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 [14C]BHA and [14C]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 phenoxy radical with BHT or BHT phenoxy radical. These results suggest a potential role for phenoxy 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)* 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

*The abbreviations used are: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; HPLC, high performance liquid chromatography.
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 I1 was identical to a BHA dimer 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 14C-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 O2 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

EXPERIMENTAL PROCEDURES

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-butyl-dibenzo[furan], 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.

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![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.](image1)

![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.](image2)
The effects of these same compounds on the covalent binding of BHT to protein catalyzed by prostaglandin H synthase were 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 BHA 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. 6A). 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. 6B, 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 [14C]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.

**TABLE I**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>nmmol BHT bound/mg protein</th>
<th>% of complete system</th>
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</thead>
<tbody>
<tr>
<td>BHA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.19 ± 0.01</td>
<td>100</td>
</tr>
<tr>
<td>Complete system</td>
<td>6.66 ± 0.67 100</td>
<td></td>
</tr>
<tr>
<td>+ Indomethacin 0.25 ± 0.04 1</td>
<td></td>
<td></td>
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<tr>
<td>+ Glutathione 2.08 ± 0.12 30</td>
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<td></td>
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<tr>
<td>+ BHT 2.35 ± 0.12 33</td>
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<tr>
<td>+ Ascorbate 3.93 ± 0.27 58</td>
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<td></td>
</tr>
<tr>
<td>+ Methimazole 5.20 ± 0.16 78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Propylthiouarcil 4.02 ± 0.25 59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BHT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.18 ± 0.02</td>
<td>100</td>
</tr>
<tr>
<td>Complete system</td>
<td>1.65 ± 0.09 100</td>
<td></td>
</tr>
<tr>
<td>+ Indomethacin 0.45 ± 0.01 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Glutathione 0.18 ± 0.01 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ BHA 6.03 ± 0.31 439</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Ascorbate 1.49 ± 0.04 89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Methimazole 2.86 ± 0.12 182</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Propylthiouarcil 1.40 ± 0.12 83</td>
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(37). The effects of these compounds on the covalent binding of BHT, however, revealed some interesting differences when compared to their effects on BHA. Ascorbate and propylthiouarcil 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 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.

**TABLE II**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>nmmol BHT bound/mg protein</th>
<th>% of BHT alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHT alone</td>
<td>4.35 ± 0.12</td>
<td>100</td>
</tr>
<tr>
<td>+ BHA 10 µM 11.41 ± 0.33 262</td>
<td></td>
<td></td>
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<tr>
<td>25 µM 14.77 ± 0.65 340</td>
<td></td>
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<tr>
<td>50 µM 14.89 ± 0.53 342</td>
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<tr>
<td>100 µM 15.13 ± 0.39 372</td>
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<td></td>
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<tr>
<td>250 µM 16.87 ± 0.06 388</td>
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<tr>
<td>500 µM 5.15 ± 0.16 118</td>
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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.
Peroxidative Metabolism of BHA and BHT

**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.

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.

The prostaglandin H synthase-dependent metabolism of [14C]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 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.

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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 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. 9A). 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. 9B). 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-[35S]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...
BHA would inhibit the peroxidase-dependent oxidation of 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 (phenoxy 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 BHT, BHT was metabolized to a reactive intermediate (phenoxy radical), which was capable of covalently binding to cellular macromolecules. Previous studies on the BHT phenoxy 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 phenoxy 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 phenoxy 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 BHT and 4-methoxyphenol (48). These observations suggest that peroxidase-mediated activation of antioxidants might be important in vivo in some of the toxic or carcinogenic processes mentioned above.

REFERENCES


Continued on next page.
**Peroxidative Metabolism of BHA and BHT**

**MINIREVIEW**

### Supplementary Material to:

The Peroxidase-Dependent Activation of Hydroxyalkylalkanones and Narkotic Eicosanoids to Metabolites of BHA and BHT: A Chemical-Teased Interaction

David C. Thompson, Young-Nam Cho, and Michael A. Travis

**Materials:**
- BHA, BHT, glutathione, mercaptoethanol, phosphatidylcholine (PC), arachidonic acid, N-acetyl cysteine, hydrogen peroxide, and sodium iodide.

**Experimental Procedures:**

To prepare enzyme reaction mixtures, the following components were added:
- 250 mM BHA or BHT in 100 mM sodium phosphate (pH 7.0) and 100 mM sodium iodide.
- 100 mM hydrogen peroxide.
- 100 mM potassium iodide.
- 100 mM N-acetyl cysteine.

**Results:**

1. **Reactions:**
   - The reactions were initiated by the addition of 0.05 M hydrogen peroxide and 0.1 M potassium iodide.
   - The reactions were monitored by the absorbance at 1645 nm, which is characteristic of BHT-quinone methide.
   - The formation of BHT-glutathione conjugates was also monitored by TLC and HPLC.

2. **Metabolites:**
   - The identities of the metabolites were confirmed by mass spectrometry and NMR spectroscopy.
   - The formation of BHT-glutathione conjugates was also confirmed by HPLC and TLC.

**Discussion:**

- The results of the study indicate that BHA and BHT are metabolized in the body via peroxidative pathways.
- The formation of BHT-glutathione conjugates is important in the detoxification of these compounds.
- The study also provides insights into the potential mechanisms of action of these compounds in the body.

**Conclusion:**

The results of this study suggest that the peroxidative pathways are important for the metabolism of BHA and BHT in vivo. Further studies are needed to fully understand the role of these pathways in the metabolism of these compounds.
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⁻¹ cm⁻¹. 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 1H NMR spectral characteristics. Compounds were dissolved in CDCl₃. Chemical shifts are listed as ppm, relative to tetramethylsilane. Each spectra also contains an expansion of the aromatic region.

**BHA dimer I -**
- 1.24 - s, 9H, t-butyl
- 1.46 - s, 9H, t-butyl
- 1.57 - s, 9H, t-butyl
- 6.25 - s, 1H, hydroxyl
- 6.50 - d, 1H, vinylic
- 6.67 - d, 1H, aromatic
- 7.00 - d, 1H, vinylic

**BHA dimer II -**
- 3.65 - s, 19H, t-butyl
- 3.79 - s, 9H, methoxyl
- 4.05 - d, 2H, aromatic
- 5.62 - d, 2H, aromatic

**BHA dimer III -**
- 1.24 - s, 9H, t-butyl
- 1.46 - s, 9H, t-butyl
- 1.57 - s, 9H, t-butyl
- 4.05 - d, 2H, methoxyl
- 5.00 - s, 1H, hydroxyl
- 6.19 - d, 1H, aromatic
- 6.57 - 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 ion (M⁺) observed for the three BHA metabolites were: BHA dimer I - 147, II - 358, and III - 358.