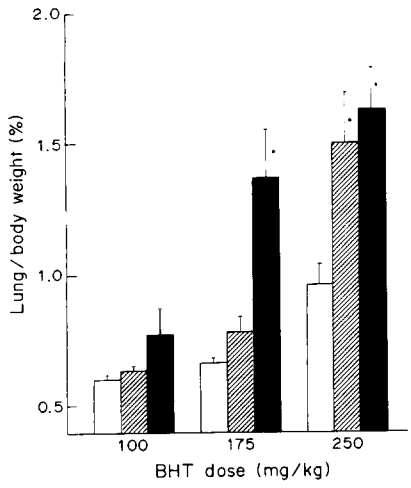


Fig. 1. Effects of 0 (□), 50 (▨) and 250 (■) mg BHA/kg given sc to male CD-1 mice on lung toxicity (effect on lung/body weight ratio) induced by a subsequent ip injection of 100–250 mg BHT/kg. Asterisks indicate values differing significantly (*$P < 0.01$) from that recorded with BHT alone.

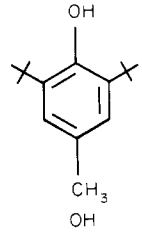
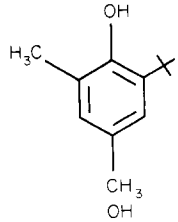
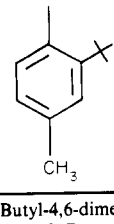
mice are exposed to hyperbaric oxygen after administration of BHT, pulmonary fibrosis ensues (Witschi, Haschek, Klein-Szanto & Hakkinen, 1981).

A preliminary experiment was carried out to determine a threshold BHT dose that would cause little or no lung toxicity (measured as an increase in lung/body weight ratio 4 days after treatment). Using 4–5-wk-old male CD-1 mice, the normal (untreated) lung/body weight ratio was always near 0.6%. The highest dose of BHT that caused no significant increase in lung weight was 175 mg/kg. Above doses of 175 mg/kg, the lung/body weight ratio climbed rapidly, reaching a plateau around 1.5% at 400 mg/kg. Higher doses of BHT did not increase this ratio. Thus, three doses at or near the threshold dose of BHT were chosen for this experiment: 100, 175 and 250 mg/kg. As shown in Fig. 1, subcutaneous injection of 50 or 250 mg BHA/kg significantly enhanced the lung/body weight ratio compared to animals receiving BHT alone. BHA did not further enhance the lung/body weight ratio above 1.5% if higher doses of BHT were used. BHA had no effect on lung weight by itself at doses up to 500 mg/kg.

The mode of BHA administration (i.e. subcutaneous injection) was chosen to minimize any intestinal or hepatic metabolism of BHA prior to its reaching the lung. When BHA was given by intraperitoneal injection, an enhancing effect on BHT-induced lung injury was observed, but was much less dramatic (not shown).

BHT has been shown to increase the incidence of lung tumours in mice given a single initiating dose of a carcinogen such as urethane, dimethylnitrosamine, benzo[*a*]pyrene or 3-methylcholanthrene (Witschi, 1985). A tumour-promoting effect of BHT can be elicited no matter whether BHT is administered by injection, by gavage or in the diet. BHT can act as a tumour promoter in at least two species, the rat and the mouse, and in several organs such as the liver, bladder and gastro-intestinal tract, in addition to the lung. Malkinson & Beers (1984) have compared BHT and two analogues, 2-*tert*-butyl-4,6-dimethylphenol (BDMP) and 2-*tert*-butyl-4-methylphenol (BMP), for their relative abilities to induce lung toxicity and promote urethane-induced tumorigenesis in mice.

Table 4. Ability of BHT and two analogues to induce mouse lung toxicity, tumour promotion and quinone methide formation

Compound	Structure	Lung toxicity*	Tumour promotion*	Quinone methide formation
BHT		+	++	++
BDMP		++	+	+
BMP		+++	−	−

BDMP = 2-*tert*-Butyl-4,6-dimethylphenol BMP = 2-*tert*-Butyl-4-methylphenol
*Data from Malkinson & Beers (1984).

Their results are briefly summarized in Table 4. BMP was observed to be the most toxic compound to mouse lung while BHT was the most effective tumour promoter. These authors suggested that their data were consistent with the hypothesis that different metabolites are responsible for causing lung toxicity and tumour promotion. They further reported a strain of mouse that was sensitive to the toxic effects of BHT but not to the promotion effects, suggesting a genetic deficiency in the ability of these mice to form the promoting metabolite.

Since BHT-quinone methide has been suggested to be the metabolite responsible for the toxic effects of BHT, and since we have a simple method for observing the formation of BHT-quinone methide, we tested the ability of BHT, BDMP and BMP to form a quinone methide. Chemically, all three compounds should be able to form a quinone methide. However, this was not the case. BHT was converted into quinone methide most efficiently, BDMP formed only a small amount of quinone methide, while BMP did not form any detectable quinone methide. If this spectral assay is an accurate measure of the ability of these compounds to form quinone methide, our findings suggest that the pulmonary toxicity of BMP may be related to a metabolite other than quinone methide.

In summary, we have presented evidence that: (1) BHA can enhance the *in vitro* metabolic activation of BHT to BHT-quinone methide; (2) BHA can enhance the *in vivo* lung toxicity of BHT in mice; (3) other phenolic compounds also enhance the peroxidative activation of BHT; (4) peroxidase enzymes from various mammalian tissues, including human tissue, can also catalyse the interaction between BHA and BHT.

What, if any, relevance do these observations have in assessing the possible human risk of exposure to BHT? Humans ingest up to 0.5 mg BHT/kg/day (Gosselin, Smith & Hodge, 1984). Doses of BHT that enhance tumorigenesis have been reported to be around 35 mg/kg/day for a period of 2 wk in mice (Witschi, 1985). This represents a cumulative dose of approximately 450–500 mg/kg. Overt lung toxicity is caused by high single doses of BHT ($\geqslant$400 mg/kg), although there are large differences between strains of mice (Kawano, Nakao & Hiraga, 1981). Microscopic lung damage is observed at doses as low as 40 mg/kg (Marino & Mitchell, 1972). Thus, the average daily intake of BHT in humans is generally far less than the doses used to elicit toxicity or promote tumorigenesis in mice. However, as we have demonstrated, microsomal enzymes from human tissues can metabolize BHT to a reactive product(s) in a manner similar to that of microsomes from susceptible animal tissues. Therefore, while the doses that humans ingest may not cause overt tissue toxicity, it is possible that at the level of human exposure the metabolism of BHT might lead to more subtle forms of cellular toxicity. Similarly, the chronic formation of non-cytotoxic amounts of BHT-quinone methide could enhance the development of tumours or alter cell defence mechanisms so that the ability of a cell to respond to other environmental stresses is compromised. Such responses may be particularly important in the small intestine, a tissue which is very active in metabolizing BHT (Table 3) and would probably be exposed to the highest concentrations of BHA and BHT.

Finally, while food and cosmetic products are the major source of exposure of humans to BHT, there have been recent reports of persons ingesting up to 6 g BHT/day for the relief of herpes virus symptoms (Shlian & Goldstone, 1986). In a 70-kg (150-lb) human this would be the equivalent to a dose of 86 mg/kg. BHT capsules (usually sold as 250 mg BHT/capsule in a gelatin coating) are now available in many health-food stores. More signs of human BHT toxicity will probably appear if this practice continues.

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REFERENCES

Bauer R. H. & Coppinger G. M. (1963). Chemistry of hindered phenols. *Tetrahedron* **19,** 1201.

Becker H. D. (1965). Quinone dehydrogenation. I. The oxidation of monohydric phenols. *J. org. Chem.* **30,** 982.

Chen C. & Shaw Y. S. (1974). Cyclic metabolic pathway of a butylated hydroxytoluene by rat liver microsomal fractions. *Biochem. J.* **144,** 497.

Cook C. D., Nash N. G. & Flanagan H. R. (1955). Oxidation of hindered phenols. III. The rearrangement of the 2,6-di-*tert*-butyl-4-methylphenoxy radical. *J. Am. chem. Soc.* **77,** 1783.

Filar L. J. & Winstein S. (1960). Preparation and behavior of simple quinone methides. *Tetrahedron* **25,** 9.

Kawano S., Nakao T. & Hiraga K. (1981). Strain differences in butylated hydroxytoluene-induced deaths in male mice. *Toxic. appl. Pharmac.* **61,** 475.

Kehrer J. P. & Witschi H. (1980). Effects of drug metabolism inhibitors on butylated hydroxytoluene-induced pulmonary toxicity in mice. *Toxic. appl. Pharmac.* **53,** 333.

Kharasch M. S. & Joshi B. S. (1957). Reactions of hindered phenols. II. Base-catalysed oxidations of hindered phenols. *J. org. Chem.* **22,** 1439.

Kurechi T. & Kato T. (1982). Studies on the antioxidants. XV. Combination effects of butylated hydroxyanisole, butylated hydroxytoluene and their analogs on hydrogen donation to 2,2-diphenyl-1-picrylhydrazyl. *Chem. Pharm. Bull., Tokyo* **29,** 3012.

Kurechi T. & Kato T. (1982). Studies on the antioxidants. XVI. Synergistic reaction between butylated hydroxyanisole and butylated hydroxytoluene in hydrogen donation to 2,2-diphenyl-1-picrylhydrazyl. *Chem. Pharm. Bull., Tokyo* **30,** 2964.

Kurechi T., Kikugawa K. & Kato T. (1980). Studies on the antioxidants. XIII. Hydrogen donating capability of antioxidants to 2,2-diphenyl-1-picrylhydrazyl. *Chem. Pharm. Bull., Tokyo* **28,** 2089.

Kurechi T., Kikugawa K., Kato T. & Numasato T. (1980). Studies on antioxidants. XII. Combination effects of antioxidants on the reduction of ferric ions. *Chem. Pharm. Bull., Tokyo* **28,** 2228.

Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265.

Malkinson A. M. & Beers D. S. (1984). Pharmacologic and genetic studies on the modulatory effects of butylated hydroxytoluene on mouse lung adenoma formation. *J. natn. Cancer Inst.* **73,** 925.

Marino A. A. & Mitchell J. T. (1972). Lung damage in mice following intraperitoneal injection of butylated hydroxytoluene. *Proc. Soc. Exp. biol. Med.* **140,** 122.

Marnett L. J. & Eling T. E. (1983). Cooxidation during prostaglandin biosynthesis: A pathway for the metabolic activation of xenobiotics. In *Reviews in Biochemical Toxicology* 5. Edited by E. Hodgson, J. R. Bend & R. M. Philpot. p. 135. Elsevier, New York.

Mizutani T., Ishida I., Yamamoto K. & Tajima K. (1982). Pulmonary toxicity of butylated hydroxytoluene and related alkylphenols: Structural requirements for toxicity potency in mice. *Toxic. appl. Pharmac.* **62,** 273.

Mizutani T., Nomura H., Yamamoto K. & Tajima K. (1984). Modification of butylated hydroxytoluene-induced pulmonary toxicity in mice by diethyl maleate, buthionine sulfoximine, and cysteine. *Toxicology Lett.* **23,** 327.

Mizutani T., Yamamoto K. & Tajima K. (1983). Isotope effects on the metabolism and pulmonary toxicity of butylated hydroxytoluene in mice by deuteration of the 4-methyl group. *Toxic. appl. Pharmac.* **69,** 283.

Nakagawa Y., Suga T. & Hiraga K. (1984). Preventive effect of cysteine on butylated hydroxytoluene-induced pulmonary toxicity in mice. *Biochem. Pharmac.* **33,** 502.

Nakagawa Y., Tayama K., Nakao T. & Hiraga K. (1984). On the mechanism of butylated hydroxytoluene-induced hepatic toxicity in rats. *Biochem. Pharmac.* **33,** 2669.

Shlian D. M. & Goldstone J. (1986). Toxicity of butylated hydroxytoluene. *New Engl. J. Med.* **314,** 648.

Smith L. J. (1983). The effect of methylprednisolone on lung injury in mice. *J. lab. clin. Med.* **101,** 629.

Subrahmanyam V. V. & O'Brien P. J. (1985). Phenol oxidation product(s), formed by a peroxidase reaction, that bind to DNA. *Xenobiotica* **15,** 873.

Tajima K., Yamamoto K. & Mizutani T. (1981). Biotransformation of butylated hydroxytoluene (BHT) to BHT-quinone methide in rats. *Chem. pharm. Bull., Tokyo* **29,** 3738.

Tajima K., Yamamoto K. & Mizutani T. (1983). Identification and determination of glutathione conjugate formed from butylated hydroxytoluene in rats. *Chem. pharm. Bull., Tokyo* **31,** 3671.

Tajima K., Yamamoto K. & Mizutani T. (1985). Formation of a glutathione conjugate from butylated hydroxytoluene by rat liver microsomes. *Biochem. Pharmac.* **34,** 2109.

Takahashi O. & Hiraga K. (1978). Dose-response study of hemorrhagic death by dietary butylated hydroxytoluene (BHT) in male rats. *Toxic. appl. Pharmac.* **43,** 399.

Takahashi O. & Hiraga K. (1979). 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone: A hepatic metabolite of butylated hydroxytoluene in rats. *Fd Cosmet. Toxicol.* **17,** 451.

Takahashi O. & Hiraga K. (1981). Inhibition of phylloquinone epoxide-dependent carboxylation of microsomal proteins from rat liver by 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone. *Fd Cosmet. Toxicol.* **19,** 701.

Thompson D. C., Cha Y. N. & Trush M. A. (1986). The peroxidative activation of butylated hydroxytoluene to BHT-quinone methide and stilbenequinone. In *Biological Reactive Intermediates*, III. Edited by J. J. Kocsis, D. J. Jollow, C. M. Witmer, J. D. Nelson & R. Snyder. p. 301. Plenum Press, New York.

Turner A. B. (1964). Quinone methides. *Q. Rev.* **18,** 347.

Witschi H. P. (1985). Enhancement of lung tumor formation in mice. In *Carcinogenesis*, Vol. 8. Edited by M. J. Moss, D. G. Kaufman, J. M. Siegfried, V. E. Steele & S. Nesnow. p. 147. Raven Press, New York.

Witschi H. P., Haschek W. M., Klein-Szanto A. J. P. & Hakkinen P. J. (1981). Alteration of diffuse lung damage by oxygen: Determining variables. *Am. Rev. resp. Dis.* **123,** 98.

Witschi H. & Saheb W. (1974). Stimulation of DNA synthesis in mouse lung following intraperitoneal injection of butylated hydroxytoluene. *Proc. Soc. exp. biol. Med.* **147,** 690.

QUESTIONS AND ANSWERS

John Daniel, Life Sciences Research, Chelmsford: I would just like to make a statement and perhaps you would react to it. In 1966, I reported the formation of the carboxylic acid from BHT in the rats which was based upon, I presume, the formation of a quinone methide, which then reacted non-enzymatically with glutathione or cystine. I also reported at that time, the failure to observe such a metabolite in man and that the major route to metabolism in man, as Dr Conning showed this morning, was the formation of a dicarboxylic acid. Would you like to put your comments into perspective now for human risk assessment?

M. Trush: All I can say is that in our studies we can actually demonstrate a direct interaction between a quinone methide and glutathione. It would be interesting to know if humans can, in fact, metabolize BHT to quinone methide and how well they can do it. In going back into the literature it's questionable how well even some of the animal tissues can metabolize it to its quinone methide in the absence of one of these activator compounds. And you can see that where we had absence of some activator compound, there was actually very little metabolism and covalent binding of BHT in and of itself. But in the presence of these other compounds, there is a dramatic increase in that.

A. Sevanian, USC Institute of Toxicology: I think your proposal here of the redox coupling between BHA and BHT and quinones is an interesting and fascinating one. I wonder if I could prod you into a little more speculation. Could it be possible that compounds such as BHA or BHT, when they undergo redox cycling, could set up a set of redox cascades with endogenous compounds of suitable redox poise relative to them, such as catechols, ascorbic acid and other things, which could then precipitate a whole series of cascade reactions? Therefore, BHA and BHT may not necessarily have to be the agents that deplete the glutathione pool?

M. Trush: Right. That's possible. We've also done some reading about the quinone methides, which prompted thought about alternative targets, such as NADPH oxidation. We looked for that and didn't find it. We even looked for the possible intercovalent binding of quinone methide to NADPH because of the possibility it could be binding to phosphate groups. It's very possible you could set up a redox cycle. Another possibility we've also thought about and haven't tested is that there may be endogenous compounds which could drive the

actual metabolic activation of BHT and could serve as sort of a role of BHA in this system. So there are other considerations one needs to think about once you generate the reactive intermediate and what could happen in terms of toxicity.

M. Simic, National Bureau of Standards: Just a comment. We have measured the redox potentials of BHT and BHA, and the redox potential of BHT is higher than that of BHA. So the flow of electrons is going to go from BHA to BHT.

M. Trush: Based upon that, yes. But based upon our results, they are consistent with the data we're presenting in the formation of the products we're seeing.

M. Simic: That's interesting, but then you have to consider perhaps some different mechanisms.