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The Peroxidase-dependent Activation of Butylated Hydroxyanisole and Butylated Hydroxytoluene (BHT) to Reactive Intermediates

FORMATION OF BHT-QUINONE METHIDE VIA A CHEMICAL-CHEMICAL INTERACTION*

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The food antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are shown to be metabolized to covalent binding intermediates and various other metabolites by prostaglandin H synthase and horseradish peroxidase. BHA was extensively metabolized by horseradish peroxidase (80% conversion of parent BHA into metabolites) resulting in the formation of three dimeric products. Only two of these dimers were observed in prostaglandin H synthase-catalyzed reactions. In contrast to BHA, BHT proved to be a relatively poor substrate for prostaglandin synthase and horseradish peroxidase, resulting in the formation of a small amount of polar and aqueous metabolites (23% conversion of parent BHT into metabolites). With arachidonic acid as the substrate, prostaglandin H synthase catalyzed the covalent binding of [^{14}C]BHA and [^{14}C]BHT to microsomal protein which was significantly inhibited by indomethacin and glutathione. The covalent binding of BHA and its metabolism to dimeric products were also inhibited by BHT. In contrast, the addition of BHA enhanced the covalent binding of BHT by 400%. Moreover, in the presence of BHA, the formation of the polar and aqueous metabolites of BHT was increased and two additional metabolites, BHT-quinone methide and stilbenequinone, were detected. The increased peroxidase-dependent oxidation of BHT in the presence of BHA is proposed to occur via the direct chemical interaction of BHA phenoxyl radical with BHT or BHT phenoxyl radical. These results suggest a potential role for phenoxyl radicals in the activation of xenobiotic chemicals to toxic metabolites.

Various peroxidase enzymes have been implicated in the bioactivation of xenobiotics (1). For example, prostaglandin H synthase has been shown to cooxidize a spectrum of xenobiotic compounds, including carcinogens, to potentially harmful reactive intermediates (2). The majority of these compounds are cooxidized by virtue of their ability to serve as reducing cofactors for the peroxidase moiety of the enzyme. Antioxidants are good electron donors and therefore are likely substrates for peroxidase enzymes.

Butylated hydroxyanisole (BHA)[1] and butylated hydroxytoluene (BHT) are phenolic antioxidants that are widely used in the food industry. It has been estimated that man consumes as much as 0.5 mg/kg body weight/day of these compounds (3). Although BHA and BHT are generally recognized as safe by the Food and Drug Administration, several reports have established that these antioxidants are involved as causative agents in a number of toxic and carcinogenic processes in animals. For example, BHT elicits the destruction of type I alveolar and pulmonary endothelial cells in the mouse lung (4, 5). It has been suggested that BHT-induced lung damage is due to the cytochrome P-450-dependent biotransformation of BHT into BHT-quinone methide (6, 7). BHT also causes hemorrhagic death in rats (8) and liver necrosis in rats (9) and mice (10). In addition, BHT has a tumor-enhancing effect on a variety of carcinogens in both mice and rats (11–15) and is a hepatic carcinogen in male mice (16) and F1 generation rats (17). BHA, on the other hand, is carcinogenic to the forestomach of rats and hamsters (18, 19), acts as a tumor promoter (14), and causes hemorrhagic lung damage in rats (20).

Although the above toxic and carcinogenic properties of BHA and BHT have been well described, little is known about the exact mechanisms of how these compounds cause toxicity or carcinogenicity. The cytochrome P-450-dependent metabolism of these compounds has been thoroughly investigated (21–24), whereas their peroxidase-dependent metabolism has not been well studied. While BHA was previously shown to be metabolized by peroxidases to a dimeric product (25–27), the metabolism of BHT by peroxidases has not been reported. As such, this study compares the metabolism and activation of BHA and BHT by two model peroxidase enzymes: horseradish peroxidase and prostaglandin H synthase. We were particularly interested in determining if BHT-quinone methide was formed during the peroxidase-mediated oxidation of BHT. Since peroxidases are found in high concentrations in certain organs (1), the peroxidative activation of BHA or

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[1] The abbreviations used are: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; HPLC, high performance liquid chromatography.

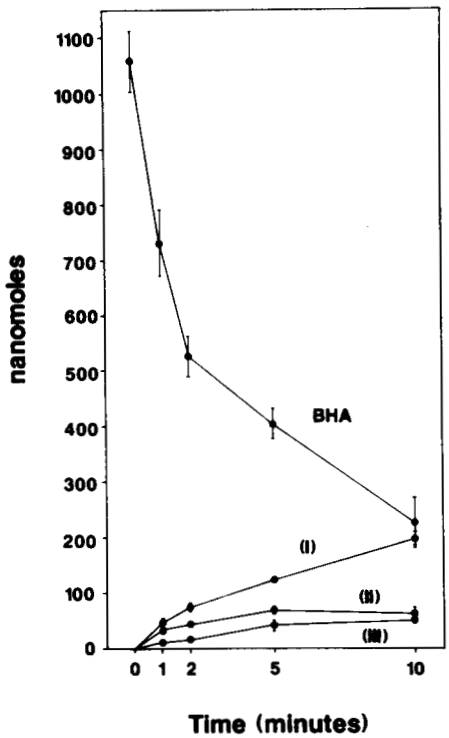

FIG. 3. **Horseradish peroxidase-dependent metabolism of BHA.** Reactions contained 1 mM (1000 nmol) BHA, 100 μg of horseradish peroxidase, and 0.9 mM hydrogen peroxide in 1 ml of 0.01 M phosphate buffer, pH 7. The incubations were carried out at 25 °C for various lengths of time when they were stopped by the addition of 0.5 ml of 5% trichloroacetic acid. Product formation was analyzed by HPLC, as described under "Experimental Procedures." *Points* represent the mean ± standard error of triplicate samples.

BHT could be relevant to the extrahepatic toxicity and carcinogenicity elicited by these agents.

EXPERIMENTAL PROCEDURES[2]

RESULTS

The horseradish peroxidase-catalyzed metabolism of BHA was investigated using HPLC. In ethyl acetate extracts from these incubations we observed three peaks which eluted from the HPLC column at 11 (I), 16 (II), and 23 (III) min. These peaks were collected and analyzed by NMR and mass spectrometry to determine their structures; all three peaks were found to be dimeric metabolites of BHA (Figs. 1 and 2, Miniprint). Dimer II was identical to a BHA dimer reported in previous studies on BHA metabolism by peroxidases (25–27). The major product observed in our studies, dimer I (2,4-dihydro-4-hydroxy-8-methoxy-2-oxo-4,6-di-*tert*-butyldibenzofuran), and a minor metabolite, dimer III (2′,3-di-*tert*-butyl-2-hydroxy-4′,5-dimethoxybiphenyl ether), have not previously been reported as biological metabolites of BHA. The time-dependent formation of these three metabolites, as well as the disappearance of parent BHA, is shown in Fig. 3. BHA dimer I was the major metabolite formed, followed by dimers II and III. The formation of these three products accounted for approximately 80% of the disappearance of parent BHA. Although *tert*-butylhydroquinone has been reported to be a peroxidase metabolite of BHA (27), none was detected under our incubation conditions.

In reactions using prostaglandin H synthase we observed the formation of dimers I and II from BHA, whereas no dimer III was detected. Both arachidonic acid and hydrogen peroxide were capable of supporting the oxidation of BHA by prostaglandin H synthase (Fig. 4), indicating that this oxidation occurs during the peroxidase portion of the enzyme reaction. When arachidonic acid was used as the substrate, it was necessary to decrease the concentration of BHA to 100 μM since we observed that higher concentrations of BHA were in fact inhibitory.

Covalent binding of chemicals to biomolecules has been a commonly used method to demonstrate the bioactivation of a number of xenobiotics. Using ^{14}C-labeled BHA or BHT in prostaglandin H synthase-catalyzed incubations, we observed that both antioxidants covalently bound to microsomal protein in a concentration-dependent manner (Fig. 5). Covalent binding occurred in the presence of either arachidonic acid or hydrogen peroxide as substrate (not shown). At equimolar concentrations BHA yielded more covalently bound product than did BHT. At concentrations of BHA above 100 μM, however, covalent binding began to be inhibited, while BHT covalent binding continued to increase up to 500 μM BHT. Similar concentration-dependent effects of BHA and BHT were observed with O_2 consumption as an end point (not shown).

The effects of indomethacin, glutathione, and various other compounds on the prostaglandin H synthase-catalyzed covalent binding of BHA or BHT to protein are presented in Table I. Indomethacin and glutathione were effective inhibitors of the arachidonic acid-dependent prostaglandin H synthase-catalyzed covalent binding of both BHA and BHT. At a concentration of 100 μM, ascorbate as well as methimazole, propylthiouracil, and BHT inhibited the binding of BHA. Methimazole and propylthiouracil have previously been used as inhibitors of prostaglandin H synthase-catalyzed reactions

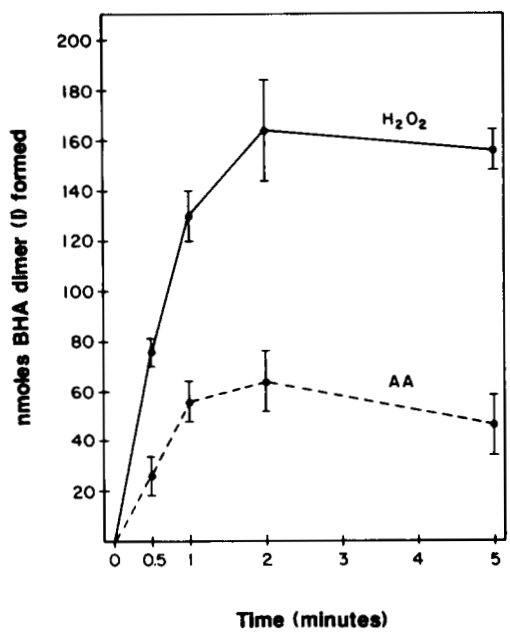

FIG. 4. **Prostaglandin H synthase-dependent formation of BHA dimer I.** Reactions contained 1 mg of ram seminal vesicle microsomal protein, 1 mM BHA, and 0.9 mM hydrogen peroxide in 1 ml of 0.1 M Tris buffer, pH 8. When arachidonic acid (*AA*) was used as substrate (110 μM), the BHA concentration was 100 μM. Reactions were carried out and analyzed as described in the legend to Fig. 3.

[2] Portions of this paper (including "Experimental Procedures" and Figs. 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

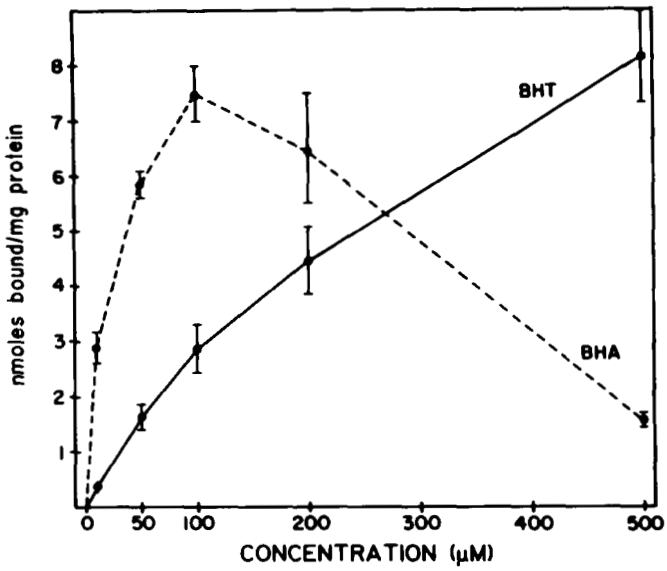

FIG. 5. **Concentration-dependent covalent binding of [^{14}C] BHA or [^{14}C]BHT to protein catalyzed by prostaglandin H synthase.** Reaction conditions are described under "Experimental Procedures." Points represent the mean ± standard error of triplicate samples.

TABLE I

Effect of various agents on the covalent binding of BHA and BHT to protein catalyzed by prostaglandin H synthase

Reactions contained 50 μM BHA or BHT (0.5 μCi), 1 mg of ram seminal vesicle microsomes, and 100 μM of all test agents except glutathione (1 mM) in 1 ml of 0.1 M Tris buffer, pH 8. Reactions were initiated by the addition of 110 μM arachidonic acid (AA) and incubated at 25 °C for 10 min. Values represent the mean ± standard error for triplicate samples.

Reaction	nmol bound/mg protein	% of complete system
BHA		
Control (−AA)	0.19 ± 0.01	
Complete system	6.66 ± 0.87	100
+ Indomethacin	0.25 ± 0.04	1
+ Glutathione	2.08 ± 0.12	30
+ BHT	2.35 ± 0.12	33
+ Ascorbate	3.93 ± 0.27	58
+ Methimazole	5.20 ± 0.16	78
+ Propylthiouracil	4.02 ± 0.25	59
BHT		
Control (−AA)	0.18 ± 0.02	
Complete system	1.65 ± 0.09	100
+ Indomethacin	0.45 ± 0.01	18
+ Glutathione	0.18 + 0.01	0
+ BHA	6.63 + 0.31	439
+ Ascorbate	1.49 ± 0.04	89
+ Methimazole	2.86 ± 0.12	182
+ Propylthiouracil	1.40 ± 0.12	83

(37). The effects of these same compounds on the covalent binding of BHT, however, revealed some interesting differences when compared to their effects on BHA. Ascorbate and propylthiouracil were weak inhibitors of BHT covalent binding, while BHA and methimazole enhanced rather than inhibited the covalent binding of BHT. In the presence of BHA, the covalent binding of BHT was enhanced by approximately 400%. Table II illustrates the magnitude of stimulation of BHT covalent binding by various concentrations of BHA.

The horseradish peroxidase-catalyzed metabolism of BHT was studied in both the presence and absence of BHA. Using HPLC conditions identical to those used to detect dimeric metabolites of BHA, no peaks representing BHT metabolites were observed whether the reactions were conducted with

TABLE II

Stimulation of prostaglandin H synthase-dependent covalent binding of BHT by BHA

Reactions were initiated by the addition of 110 μM arachidonic acid to tubes containing 100 μM BHT (0.5 μCi), 1 mg of ram seminal vesicle microsomes and various concentrations of BHA in 1 ml of 0.1 M Tris buffer, pH 8. Reactions were incubated at 25 °C for 10 min. Values represent mean ± standard error of triplicate samples. Note that the prostaglandin H synthase used in this experiment had a higher specific activity than that which was used in the experiments presented in Table I and Fig. 5.

Reaction		nmol BHT bound/mg protein	% of BHT alone
BHT alone		4.35 ± 0.12	100
+ BHA	10 μM	11.41 ± 0.33	262
	25 μM	14.77 ± 0.65	340
	50 μM	14.89 ± 0.83	342
	100 μM	15.19 ± 0.39	372
	250 μM	16.87 ± 0.06	388
	500 μM	5.15 ± 0.16	118

BHT alone or in the presence of BHA. Incubations containing both antioxidants, however, were observed to turn yellow in color. This metabolite was purified by preparative TLC and identified as stilbenequinone, a dimeric metabolite of BHT.

The horseradish peroxidase-catalyzed oxidation of BHT was also directly monitored by UV-visible spectroscopy. Reaction mixtures were repetitively scanned over the UV-visible range of 250–500 nm (see Fig. 6*A*). In reactions containing both BHA and BHT, two peaks were observed, one at 300 nm (BHT-quinone methide) and one at 460 nm (stilbenequinone). In the absence of BHA, no BHT-quinone methide or stilbenequinone peaks were detected (not shown). The appearance of these peaks was time-dependent; BHT-quinone methide formed first, followed by stilbenequinone. The kinetics of this reaction are better illustrated in Fig. 6*B*, which follows the formation and disappearance of both of these products over 20 min. The rate of formation of BHT-quinone methide was maximal during the first min of the reaction (42.3 nmol/min) and the maximal concentration was reached between 1 and 2 min. Thereafter, the concentration of BHT-quinone methide decreased, reaching a relatively constant rate of disappearance of approximately 0.30 nmol/min between 10 and 20 min. On the other hand, stilbenequinone did not begin to be formed until 1 min after the reaction had been initiated, which was the time point at which BHT-quinone methide concentration was maximal. The rate of formation of stilbenequinone was maximal from about 2 min until about 6 min (5.7 nmol/min), when it gradually slowed to a rate of approximately 0.17 nmol/min between 10 and 20 min. Between 10 and 20 min, the rate of disappearance of BHT-quinone methide was approximately equivalent to the rate of formation of stilbenequinone (2 nmol of BHT-quinone methide forming 1 nmol of stilbenequinone). The final concentration of stilbenequinone at 20 min was 30 μM, which represented 30% of the BHT initially present in the reaction. Synthetic BHT-quinone methide reportedly forms equimolar amounts of BHT dimer and stilbenequinone upon standing (38). Small, variable amounts of BHT dimer were detected on TLC from a horseradish peroxidase-catalyzed reaction when [^{14}C]BHT was used. However, we also observed that BHT dimer can be further oxidized to form stilbenequinone by horseradish peroxidase in the presence of BHA. Therefore, some of the stilbenequinone observed in this spectral assay may represent BHT dimer which was further oxidized to form stilbenequinone.

Prostaglandin H synthase also catalyzed the formation of BHT-quinone methide from BHT in the presence of BHA which could be detected spectrally (not shown). In contrast to the horseradish peroxidase system, no stilbenequinone was

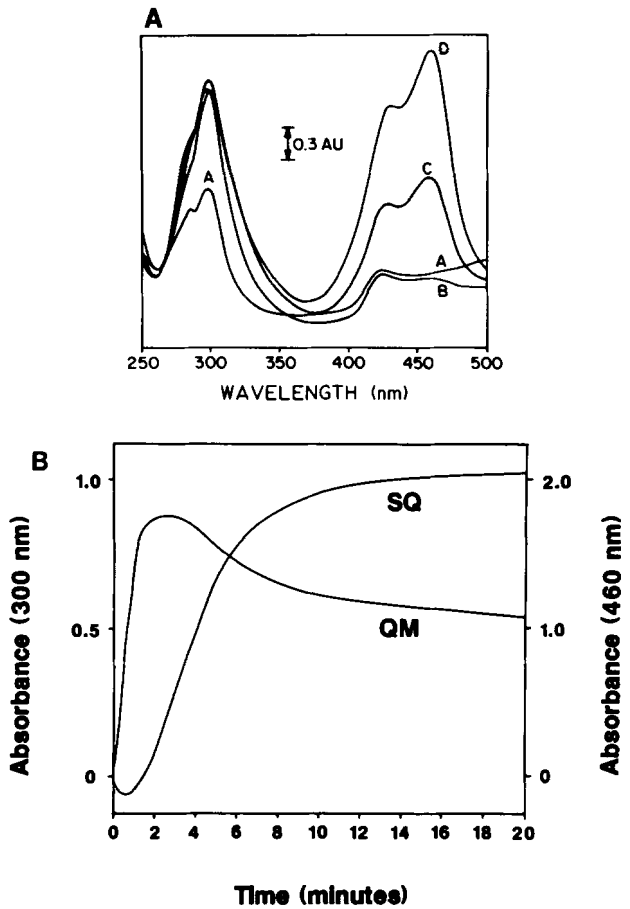

FIG. 6. **Spectrophotometric demonstration of the formation of BHT-quinone methide and stilbenequinone during the horseradish peroxidase-catalyzed oxidation of BHT in the presence of BHA.** The reaction contained 100 μg of horseradish peroxidase, 100 μM BHA, and 200 μM BHT in 1 ml of 0.01 M phosphate buffer, pH 7, and was initiated by the addition of 0.9 mM hydrogen peroxide. *A*, scan of reaction products at various time points after start of reaction. Absorption maxima: BHT-quinone methide (300 nm), stilbenequinone (460 nm). *Traces A–D* represent scans initiated at various times after start of reaction: *trace A*, 0 s; *trace B*, 30 s; *trace C*, 60 s; and *trace D*, 90 s. Each scan took approximately 25 s and began at 500 nm. *AU*, absorption units. *B*, kinetics of the horseradish peroxidase-catalyzed formation of BHT-quinone methide and stilbenequinone. Reaction conditions were the same as in *A* except 180 μM hydrogen peroxide was used. The formation and/or disappearance of BHT-quinone methide (*QM*, 300 nm) and stilbenequinone (*SQ*, 460 nm) were followed over a time period of 20 min.

detected spectrally in the prostaglandin H synthase-catalyzed reactions, presumably because of the higher concentration of protein (1 mg/ml) used. If exogenous protein (1 mg/ml bovine serum albumin) was added to a horseradish peroxidase-catalyzed reaction, stilbenequinone formation was greatly inhibited (not shown).

In addition to horseradish peroxidase and prostaglandin H synthase, lactoperoxidase was used to investigate the nature of the interaction between BHA and BHT which results in the formation of BHT-quinone methide. Lactoperoxidase catalyzes the one-electron oxidation of xenobiotics in a manner similar to other peroxidases (39). However, in the presence of iodide, the reaction shifts to a two-electron pathway. This is due to preference of the enzyme for iodide as a reducing cofactor. In incubations with lactoperoxidase, in the absence of iodide, BHA was necessary to see the formation of BHT-quinone methide, similar to horseradish peroxidase and prostaglandin H synthase (not shown). However, in the presence of iodide, BHT-quinone methide was formed directly from BHT (Fig. 7). When BHA was added to these incubations, the formation of BHT-quinone methide was delayed. This observation suggests that the oxidized metabolite of BHA which enhances the oxidation of BHT is a one-electron oxidation product (the phenoxy radical) rather than a two-electron oxidation product.

The prostaglandin H synthase-dependent metabolism of [^{14}C]BHT was also investigated by TLC. In Fig. 8 the profile of products formed from three sets of reactions are shown: (*a*) BHT alone, (*b*) BHT and BHA, and (*c*) BHT, BHA, and glutathione. As shown, with BHT alone (100 μM) less than 25% of the parent compound was metabolized. Products de-

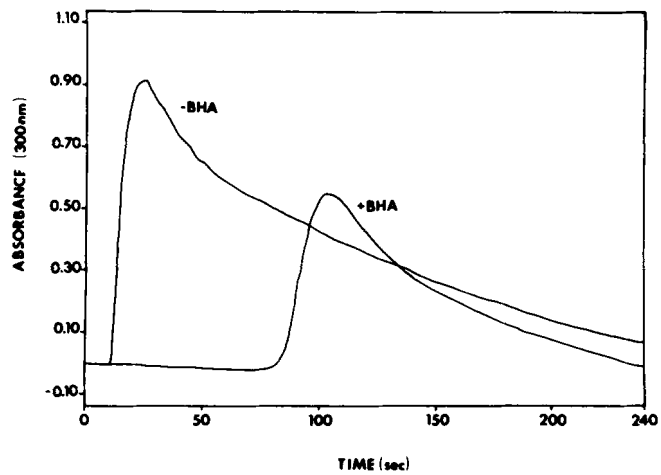

FIG. 7. **Effect of BHA on the lactoperoxidase-catalyzed formation of BHT-quinone methide.** Incubations contained 1 μg/ml lactoperoxidase, 200 μM BHT, 100 μM BHA (where indicated), and 1 mM potassium iodide in a total of 2 ml of 0.1 M phosphate buffer, pH 7. Hydrogen peroxide (250 μM) was added to incubations approximately 10 s after recording of absorbance began.

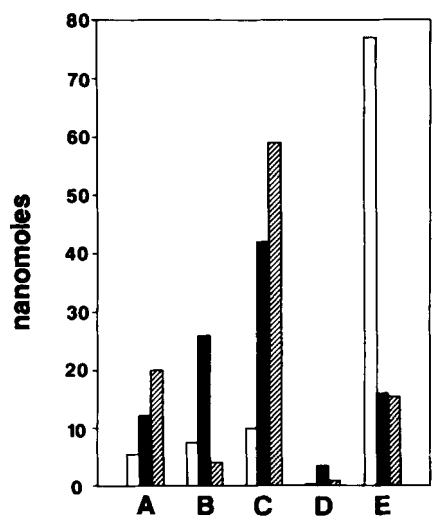

FIG. 8. **TLC of products formed from the prostaglandin H synthase-catalyzed metabolism of BHT.** Reactions contained 110 μM arachidonic acid, 100 μM BHT (0.25 μCi), and 1 mg of ram seminal vesicle microsomes in 1 ml of 0.1 M Tris buffer, pH 8. Some reactions also contained 100 μM BHA or 1 mM glutathione. □, BHT alone; ■, BHT and BHA; ▨, BHT, BHA, and glutathione. *A*, aqueous metabolite; *B*, covalently bound metabolite; *C*, polar metabolite (R_F = 0); *D*, stilbenequinone (R_F = 0.49); and *E*, BHT (R_F = 0.81). Chromatograms were developed on silica gel in heptane/benzene (1:1). Total nanomoles BHT present at the start of each reaction was 100. Values represent the mean from at least six reactions.

tected were the previously mentioned covalently bound metabolite and two unidentified metabolites: a polar metabolite which was extracted into the organic phase but stayed at the origin of the TLC plate when chromatographed, and an aqueous metabolite representing counts which remained in the aqueous phase. When 100 μM BHA was added to the reaction the formation of each of these products was greatly increased, and a small amount of stilbenequinone was detected. The percentage of parent BHT metabolized was increased from 23 to 85%. In the presence of 1 mM glutathione the amounts of aqueous and polar metabolites formed were further increased, indicating the probable formation of a BHT-glutathione conjugate. Likewise, the amounts of covalently bound product and stilbenequinone formed were decreased.

Glutathione has been shown to play an important role in modifying some of the toxic effects of BHT on rat liver and mouse lung (40–42), presumably through its direct interaction

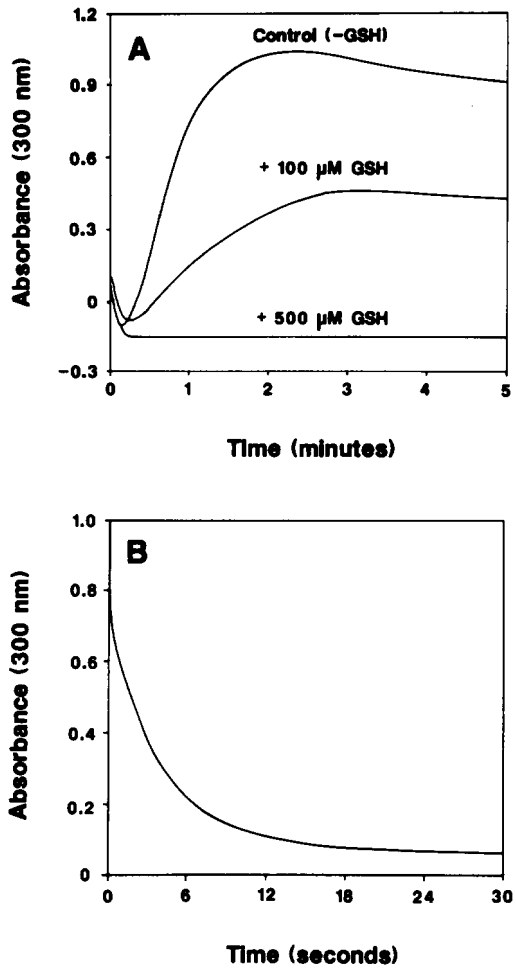

FIG. 9. **Horseradish peroxidase-catalyzed formation of BHT-quinone methide in the presence of glutathione.** Reactions contained 100 μg of horseradish peroxidase, 100 μM BHA, 200 μM BHT, and 0.9 mM hydrogen peroxide in 0.01 M phosphate buffer, pH 7. *A*, various concentrations of glutathione (*GSH*) were added to reactions just before the hydrogen peroxide. *B*, effect of glutathione on the disappearance of BHT-quinone methide. When the concentration of BHT-quinone methide had reached its peak (at approximately 2 min in *A*), 1 mM glutathione was quickly added and the rate of absorbance change at 300 nm monitored. The time of addition of glutathione is equivalent to 0 s. The absorbance at the time of addition of glutathione was 1.0, and approximately 2–4 s elapsed between the addition of glutathione and the start of the recording of the absorbance changes.

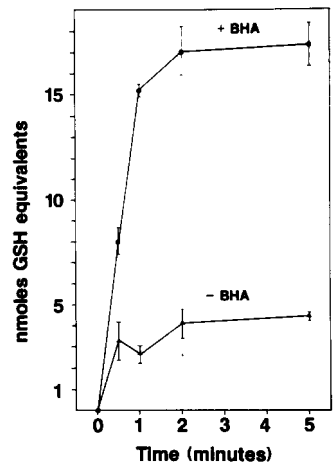

FIG. 10. **Horseradish peroxidase-catalyzed formation of BHT-glutathione (*GSH*) conjugate.** Reactions contained 100 μM BHA and/or BHT, 100 μM [^{35}S]glutathione (0.25 μCi), 100 μg of horseradish peroxidase, and 0.9 mM hydrogen peroxide in 1 ml of 0.01 M phosphate buffer, pH 7. Incubations were carried out at 25 °C for various lengths of time. BHT-glutathione was quantified by TLC and is expressed as nanomoles of GSH equivalents. Values represent the mean ± standard error for triplicate samples.

with BHT-quinone methide. Accordingly, we measured the effect of glutathione on both the formation of BHT-quinone methide as well as its direct interaction with BHT-quinone methide (Fig. 9). When glutathione was present at the beginning of the reaction it was able to prevent the formation of BHT-quinone methide in a concentration-dependent manner (Fig. 9*A*). 100 μM glutathione inhibited the rate of BHT-quinone methide formation by approximately 50%, while 500 μM was completely inhibitory. The formation of stilbenequinone was similarly inhibited (not shown). If 1 mM glutathione was added to the reaction mixture after BHT-quinone methide had already been formed (at approximately 2 minutes after starting the reaction, a time when the concentration of BHT-quinone methide was highest), the peak representing BHT-quinone methide rapidly disappeared (Fig. 9*B*). This disappearance of BHT-quinone methide could be due to either the formation of a BHT-glutathione conjugate or possibly the formation of oxidized glutathione and the reduction of the quinone methide back to BHT. The horseradish peroxidase-catalyzed formation of a BHT-[^{35}S]glutathione conjugate was measured in the presence and absence of BHA (Fig. 10). In the presence of BHA almost four times as much BHT-glutathione conjugate was formed as compared to that in the absence of BHA. These data demonstrate that BHT-quinone methide is highly reactive toward glutathione and that this interaction results in the formation of a BHT-glutathione conjugate.

DISCUSSION

Both BHA and BHT have been used to block the actions of peroxidase enzymes, including their ability to catalyze the oxidation of xenobiotics (43). Surprisingly, the possibility that BHA or BHT are themselves oxidized during such reactions has not been addressed. In this study we have investigated and compared the peroxidative metabolism of two commonly used phenolic antioxidants, BHA and BHT, and have shown that both horseradish peroxidase and the peroxidase component of prostaglandin H synthase are capable of oxidizing BHA and BHT to reactive intermediates which can covalently bind to protein or form dimeric products. In addition, we observed a chemical-chemical interaction between BHA and

BHT resulting in a significant stimulation of BHT oxidation and the formation of the potentially toxic BHT-quinone methide.

From a horseradish peroxidase-catalyzed reaction three dimeric products of BHA were identified, whereas with prostaglandin H synthase only two dimers were found. Two of these dimeric products, BHA dimers I and III, have not been reported previously in biological incubations. In studying the metabolism of BHA by horseradish peroxidase and rat intestinal peroxidase, Sgaragli *et al.* (25) reported the formation of a BHA dimer (equivalent to dimer II). This same dimeric product has also been reported by Guarna *et al.* (26) and Rahimtula (27). The formation of this dimer was detected using TLC or gas chromatography. In contrast, we used HPLC to separate the products of BHA metabolism, as well as different reaction conditions, which might account for the fact that we obtained two additional dimeric products.

Compared with BHA, BHT is a much poorer substrate for peroxidase. This is evident from the observations that dimeric products are readily formed from BHA alone but not from BHT alone. In prostaglandin H synthase-dependent incubations with BHT alone, only 23% of the parent compound was converted into metabolites, compared with approximately 80% of BHA. Also, using equimolar concentrations of these antioxidants, greater amounts of BHA were covalently bound to protein than BHT. The binding of BHT only surpassed BHA binding at concentrations of BHA which inhibited prostaglandin H synthase activity. This observation is in agreement with other reports in the literature which suggest that BHA is a better cofactor (electron donor) for peroxidase enzymes than is BHT (44).

The most significant observation from this study is the stimulation of BHT metabolism by BHA. We anticipated that BHA would inhibit the peroxidase-dependent oxidation of BHT through competition for the binding site for electron donating cofactors on the peroxidase. Instead, we observed that BHA markedly stimulated the covalent binding of BHT (400%) and the formation of BHT-quinone methide and stilbenequinone. Although the direct formation of BHT-quinone methide in *in vitro* incubations has never been reported, the quantities of BHT-quinone methide formed in the presence of BHA were of sufficient concentration that we were able to observe it spectrally. Previous studies have detected this metabolite indirectly through trapping or directly, using gas chromatography-mass spectrometry, in the bile of rats injected with extremely large quantities of BHT (7, 45).

Fig. 11 summarizes our results on the peroxidative activation of BHA and BHT and also details a possible mechanism for the formation of BHT-quinone methide and stilbenequinone from peroxidative reactions in the presence of both BHA and BHT. In the schematic, when BHA is present in peroxidase incubations in the absence of BHT, BHA is metabolized to a reactive intermediate (phenoxyl radical), which subsequently dimerizes or covalently binds to cellular macromolecules. In the presence of BHT, however, BHA is recycled back to the parent compound. Using HPLC we observed that very little parent BHA (<10%) was metabolized in horseradish peroxidase-catalyzed reactions in the presence of BHT and that the production of BHA dimers was completely inhibited (not shown). We have shown in Table I that BHT also inhibits BHA covalent binding by approximately 70%. These data all indicate that BHA is not consumed in these reactions in the presence of BHT. Similarly, in the absence of BHA, BHT was metabolized to a reactive intermediate (phenoxyl radical), which was capable of covalently binding to cellular macromolecules. Previous studies on the BHT phenoxyl radical indicate that it disproportionates into BHT-quinone methide and the parent compound (38). However, in our spectral assay, we could not detect any BHT-quinone methide from BHT alone (limit of detection was approximately 1 μM). In the presence of BHA the metabolism of BHT was enhanced to form BHT-quinone methide and its subsequent dimerization product, stilbenequinone. Since BHT-quinone methide is highly reactive, its increased formation was likely responsible for the enhanced covalent binding of BHT to protein observed in the presence of BHA (Tables I and II).

A crucial point in this mechanism is the direct interaction of an oxidized metabolite of BHA with BHT. Our data with lactoperoxidase suggest that the phenoxyl radical of BHA is the species which interacts with BHT. The occurrence of such an interaction has been suggested previously in the chemical literature. Using *tert*-butyl hydroperoxyl radical as the oxidizing species, Kurechi and Kato (46) suggested that the BHA phenoxyl radical may directly interact with BHT. These authors suggested that such an interaction is the molecular basis for the synergism between BHA and BHT seen in

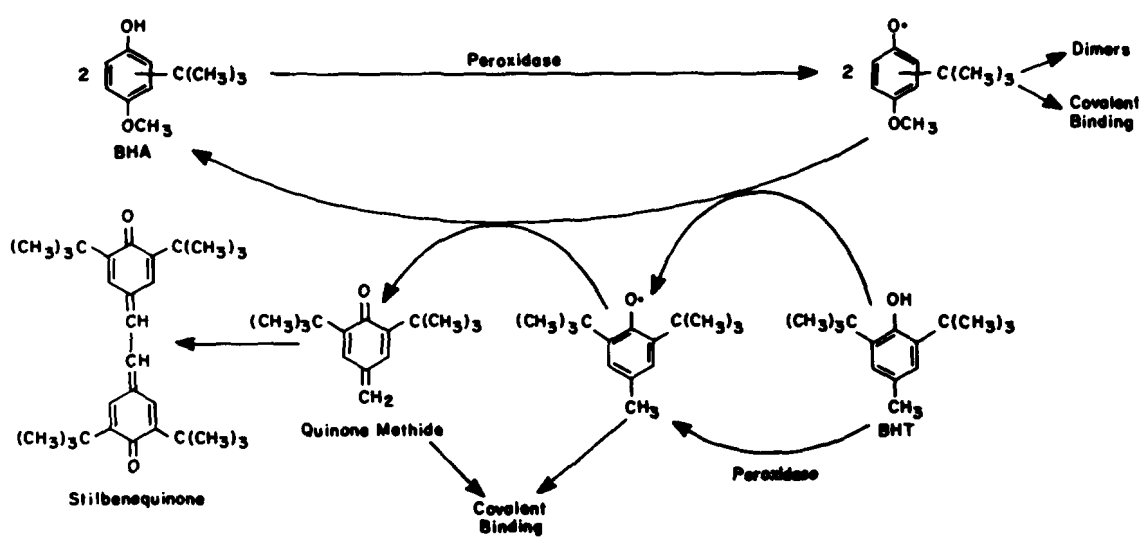

FIG. 11. **Possible mechanism for the formation of BHT-quinone methide and stilbenequinone from the peroxidative interaction of BHA with BHT.**

antioxidation experiments. On the basis of kinetic experiments on the inhibition of cumene oxidation by BHA and BHT, Ivanova *et al.* (47) also suggested that a rapid one-electron transfer occurs between BHT and the phenoxyl radical of BHA. A similar result was reported for the combination of BHT and 4-methoxyphenol (48). These observations and our suggested mechanism (Fig. 11) imply the formation of phenoxyl radicals from BHA and BHT. Valoti *et al.* (49) have recently demonstrated the formation of the primary radicals from BHA and BHT in a horseradish peroxidase-catalyzed reaction.

The peroxidase-mediated formation of BHT-quinone methide is of interest from a toxicologic standpoint since this metabolite has been suggested to be responsible for the pulmonary damage elicited by BHT in mice (7). The ability of BHA to stimulate the *in vitro* formation of BHT-quinone methide suggests that coadministration of BHA with BHT may exacerbate BHT-induced pulmonary toxicity *in vivo*. Indeed, we have recently completed studies which demonstrate that BHA can enhance the pulmonary toxicity of subthreshold doses of BHT and that this might be due to the increased formation of BHT-quinone methide (50, 51). In addition, we have observed that aspirin inhibits BHT-induced lung toxicity in mice,[3] and aspirin has also been reported to inhibit BHA-induced hyperplasia in rat forestomach (52). These observations suggest that peroxidase-mediated activation of antioxidants might be important *in vivo* in some of the toxic or carcinogenic processes mentioned above.

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[3] D. C. Thompson, Y.-N. Cha, and M. A. Trush, unpublished observations.

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MINIPRINT SECTION

Supplementary Material to:

The Peroxidase-Dependent Activation of Butylated Hydroxyanisole and Butylated Hydroxytoluene to Reactive Intermediates: *Formation of BHT-Quinone Methide Via a Chemical-Chemical Interaction*

David C. Thompson, Young-Nam Cha, and Michael A. Trush

Experimental Procedures

Materials:

BHA, BHT, glutathione, methimazole, propylthiouracil (6-n-propyl-2-thiouracil), lithium aluminum hydride, lithium aluminum deuteride, potassium iodide, horseradish peroxidase (type II), lactoperoxidase and hydrogen peroxide (30% solution) were obtained from Sigma (St. Louis, MO). Ascorbic acid and BHT acid (3,5-di-tert-butyl-4-hydroxybenzoic acid) were obtained from Aldrich (Milwaukee, WI). Di-tert-butyl phenol was purchased from Pfaltz and Bauer (Stanford, CT). Indomethacin (sodium trihydrate) was a generous gift of Merck, Sharp and Dohme (West Point, PA). Arachidonic acid was obtained from Nu Chek Prep (Elysian, MN). (Ring U-^{14}C) BHA (11.1 mCi/mmole) and BHT (20 mCi/mmole) were purchased from Amersham (Arlington Heights, IL) while ^{35}S-glutathione (150 Ci/mmole) was from New England Nuclear (Boston, MA). These compounds were $\geq$ 98% pure as determined by TLC and HPLC.

PHS Enzyme Preparation:

Ram seminal vesicles were obtained either from local sheep farmers and slaughterhouses or from Dr. Lawrence Marnett, Wayne State University. Vesicles were stored frozen at -80°. Microsomes were prepared as a 25% homogenate in 0.15 M KCl adjusted to pH 7.8 with dibasic sodium phosphate. The homogenate was spun at 9,000 g for 20 minutes. The pellet was washed with 0.15 M KCL buffer and spun again. The combined supernatants were filtered through cheesecloth and then spun at 100,000 g for 1 hour. The final microsomal pellet was resuspended in 0.15 M KCl buffer at a protein concentration of approximately 10 mg/ml and then rapidly frozen in methanol/dry ice and stored at -80°. The specific activity of PHS microsomes was measured by monitoring oxygen consumption (28).

Covalent Binding to Protein:

Reactions were initiated by the addition of hydrogen peroxide (0.9 mM) or arachidonic acid (110 μM) and allowed to proceed for 10 minutes at 25°. Each tube contained 0.5 μCi of ^{14}C-labeled BHA or BHT (diluted to the appropriate concentration with cold antioxidant) and 1 mg of ram seminal vesicle microsomes in a total volume of 1 ml of 0.1 M Tris buffer (pH 8.0). BHA, BHT and other test compounds were dissolved in DMSO, water, or methanol and added to the reaction in a volume not exceeding 10 μl. These solvents had no effect on the covalent binding of BHA or BHT. Reactions were stopped by the addition of 4 ml methanol and subsequently centrifuged at 2300 rpm for 10 minutes to pellet the protein. The pellets were repeatedly extracted with 2 ml methanol or methanol/ether (3:1) until no further radioactivity could be extracted (approximately 12-15 washes). Pellets were dissolved in 1 ml of 1 N NaOH by heating at 60° for 1 hour. A 0.5 ml aliquot was added to 10 ml ACS (Amersham) scintillation fluid and the radioactivity measured on a Packard Tri-Carb 300 Liquid Scintillation Counter. Another aliquot (50 μl) was used to determine protein concentration using the Lowry procedure (29).

Identification and Analysis of BHA Metabolites:

BHA dimer (2,2'-diol-3,3'-[1,1-dimethylethyl]-5,5'-dimethoxybiphenyl) was synthesized by the method of Hewgill and Hewitt (30). The proton nuclear magnetic resonance (NMR) and mass spectra (MS) of this compound (designated BHA dimer II) are shown in Figures 1 and 2. Electron impact MS analyses were carried out by Dr. Catherine Fenselau and associates of the Middle Atlantic Mass Spectrometry Laboratory, a National Science Foundation Shared Instrumentation Facility, or by the University of Minnesota Mass Spectrometry Service Laboratory. NMR spectra were determined by Dr. Lou-Sing Kan of the Johns Hopkins University Biophysics NMR Center (supported by GM 27512).

Metabolites of BHA were detected using HPLC. The system consisted of a Waters Associates Model 6000 A solvent delivery system and Model U6K injector, a Kratos SF 769 Z variable wavelength UV-visible spectrophotometer, and a Hewlett Packard model 3390 A integrator. A Lichrosorb Si 60 (10 μm, Merck) reverse phase C-18 column and a C-18 guard column were used with 80% methanol (isocratic) as the mobile phase. The flow rate was set at 1 ml/min and the UV-detector at 280 nm. Incubations with PHS were initiated by the addition of 0.9 mM hydrogen peroxide or 110 μM arachidonic acid and allowed to proceed for 10 minutes at 25°. Each tube contained 1 mg ram seminal vesicle microsomes and either 100 μM (arachidonic acid) or 1 mM BHA (hydrogen peroxide) in a total volume of 1 ml of 0.1 M Tris buffer (pH 8.0). Incubations with HRP contained 100 μg HRP (15 units) and 1 mM BHA in a total volume of 1 ml of 0.01 M phosphate buffer, pH 7.0 and were initiated by the addition of 0.9 mM hydrogen peroxide. All reactions were stopped by the addition of 0.5 ml of 5% trichloroacetic acid or 1 N perchloric acid and extracted with 3 ml ethyl acetate. Aliquots (20 μl) were directly assayed for BHA metabolites. Dimer formation was quantified by constructing a standard curve using the synthetic dimer and by using radiolabeled BHA. Efficiency of extraction was monitored by spiking each sample with 80 μg di-tert-butyl phenol.

Upon HPLC analysis of the horseradish peroxidase-catalyzed reaction for BHA metabolites, three peaks were detected: 11 minutes (I), 16 minutes (II) and 23 minutes (III). The peak at 16 minutes coeluted with synthetic BHA dimer. To identify the other two metabolites, the HRP reaction was proportionately scaled up and the three products separated and collected using a semi-preparative HPLC column (10 x 250 mm RP-18 Hibar, 7.5 μm, Merck) and similar chromatographic conditions except the flow rate was increased to 2 ml/min. The retention times for the three products under these conditions were 18, 23 and 31 minutes, respectively. The metabolites were collected, vacuum evaporated and subjected to NMR and MS analysis. The NMR and MS for the three metabolites are shown in Figures 1 and 2 and the dimeric structures illustrated in Figure 1 were assigned based on these spectral analyses. The spectrum for compound II was identical to synthetic BHA dimer. Dimer I was also subjected to infrared spectroscopy which revealed an absorption band at 1645 nm confirming the presence of a carbonyl functional group.

Identification and Analysis of BHT Metabolites:

Stilbenequinone (3,5,3',5'-tetra-tert-butyl-stilbene-4,4'- quinone) was synthesized according to the method of Cook et al (31). The UV-visible spectrum (in hexane) showed a maximum at 445 nm with two smaller shoulder peaks at 418 and 395 nm. BHT dimer (1,2-bis-[3,5-di-tert-butyl-4-hydroxyphenol]-ethane) was prepared by the lithium aluminum hydride reduction of stilbenequinone as described by Yohe et al (32). Deuterated BHT (d_3-BHT), in which the three hydrogens on the C-4 methyl group are replaced by deuterium, was prepared by the reduction of the methanol ester of BHT-acid by lithium aluminum deuteride following the procedure of Mizutani et al (7). The crude product was purified by flash chromatography (silica gel) using benzene/heptane (1:1) as eluent. MS analysis revealed the isotopic purity of the deuterated compound to be greater than 99%. BHT-quinone methide was synthesized according to the method of Becker (33). BHT-quinone methide has a strong ultraviolet absorption at 285 nm and in concentrated solutions ($> 10^{-2}$M) will dimerize (34). Identification of BHT-quinone methide was based on its ultraviolet absorption in isooctane and the formation of stilbenequinone and BHT dimer from concentrated solutions.

Metabolites of BHT were analyzed by HPLC, TLC and UV-visible spectroscopy. While dimeric products of BHA could be detected using HPLC, no dimeric products of BHT were detected using this method. As will be discussed later, BHT only formed detectable dimeric metabolites (stilbenequinone) in the presence of BHA. Since stilbenequinone does not absorb in the UV region but is easily separated on TLC, we measured BHT metabolism using ^{14}C-BHT and TLC. Reactions contained 0.5 μCi ^{14}C-BHT (100 μM total BHT) and 1 mg ram seminal vesicle microsomes in a total of 1 ml of 0.1 M Tris buffer (pH 8.0), and were initiated by the addition of 110 μM arachidonic acid. In addition, some reactions contained 100 μM BHA or 100 μM BHA and 1 mM glutathione. Reactions were terminated by the addition of 0.5 ml of 5% trichloroacetic acid. The incubation mixtures were extracted with 5 x 2 ml ethyl acetate and then evaporated under nitrogen. The residues were dissolved in benzene and a small aliquot spotted on a silica gel plate (Si250F-PA(19C), Baker) and developed 15 cm in heptane/benzene (1:1). The R_f values of standards and metabolites were: polar metabolite(s) - 0.0; stilbenequinone - 0.49; BHT dimer - 0.70; BHT - 0.81. Products were visualized by iodine vapor, scraped and counted for radioactivity.

The formation of BHT-glutathione conjugate was also measured by TLC. Reactions contained 100 μM BHA and/or BHT, 100 μM (0.25 μCi) of ^{35}S-glutathione and 100 μg HRP in 1 ml of 0.01 M phosphate buffer, pH 7.0. Reactions were initiated by the addition of 0.9 mM hydrogen peroxide and allowed to incubate for various lengths of time at 25°. Reactions were stopped at the indicated times with 4 ml methanol and evaporated under nitrogen. The residues were redissolved in 100 μl methanol and an aliquot spotted on a silica gel plate and developed 15 cm in n-butanol/ethanol/water (2:1:1) (35). The R_f value of the BHT-glutathione conjugate was 0.55. The preliminary identification of this compound as a glutathione conjugate was accomplished by running parallel experiments incorporating either ^{14}C-labeled BHT or ^{35}S-labeled glutathione.

A Beckman DU7 spectrophotometer or Hewlett Packard 8450A diode array spectrophotometer was used to measure the direct formation of BHT-quinone methide and stilbenequinone. Reactions contained 100 μg HRP (15 units), 0.9 mM hydrogen peroxide, 100 μM BHA and 200 μM BHT in 1 ml of 0.01 M phosphate buffer, pH 7.0. Incubations with lactoperoxidase contained 1 μg/ml lactoperoxidase, 100 μM BHA, 200 μM BHT, 1 mM potassium iodide and 250 μM hydrogen peroxide in the same buffer as the HRP incubations. The rate of formation of BHT-quinone methide was measured at 300 nm using an extinction coefficient of 27,000/M/cm (33). Identification of the reaction product (300 nm) as BHT-quinone methide (285 nm in organic solvents) was based on the following observations: a) When the product formed in aqueous solution was extracted with hexane, the absorbance shifted back to 285 nm and gave a spectrum identical to the synthetic BHT-quinone methide, b) upon standing (or allowing the reaction to continue) the product formed BHT dimer and stilbeneguinone, two compounds known to result from the dimerization of BHT-quinone methide (34), c) deuteration of BHT dramatically slowed the rate of formation of this product (rate of BHT/d_3-BHT was 2.6 for BHT-quinone methide and 4.7 for stilbenequinone), and finally d) glutathione blocked the formation of product in the this reaction and also caused the rapid disappearance of previously formed product immediately upon its addition to the reaction mixture, leading to the formation of a BHT-glutathione conjugate (see results section).

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 (36). Stilbenequinone, formed from the above HRP-catalyzed reactions with BHA and BHT, was extracted and purified using preparative TLC (1 mm plates, Kieselgel 60 F254S, Merck) and compared to the synthetic stilbenequinone (UV-Vis spectra, MS and NMR) to confirm the identity of this product.

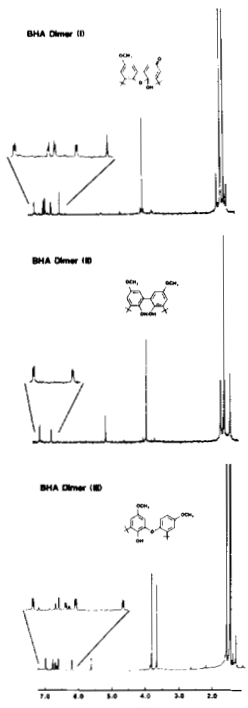

Figure 1. Proton nuclear magnetic resonance spectra of BHA metabolites resulting from an HRP-catalyzed reaction. The three BHA metabolites had the following ^{1}H NMR spectral characteristics. Compounds were dissolved in $CDCl_3$. Chemical shifts are listed as ppm, relative to tetramethylsilane. Each spectra also contains an expansion of the aromatic region.

BHA dimer I -
1.34 - s, 9H, t-butyl
1.44 - s, 9H, t-butyl
3.77 - s, 3H, methoxyl
6.25 - s, 1H, hydroxyl
6.50 - d, 1H, vinylic
6.67 - d, 1H, aromatic
6.73 - d, 1H, aromatic
7.00 - d, 1H, vinylic

BHA dimer II -
1.43 - s, 18H, t-butyl
3.78 - s, 6H, methoxyl
4.99 - s, 2H, hydroxyl
6.62 - d, 2H, aromatic
6.96 - d, 2H, aromatic

BHA dimer III -
1.42 - s, 9H, t-butyl
1.44 - s, 9H, t-butyl
3.64 - s, 3H, methoxyl
3.80 - s, 3H, methoxyl
5.60 - s, 1H, hydroxyl
6.18 - d, 1H, aromatic
6.59 - d, 1H, aromatic
6.66 - q, 1H, aromatic
6.75 - d, 1H, aromatic
6.96 - d, 1H, aromatic

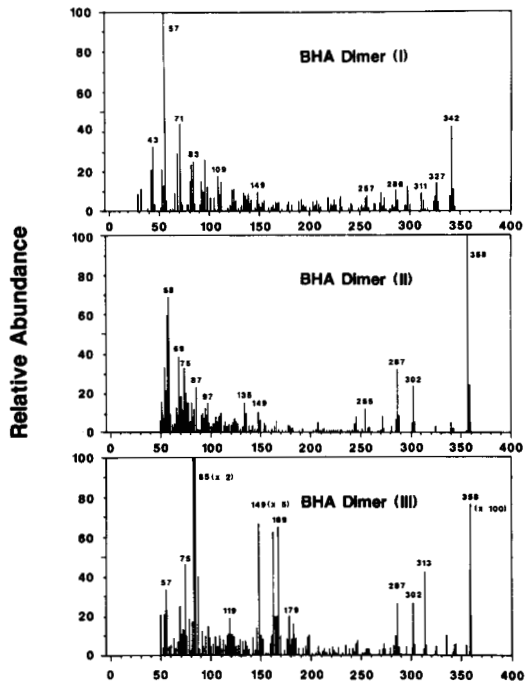

Figure 2. Electron impact mass spectra of BHA metabolites resulting from an HRP-catalyzed reaction. The parent molecular molecular ions (m/z) observed for the three BHA metabolites were: BHA dimer I - 342, II - 358, and III - 358.