Chem.-Biol. Interactions, 72 (1989) 157–173
Elsevier Scientific Publishers Ireland Ltd.

ENHANCEMENT OF THE PEROXIDASE-MEDIATED OXIDATION OF BUTYLATED HYDROXYTOLUENE TO A QUINONE METHIDE BY PHENOLIC AND AMINE COMPOUNDS

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(Received September 7th, 1988)
(Revision received March 16th, 1989)
(Accepted March 24th, 1989)

SUMMARY

We have recently demonstrated that butylated hydroxyanisole (BHA) markedly stimulates the peroxidase-dependent oxidation of butylated hydroxytoluene (BHT) to the potentially toxic BHT-quinone methide. Using both horseradish peroxidase and prostaglandin H synthase we now report the ability of a wide variety of compounds to stimulate peroxidase-dependent activation of BHT. These compounds include several phenolic compounds commonly present in pharmacologic preparations or occurring naturally in foods. The ability of a given compound to stimuate BHT oxidation was found to depend on the type of radical it forms upon peroxidase oxidation. Compounds which have been shown to form phenoxy radicals or nitrogen-centered cation radicals were observed to enhance BHT oxidation. Conversely, compounds which are known to form peroxy radicals or semiquinone radicals either inhibited or had no effect on BHT oxidation. Compounds which enhanced BHT oxidation (monitored by covalent binding of [^{14}C]BHT to protein) were also observed to stimulate the formation of BHT-quinone methide and stilbenequinone. This suggested a common mechanism of interaction of these compounds with BHT. The stimulation of BHT covalent binding by BHA was also seen in various human and animal tissues using either arachidonic acid or hydrogen peroxide as substrate. The possible toxicologic implications of the enhancement of peroxidase-catalyzed BHT oxidation to BHT-quinone methide are discussed.

Key words: Butylated hydroxytoluene – Butylated hydroxyanisole – Peroxidase – Quinone methide – Phenoxy radical

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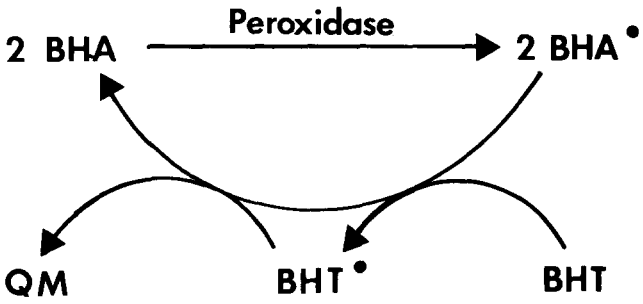
0009-2797/89/$03.50

INTRODUCTION

Quinone methides are unstable electrophilic compounds which are capable of reacting with various nucleophiles in the cell including amines, carbohydrates, phosphates, olefins and thiols [1—4]. Despite their apparent reactivity, quinone methides have only recently been implicated in the toxicity of specific xenobiotic compounds. Jurd et al. [5] reported that benzyl and cinnamyl derivatives of 2,4-di-*tert*-butylphenols and of 1,3-benzodioxoles are effective insect sterilants, presumably due to their in vivo oxidation to reactive quinone methides. Sinha and Gregory [6] suggested that quinone methide metabolites formed during the NADPH-dependent microsomal metabolism of adriamycin and daunomycin can alkylate nucleic acids and proteins, while Moore [7] has hypothesized that quinone methides are the covalent binding species formed from a variety of quinone drugs. Recently, Thompson et al. [8] have reported the formation of a quinone methide from eugenol which covalently binds to protein, forms conjugates with glutathione and is highly cytotoxic. Perhaps the best known example of toxicity associated with a quinone methide, however, is that reported for butylated hydroxytoluene (BHT). In a structure-activity study, Mizutani et al. [9] demonstrated that only phenolic compounds which were capable of forming quinone methide metabolites were able to elicit pneumotoxicity in mice. Furthermore, they synthesized deuterated BHT and demonstrated a significant decrease in toxic potency of this compound compared with BHT [10]. BHT-quinone methide may also play a role in hemorrhagic death reported in rats given high doses of BHT [11] since BHT-quinone methide inhibits phylloquinone epoxide reductase, an enzyme associated with the vitamin K-dependent synthesis of clotting factors [12]. Liver necrosis in both mice and rats given high doses of BHT has similarly been attributed to the formation of quinone methide [13,14]. Enzymatic reactions which result in the formation of quinone methides are thus of significant toxicologic interest.

We have recently reported that butylated hydroxyanisole (BHA) stimulates the peroxidase-dependent covalent binding of BHT to protein [15,16]. We were able to spectrally detect the formation of BHT-quinone methide in these reactions as well as a further dimerization product, stilbenequinone. We suggested that the formation of BHT-quinone methide came about through the direct interaction of the BHA phenoxy radical with BHT, with the subsequent reduction of the BHA radical back to the parent compound and oxidation of BHT to a phenoxy radical and then BHT-quinone methide (QM) (Scheme I). This type of mechanism prompted us to look at a variety of other phenolic and amine compounds which form peroxidase-dependent radical intermediates to see if they could also stimulate the formation of BHT-quinone methide in a manner similar to BHA. We report in this paper that a large number of compounds are able to enhance BHT-quinone methide formation, that the ability of a given compound to enhance BHT oxidation depends on the type of reactive intermediate (radical) the compound forms during peroxidase metabolism, and that all compounds which enhance BHT oxidation appear to do so via a common mechanism.



Scheme I.

METHODS

Materials

BHA, BHT, eugenol, diethylstilbestrol, acetaminophen, catechol, estradiol, phenylbutazone, phenidone (1-phenyl-3-pyrazolidone), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), quercitin, chrysin, horseradish peroxidase (HRP, type II and type VI) and hydrogen peroxide (30% solution) were obtained from Sigma (St. Louis, MO). Methyl paraben, guaiacol, ferulic acid, 2-*tert*-butyl-4-methylphenol, 6-*tert*-2,4-dimethylphenol, diphenylisobenzofuran, caffeic acid, chlorogenic acid, *tert*-butylhydroquinone (TBHQ) and propyl gallate were obtained from Aldrich (Milwaukee, WI). Arachidonic acid was obtained from Nu Chek Prep (Elysian, MN). [Ring U-^{14}C]BHT (20 mCi/mmol) was purchased from Amersham (Arlington Heights, IL). BHT-quinone methide and stilbenequinone were synthesized following published methods [17,18]. Deuterated BHT (d_3-BHT, labeled on C-4 methyl group) was prepared by the procedure of Mizutani et al. [10]. All other chemicals were of the highest grade available.

Enzyme preparations

Microsomal prostaglandin H synthase (PHS) was prepared from ram seminal vesicles as previously described [17]. Microsomal preparations were also made from sheep, guinea pig, dog, mouse, rat and human lung as well as dog bladder and rabbit kidney medulla. Rabbit kidney medullas were obtained from Pel-Freeze Biologicals (Rogers, AR). Human lung tissue was obtained from the Johns Hopkins Surgical Pathology Laboratory under the direction of Dr. Joseph Eggleston. Non-involved tissue was obtained from patients undergoing surgery for the removal of cancerous portions of lung. Dog tissues were donated by Dr. George Ikeda of the Food and Drug Administration. Sheep lung microsomes were donated by Dr. Ernst Spannhake, Johns Hopkins University. All tissues were thoroughly minced with scissors, homogenized (25% solution in 0.15 M KCl) and centrifuged for 20 min at 9000 × *g*. The supernatants were filtered through cheesecloth and then spun at 100 000 × *g* for one hour. The microsomal pellets were washed

once, suspended in 0.15 M KCl/0.05 M Tris (pH 7.5) at protein concentrations of approximately 10 mg/ml and rapidly frozen in methanol/dry ice and stored at −80 °C. Rat intestinal extracts were prepared according to the method of Kimura and Jellinck [19].

Covalent binding

Incubation vessels usually contained 100 μM (0.25 μCi) BHT, 1 mg ram seminal vesicle microsomes and 100 μM of the various test compounds in 1 ml of 0.1 M Tris buffer (pH 8.0). The reactions were initiated by the addition of 110 μM arachidonic acid and allowed to proceed for 10 min at 25 °C. BHA, BHT and other test compounds were dissolved in dimethyl sulfoxide, water, methanol or acetone and added to the reaction in a volume not exceeding 10 μl. These solvents had no effect on the covalent binding reaction. Reactions were stopped with 4 ml methanol and the amount of BHT covalently bound to protein was determined as previously described [17]. Protein was measured by the Lowry procedure [20].

Assay for formation of BHT-quinone methide and stilbenequinone

A Beckman Du7 spectrophotometer was used to measure the rate of formation of BHT-quinone methide and stilbenequinone. Reactions generally contained 100 μg HRP (15 units), 0.9 mM hydrogen peroxide, 100 μM BHA (where indicated) and 200 μM BHT in 1 ml of 0.01 M phosphate buffer (pH 7.0). Alternatively, some reactions substituted 1 mg PHS for HRP and were carried out in 1 ml of 0.1 M Tris buffer (pH 8.0). The rate of formation of BHT-quinone methide was measured at 300 nm using an extinction coefficient of 27 000 M^{-1} cm^{-1} [21] while the rate of formation of stilbenequinone was measured at 460 nm using an extinction coefficient of 72 000 M^{-1} cm^{-1} [22].

The formation of BHT-quinone methide and stilbenequinone were also monitored by normal phase HPLC with a Waters 990 diode array spectrophotometer detection system. For these assays, the incubations were stopped at the times indicated (either 1 or 10 min) by extraction with an equal volume of ethyl acetate. An aliquot of 25 μl was injected onto the column (μPorasil, 10 μm, Waters) and eluted using a mobile phase of hexane/0.1% acetic acid at a flow rate of 0.8 ml/min. Under these conditions, parent BHT had a retention time of 4.8 min, BHT-quinone methide 5.4 min and stilbenequinone 6.5 min. Stilbenequinone came off the column with the solvent front (the ethyl acetate) but could be accurately measured because of its absorption maxima (there was no absorption at 450 nm due to the solvent front). If stilbenequinone was dissolved in hexane and applied to the column, its retention time was about 11 min.

The disappearance of BHA, phenol, TMPD or TBHQ in the presence or absence of BHT was measured by reverse phase HPLC using a Beckman 5 μM column with UV detection (280 nm for BHA, phenol and TBHQ, 254 nm for TMPD) and eluted with various concentrations of a methanol/water mobile phase. Incubations for these measurements were adjusted (by lower-

ing HRP concentration) so that the parent compounds were metabolized by approximately 50% when the reactions were stopped after 1–5 min (by the addition of 5% trichloroacetic acid, final concentration). Incubations with TMPD were stopped by rapid freezing in liquid nitrogen. Incubations with BHA contained 10 μg/ml HRP, while phenol and TBHQ incubations contained 1 μg/ml HRP and TMPD incubations 0.1 μg/ml HRP. In all incubations the concentration of test compounds, BHT and hydrogen peroxide was 1 mM (except phenol, 0.5 mM). The reactions were carried out in 10 mM phosphate (pH 7).

RESULTS

Effects of BHA on BHT metabolism by peroxidases

The stimulatory effects of BHA on the peroxidative metabolism of BHT can be seen in Table I. In reactions containing HRP, the presence of 100 μM BHA markedly stimulated the rate of formation of BHT-quinone methide and its dimerization product stilbenequinone. In the absence of BHA neither of these two products could be detected (limit for detection of BHT-quinone methide was approximately 1 μM). In HRP-catalyzed reactions containing deuterated BHT, the rates of formation of these two metabolites were slowed. Isotope effects of 2.6 and 4.7 were observed for the reactions involving the removal of one deuterium atom (BHT-quinone methide) and two deuterium atoms (stilbenequinone), respectively. In reactions containing PHS, BHA stimulated the covalent binding of [^{14}C]BHT to microsomal protein by approximately 4-fold. BHA also enhanced the PHS-catalyzed formation of BHT-quinone methide, but little stilbenequinone was observed. This was probably due to the presence of a high concentration of microsomal protein in

TABLE I

EFFECT OF BHA ON THE IN VITRO PEROXIDATIVE METABOLISM OF BHT

Reaction	Covalent binding[a]	BHT-quinone methide[b]	Stilbene-quinone[b]
BHT alone	6.9 ± 0.4	n.d.	n.d.
BHT + BHA	26.2 ± 0.9	348 ± 3	35 ± 0.1
d_3-BHT + BHA	–	133 ± 3	7 ± 0.3

[a] Incubations for covalent binding contained 100 μM BHT (0.25 μCi), ± 100 μM BHA, 1 mg ram seminal vesicle microsomes and 110 μM arachidonic acid in 1 ml 0.1 M Tris buffer (pH 8). Reactions were incubated at 25°C for 10 min. Values represent nmol BHT bound/mg protein (mean ± S.E.).

[b] Incubations for measuring the formation of BHT-quinone methide or stilbenequinone contained 100 μM BHA, 200 μM BHT, 100 μg horseradish peroxidase (15 units), and 0.9 mM hydrogen peroxide in 1 ml of 0.01 M phosphate buffer (pH 7). Values represent nmoles product formed/min/mg horseradish peroxidase (mean ± S.E.). n.d., not detected.

these reactions (1 mg/ml) which would react with the BHT-quinone methide and prevent its dimerization. The formation of BHT-quinone methide by HRP and PHS was observed to be dependent on enzyme concentration as depicted in Fig. 1.

The comparative abilities of various animal tissue peroxidases to activate BHT to a covalently bound product was assessed using either hydrogen peroxide or arachidonic acid as substrate (Table II). These tissues included lung microsomes from various species including rat, mouse, sheep, guinea pig, dog and human, as well as microsomal preparations from dog bladder and rabbit kidney medulla and an extract of rat intestine. In each case the presence of BHA stimulated the covalent binding of BHT. The enhancement by BHA in the hydrogen peroxide-dependent reactions was relatively constant in all tissues (500–600% of the value of BHT alone) whereas there was greater variation in the arachidonic acid-dependent reactions (246% for guinea pig lung vs. 1800% for dog bladder). These data indicate that the peroxidase-dependent stimulation of BHT covalent binding by BHA is not peroxidase specific and occurs in a variety of tissues, including human lung.

Effect of various compounds on peroxidase-dependent BHT metabolism

The effect of various compounds on the peroxidative oxidation of BHT was assessed by measuring their effect on the PHS-catalyzed covalent bind-

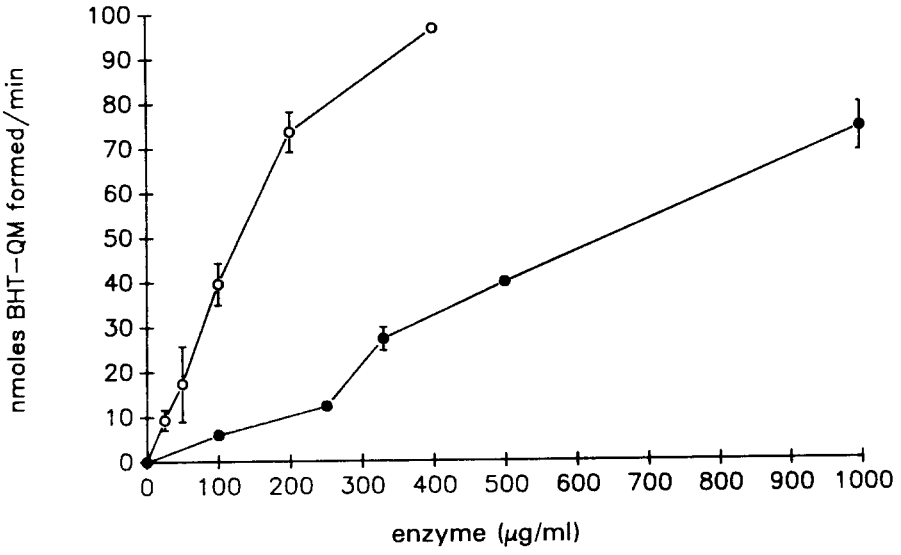


Fig. 1. Formation of BHT-quinone methide catalyzed by HRP (O) and PHS (●). HRP reactions contained 100 μM BHA, 200 μM BHT, 0.9 mM hydrogen peroxide and various enzyme concentrations in 1 ml of 0.01 M phosphate buffer (pH 7). PHS reactions contained 100 μM BHA, 200 μM BHT, 110 μM arachidonic acid and various concentrations of ram seminal vesicle microsomes in 1 ml of 0.1 M Tris buffer (pH 8). Rate of formation of BHT-quinone methide was measured as described in Methods.

TABLE II

PEROXIDASE-DEPENDENT COVALENT BINDING OF BHT TO PROTEIN BY MICROSOMES FROM VARIOUS ANIMAL TISSUES

Tissue	BHT	BHT/BHA	% of BHT alone
Arachidonic acid-dependent			
Human lung	0.10 ± 0.01[a]	0.35 ± 0.05	350
Sheep lung	0.18 ± 0.02	1.63 ± 0.06	906
Guinea pig lung	0.15 ± 0.02	0.37 ± 0.12	246
Dog lung	n.d.	0.68 ± 0.08	>1000
Dog bladder	0.44 ± 0.05	7.90 ± 0.65	1795
Rabbit kidney medulla	n.d.	0.69 ± 0.23	>1000
Hydrogen peroxide-dependent			
Rat lung	0.06 ± 0.02	0.32 ± 0.04	533
Mouse lung	0.13 ± 0.01	0.80 ± 0.03	615
Human lung	1.03 ± 0.01	6.14 ± 0.03	596
Rat intestine	4.33 ± 0.51	27.66 ± 4.2	639

[a] Values represent nmol BHT bound/mg protein (mean ± S.E. of triplicate reactions). Reactions contained 1 mg microsomal protein in 1 ml of 0.1 M phosphate buffer (pH 7.5). Reactions were initiated by the addition of 110 μM arachidonic acid or 0.9 mM hydrogen peroxide. BHA and BHT concentrations were 100 μM (0.25 μCi [^{14}C]BHT/tube). Incubations were 10 min at 25°C except for hydrogen peroxide-dependent human lung (30 min). n.d., not detected.

ing of [^{14}C]BHT to microsomal protein. Table III lists the effects of 12 compounds on BHT covalent binding. With the exception of two BHT analogs, each of these compounds has previously been shown to be metabolized by peroxidase enzymes to various reactive intermediates [23–30]. The compounds are grouped in the table according to the type of radical they form: peroxy, nitrogen-centered cation radical or phenoxy. Compounds which formed nitrogen-centered cation radicals (phenidone and TMPD) or phenoxy radicals (phenol, acetaminophen, estradiol, serotonin, diethylstilbestrol, and 2 analogs of BHT) were observed to stimulate BHT covalent binding. In contrast, compounds which formed peroxy radicals (phenylbutazone and diphenylisobenzofuran) either inhibited or had no effect on BHT covalent binding. The best stimulator of all the compounds tested was BHA (+392% of control).

To further explore the ability of compounds having phenolic groups to enhance the activation of BHT, the effects of various mono- and polyhydroxylated aromatic compounds on BHT covalent binding was examined. As shown in Table IV, a striking difference was seen between these two groups of compounds. The monohydroxylated compounds uniformly stimulated BHT covalent binding while the polyhydroxylated compounds inhibited. In several cases (i.e. BHA and TBHQ, guaiacol and catechol, and ferulic acid and caffeic acid) the only structural difference between a compound which stimulated BHT covalent binding and one which inhibited was the

TABLE III

MODIFICATION OF PHS-CATALYZED COVALENT BINDING OF BHT BY VARIOUS COMPOUNDS

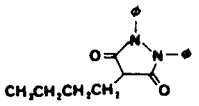
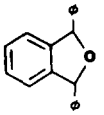
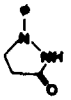
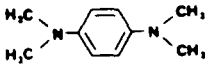
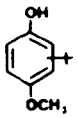
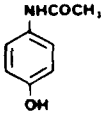
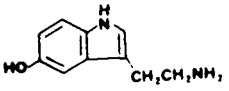
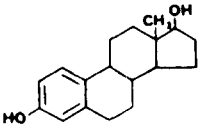
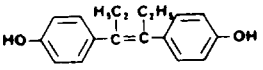
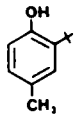
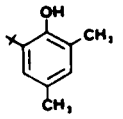
Reaction	Structure	% of complete system	Type of radical formed from added compound	Reference
Complete system[a]		100	–	–
+ Phenylbutazone		50	Peroxy	23
+ Diphenylisobenzofuran		93	Peroxy	23
+ Phenidone		194	N-cation	24
+ TMPD		180	N-cation	25
+ BHA		392	Phenoxy	26
+ Phenol		191	Phenoxy	27
+ Acetaminophen		160	Phenoxy	28
+ Serotonin		179	Phenoxy	29
+ Estradiol		145	Phenoxy	30
+ Diethylstilbestrol		252	Phenoxy	30

TABLE III (*continued*)

Reaction	Structure	% of complete system	Type of radical formed from added compound	Reference
+ 2-*t*-Butyl-4-methylphenol		288	Phenoxy	?
+ 6-*t*-Butyl-2,4-dimethylphenol		305	Phenoxy	?

[a] Complete system contained 100 μM (0.25 μCi) BHT, 1 mg ram seminal vesicle microsomes and 110 μM arachidonic acid in 1 ml of 0.1 M Tris buffer (pH 8). 100% = 6.14 ± 0.12 nmol BHT bound/mg protein in a 10-min incubation at 25°C. The concentration of all modifying agents was 100 μM. Values represent the mean of at least three samples. The standard error was less than 10% of the mean in all cases.

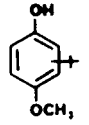
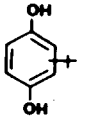
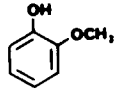
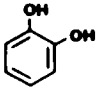
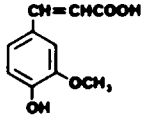
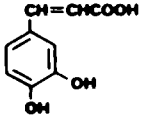
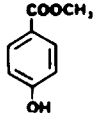
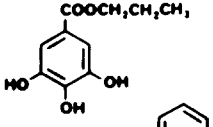
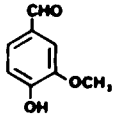
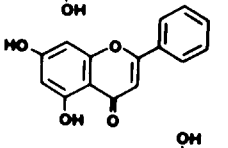
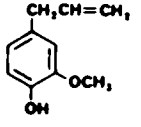
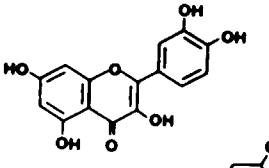
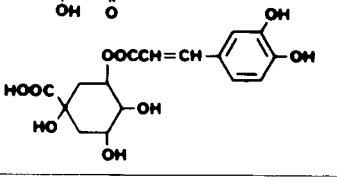
substitution of a methoxyl group with an hydroxyl group. Compounds with two *ortho* or *para* hydroyxl groups on the same aromatic ring are known to form semiquinone radicals as contrasted with the monohydroxylated compounds which form phenoxy radicals upon peroxidative metabolism [31]. Thus, the ability of various compounds to stimulate or inhibit BHT oxidation could be conveniently divided according to the type of radical the compound forms when oxidized by peroxidases. Those compounds forming peroxy or semiquinone radicals were inhibitors of BHT metabolism, while compounds forming phenoxy or nitrogen-centered cation radicals were stimulators of BHT metabolism.

Since all compounds in Tables III and IV were tested at one concentration (100 μM) it was important to ensure that compounds which we identified as inhibitors were inhibitory over a wide concentration range. In Table V the concentration-dependent inhibitions of BHT covalent binding by TBHQ, caffeic acid and chlorogenic acid are shown. At all concentrations tested (10–500 μM) these compounds were either inhibitory or had no effect.

Compounds which stimulated BHT covalent binding were also able to stimulate the formation of BHT-quinone methide and stilbenequinone. The measurement of BHT-quinone methide by normal phase HPLC is illustrated in Fig. 2. It can be seen in panel A that incubations with BHT alone produce negligible amounts of BHT-quinone methide (peak at 5.4 min). In panels B–D substantial amounts of BHT-quinone methide are produced in the presence of BHA (panel B), phenol (panel C) and TMPD (panel D). These three compounds all stimulated the covalent binding of BHT (Table III). The quantity of the BHT-quinone methide formed was proportional to the ability of these

TABLE IV

MODIFICATION OF PHS-CATALYZED COVALENT BINDING OF BHT BY VARIOUS MONO- AND POLYHYDROXYLATED COMPOUNDS

Monohydroxylated compound	Structure	% of control[a]	Polyhydroxylated compound	Structure	% of control[a]
BHA		425	TBHQ		60
Guaiacol		178	Catechol		18
Ferulic acid		155	Caffeic acid		28
Methylparaben		209	Propyl gallate		50
Vanillin		231	Chrysin		91
Eugenol		296	Quercitin		39
			Chlorogenic acid		58

[a] Reaction conditions were the same as those described in Table III. 100% value for BHT control reaction was 8.41 ± 0.14 nmol BHT bound/mg protein. Values represent the mean of at least three samples. The standard error was less than 10% of the mean in all cases.

TABLE V

CONCENTRATION-DEPENDENT INHIBITION OF THE COVALENT BINDING OF BHT BY TBHQ, CAFFEIC ACID AND CHLOROGENIC ACID

Reaction conditions were the same as those described in Table III. 100% value for BHT control reaction = 8.04 ± 0.17 nmol BHT bound/mg protein. Values represent the mean from two samples.

Inhibitor conc. (μM)	% of control
TBHQ	
10	117
50	65
100	64
250	15
500	2
Caffeic acid	
10	93
50	45
100	37
250	14
500	4
Chlorogenic acid	
10	83
50	62
100	69
250	43
500	17

compounds to stimulate BHT covalent binding. In contrast, TBHQ (panel E) did not stimulate the formation of BHT-quinone methide and is an inhibitor of BHT covalent binding (Table IV). Table VI compares the amounts of BHT-quinone methide and stilbenequinone formed in these HRP-catalyzed reactions. Compounds which formed BHT-quinone methide also formed stilbenequinone and increased the percent of BHT metabolized. With BHT alone, only 15% of the parent compound had disappeared after a 10-min incubation, compared with 90% disappearance in the presence of BHA. Phenol and TMPD also stimulated BHT metabolism while TBHQ inhibited BHT metabolism.

The stimulation of BHT metabolism by these compounds implies that the stimulatory compounds may be recycled back to the parent compound in the presence of BHT. The data presented in Table VII demonstrate that BHT inhibits the disappearance of parent compound for each of the stimulatory compounds BHA, phenol and TMPD. Considered together with the data in Table VI, this confirms our suggested mechanism that an oxidized metabolite

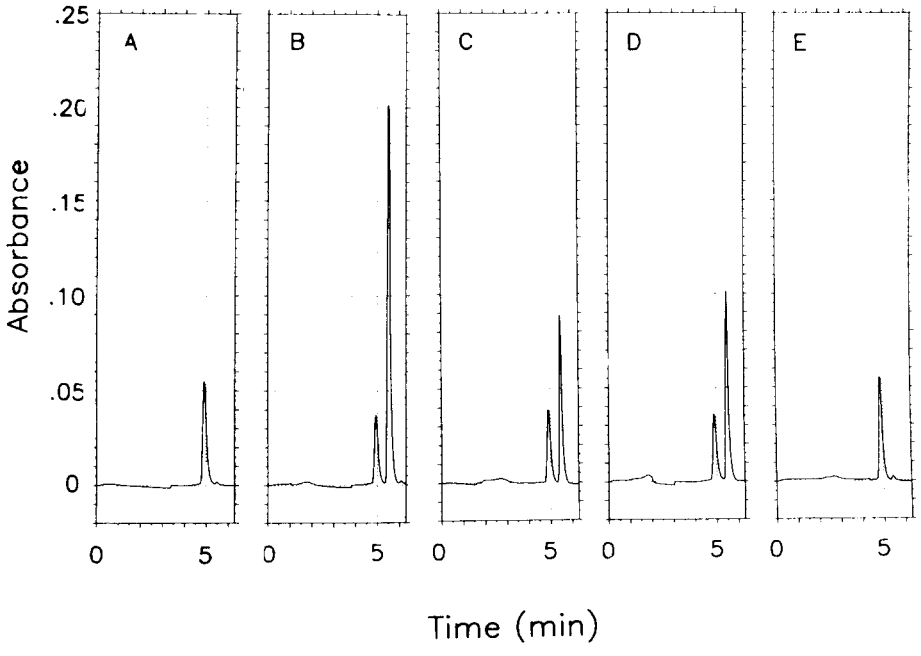


Fig. 2. Formation of BHT-quinone methide in HRP-dependent reactions in the presence of BHA, phenol, TMPD or TBHQ. Reactions contained 200 μM BHT, 100 μg HRP, 180 μM hydrogen peroxide and 100 μM of test compound in a total volume of 1 ml of 10 mM phosphate buffer (pH 7). Reactions were extracted with 1 ml ethyl acetate after incubation for 1 min and injected directly onto a normal phase HPLC column. Retention times for BHT and BHT-quinone methide were 4.8 and 5.4 min, respectively. Traces represent A: BHT alone, B: BHT + BHA, C: BHT + phenol, D: BHT + TMPD, and E: BHT + TBHQ.

TABLE VI

FATE OF BHT IN HRP-DEPENDENT REACTIONS IN THE PRESENCE OF BHA, TMPD, PHENOL OR TBHQ

Reaction	μM BHT remaining[a]	μM BHT-quinone methide formed	μM SQ formed
Control	200 ± 11	n.d.	n.d.
BHT alone	170 ± 11	n.d.	n.d.
+ BHA	19 ± 1	92 ± 4	67 ± 2
+ Phenol	59 ± 2	38 ± 4	5 ± 0.2
+ TMPD	116 ± 3	48 ± 8	4 ± 0.5
+ TBHQ	213 ± 5	n.d.	n.d.

[a] Reactions contained 180 μM hydrogen peroxide, 200 μM BHT, 100 μg horseradish peroxidase in 1 ml of 0.01 M phosphate buffer (pH 7). Concentration of test agents was 100 μM. The control incubation did not contain hydrogen peroxide. n.d. indicates not detected. Values represent mean ± S.E. of at least three reactions. Reactions were terminated after 10 min by the addition of 1 ml ethyl acetate, except for BHT-quinone methide measurements which were terminated after 1 min. Reaction products were analyzed by normal phase HPLC.

TABLE VII

FATE OF BHA, PHENOL, TMPD AND TBHQ IN HRP-DEPENDENT REACTIONS IN THE PRESENCE OR ABSENCE OF BHT

Reaction conditions are described in Methods. Disappearance of parent compounds was measured by HPLC. Values represent mean ± S.E. of at least three reactions.

Compound	% Parent compound remaining	
	− BHT	+ BHT
BHA	58.2 ± 1.5	82.4 ± 5.2
Phenol	54.3 ± 1.6	67.1 ± 1.1
TMPD	52.6 ± 3.1	99.4 ± 1.1
TBHQ	42.5 ± 0.3	77.7 ± 1.0

of the stimulatory compounds is being recycled (reduced) back to the parent compound in the presence of BHT with concomitant enhanced oxidation of BHT. TBHQ metabolism was also inhibited by BHT, but this is probably due to competition with BHT for the peroxidase active site, since TBHQ does not stimulate BHT oxidation.

DISCUSSION

In this study we investigated various factors which modulate the peroxidase-catalyzed activation and oxidation of BHT. We observed that BHA, another phenolic antioxidant, increased both the covalent binding of BHT and the formation of BHT-quinone methide, a reaction which could be diminished by deuterating BHT. In addition to HRP and PHS, peroxidase activity associated with a number of tissues catalyzed the activation or oxidation of BHT. BHA also enhanced these reactions. The oxidation of BHT by peroxidases thus represents an alternative pathway for its activation in tissues apart from the better characterized cytochrome *P*-450 system.

We also observed that, in addition to BHA, a number of other compounds could similarly stimulate BHT oxidation. These included a variety of phenolic compounds as well as phenidone and the aromatic amine, TMPD. Many of the compounds tested are present in commonly used medicines (e.g. acetaminophen, guaiacol and eugenol) or food products and therefore humans and animals are likely to be exposed to these compounds simultaneously with BHT, which is used as an antioxidant in foods at concentrations up to 0.02% based on the fat content [32]. Likewise, endogenous phenolic compounds could also serve as activators of BHT oxidation. For example, serotonin, which increased the PHS-catalyzed covalent binding of BHT (Table III), is known to accumulate in pulmonary endothelial cells, which are susceptible to BHT-dependent damage. These data suggest that the simultaneous presence of BHT and an activator chemical, either exogenous or endogenous,

in a cell or tissue with significant peroxidase activity might result in the enhanced formation of BHT-quinone methide and toxicity. In fact, we have recently demonstrated that the coadministration of BHA and BHT to mice significantly enhanced the BHT-dependent pulmonary toxicity which is believed to be mediated by BHT-quinone methide [33,34].

Our results demonstrate that compounds which enhance BHT covalent binding also enhance the formation of BHT-quinone methide and stilbenequinone. This implies that they are enhancing BHT oxidation through a common mechanism. Data from Tables III and IV demonstrate that there are rather strict requirements for the ability of a compound to enhance BHT oxidation. The only common feature of the various compounds tested was the type of radical formed upon peroxidase oxidation. Thus, only compounds which formed phenoxy or nitrogen-centered cation radicals were able to stimulate BHT oxidation. Compounds which formed peroxy or semiquinone radicals were unable to stimulate BHT oxidation. The mechanism for stimulation of BHT oxidation by BHA which we have advanced previously [17] requires that BHA be preferentially oxidized to a phenoxy radical which then reacts with BHT. Our results here must likewise require that each of these compounds be preferentially oxidized by the peroxidase compared to BHT. Markey et al. [35] have recently compared the ability of several peroxidase substrates to serve as reducing cofactors for PHS. Of the compounds common to their paper and ours, BHA, acetaminophen, phenol and guaiacol are all listed as better peroxidase cofactors than BHT, which is listed as a poor cofactor. This supports our proposed mechanism.

Our results indicate that BHT interacts differently with peroxy and semiquinone radicals than with phenoxy and nitrogen-centered cation radicals. The inhibition of BHT covalent binding by peroxy radicals may be due to competition between BHT and these compounds for the peroxidase or conversely, to the formation of adducts with the peroxy radicals. BHT has previously been shown to form adducts with specific peroxy radicals [36], and the ability of BHT to prevent lipid peroxidation by terminating peroxy radical chain reactions is well known [32].

Finally, radical-mediated chemical interactions have recently been shown to result in the activation of specific xenobiotic compounds to reactive intermediates. Previous reports in the literature have dealt with the abilities of oxygen radicals or peroxy radicals to activate secondary compounds. Reactive oxygen species have been implicated in the metabolic activation of dopa [37], bleomycin [38] and vanadate [39]. The extensive work of Marnett's laboratory [40—42] has involved the ability of peroxy radicals to epoxidate polycyclic aromatic hydrocarbons while Reed et al. reported similar reactions involving peroxy radicals derived from bisulfite or phenylbutazone [43,44]. Phenol and phenolic compounds have been shown to stimulate the peroxidase-mediated oxidation of a variety of compounds [45—51]. Other examples of radical-mediated interactions include the stimulation of catecholamine oxidation by the peroxidase-mediated formation of chlorpromazine radical [52]. This report demonstrates that phenoxy radicals and nitrogen-centered cation

radicals are capable of activating BHT to its ultimate toxic metabolite, BHT-quinone methide, and thus our work represents a significant extension of this novel field of chemical interactions.

ACKNOWLEDGEMENTS

This research was supported in part from the following sources: NIOSH OH 01833, NIH ES07141 and ES05131, the American Cancer Society SIG-3 and Johns Hopkins CAAT.

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