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RELATIONSHIP OF MEMBRANE FLUIDITY, CHEMOPROTECTION, AND THE INTRINSIC TOXICITY OF BUTYLATED HYDROXYTOLUENE

HOWARD G. SHERTZER,*† GERARD L. BANNENBERG,‡ MATS RUNDGREN‡ and PETER MOLDÉUS‡

* Department of Environmental Health, University of Cincinnati Medical Center, Cincinnati, OH 45267-0056, U.S.A.; and ‡ Department of Toxicology, Karolinska Institute, S-104 01, Stockholm, Sweden

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Abstract—In isolated rat hepatocytes, many chemicals elicit toxicity which is inhibitable by antioxidants such as butylated hydroxytoluene (BHT). Although BHT protection is evident at concentrations of less than about 50 nmol/mg protein, higher concentrations exhibit intrinsic concentration-dependent toxicity, which involves mitochondrial dysfunction. We evaluated the possibility that both chemoprotection and intrinsic toxicity could be explained by a common mechanism involving alterations in the physical properties of cellular membranes. In the red blood cell (RBC) osmotic fragility assay, BHT at less than 60 nmol/mg protein protected against osmotic fragility; however, BHT at higher concentrations enhanced osmotic fragility such that total osmolysis occurred at 135 nmol/mg. The BHT-mediated alterations in osmotic fragility correlated with changes in membrane fluidity, determined by fluorescence polarization of the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene. Protection from osmolysis correlated with decreased fluidity, while enhanced RBC fragility correlated with increased fluidity. In rat hepatocyte suspensions, high BHT concentrations also permeabilized the plasma and mitochondrial membranes to enzyme leakage, and these effects were accompanied by enhanced membrane fluidity. Although other mechanisms may be operative, alterations in membrane fluidity appear to be, in part, responsible for the observed chemoprotective effects at low concentrations, and intrinsic toxicity at higher concentrations of BHT.

In processed foods, cosmetics and petroleum products, lipophilic chain-breaking antioxidants are commonly used as preservatives. Among these, butylated hydroxytoluene (BHT§) is a phenolic free radical scavenger that has undergone extensive evaluation for its abilities to influence the toxicity, mutagenicity and carcinogenicity of other chemicals [1–4], and for its own intrinsic toxicity [5–8].

In rats, high BHT dosages produce an inhibition of hepatic prothrombin synthesis and hemorrhagic death [9]. Furthermore, BHT enhances freeze–thaw damage in mammalian cells [10], and pulmonary toxicity in mice resulting from damage to the type I pneumocyte [11]. Such toxicity appears to be due, in part, to the quinone methide metabolite of BHT, which is extremely reactive toward thiol groups. However, BHT is known also to interact directly with biological membranes to produce alterations in fluidity and phase transitions [12, 13]. Such effects have important toxicological implications which have not been carefully characterized. This paper reports on the influence of BHT on membrane fluidity and the relationship between fluidity alterations and the known chemopreventive and toxicological properties of this multifaceted compound.

† Corresponding author.

§ Abbreviations: BHT, butylated hydroxytoluene (2,6-di-*tert*-butyl-4-methylphenol); DPH, 1,6-diphenyl-1, 3, 5-hexatriene; DMSO, dimethyl sulfoxide; GDH, glutamate dehydrogenase; LDH, lactate dehydrogenase; MMS, methylmethanesulfonate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

METHODS

Materials. BHT, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), neutral red, *p*-nitrophenylphosphate disodium, Triton X-100, digitonin, and crude egg and crude soybean phospholipids were obtained from the Sigma Chemical Co., St Louis, MO. Methylmethane sulfonate (MMS) was from the Eastman Kodak Co., Rochester, NY, while paracetamol (acetaminophen) and CCl_4 were from the Aldrich Chemical Co., Milwaukee, WI. DPH was obtained from Molecular Probes, Inc., Eugene, OR. All other chemicals were of the highest quality available. All solvents were of reagent grade quality or better.

Hepatocytes. Male Wistar rats (*ca.* 225 g) were allowed food and water *ad lib*. For the experiment shown in Fig. 1, hepatocytes were prepared by collagenase (Worthington Biochemicals, Freehold, NJ) treatment and suspended in a modified Krebs buffer as previously described [14]. Cells were incubated in a rotary shaker at 80 cycles/min at 37° under a constant flow of humidified carbogen (95% oxygen, 5% carbon dioxide). For the other experiments using hepatocytes, cells were prepared following collagenase (grade II, Boehringer Mannheim, F.R.G.) digestion as described [15]. Cells were suspended in Krebs buffer containing

0.3% (w/v) HEPES and 1% (w/v) bovine serum albumin (pH 7.4), and incubated in rotating round-bottom flasks at 37° under a carbogen atmosphere. The differences between the two hepatocyte preparations are considered to be minor for the purposes of the experiments conducted. BHT was added as a solution in dimethyl sulfoxide (DMSO) with the concentration of DMSO never exceeding 0.5%. Cell viability was assessed by 0.2% trypan blue exclusion. The initial viability of hepatocytes prepared by either method was approximately 90%.

Red blood cell (RBC) osmotic fragility assay. The assay has been described previously [16]. Briefly, blood samples were removed from anesthetized rats into a heparinized syringe. Blood was diluted 20-fold with 140 mM NaCl, 10 mM sodium citrate and 5 mM glucose, pH 7.4, and kept on ice no longer than 2 hr. To 0.75-mL aliquots of diluted blood were added 10 μL DMSO or various concentrations of a test compound in DMSO. After 1 min and gentle swirling, 0.75 mL of distilled water was added forcefully in order to achieve rapid and complete mixing. Absorbance at 656 nm was used to monitor turbidity which is inversely proportional to the degree of osmolysis. The initial osmolysis in the absence of test compound was normally 65–70%. The absorbance value for 100% lysis was obtained after diluted RBCs were sonicated for 5 sec at 50% power, using a Kontes ultrasonic cell disrupter.

Membrane preparations. Mitochondria were prepared essentially as described [17], except that 3 mM HEPES buffer was used and the mitochondria were pelleted at 8000 *g* for 10 min. Lysosomes were prepared by the same method, with the post-mitochondria supernatant fraction pelleted at 25,000 *g* for 10 min. Incubations of mitochondria and lysosomes were done at 37° in a buffer containing 210 mM mannitol, 70 mM sucrose and 3 mM HEPES-KOH, pH 7.1.

Crude egg and soybean phospholipids were purified by dissolving 1 g in 0.5 mL $CHCl_3$. Acetone (20 vol.) was added to precipitate the phospholipids, while neutral lipids, flavonoids and tocopherols remained in solution. The process was repeated twice and the precipitate was washed with acetone. The phospholipids were dissolved in $CHCl_3$ and stored at −20°. Vesicles were prepared by sonicating 1 mg phospholipid (dried under a stream of nitrogen) in 1 mL of 0.9% NaCl, 10 mM HEPES-KOH (pH 7.4) until the suspension became translucent.

Membrane fluidity. The fluorescent hydrocarbon 1,6-diphenyl-1,3,5-hexatriene (DPH) was used as a probe for monitoring fluidity in the various membrane preparations used in this study. Membranes were labeled by adding 1 μL of 1 mM DPH in tetrahydrofuran per mL of membrane suspension. The membrane suspensions contained 1 mg protein/mL in the case of erythrocytes, hepatocytes or subcellular membranes, or 1 mg phospholipid/mL in the case of purified phospholipid vesicles. Suspensions of unlabeled membranes containing tetrahydrofuran without DPH were used as reference samples. Fluorescence was determined using a Shimadzu RF510LC equipped with polarizing filters on the excitation and emission sides. Determinations were made with the filters in parallel and perpendicular orientations, with excitation = 357 nm and emission = 428 nm. When using blood, excitation = 370 nm and emission = 460 nm, due to interference by hemoglobin at the standard wavelengths. Fluorescence intensity measurements were performed at 25°, 5–10 sec after exposing the samples to the excitation light. This prevented reversible bleaching of DPH that may result from photoisomerization that occurs after prolonged exposure to light [18]. Background fluorescence values without DPH, but with other components such as BHT, were subtracted in each plane of polarization. The perpendicular component of fluorescence intensity was corrected for the intrinsic light polarization of the fluorometer [19], and the fluorescence anisotropy (*r* value) was calculated from the equation: $r = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$ [20], where $I_\parallel$ and $I_\perp$ are the fluorescence intensities in the parallel and perpendicular orientations of the polarizing filters, respectively.

Assays. Hepatocyte viability was determined by measuring lactate dehydrogenase (LDH) activity associated with the hepatocytes and the suspending medium [21], and calculating the percentage of total LDH activity that had leaked from the hepatocytes into the suspending medium. This provides an estimate of the integrity of the hepatocellular membrane.

The reduction of MTT to a purple-colored formazan product, as described in Ref. 22, was used to measure mitochondrial electron transport. Samples of 200 μL hepatocyte suspension were incubated for 10 min at 37° in the presence of 0.57 mM MTT. The reaction was stopped by adding 1.0 mL of DMSO, and after adding 50 μL of 50 mM glycine buffer, pH 10.5, the absorbance of the formazan was determined at 560 nm.

The uptake of neutral red by hepatocytes was used to measure lysosomal integrity. To samples of 200 μL hepatocyte suspension, 50 μL of 0.036% (w/v) neutral red dissolved in saline containing 16 mM HCl was added and incubated for 20 min at 37°. The incubation was stopped by adding 1 mL of ice-cold Krebs buffer, pH 7.4. The samples were centrifuged and the pellets dissolved in 1 mL of a 1:1 mixture of ethanol and 0.1 M NaH_2PO_4, pH 4.5. After addition of 50 μL of 10% trichloroacetic acid (TCA), mixing and centrifugation, the color in the supernatant solution was determined at 542 nm [23].

Lysosomal integrity was measured by leakage of *p*-nitrophenylphosphate acid phosphatase (PNPP) as described [22]. The integrity of the mitochondrial inner membrane was assessed by the latency of the mitochondrial matrix enzyme glutamate dehydrogenase (GDH). GDH activities were measured by enzymatic analysis, utilizing 2-oxoglutarate as substrate and determining the rate of NADH oxidation at 340 nm [24]. The leakage of GDH from mitochondria in isolated hepatocytes was calculated from the activity of GDH in the mitochondrial, cytosolic and extracellular compartments, which were separated by digitonin fractionation and centrifugation, as described [25]. Samples of 1.0 mL hepatocyte suspensions were incubated for 3 min in the presence of either 0.2 mg/mL digitonin, 0.1% Triton X-100 or control addition of Krebs buffer,

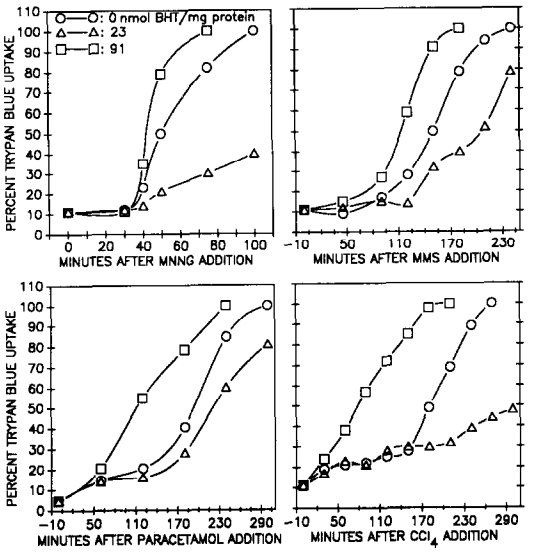

Fig. 1. Effect of BHT on hepatocyte killing by various toxicants. The panels reveal the effects of 23 nmol BHT/mg protein (Δ) and 91 nmol BHT/mg protein (□) on the rate of toxicant-mediated cell killing (○). The four toxicants utilized were 0.5 mM MNNG (top left panel), 0.75 mM MMS (top right panel), 5 mM paracetamol (bottom left panel), and 8 mM CCl_4 (bottom right panel). Incubations were performed at 2×10^6 cells/mL. The medium with paracetamol also contained 100 mM acetone. The results are representative of three separate experiments with each toxicant.

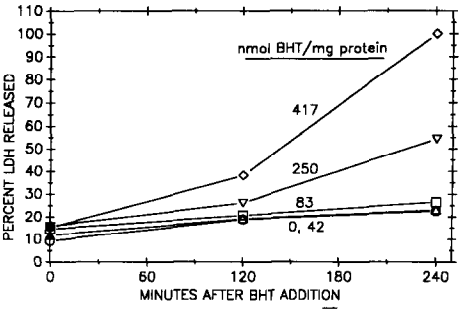

Fig. 2. Effect of BHT on hepatocyte viability. Hepatocytes were incubated at a density of 10^6 cells/mL. The results are representative of three separate experiments, and are expressed as the percentage of total LDH that had leaked from the hepatocytes to the incubation medium.

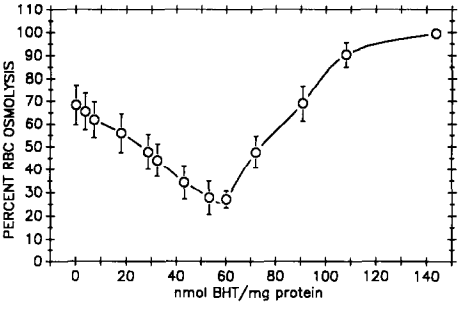

Fig. 3. Effect of BHT on osmotic fragility of rat erythrocytes. The percent osmolysis after the addition of water to a suspension of RBCs was used as an index of osmotic fragility. The results shown are the average of five independent determinations on five different blood samples ± SD.

and viable cells and whole organelles were subsequently centrifuged (2 min, 12,000 *g*, Ole Dich microcentrifuge 154) through a layer of an oil-mixture (silicon oil, density 1.05 g/L and white light paraffin oil, viscosity 125/135, 6:1 ratio, final density 1.04 g/mL) into glycerol (density 1.10 g/mL). The difference of GDH activity in the supernatant of Triton X-100-treated and digitonin-treated hepatocytes reflects the activity of GDH in the cytosol due to rupture of the mitochondrial membrane. Digitonin completely lysed the cell membrane, since 100% of intracellular LDH was found in the supernatant, as was the case with Triton X-100-treated hepatocytes. Digitonin treatment resulted in approximately 20% lysis of mitochondria, as measured by GDH activity in the supernatant, compared to complete lysis of mitochondria in hepatocytes treated with Triton X-100. In contrast to the mitochondrial membrane, digitonin treatment did not selectively permeabilize the cell membrane versus the lysosomal membrane, as lysosomal PNPP leakage occurred parallel to LDH leakage following digitonin treatment.

Statistics. Data were analyzed by one-way analysis of variance. When indicated by ANOVA, differences between group means were evaluated by Student's *t*-test (one-sided) based on the mean square residual from the analysis of variance in question. Statistics were evaluated using the Number Cruncher Statistical System (NCSS, Kaysville, UT).

RESULTS

Addition of BHT to rat hepatocytes (10^6 cells/mL ≈ 1.2 mg protein/mL) has been shown previously to be cytotoxic in excess of 100 μM [5], as well as protective against the cytotoxicity of MNNG and MMS at less than 50 μM BHT [16]. Figure 1 shows that while 23 nmol BHT/mg protein protected hepatocytes from MNNG, MMS and paracetamol toxicity, 91 nmol/mg exacerbated toxicity. BHT alone was marginally cytotoxic at 83 nmol/mg protein, but exhibited pronounced toxicity at higher concentrations (Fig. 2). We have demonstrated previously that for many compounds, including BHT, protection against MNNG or MMS toxicity in hepatocytes correlates with their abilities to stabilize the RBC membrane against osmotic lysis [16]. Therefore, we utilized this RBC osmotic fragility assay to determine the effects of BHT at concentrations which produced toxicity in hepatocytes. The results in Fig. 3 indicate a concentration-dependent protection from osmolysis at low BHT concentration, with an optimum at about 60 nmol BHT/mg protein, followed by a sharp increase in osmotic fragility, such that total lysis

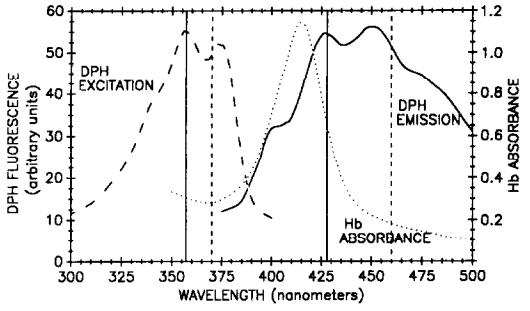

Fig. 4. Excitation and emission fluorescence spectra of DPH. The excitation spectrum (emission at 428 nm) and emission spectrum (excitation at 357 nm) for DPH in hexane are indicated as dashed and solid lines, respectively. The absorbance curve for rat RBCs (lysed by sonication) is depicted by the dotted line. The standard values for determining DPH fluorescence (excitation = 357 nm; emission = 428 nm) are shown by solid vertical lines. The values used in the present study (excitation = 370 nm; emission = 460 nm) are indicated by dashed vertical lines.

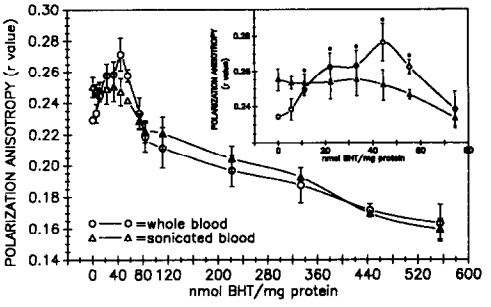

Fig. 5. Influence of BHT on erythrocyte membrane fluidity. The polarization anisotropy value for DPH in a red blood cell suspension is shown at various BHT concentrations. Sonication (where indicated) was performed prior to the addition of DPH, and samples were incubated for 30 min at room temperature prior to assay. The inset shows an expanded portion of the graph at 0–80 nmol BHT/mg protein. Values shown are means of three independent determinations ± SD. The asterisks in the inset indicate that, for whole blood, the mean values are significantly different from the mean value obtained in the absence of BHT, at $P < 0.05$.

occurred at about 135 nmol BHT/mg protein. BHT (up to 367 nmol/mg protein) did not produce lysis until the addition of water, indicating that the effect was due to osmotic fragility, rather than direct disruption of the membrane.

To assess whether the effect of BHT on RBC osmotic fragility was mediated by alterations in membrane fluidity, we utilized the hydrophobic fluorescent probe DPH to determine polarization anisotropy. Normally, DPH is monitored at excitation and emission wavelengths of 357 and 428 nm, respectively. However, hemoglobin interfered with the assay at these wavelengths (Fig. 4). The excitation and emission spectra of DPH (Fig. 4) revealed broad bands; therefore, wavelengths of 370 nm excitation (an absorbance minimum for hemoglobin) and 460 nm emission (low hemoglobin absorbance and high DPH fluorescence intensity) were chosen as those exhibiting minimal interference in the assay. As shown in Fig. 5, BHT elicited a decrease in intact RBC membrane fluidity (increase in r value) in the range of 40 nmol/mg protein, followed by a return to control fluidity levels at 65 nmol/mg. At higher BHT concentrations, a continuous increase in fluidity was observed. It is noteworthy that sonication abolished the fluidity changes observed with low BHT concentrations, but had no effect on those effects produced by BHT exceeding 65 nmol/mg protein. When crude egg phospholipid or soybean phospholipid vesicles, or rat hepatic microsomes were used in place of RBCs, no fluidity decreases were observed at low BHT concentrations (data not shown); however, fluidity enhancement at higher BHT concentrations was similar to the curve for sonicated RBCs.

To establish the relevance of membrane fluidity alterations in RBCs to chemoprotective and toxic effects of BHT in hepatocytes, we followed changes in polarization anisotropy over time in hepatocytes in the presence of various BHT concentrations (Fig. 6). In the absence of BHT, membrane fluidity

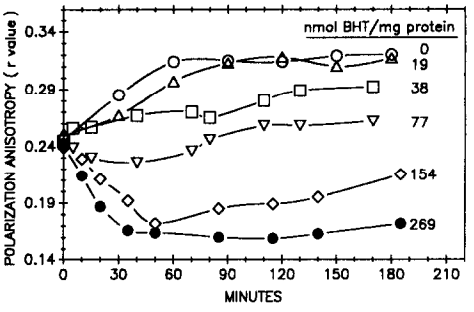

Fig. 6. Effect of BHT on membrane fluidity in hepatocytes. DPH was added to hepatocyte suspensions (10^6 cells/mL) in the presence of BHT at the indicated concentrations. The hepatocytes continued to be incubated at 37° under a carbogen atmosphere as described in Methods. At various times, the cells were sampled and fluorescence polarization was determined. Background fluorescence of cells without DPH was subtracted in each plane of polarization and r values were calculated. Each measurement was taken in duplicate. The values shown are representative from two independent experiments.

decreased with time to a minimum at 60 min. Increasing BHT concentrations up to 77 nmol/mg protein diminished this effect, while higher cytotoxic concentrations produced enhanced fluidity. It should be noted that the increase in fluidity at high BHT concentrations was time dependent requiring about 50 min to reach a maximum. Since it has been demonstrated [5] that BHT acts as a mitochondrial uncoupler at high concentrations, we examined the effect of BHT on mitochondrial membrane stability in hepatocytes. Figure 7 shows that >77 nmol BHT/mg protein produced an increase in the cytosolic activity of the mitochondrial matrix enzyme GDH indicative of inner membrane leakiness. The

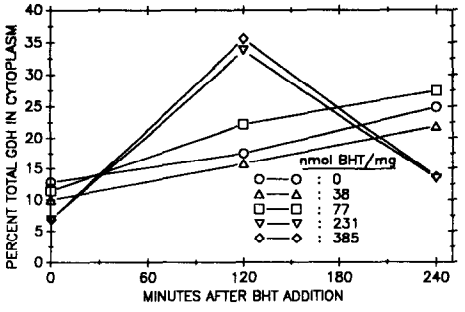

Fig. 7. Release of mitochondrial glutamate dehydrogenase into hepatocyte cytosol. Hepatocytes (10^6 cells/mL) were incubated in the presence of various BHT concentrations as shown. At 0, 120 and 240 min, cells were assayed for GDH activity as described in Methods. The percentage of total cellular GDH in the cytoplasm represents that fraction of GDH released by digitonin treatment. Each assay was performed in duplicate. Representative results from two independent experiments are shown.

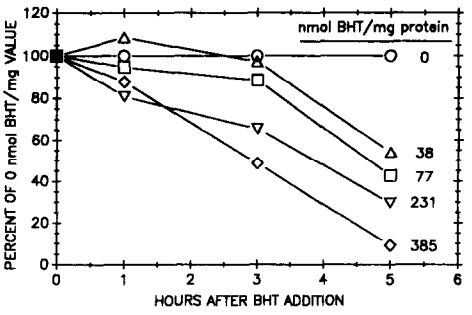

Fig. 9. Inhibition of MTT reduction by BHT in hepatocytes. Hepatocytes (10^6 cells/mL) were incubated with various concentrations of BHT as indicated. At 0, 1, 3 or 5 hr, cells were incubated with MTT at 37° as described in Methods. The rate of reduction of MTT at each time point is shown as a percent of the value obtained at that time in the absence of BHT. Each assay was performed in duplicate. Representative results from two independent experiments are shown.

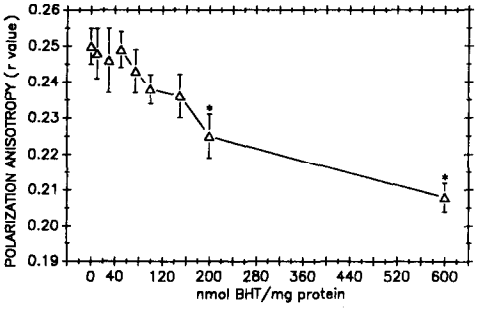

Fig. 8. Enhancement by BHT of membrane fluidity in isolated mitochondria. DPH was added to mitochondria in the presence of the indicated concentrations of BHT. After 30 min of incubation at room temperature, suspensions were assayed for fluorescence polarization. Fluorescence obtained in each plane of polarized light, in the absence of DPH, was subtracted from values obtained in the presence of DPH, prior to calculating the r value. Values shown are the means of four independent determinations ± SD. The asterisks indicate that the mean values are significantly different from the mean value obtained in the absence of BHT, at $P < 0.05$.

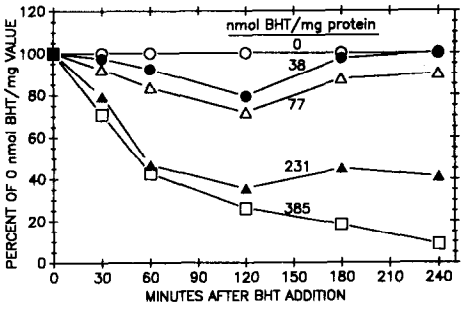

Fig. 10. Effect of BHT on neutral red uptake by hepatocytes. Hepatocytes (10^6 cells/mL) were incubated with various concentrations of BHT as shown. At the indicated times, cells were incubated with neutral red at 37° as described in Methods. The rate of uptake of neutral red by hepatocytes is shown as a percent of the values obtained at that time in the absence of BHT. Each assay was performed in duplicate. Representative results from two independent experiments are shown.

subsequent decrease in cytosolic GDH at 240 min was due to cell death and loss of plasma membrane integrity (Fig. 2). Enhanced membrane fluidity produced by BHT in isolated mitochondria (Fig. 8) correlated with the observed loss of structural integrity and GDH leakage.

Although membrane fluidity changes appear to be responsible in part for BHT-mediated hepatotoxicity, other mechanisms may be operative. Such would seem to be the case for the inhibition of reduction of the tetrazolium salt MTT [22] by the mitochondrial electron transport chain (Fig. 9). This effect was manifest at low nontoxic concentrations of BHT. Other organelles may also be involved in the mechanism of BHT toxicity. The weakly basic dye, neutral red is taken up by lysosomes of intact cells, with uptake driven by the pH gradient across the lysosomal membrane [26]. Such uptake in hepatocytes was inhibited slightly by BHT at subtoxic concentrations, and this effect was reversible (Fig. 10). In contrast, cytotoxic concentrations of BHT produced large, irreversible inhibition in the uptake of neutral red by hepatocytes. The inhibition of neutral red uptake was not accompanied by the release to the cytoplasm of the intralysosomal enzyme p-nitrophenylphosphate acid phosphatase (data not shown).

DISCUSSION

The impact of BHT on human health is of fundamental concern because of its widespread use as a preservation in the food and cosmetic industries. BHT is a well-characterized phenolic chain-breaking

antioxidant [27] which has been shown to induce enzymes associated with glutathione metabolism as well as NAD(P)H: quinone oxidoreductase [28–30]. Such antioxidant and enzyme inducing properties have been related to chemoprotection afforded by BHT against chemical toxicity, mutagenesis and carcinogenesis [3, 4, 16, 31, 32]. However, BHT may also promote biological damage, including pulmonary toxicity and tumor formation [2, 33, 34] and hepatoxicity [5, 6]. These adverse responses to BHT have been attributed in part to the metabolism of BHT to a sulfhydryl reacting quinone methide [11, 35–37], which is capable of reacting with critical cellular thiol groups.

Another phenomenon that appears to be associated with BHT toxicity is the enhancement of fluidity in the hydrocarbon but not the polar regions of biological membranes [12]. By this mechanism, BHT was shown to decrease freeze–thaw survival as well as enhance cellular centrifugal damage in fibroblasts. These results suggest that BHT weakens the cell membrane to physical stress and would explain the increase in osmotic fragility of erythrocytes produced by BHT in the present study. The sensitization of RBC to osmotic lysis could also help to explain the hemorrhagic death produced by BHT in rats [9], although this effect appears to be partly mediated by an inhibition of blood clotting through the BHT quinone methide inhibition of phylloquinone epoxide reductase [38]. Although in the present study BHT did not produce the spontaneous lysis of erythrocytes, increased susceptibility to physical stress would produce rupture during RBC deformation such as occurs during passage through capillaries. Unfortunately, Takahashi and Hiraga [9] did not monitor hematocrit in the course of hemorrhagic damage produced by BHT.

Furthermore, in the course of producing the membrane structural alteration associated with an increase in membrane fluidity, BHT also produced substantial leakiness in the plasma and especially the mitochondrial membranes of hepatocytes. Such enzyme sized pores, whether of a transient or fixed nature, would certainly account for the uncoupling of oxidative phosphorylation and the dissipation of mitochondrial membane potential observed previously [5]. In that study, BHT toxicity did not appear to require cytochrome P450 metabolism, an observation that would support a physical mechanism of toxicity for BHT such as enhancement of membrane fluidity.

The present study also supports the previous suggestion [5] that mitochondria represent a major organellar target for BHT toxicity. This implication is based on the BHT concentration-dependent release of GDH to the cytoplasm and the inhibition of MTT reduction in isolated hepatocytes. MTT reduction is 90–95% mitochondrial specific, based on the low rates of MTT reduction in post-mitochondrial supernatant and the rapid inhibition of reduction in isolated hepatocytes by cyanide or a change to an anerobic atmosphere (data not shown). It would not be expected that alterations in membrane fluidity are related to the inhibition of mitochondrial respiration by BHT. Rather, this effect is possibly mediated by BHT quinone methide binding to critical respiratory chain thiols.

Although some of the protection afforded by BHT in various biological systems may be attributable to free radical scavenging and enzyme induction, this is not the complete explanation. For example, protection by various antioxidants, including BHT, against MNNG and MMS cytotoxicity in rat hepatocytes was better correlated with their abilities to protect against osmotic fragility than with their effectiveness as antioxidants [16]. Although in erythrocytes a correlation was observed between protection against osmotic fragility and decreased membrane fluidity, this was not observed in any of the hepatocyte membranes. The reason may be related to the abolishment of BHT-mediated decreased membrane fluidity in erythrocytes by sonication. Thus, membrane curvature or surface properties may be important in the mechanism of BHT cytoprotection, and these properties might be altered during mitochondrial preparation. In isolated hepatocytes, endogenous phospholipases and proteases are quite active, even in the absence of cytotoxicants [39]. We speculate that autolysis could alter the chemical composition of cellular membranes, resulting in a decrease in membrane fluidity in control cells. If BHT at low concentrations were to decrease membrane fluidity, such changes could be masked by the spontaneous decrease observed. Thus, direct evidence for decreased membrane fluidity being associated with chemoprotection in hepatocytes has yet to be obtained.

It is difficult to assess lysosomal involvement in the mechanism of cytotoxicity by BHT. Although the data suggest that the intralysosomal pH is increased by BHT, the data do not indicate that intralysosomal acid hydrolases are released into the hepatocyte cytosol. The toxicological relevance of these data is unclear.

In conclusion, the protection and toxicity that result from exposure to BHT has a complex etiology that results from its action as an antioxidant, ability to induce enzymes, production of reactive metabolites and alterations in membrane fluidity. The concentration–response effect of BHT on osmotic fragility and membrane fluidity in the erythrocyte, and some of these data in the hepatocyte, indicate that membrane fluidity changes are involved in cytotoxicity and possibly also in chemoprotection by BHT.

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