ABSTRACT

Effects of feeding mice and rats with 2(3)-tert-butyl-4-hydroxyanisole (BHA) and 3,5-di-tert-butyl-4-hydroxytoluene (BHT), the two most commonly used food-additive phenolic antioxidants with known anticarcinogenic properties but with only minor differences in their chemical structures, have been compared to search for common effects between the two agents in two different rodent species and then applied toward better understanding of the mechanisms involved in their protective actions. In liver microsomes of treated mice, both BHA and BHT enhanced the relative activity of aniline ring hydroxylation but decreased the relative benzo[a]pyrene monooxygenase activities. However, in rats, although aniline ring hydroxylation activity was decreased by both compounds, the decrease of benzo[a]pyrene monooxygenase activity was observed only with BHT. Thus, common effects could not be recognized at the microsomal mixed-function oxidase level.

Contrary to expectations based on chemical structures, BHT feeding elevated the epoxide hydrolase activity to an even greater extent than that produced by BHA, especially in rats. However, enzyme activities involved in the glucuronide conjugation system (uridine diphosphate:glucuronyl transferase, uridine diphosphoglucose dehydrogenase, and quinone reductase) are all elevated by both antioxidants in both rodent species. With BHA treatment, the levels of acid-soluble thiols were increased in both rats and mice. However, with BHT, the level was increased only in mice but not in rats. Similar trends were produced for glucose-6-phosphate dehydrogenase activity, but glutathione reductase activity was increased even for BHT-treated rats. Additionally, the glutathione S-transferase activities were also increased by both antioxidant treatments and in both rodent species. Based on these results, the elevations of epoxide hydrolase activity along with the enhanced conjugation and glutathione oxidation and reduction conjugation system enzyme activities were common to both compounds in both rodent species. This suggests their involvement in anticarcinogenic mechanisms. Increases of these detoxification enzyme activities appeared to be all designed to accelerate the elimination of administered antioxidants but, inadvertently, conferring protective effects from xenobiotics such as carcinogens.

INTRODUCTION

Dietary administrations of BHA, 3 BHT, and some other food additives antioxidants protect against tumor induction by chemical carcinogens of diverse structures (i.e., benzo[a]pyrene, dimethylaminocarboxazone, fluororenylacetamide) in a variety of rodent tissues (42, 48, 49). Recent studies dealing particularly with BHA have demonstrated (a) reduction of mutagenic activity arising from benzo[a]pyrene and several drugs (3); (b) reduction in the binding of benzo[a]pyrene metabolites arising from liver microsomes of treated mice to purified calf thymus DNA (44) and to hepatocyte DNA (15); (c) alterations in metabolic profile of benzo[a]pyrene by liver microsomes (16, 24) and by isolated hepatocytes (15) from treated mice; (d) elevations of various enzyme activities catalyzing the inactivation of reactive electrophiles (4-6, 8-10, 16, 17, 31, 33); (e) alterations of microsomal mixed-function oxidase activities with various substrates (8, 16) which may support the observed changes of metabolic profiles; and (f) increased concentration of nonprotein thiol compounds in liver and other tissues (3, 5). Therefore, the protective effects of BHA could be accounted for, at least in part, by its ability to enhance the activities of a variety of detoxification enzymes or by shifting the metabolic profile in such a manner that lowers the intracellular concentration of reactive products.

However, the multiplicity of the effects of BHA treatments does not indicate which or what combination of altered enzyme activities is responsible for the anticarcinogenic properties of this antioxidant. In an effort to understand the biochemical basis of BHA-dependent protective action, we have selected another anticarcinogenic phenolic antioxidant BHT and compared its effects with those of BHA. The strategy involved was to search for common effects. Previous studies dealing with this principle by comparing with the effects of few well-known inducers of hepatic drug metabolism have uncovered some differences. 4

Another purpose for selecting BHT for this study was to test the hypothesis that, because of the absence of free double bond carbon atoms in this compound, it could not form arene oxide and, thus, should not enhance epoxide hydrolase activity. On the other hand, since the BHA has such carbon atoms, it suggested the possibility that BHA epoxide could have formed and caused marked elevation of the enzyme activity. If this were the case, the increase of this enzyme activity would be unique to BHA feeding and most probably would not be involved in anticarcinogenic mechanism of this antioxidant.

As was mentioned earlier, the anticarcinogenic effects were also observed with rats (48). However, our previous experiences with this rodent species have indicated either a complete lack of or much suppressed responses to BHA treatments (10).

U9PQDH, UDP-glucose dehydrogenase; GST, glutathione S-transferases; UDPGGA, UDP-glucuronic acid.


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2 To whom requests for reprints should be addressed, at Department of Pathobiology, The Johns Hopkins University, 615 North Wolfe Street, Baltimore, Md. 21205.
3 The abbreviations used are: BHA, 2(3)-tert-butyl-4-hydroxyanisole; BHT, 3,5-di-tert-butyl-4-hydroxytoluene; GSH, glutathione; QR, quinone reductase; UD

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Y-N. Cha and H. S. Heine

Therefore, we have also included rats in this study and compared them with mice. If some common effects could be recognized in these 2 rodent species, this could also provide indications for the protective mechanism.

This report, then, compares the effects resulting from dietary administration of 2 structurally different phenolic food additive antioxidants (BHA and BHT) which have been demonstrated to protect mice and rats from several carcinogenic compounds. To understand the underlying mechanisms of various biochemical effects which may explain the protective action, we have attempted to correlate results with what is so far known about the metabolism of these anticarcinogenic antioxidants (7).

MATERIALS AND METHODS

Treatment of Animals. Female CD-1 mice (4 to 5 weeks old) and female Sprague-Dawley rats (~120 g) (Charles River Breeding Laboratories, Wilmington, Mass.) were housed in hanging stainless steel wire cages with 12-hr light-dark cycles with free access to Purina laboratory chow (Code 5001) and tap water for 1 to 2 weeks of acclimation. Subsequently, pelletized diets (Purina) containing 0.75% BHA and 0.5% BHT (Sigma Chemical Co.) were given to experimental animals (4 mice or 4 rats/cage).

Enzyme Assays. Animals were sacrificed by either decapitation (rats) or cervical dislocation (mice), and their gross body and liver weights were determined. At this time, lungs, kidneys, and upper small intestinal mucosa were also collected for determination of acido-soluble thiol levels (data not presented). The livers were homogenized individually in 35 ml of ice-cold sucrose (0.25 M) using a Teflon-glass homogenizer (6 strokes). All subsequent steps for isolation of microsomes and cytosol fractions were as described previously (8). Protein concentrations were determined by the method of Lowry et al. (27) using crystalline bovine serum albumin (Sigma) as the standard.

In the isolated microsomes, the levels of cytochromes P-450 and b5 were measured by the method of Omura and Sato (37, 38) utilizing an Aminco Model DW-2a spectrophotometer (American Instrument Co., Silver Spring, Md.) operating in split-beam scanning mode. Assays of NADPH and NADH cytochrome c reductases were carried out according to Maestra et al. (29) using the spectrophotometer in double-beam time-base mode. Activities of microsomal mixed-function oxidases were measured using either p-nitroanisole (8 mM), amphetamine (5 mM), 7-ethoxycoumarin (147 nM), or [14C]benzo(a)pyrene (100 μM) as substrates according to the methods described by Nash (36), Schenkman et al. (41), Ulrich and Weber (46), and DePierre et al. (14), respectively. The epoxide hydrolase activity was measured radiometrically using the [14C]epoxycoumarin as the substrate (10), and the activity of UDP-glucuronyl transferase was determined with p-nitrophenol as a substrate according to Mills and Smith (30).

Activities of several cytosolic enzymes such as glucose-6-phosphate dehydrogenase (26), GSSG reductase (40), UDPG DH (26), QR (8), and GST (19) were also measured.

Acid-soluble thiol levels of rat and mouse tissues were determined according to Grassetti et al. (18), and at least 95% of the thiol levels could be accounted for GSH when measured according to Tietze (45).

Materials. Both the BHA and BHT were purchased from Sigma and sent to the Ralston-Purina Co. to make up the 0.75% BHA and 0.5% BHT pellet diets used in this study. Various substrates for enzyme assays were obtained from Sigma [e.g., cytochrome c, glucose 6-phosphate, p-nitroanisole, 7-ethoxycoumarin, aniline, benzo(a)pyrene, etc.] and from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.) (e.g., NAD+, NADH, NADP+, NADPH, UDP-glucose, and UDPGA). The [14C]benzo(a)pyrene (Amersham-Searle) and styrene oxide (New England Nuclear) were repurified according to DePierre et al. (14) before being diluted with respective unlabeled substrates to the desired specific activities.

RESULTS

As was observed previously with BHA (8, 28, 39), dietary administration of BHT to mice and rats has also produced marked enlargements of the liver (1, 25). Although the increase in liver size is more pronounced by BHA feeding in mice, the BHT feeding produced greater proportional enlargements in rats. As a result, the relative liver weight in rats (normally 4.0% of body weight) is increased to 6.2% (Table 1).

Activities of Microsomal Drug-metabolizing Enzyme System. Table 2 shows the effects of feeding 2 structurally different antioxidants on specific activities and contents of enzymes involved in hepatic microsomal oxidation of various drugs. The effects produced in mice and rats are included in the table for comparison. In general, the basal enzyme activities obtained from rats were higher. Although the BHA feeding did not significantly elevate NADPH cytochrome c reductase activity, it was increased by BHT feeding. Concentrations of cytochrome P-450 were not changed in mice but were significantly increased for rats by BHA feeding. With BHT, however, marked elevations of the cytochrome level were observed in both rodent species. Furthermore, while the activity of NADH cytochrome c reductase was not changed, the concentrations of cytochrome b5 were moderately increased by both BHA and BHT and also in both rodent species (23).

Table 1

<table>
<thead>
<tr>
<th>Diet</th>
<th>Structure</th>
<th>Mice</th>
<th>Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.1 ± 0.4</td>
<td>4.0 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>BHA</td>
<td>7.7 ± 0.6</td>
<td>5.7 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>BHT</td>
<td>6.7 ± 0.3</td>
<td>62 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

*Concentrations and duration of treatments are given in the text.
**Mean ± S.D.
TB, tert-butyl side chain.
that BHA could serve as an alternate substrate of O-demethylation as well as an inducer of the enzyme activity by chronic administrations. Thus, it was reasoned and expected that only the feeding of structurally similar BHA, but not the dissimilar BHT, would elevate the O-demethylation activity of p-nitroanisole. Results showed otherwise, and in mice, the enzyme activity was increased only marginally with BHA, but substantially by BHT. Again, in rats, the O-demethylase activity was not responsive to these antioxidant treatments. Upon further reasoning, the higher levels of basal activity of these enzymes in rats were considered to be the reason for such lack of responses. Thus, we have selected another substrate, the 7-ethoxycoumarin, to determine the O-deethylating enzyme activity which in mice is 5 times higher than that of rats. Feeding the BHA caused a 2.2-fold further increase of the enzyme activity in mice, but only marginally in rats. Conversely, BHT produced only slight increases of mouse enzyme activity but caused a 2-fold elevation in rats. This indicated that rats are indeed responsive but only to some of the antioxidants. Additionally, when the effects of these antioxidant treatments were compared for changes in overall metabolism of benzo(a)pyrene, yet another type of result was obtained. Dietary administrations of both BHA and BHT to mice caused decreases of benzo(a)pyrene metabolism. However, the same treatment to rats produced either a slight increase or no change.

**Glucuronide Conjugation Enzyme System.** The activity of liver microsomal epoxide hydrolase, responsible for the formation of trans-dihydrodiols to be conjugated with UDPGA by the transferase, was measured using styrene oxide, and results are shown in Table 4. Basal enzyme activities were 3.7 and 9.3 nmol/min/mg in mouse and rat, respectively. These values, when compared to those that have been reported previously (10), are 3 to 4 times greater. However, we have been able to reduce the basal activities to the same low levels observed earlier by chronic feeding of purified diet (Nutritional Biochemicals) instead of Purina laboratory chow. With BHA mixed in Purina diet, enzyme activities were increased up to 16 (mouse) and 30 (rat) nmol/min/mg, and with BHT treatments, these were elevated to 11 (mouse) and 50 (rat) nmol/min/mg (23). Another microsomal enzyme, namely the UDP-glucuronyl transferase activity was also measured using p-nitrophenol as substrate. This was increased in mouse by feeding BHA but not BHT. In rats, where basal activity of this enzyme was one-half of that in mice, both the BHA and BHT (28) diets elevated the enzyme activity to equal levels (2.5-fold).

The activity of UDP:glucose dehydrogenase, a cytosolic enzyme requiring the reduction of NAD to NADH and responsible for production of UDPGA to be used as a cosubstrate toward glucuronide formation by the transferase, was increased moderately in both rats and mice by feeding both antioxidants. Activity of another cytosolic enzyme, the dicoumarol-sensitive NADH:QR measured using 2,6-dichlorophenolindophenol, was markedly increased by feeding BHA (6), but only moderately by BHT, in both mice and rats. The possible linkages between these 2 cytosolic enzyme activities will be discussed.

**Glutathione Oxidation-Reduction and Conjugation Enzyme System.** The feeding of both BHA and BHT produced moderate increases (2-fold) of glucose-6-phosphate dehydrogenase activity in mice (8). In rats, although basal activity of this enzyme was 6 times that of mice, it was further elevated by BHA but not by BHT (Table 5). Unlike glucose-6-phosphate dehydro-

### Table 2

<table>
<thead>
<tr>
<th>Diet</th>
<th>Animal</th>
<th>NADPH c reductase</th>
<th>P-450</th>
<th>NADH c reductase</th>
<th>b5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Mouse</td>
<td>225 ± 13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.53 ± 0.08</td>
<td>2.0 ± 0.4</td>
<td>0.36 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>300 ± 50</td>
<td>0.65 ± 0.12</td>
<td>3.5 ± 0.5</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>BHA</td>
<td>Mouse</td>
<td>263 ± 38&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.56 ± 0.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.4 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.58 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>275 ± 25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.87 ± 0.08</td>
<td>4.8 ± 0.5</td>
<td>0.70 ± 0.05</td>
</tr>
<tr>
<td>BHT</td>
<td>Mouse</td>
<td>388 ± 19</td>
<td>0.94 ± 0.07</td>
<td>2.2 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.51 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>450 ± 56</td>
<td>1.37 ± 0.15</td>
<td>3.7 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.94 ± 0.05</td>
</tr>
</tbody>
</table>

<sup>a</sup> NADPH c reductase, NADPH cytochrome c reductase (nmol cytochrome c reduced per min per mg); P-450, cytochrome P-450 (nmol/mg); NADH c reductase, NADH cytochrome c reductase (μmol cytochrome c reduced per min per mg); b5, cytochrome b5 (nmol/mg).

<sup>b</sup> Mean ± S.D.

<sup>c</sup> Not significantly different from respective controls.

### Table 3

<table>
<thead>
<tr>
<th>Diet</th>
<th>Animal</th>
<th>ANH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p-NA O-DM</th>
<th>7-EC O-DE</th>
<th>BPMO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Mouse</td>
<td>101 ± 24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.8 ± 3.7</td>
<td>938 ± 150</td>
<td>88 ± 7</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>130 ± 29</td>
<td>67.7 ± 1.5</td>
<td>167 ± 40</td>
<td>261 ± 41</td>
</tr>
<tr>
<td>BHA</td>
<td>Mouse</td>
<td>182 ± 34</td>
<td>39.4 ± 2.5</td>
<td>2066 ± 79</td>
<td>63 ± 3</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>125 ± 10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>66.4 ± 7.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>208 ± 50&lt;sup&gt;c&lt;/sup&gt;</td>
<td>319 ± 29</td>
</tr>
<tr>
<td>BHT</td>
<td>Mouse</td>
<td>254 ± 34</td>
<td>64.0 ± 8.6</td>
<td>1203 ± 95</td>
<td>64 ± 3</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>173 ± 24</td>
<td>72.6 ± 7.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>328 ± 51</td>
<td>289 ± 38&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> ANH, aniline hydroxylase (nmol p-aminophenol produced per min per mg); p-NA O-DM, p-nitroanilide Oxidemethylase (nmol HCHO per min per mg); 7-EC O-DE, 7-ethoxycoumarin O-deethylase (pmol 7-hydroxycoumarin formed per min per mg); BPMO, benzo(a)pyrene monooxidase (pmol/min/mg).

<sup>b</sup> Mean ± S.D.

<sup>c</sup> Not significantly different from respective controls.


**Table 4**

Effects of feeding BHA and BHT on epoxide hydrolase and glucuronidation system enzyme activities

<table>
<thead>
<tr>
<th>Diet</th>
<th>Animal</th>
<th>EH[a]</th>
<th>UDPG-Tr</th>
<th>UDPGDH</th>
<th>NADH-QR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Mouse</td>
<td>3.7 ± 0.7[b]</td>
<td>27.9 ± 4.9</td>
<td>47.6 ± 5.5</td>
<td>160 ± 12</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>9.3 ± 1.2</td>
<td>15.0 ± 4.7</td>
<td>63.5 ± 8.1</td>
<td>538 ± 30</td>
</tr>
<tr>
<td>BHA</td>
<td>Mouse</td>
<td>15.9 ± 2.3</td>
<td>48.3 ± 4.5</td>
<td>106.3 ± 11.1</td>
<td>991 ± 25</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>29.2 ± 5.5</td>
<td>36.5 ± 5.0</td>
<td>92.0 ± 9.5</td>
<td>2556 ± 136</td>
</tr>
<tr>
<td>BHT</td>
<td>Mouse</td>
<td>10.9 ± 1.2</td>
<td>29.8 ± 2.5[c]</td>
<td>68.2 ± 8.5</td>
<td>396 ± 23</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>49.4 ± 7.1</td>
<td>36.8 ± 6.3</td>
<td>100.0 ± 9.8</td>
<td>1180 ± 81</td>
</tr>
</tbody>
</table>

[a] EH, epoxide hydrolase (nmol styrene diol per min per mg); UDPG-Tr, UDP glucuronyl transferase (nmol p-nitrophenol conjugated per min per mg); UDPGDH (nmol NADH per min per mg); NADH-QR, NADPH:(quinone acceptor) oxido-reductase (nmol dichloroindophenol reduced per min per mg using NADH).

[b] Mean ± S.D.

c Not significantly different from respective controls.

**Table 5**

Effects of feeding BHA and BHT on activities of glutathione oxidation-reduction and conjugation enzyme system

<table>
<thead>
<tr>
<th>Diet</th>
<th>Animal</th>
<th>G6PD[a]</th>
<th>GR</th>
<th>Thiol</th>
<th>CDNB</th>
<th>DCNB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Mouse</td>
<td>9.7 ± 1.3[b]</td>
<td>18.7 ± 1.9</td>
<td>8.8 ± 1.1</td>
<td>1810 ± 86</td>
<td>31.1 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>59.5 ± 7.5</td>
<td>21.5 ± 3.8</td>
<td>7.3 ± 0.5</td>
<td>1298 ± 37</td>
<td>21.4 ± 1.3</td>
</tr>
<tr>
<td>BHA</td>
<td>Mouse</td>
<td>20.2 ± 3.3</td>
<td>47.7 ± 8.4</td>
<td>15.7 ± 0.5</td>
<td>1682 ± 582</td>
<td>159.0 ± 6.4</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>137.1 ± 11.7</td>
<td>38.0 ± 6.6</td>
<td>9.7 ± 0.6</td>
<td>3188 ± 84</td>
<td>43.7 ± 2.3</td>
</tr>
<tr>
<td>BHT</td>
<td>Mouse</td>
<td>19.0 ± 2.4</td>
<td>51.5 ± 5.7</td>
<td>10.4 ± 0.6</td>
<td>9060 ± 457</td>
<td>93.5 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>57.1 ± 5.9[c]</td>
<td>59.7 ± 5.7</td>
<td>6.1 ± 0.7[c]</td>
<td>3654 ± 151</td>
<td>46.2 ± 0.9</td>
</tr>
</tbody>
</table>

[a] G6PD, glucose-6-phosphate dehydrogenase (nmol NADPH formed per min per mg); GR, glutathione reductase (nmol NADPH oxidized per min per mg); Thiol, nonprotein thiol levels (¿umol/g liver); GST (nmol conjugate formed per min per mg) using substrates like chlorodinitrobenzene (CDNB) and dichloroindophenol (DCNB).

[b] Mean ± S.D.

c Not significantly different from respective controls.

genase, constitutive levels of glutathione reductase activity were the same between mice and rats, and the activities of this enzyme were increased modestly (2- to 3-fold) by both antioxidant treatments and in both rodent species. Increases of both of these enzyme activities in mouse liver by BHA feeding appeared to support the elevation of acid-soluble thiol levels; 95% of this could be accounted by GSH. However, in BHT-treated mice, increases of these enzyme activities were not reflected by elevated thiol levels. Furthermore, thiol levels in rats fed BHA showed slight increases which were barely significant, and those fed BHT were not changed at all.

Dietary administrations of both BHA (4, 31) and BHT to mice resulted in marked elevations (5- to 9-fold) of the specific activities of GST which were measured with chlorodinitrobenzene and dinitrochlorobenzene. However, although basal enzyme activities in rats were considerably lower than those in mice, the same antioxidant treatments to rats produced only moderate increases (2- to 3-fold). It was also interesting to note differences in the magnitude of increases depending on whether the enzyme activity was determined with chlorodinitrobenzene (9-fold) or dinitrochlorobenzene (5-fold), especially in BHA-treated mice.

**DISCUSSION**

Both BHA and BHT are phenolic antioxidants (Table 2) which are widely used as additives by the food industry to stabilize processed food products from free radical-induced damages (11, 20). Associated with their uses, there have been several studies dealing with their metabolism in rodents as well as in primates (reviewed in Ref. 7). Although major differences exist between animals in their pattern of metabolism and rates of excretion, BHA was metabolized and excreted more rapidly than was BHT. Rodents, in general, rapidly excreted BHA primarily as gluturonic conjugates and small amounts as sulfate esters, free phenols, and tert-butyl hydroquinone. BHT was excreted at much slower rates as either free BHT, 3,5-di-tert-butyl hydroxybenzoic acid, and their glucuronide conjugates, BHT:mercapturic acid, 3,5-di-tert-butyl-4-hydroxybenzyl alcohol and benzaldehyde, as well as 1,2-bis(3,5-di-tert-butyl-4-hydroxyphenyl)ethane and its glucuronic conjugate.

The 2 antioxidants used in this study have been demonstrated to protect mice and rats from carcinogenic effects produced by a variety of carcinogens [i.e., benzo(a)pyrene, dimethylnitrosamine, urethan, fluorouracil, and dimethylhydrazine] at various anatomical sites (i.e., liver, lung, colon, forestomach, mammary gland) (48). The protective effect of BHA has been attributed to the marked elevation of enzyme activities catalyzing the conjugation reactions which may lead to a more rapid detoxification of reactive metabolites arising from precarcinogens by metabolic activation (4-6, 8-10, 15-17, 31, 33). Additionally, growing indirect evidence indicated that discrete changes may have occurred in either the catalytic activity or the concentration of specific molecular forms of cytochrome P-450 without any significant changes in the overall level of this cytochrome (8, 15). BHA administration caused a selective decrease in activities (or concentrations) of cytochrome catalyzing the overall metabolism of benzo(a)pyrene and, yet, no changes in the catalytic activity (or concentration) of the cytochrome involved in O-
demethylation of p-nitroanisole. Thus, feedings of these 2 structurally different anticarcinogenic antioxidants appeared to commonly produce increases in ring-hydroxylation enzyme activity but decreases in overall metabolism of benzo(a)pyrene. Based on such analogies, these commonly shared effects between the 2 antioxidants may also be involved in the anticarcinogenic actions produced by both of these food additives. Such changes of relative enzyme activities may be caused by: (a) marked increases in the overall concentrations of cytochrome P-450 without accompanying comparable increases of these oxidase activities (i.e., with BHT); (b) selective decreases in the levels of specifically involved forms of cytochromes but increases of noninvolved cytochromes; or (c) selective inhibition of the oxidases by antioxidants. In any case, such differential effects produced between these compounds make it difficult to identify the common mechanisms involved in anticarcinogenic actions at the microsomal mixed-function oxidase level. Efforts to deal with such complexities by purifications and reconstitutions of specifically affected mixed-function oxidase systems in both rodent species are in progress.

Comparing the chemical structures of BHA and BHT, there are 2 major differences: anisole versus toluene as well as one versus 2 tert-butyl side chains in BHA and BHT, respectively (Table 1). Based on these differences, we had expected that BHA, but not BHT, would serve as a substrate for O-demethylation reaction and that the chronic administration would lead to a marked elevation of this enzyme activity. Additionally, we had also assumed that the availability of an open double bond in the benzene ring of BHA could form BHA epoxide by mixed-function oxidase reaction. Subsequently, the epoxide would serve as a substrate and inducer for epoxide hydrolase. Based on this analogy, we had expected that only BHA, but not BHT, feeding would elevate this enzyme activity by the mechanism of substrate induction.

Experimental supports indicating the BHA is being O-demethylated came from the identification of tert-butyl hydroquinone as a metabolite of BHA (2) and from the differing inhibitory constants of this compound on p-nitroanisole metabolism ("Results"). Although there is yet no evidence indicating the existence of BHA epoxide, we hope to identify this metabolite at a later study. In any case, results obtained in this study show that O-demethylase activity was not enhanced by BHA feeding and that dietary BHT also caused marked elevation of epoxide hydrolase activity in mice and rats (22, 23) to an even greater extent than that produced by BHA administration (Table 4).

Results shown in Table 4 indicate that activities of NADH:QR, UDPGDH, and UDP-glucuronyl transferases are all elevated by feeding both of these phenolic antioxidants. A schematic diagram illustrating the permissive role of QR toward the synthesis of UDPGA via UDPGDH is presented in Chart 1. The UDPGDH catalyzes oxidation of UDP-glucose to UDPGA by reducing NAD to NADH. In the normal cell, this enzyme reaction appears to be controlled by the NADH/NAD ratio and is rate limiting. Consequently, the concentration of UDPGA, a cofactor required for glucuronidation, is maintained at low level (32). However, the presence of QR, among many other enzymes within cytosol, would oxidize the accumulated NADH back to NAD (only when quinone substrates are provided) and would permit continued synthesis of UDPGA. The elevation of UDPGDH activity by BHA would not be useful for increased synthesis of UDPGA, unless the markedly increased QR activities for accelerated synthesis of UDPGA via UDPGDH and for increased rate of ethanol (or aldehyde) oxidation in isolated hepatocytes. Also, the enhanced UDP-glucuronyl transferase (UDPG TR) activity together with greater available amounts of UDPGA would readily conjugate phenolic substances like BHA, BHT, and tert-butyl hydroquinone (TBHQ). Furthermore, the toxic acetaldehyde produced from accelerated ethanol oxidation would be detoxified either by elevated GSH and GST (49) or by efficiently turning-over aldehyde dehydrogenase (ALO. DH) activity to an innocuous acetate. †, enzymes elevated by feeding BHA or BHT. TBQ, tert-butyl quinone; ALC. DH, alcohol dehydrogenase.

Chart 1. Schematic diagram for the possible involvement of elevated QR activities for accelerated synthesis of UDPGA via UDPGDH and for increased rate of ethanol (or aldehyde) oxidation in isolated hepatocytes. Also, the enhanced UDP-glucuronyl transferase (UDPG TR) activity together with greater available amounts of UDPGA would readily conjugate phenolic substances like BHA, BHT, and tert-butyl hydroquinone (TBHQ). Furthermore, the toxic acetaldehyde produced from accelerated ethanol oxidation would be detoxified either by elevated GSH and GST (49) or by efficiently turning-over aldehyde dehydrogenase (ALO. DH) activity to an innocuous acetate. †, enzymes elevated by feeding BHA or BHT. TBQ, tert-butyl quinone; ALC. DH, alcohol dehydrogenase. Glucuronides were made functional by the presence of quinones; in this case, perhaps the tert-butyl quinone generated from BHA itself. Furthermore, both the administered antioxidants are phenolic in nature and would serve as excellent substrates for glucuronidation. In fact, glucuronides of these compounds have been identified to exist in large quantities and proportions of excreted metabolites (12, 13). Under such circumstances of increased demand, additional UDPGA could be provided by the markedly elevated UDPGDH and QR activities but only in the presence of quinones generated from BHA. This would then accelerate the overall rate of glucuronide formation and thus elimination of BHA from cells.

In fact, in a recent study using harmol and paracetamol as substrates of glucuronidation in isolated hepatocytes obtained from BHA-fed mice, we have demonstrated 5-fold increases of glucuronide formation (33). Furthermore, we have also obtained preliminary results indicating that addition of quinones (menadione or benzoquinone) to hepatocytes further enhanced this reaction. Studies of Moldeus et al. (32) have demonstrated that formation of paracetamol and harmol glucuronides in isolated hepatocytes was severely inhibited by the presence of ethanol. As for the mechanism of this inhibitory effect, the excess of NADH produced during ethanol oxidation exerted the product inhibition on synthesis of UDPGA at the UDPGH level (Chart 1). This inhibitory effect of ethanol on conjugation was abolished by addition of small amounts of either menadione

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or benzoquinone. Therefore, although it remains to be experimentally proven, in the presence of quinone substrates, QR aids the production of UDPGA via UDPGDH. In doing so, the system balances the intracellular ratios of NADH to NAD and accelerates the glucuronidation cycle and consequently the elimination of phenolic antioxidants and metabolites.

While the synthesis and degradation of GSH are considered primarily the responsibility of γ-glutamyl cycle, results shown in Table 5 indicate considerable increases of enzyme activities involved in the regeneration of GSH from the oxidized GSH dimer GSSG, especially for BHA-treated mice. This may have contributed to the increased thiol levels as well as the increased ratio between reduced and oxidized GSH (33).

Cellular GSH, by itself or together with GST, can act as noncritical nucleophile for addition reactions and play a major role in deactivation of electrophilic compounds (43). Such formation of thioester conjugates represents the first step in mercapturic acid bioynthesis. These conjugates are typically less toxic than the original reactive compounds and are more readily eliminated from the body (Chart 1). Furthermore, the binding actions of GST represent an equally if not more important function to serve as expendable "buffer" protein for reactive metabolites (21). In these contexts, the concentrations of GSH (5 to 10 μm) and of GST protein (3 to 10% of cytosolic protein) in normal mammalian liver are extraordinarily high and yet further elevated by dietary antioxidants (Table 5) (3-5, 31, 33). Although the underlying mechanisms for these increases are not known, it is plausible that large quantities of stable free radicals of BHA and BHT produced from hydrogen abstraction of these antioxidants could provide inductive pressures for the elevation of GSH and GST. Support for this hypothesis came from the identification of BHT-mercapturic acid in urine (12, 13) and from recent studies of Nakagawa et al. (34, 35) indicating the presence of these stable free radical metabolites.

In summary, enhancements of this elimination pathway involving GSH and GST (Table 5), in addition to that of the glucuronide conjugation pathway (Table 4), appeared to be all designed to accelerate the elimination of administered dietary antioxidants. Elevations of these detoxification enzyme systems inherent in living cells may then confer serendipitous protection from environmental toxic agents including chemical carcinogens and acetaldehyde (Chart 1). In this connection, it was interesting to note that ethanol-induced hepatocyte toxicity could be prevented in cells isolated from BHA-pretreated animals.

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References

Comparative Effects of Dietary Administration of $2(3)$-\textit{tert}-Butyl-4-hydroxyanisole and $3,5$-di-$\textit{tert}$-Butyl-4-hydroxytoluene on Several Hepatic Enzyme Activities in Mice and Rats

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