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CYTOTOXICITY OF BUTYLATED HYDROXYANISOLE AND BUTYLATED HYDROXYTOLUENE IN ISOLATED RAT HEPATOCYTES

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Abstract—The effects of the antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on isolated rat hepatocytes were investigated. Both antioxidants were observed to be cytotoxic in a concentration-dependent manner at concentrations ranging from 100 to 750 μM. At equimolar concentrations BHT was more cytotoxic than BHA. Their toxicity appeared to be independent of their metabolism to reactive intermediates since inhibitors of cytochrome P-450 (metyrapone, SKF 525-A and piperonyl butoxide) had no effect on the cytotoxicity and *N*-acetylcysteine was also without protective effect. In addition, deuterated BHT was equitoxic with BHT. Only low temperature incubation (4°), which has previously been shown to inhibit the insertion of these compounds into biomembranes, was effective in inhibiting the cytotoxic effects. Using isolated rat liver mitochondria we observed that both BHA and BHT inhibited respiratory control primarily by stimulating state 4 respiration and thus acting as membrane uncouplers. BHA and BHT also effectively dissipated membrane potential across the mitochondrial membrane and caused the release of calcium and mitochondrial swelling. These mitochondrial effects were reflected by a rapid decrease in ATP levels in intact hepatocytes which preceded cell death. These results suggest that the observed cytotoxicity of BHA and BHT to hepatocytes is related to their effects on biomembranes and mitochondrial bioenergetics.

The lipophilic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) exert multifaceted effects on cellular functions, both *in vitro* and *in vivo*. For instance, they can influence cellular events through their metabolism [1], their antioxidant activity [2] or, as has more recently been shown, their interaction with biomembranes [3].

BHA and BHT are well known for their ability to inhibit the autocatalytic chain reactions which initiate and propagate lipid peroxidation and have been widely studied for their abilities to induce xenobiotic metabolizing enzymes and protect against certain types of toxins and carcinogens [4, 5]. On the other hand, BHT has been shown to enhance carcinogenesis, cause pulmonary damage in mice, and liver necrosis and hemorrhagic death in rats while BHA induces neoplasia in rat forestomach [6–10]. These effects are thought to be due to the formation of reactive metabolites from each compound.

Biomembrane effects of these antioxidants include inactivation of lipid-containing viruses [11], protection of cells from killing at low temperature [12], lowering the gel to liquid crystalline phase transition of lipids [13], increasing fluidity of cellular membranes in hydrocarbon but not polar regions [14] and perturbing phospholipid packing [15]. These effects on membranes are paradoxical because on the one hand antioxidants prevent damage to lipid membranes by terminating free radical chain reactions, while on the other hand they can cause damage of their own by intercalating into the hydrophobic phase and possibly interfering with membrane bound enzyme function and membrane integrity [16–18].

In the course of a series of experiments involving the antioxidants BHA and BHT in isolated rat hepatocytes we observed significant cytotoxic effects from each antioxidant alone. The present report concerns our investigation into the mechanism of this cytotoxicity. Our results suggest that the cytotoxic effects of these compounds are mediated not through the formation of reactive metabolites but, rather, by their biomembrane effects.

MATERIALS AND METHODS

Chemicals. Collagenase (Grade II), ATP, ADP and antimycin A were obtained from Boehringer Mannheim (F.R.G.). BHA, BHT, DMSO (dimethyl sulfoxide), succinate, rotenone, arsenazo III and *N*-acetylcysteine were from Sigma (St Louis, MO). SKF 525-A was from Smith-Kline & French Laboratories. Piperonyl butoxide was from ICN Pharmaceuticals. Dinitrophenol was purchased from Merck while metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) was obtained from Ciba Geigy. Deuterated BHT was synthesized according to Mizutani *et al.* [19]. Tetraphenylphosphonium chloride (TPP^+) was obtained from Fluka. Other chemicals were of the highest purity obtained from local suppliers.

Preparation and incubation of hepatocytes. Male Sprague–Dawley rats (*ca* 200 g), given food and water *ad libitum*, were used for these experiments. Hepatocytes were isolated by collagenase perfusion of the liver as previously described [20]. Cells prepared by this method were routinely found to be 90–95% viable. Cells were incubated at a concentration of 1×10^6 cells per ml in rotating round bottom flasks

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at 37° in Krebs–Henseleit buffer, pH 7.4, supplemented with 12.5 mM Hepes. The flasks were incubated in an atmosphere of 95% oxygen/5% carbon dioxide. BHA and BHT were dissolved in DMSO. All other test compounds were dissolved in either DMSO or buffer. The final concentration of DMSO in each incubation was 2% (v/v). Cell viability was assessed by trypan blue exclusion.

Preparation of mitochondria. Liver mitochondria were isolated by a standard method [21] in a medium containing 0.25 M sucrose, 5 mM Tris–HCl (pH 7.4) and 0.1 mM EDTA. EDTA was omitted in a final wash and resuspension. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard [22].

Measurement of respiration rates. Respiration rates of mitochondria were measured polarographically [23] at 25° using a Clark-type oxygen electrode and a Yellow Springs Instruments Model 53 Oxygen Monitor. The respiration buffer (3 ml) contained 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$ and 5 mM potassium phosphate (pH 7.4). The respiration substrate was 5 mM succinate and the amount of mitochondria added was 1 mg protein/ml. Rates of state 3 and state 4 respiration (natoms O consumed/min/mg protein) were measured in the presence or absence of 150 μM ADP, respectively. Respiratory control index was calculated as the ratio of state 3/state 4 respiration. Thus 100% inhibition of respiratory control indicates that no change in rate of oxygen consumption occurred following the addition of 150 μM ADP. Respiration in intact hepatocytes was also measured polarographically. Incubations were carried out at 37° in the Krebs buffer described earlier at a cell concentration of 5×10^5 cells/ml.

Measurement of membrane potential. Membrane potential ($\Delta\Psi$) across the mitochondrial membranes was monitored at 25° with a TPP^+ electrode [24]. The reaction medium (1.5 ml) contained 210 mM mannitol, 70 mM sucrose, 10 mM Hepes and 20 mM NaCl (pH 7.4). 10 μM TPP^+ and 1 mg/ml mitochondrial protein were added. The mitochondria were energized with 5 mM succinate to generate a transmembrane potential. After stabilization occurred (*ca* 3–4 min) BHA or BHT were added.

Measurement of calcium release and mitochondrial swelling. One milligram of mitochondrial protein was incubated in a cuvette containing 1 ml of the following medium at 25°: 210 mM mannitol, 70 mM sucrose, 10 mM Hepes, pH 7.4, supplemented with 5 mM succinate and 3 μM rotenone. For the calcium release experiments, 50 μM calcium chloride was added in the presence of 60 μM arsenazo III. After 5 min BHA or BHT were added and the change in absorbance at 654–685 nm followed using a Sigma ZWS II spectrophotometer. Antimycin A (5 μM) was added after BHA or BHT to ensure that these compounds did not interfere with the calcium measurement and to cause full release of calcium from mitochondria. Mitochondrial swelling was monitored on the same instrument as apparent absorbance changes at 540 nm (double beam mode) under the same conditions except without any arsenazo III present.

Measurement of adenine nucleotides. Levels of adenine nucleotides in hepatocytes were measured using high pressure liquid chromatography following the procedure of Jones *et al.* [25] as modified by Andersson and Uhlig [26].

Statistical analysis. All experiments were repeated two to six times. In Figs 1–3 data points represent mean ± SE from at least three experiments. In Figs 4, 5 and 7 data from an individual, representative experiment are presented, while in Fig. 6 data points represent the means from two experiments.

RESULTS

Effects on hepatocytes

Addition of BHT or BHA to isolated hepatocytes resulted in a concentration-dependent increase in cytotoxicity (Fig. 1a, b). The onset of cell death (assessed by % trypan blue uptake) was preceded by the appearance of blebs in the case of both compounds (not shown). At a given concentration BHT was always observed to be more cytotoxic than BHA.

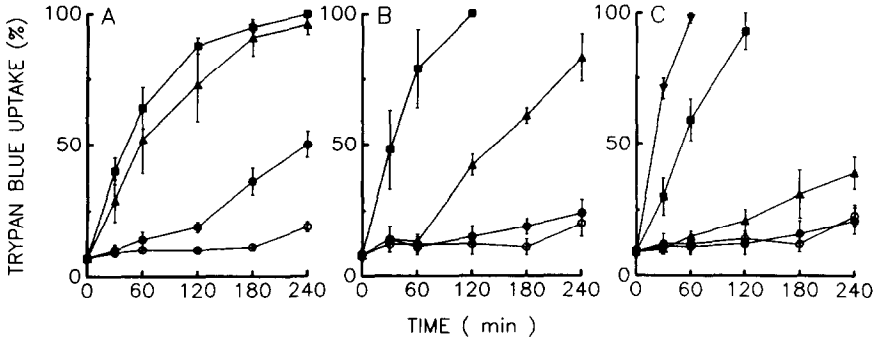

Fig. 1. Effects of BHA and BHT on hepatocyte viability. Freshly isolated hepatocytes (1×10^6 cells/ml) were incubated at 37° with various concentrations of BHA, BHT or combinations of both antioxidants for periods up to 4 hr. Cell viability was assessed by counting the percentage of cells which excluded trypan blue. Panel (A) BHT alone. Key: ○, Control (no BHT); ●, 250 μM BHT; ▲, 375 μM BHT; ■, 500 μM BHT. Panel (B) BHA alone. Key: ○, Control (no BHA); ●, 250 μM BHA; ▲, 500 μM BHA; ■, 750 μM BHA. Panel (C) BHA + BHT. Key: ○, control (no BHA or BHT); ●, 50 μM BHA + 50 μM BHT; ▲, 100 μM BHA + 100 μM BHT; ■, 250 μM BHA + 250 μM BHT; ▼, 500 μM BHA + 500 μM BHT.

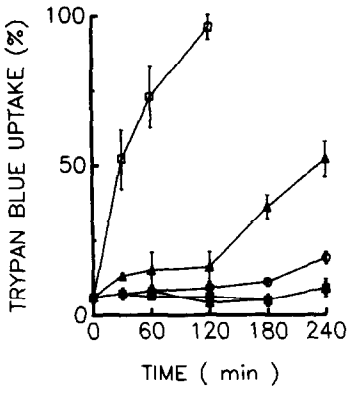

Fig. 2. Effect of temperature on BHT cytotoxicity. Incubations containing 250 or 500 μM BHT were incubated at 4° or 37°. Key: ▲, 250 μM BHT at 4°; △, 250 μM BHT at 37°; ■, 500 μM BHT at 4°; □, 500 μM BHT at 37°; ○, control cells at 37°.

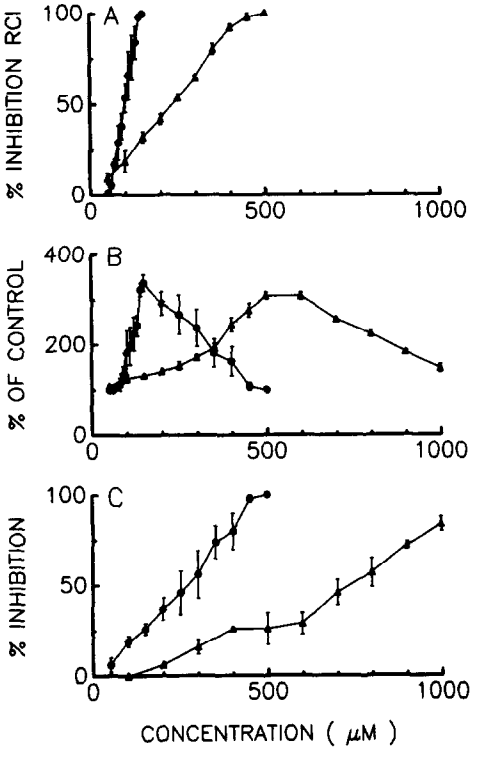

Fig. 3. Effects of BHA or BHT on mitochondrial respiration. The incubation medium consisted of 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, 5 mM potassium phosphate (pH 7.4), 5 mM succinate and 1 mg/ml mitochondria. Temperature, 25°, volume 3 ml. For measurement of state 3 respiration, antioxidants were preincubated with mitochondria for 3 min before the addition of 150 μM ADP. Each point is a mean ± SE of three separate experiments. Control rates were 33.6 ± 2.4 and 136.9 ± 4.2 natoms oxygen/min/mg protein for state 4 and state 3 respiration respectively. Initial respiratory control index was thus 4.07. Panel (A) Inhibition of respiratory control index by BHA or BHT. Panel (B) Stimulation of state 4 respiration by BHA or BHT. Panel (C) Inhibition of state 3 respiration by BHA or BHT. Circles (●) represent BHT, triangles (▲) represent BHA in all panels.

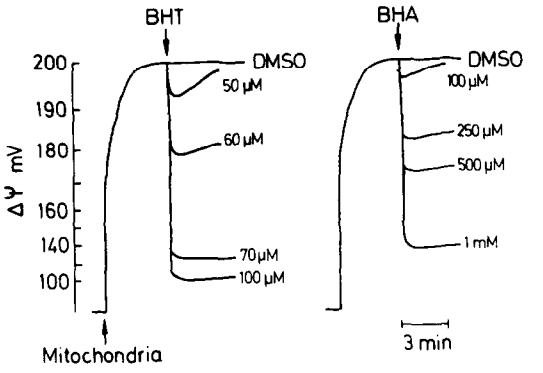

Fig. 4. Effects of BHA or BHT on the movement of TPP^+ across mitochondrial membanes. Mitochondria (1 mg protein/ml) were incubated at 25° in a buffer (1.5 ml) containing 210 mM mannitol, 70 mM sucrose, 20 mM NaCl, 10 mM Hepes (pH 7.4) and 10 μM TPP^+. 5 mM succinate was added to energize the mitochondria. After the potential had stabilized (arrow), various concentrations of BHA or BHT were added.

When combinations of antioxidants were incubated with hepatocytes, the cytotoxicity observed was greater than that observed with either compound alone at the same concentration (Fig. 1c). This effect appeared to be additive since a combination of 250 μM BHT with 250 μM BHA produced a similar cytotoxic effect as did 500 μM BHT or 750 μM BHA alone. Levels of reduced thiols decreased during the incubations concomitant with cell death, but did not precede it (not shown).

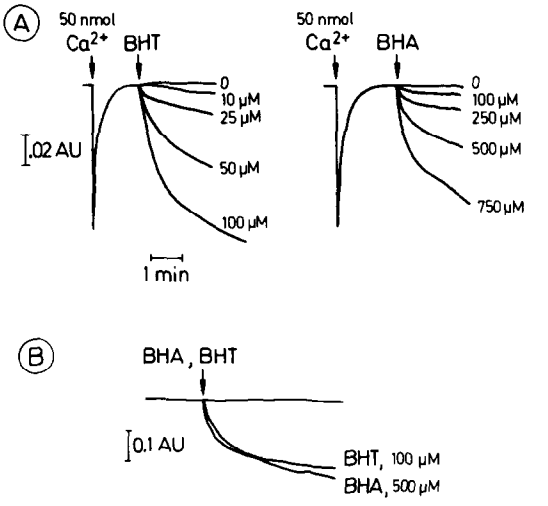

Fig. 5. The release of calcium from mitochondria and mitochondrial swelling induced by BHA or BHT. Mitochondria (1 mg protein/ml) were incubated at 25° in a buffer (1 ml) containing 210 mM mannitol, 70 mM sucrose, 10 mM Hepes (pH 7.4), 3 μM rotenone and 60 μM arsenazo III. 50 μM calcium chloride was added to load mitochondria. After loading was complete, various concentrations of BHA or BHT were added (arrow). The same buffer (without arsenazo III) was used for the swelling experiments. Panel (A) Concentration-dependent effects of BHA or BHT on mitochondrial calcium release. Panel (B) Antioxidant-induced swelling of mitochondria.

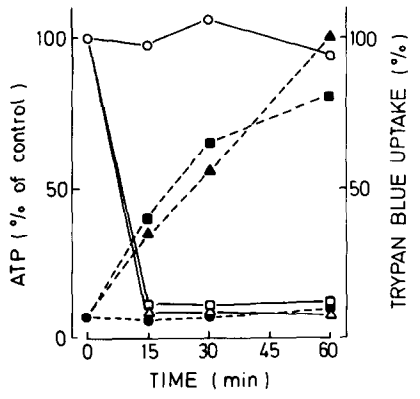

Fig. 6. Effects of BHA or BHT on hepatocyte ATP levels. Hepatocytes were incubated in the presence of 500 μM BHT or 750 μM BHA and the effects on ATP levels and cell viability measured. Control value for ATP was 15.3 nmoles/10^6 cells. Each point is a mean from two separate experiments. Open points represent ATP levels while solid points represent % trypan blue uptake. Key: ○, ●, control; □, ■, 500 μM BHT treated; △, ▲, 750 μM BHA treated cells.

Since BHA and BHT have been previously shown to be metabolized by rat liver enzymes to reactive intermediates which may be responsible for the cytotoxicity [27, 28], we tested the ability of inhibitors of cytochrome P-450 to prevent these effects in hepatocytes. At concentrations which were nontoxic to hepatocytes, neither metyrapone (1 mM), SKF 525-A (50 μM) nor piperonyl butoxide (100 μM) had an inhibitory effect on BHT toxicity (not shown). Similarly, *N*-acetyl cysteine (1 mM), which alone had no effect on cell viability, had no inhibitory effect on BHT toxicity, and deuterated BHT was equitoxic as BHT (results not shown). Deuterated BHT has previously been shown to inhibit the formation of BHT-quinone methide, the presumed toxic metabolite of BHT [19]. Incubation at low temperature (4°) was the only method employed which successfully inhibited the cytotoxicity (Fig. 2). In each experiment, similar results were obtained for hepatocytes exposed to BHA (with the exception of the experiment with deuterated BHT). Thus, the results for BHA are not shown. While low temperature inhibits most metabolic functions in hepatocytes, it has also been shown to inhibit the insertion of hydrophobic compounds into lipid membranes [29]. These results are consistent with the interpretation that metabolism is unnecessary for BHA and BHT to elicit cytotoxicity in hepatocytes.

Effects on isolated mitochondria

Sgaragli *et al.* [3] have shown that BHA and BHT induce the leakage of enzymes from rat liver mitochondria and lysosomes and also damage the cellular membrane of erythrocytes. We investigated the effects of these antioxidants on a variety of functional parameters in isolated mitochondria in order to see if this might account for their toxic effects in whole cells. The effects of BHA and BHT on the respiratory control index (state 3 respiration/state 4 respiration) of mitochondria are shown in Fig. 3a. Respiratory control was completely lost at a concentration of 150 μM BHT and 500 μM BHA. The IC_{50}s were 97.9 and 230 μM for BHT and BHA, respectively. The inhibition curves were linear for both compounds but the curve was much steeper in the case of BHT.

Inhibition of respiratory control can be due either to an inhibition of state 3 respiration (active respiration in the presence of ADP) or by a stimulation of state 4 respiration (resting respiration after the expenditure of ADP). BHA and BHT were able to both stimulate state 4 and inhibit state 3 respiration (Fig. 3b, c). The stimulation of state 4 respiration was preceded by a short lag period of approximately 1–2 min, thus all measurements were made after a preincubation time of 3 min. This lag period was not, however, observed with dinitrophenol, which was used as a positive control. The maximal stimulation of state 4 respiration was approximately 300–350% for both compounds. The addition of dinitrophenol was not able to stimulate further BHA or BHT-enhanced state 4 respiration. The stimulation of state 4 respiration occurred at lower concentrations (maximal effect of BHT was at 150 μM, while for BHA it was at 500 μM) than did the inhibition of state 3 respiration (IC_{50} for BHT was 254 μM while for BHA it was 703 μM). Thus, at lower concentrations the primary effect on respiratory control of BHA and BHT appears to be the stimulation of state 4 respiration, mimicking the effects of classic uncouplers such as dinitrophenol.

Effects on membrane potential

The effect of BHA and BHT on the potential across the mitochondrial membrane was measured using the permeant cation TPP^+. This cation is taken up from the reaction medium into the inner side of mitochondria when they are energized by succinate, indicating the formation of membrane potential. The addition of BHA or BHT induced an immediate concentration-dependent release of incorporated TPP^+ into the incubation medium, indicating release of membrane potential (Fig. 4). The concentrations of BHT which caused the release of membrane potential were somewhat lower than the concentrations necessary to inhibit respiratory control.

Effects on calcium release and mitochondrial swelling

Calcium movements across the mitochondrial membrane were monitored by measuring the amount of calcium in the incubation medium using the metallochromic indicator, arsenazo III. Both BHA and BHT caused a concentration-dependent release of calcium from mitochondria immediately after addition to the incubation medium. BHT was much more potent in this regard than was BHA (Fig. 5a). Concomitant with the release of calcium, a concentration of 100 μM BHT or 500 μM BHA elicited mitochondrial swelling (Fig. 5b).

Effects on cellular adenine nucleotide levels

In order to correlate effects seen in isolated mitochondria with cellular events, the effects of BHA and BHT on adenine nucleotide levels in intact hepatocytes were measured (Fig. 6). In hepatocytes

treated with 500 μM BHT or 750 μM BHA, ATP levels dropped to 10% of control levels within 15 min and remained at this level until the end of the experiment. At the 15 min time point, cytotoxicity was very low, thus indicating that the reduction in nucleotide levels preceded cell death.

Effects on cellular respiration

Finally, the influence of BHA and BHT on cellular respiration (oxygen consumption) was measured (Fig. 7). Hepatocytes alone exhibited a rapid, fairly linear utilization of oxygen which continued until the oxygen supply in the incubation vessel was exhausted. Within one minute after the addition of 500 μM BHT, however, there was a noticeable increase (148 ± 5%) in the rate of oxygen consumption which, after a few minutes, resulted in a striking inhibition of oxygen consumption. Similar inhibitory results were obtained with 750 μM BHA but the initial increase in rate of oxygen consumption was not apparent. These results provide evidence for a rapid effect of BHA and BHT on cell respiration which probably increases the rate of hydrolysis of ATP in the mitochondrial membrane and directly leads to the total depletion of cellular ATP (as shown in Fig. 6) and subsequent inhibition of cellular respiration.

DISCUSSION

We have presented evidence that the commonly used food antioxidants BHA and BHT are cytotoxic to freshly isolated rat hepatocytes and that this toxicity is not related to the formation of reactive metabolites, but is rather due to the effects of these compounds on lipid membranes, with particular emphasis on the mitochondrial membrane. In isolated mitochondria these compounds were observed to disrupt membrane potential, inhibit respiratory control, cause the release of calcium from mitochondrial stores and elicit mitochondrial swelling. These mitochondrial effects were reflected by a rapid decrease in ATP levels in intact hepatocytes which preceded cell death.

It was somewhat surprising that the membrane effects of these antioxidants were predominant in the cytotoxicity observed in these experiments. Cytotoxic effects due to membrane perturbation by BHA or BHT have previously only been reported in cells that are less metabolically active than liver cells, such as erythrocytes [30] and lipid-containing viruses [11]. In mammalian liver, BHA and BHT are examples of xenobiotic compounds which can exert toxic effects through their cytochrome P-450-mediated metabolism to reactive intermediates. For example, BHT has been shown to cause hepatic necrosis in both rats and mice [9, 31]. These effects are thought to depend on the formation of a reactive quinone methide from BHT which can covalently bind to protein and low molecular weight thiols [19, 32]. BHA is efficiently *O*-demethylated by hepatic microsomes, hepatocytes, and *in vivo* to form a redox active metabolite, *t*-butyl hydroquinone [33, 34]. The hepatic microsomal production of metabolites which covalently bind to protein has been demonstrated for both BHA and BHT [26, 27].

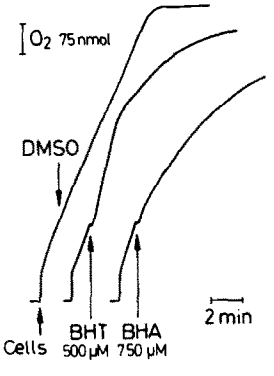

Fig. 7. Effects of BHA or BHT on cell respiration. Hepatocytes (5×10^5 cells/ml) were incubated at 37° in 3 ml of Krebs buffer containing 12.5 mM Hepes and oxygen uptake measured polarographically. Control rate of oxygen consumption was 44.7 nmoles oxygen/10^6 cells/min.

In rats, Nakagawa *et al.* [35] have shown that BHT causes liver necrosis at high single i.p. doses (*ca* 1000 mg/kg). This toxicity *in vivo* is inhibitable by colbaltous chloride suggesting that metabolism of BHT is necessary for the necrosis to occur. Similar experiments in mice, with livers compromised by buthionine sulfoximine [30], have also indicated that cytochrome P-450-dependent metabolism is necessary for the observed effect. At these doses the *in vivo* concentrations of BHT in the livers of these animals would certainly be higher than those used in the present *in vitro* experiments, yet in the *in vivo* experiments no membrane effects were reported. Although it is not known what effect, if any, large doses of BHA and BHT have on liver mitochondria *in vivo*, our results suggest that membrane effects from BHA and BHT should be considered as a possible contribution to toxic effects of antioxidants *in vivo* as well as *in vitro*.

Our results demonstrate specific effects of BHA and BHT on the mitochondrial membrane. In isolated mitochondria BHA and BHT were effective perturbers of several indices of mitochondrial membrane function—membrane potential, calcium release, swelling, and respiratory control. The concentrations at which calcium release, swelling and membrane potential were disturbed by these antioxidants were almost identical and were somewhat lower than those required for inhibition of respiratory control. For example, 100 μM BHT caused the complete release of calcium from the mitochondrial pool, completely dissipated membrane potential and caused swelling while the same concentration inhibited respiratory control by only 50%. Respiratory control is dependent on an intact membrane which can prohibit the influx of protons and thus maintain a chemiosmotic gradient through which ATP is ultimately generated. Compounds which carry protons across the inner mitochondrial membrane dissipate the proton gradient and thereby uncouple oxidation and phosphorylation. BHA and BHT appear to act similar to dinitrophenol (and other uncouplers) in this regard.

The effects of BHT on membranes have recently been studied using mammalian cells as well as model membranes. Using spin labeling techniques in a line

of Chinese hamster fibroblasts, Law *et al.* [14] observed that BHT affects the interior hydrophobic portion of the membrane more than the more polar membrane–water interface. They suggested that this might be because BHT is buried near the center of the membrane, thereby perturbing the tails of the alkyl chains with relatively little effect upon the polar head groups. BHT has also been shown to lower as well as broaden the phase transition of various membrane lipid components as measured in artificial liposomes [36]. The distribution of BHT in membranes has been suggested to be heterogeneous where the regions of highest BHT content may represent points of weakness in the membrane [15]. Cheng *et al.* [36] have also recently shown that BHT facilitates the formation of non bilayer lipid structures in model lipid bilayers (termed hexagonal phase lipids). Additionally, Singer and Wan [13], in experiments with model membranes, suggested that BHT might also affect the membrane–water interface through interactions with the hydroxyl group.

It is not known what ultimately leads to the cytotoxicity in the hepatocytes. Our results clearly show that the effects of BHA and BHT on the mitochondrial membrane result in a drastic reduction in the availability of ATP for the cell. The reduction in ATP levels may cause deleterious effects on a myriad of cellular activities, including the ability of the cell to maintain ionic gradients of calcium through the function of ATP-dependent translocases [37], the polymerization of actin and the maintenance of the cytoskeleton [38, 39], and critically impairing the ability of the cell to respond to a toxic insult through energy-requiring repair processes. These ATP-dependent effects, coupled with the loss of vital membrane functions in mitochondria and perhaps other membranes as well, could initiate the complex chain of events which results in cell death.

Obviously, one must consider possible effects on other biomembranes within the cell as well. Antioxidants have been shown to disrupt lysosomal membranes [3] and may affect the outer cellular membrane and other organelle membranes as well [29]. In separate experiments, we observed that a concentration of 500 μM BHT did not cause an increase in permeability of the outer cellular membrane to ethidium bromide, a small molecule of molecular weight 400. The endoplasmic reticulum is apparently affected little by these antioxidants since they are actively metabolized by microsomal fractions although it was recently shown that BHA and BHT are potent inhibitors of sarcoplasmic reticulum ATPase [18]. The authors suggested membrane effects as a possible mechanism of inhibition. Perhaps the sum total of these various membrane effects leads to cell death or perhaps it is related to the rise in intracellular calcium which has recently been proposed as a common mechanism of cell death [40]. In preliminary experiments we noted a rise in cytosolic calcium from 0.2 μM to over 2 μM in cells within 25 min in hepatocytes exposed to 500 μM BHT which preceded the onset of cytotoxicity in this experiment.

In summary, our results demonstrate that the phenolic antioxidants BHA and BHT are cytotoxic to freshly isolated rat hepatocytes and that their toxicity appears to be related to their effects on the mitochondrial membrane. Furthermore, these results suggest that these membrane effects occur even in cells which actively metabolize these compounds. Perturbation of membrane function may, therefore prove to be important in some of the other biological effects of these compounds.

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