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ON THE MECHANISM OF BUTYLATED HYDROXYTOLUENE-INDUCED HEPATIC TOXICITY IN RATS

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Abstract—The relations between serum transaminase activity and the hepatic contents of glutathione and lipid peroxide were examined following oral administration to rats of butylated hydroxytoluene (BHT; 500 or 1000 mg/kg). The glutathione level rapidly diminished and reached a minimum at 6 hr after BHT administration. The period of depletion was dependent on dose: restoration of the glutathione level took longer in high-dose rats than in low-dose rats. The content of hepatic lipid peroxide was not markedly changed by BHT throughout the experimental period. The activity of glutathione *S*-transferase was not affected until 12 hr after BHT administration but, thereafter, it increased with time and was accompanied by elevation of the glutathione level. Though the activities of serum glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase were not affected by low-dose BHT, they increased rapidly in the the high-dose rates after a lag period of about 6 hr and reached a maximum at 24 hr after administration; at that time, the livers of the high-dose rats showed centrilobular necrosis. The results indicate that acute hepatic injury was induced by the high-dose BHT. Pretreatment with cobaltous chloride inhibited the increase in the activities of the serum transaminases produced by the high-dose of BHT accompanying the depletion of microsomal cytochrome P-450 content and the induction of glutathione content. These observations suggest that hepatic damage was associated with prolonged depletion of glutathione rather than with lipid peroxidation in the liver, and that the activated metabolites of BHT rather than the parent compound induced the tissue damage.

Since butylated hydroxytoluene (3,5-di-*tert*-butyl-4-hydroxytoluene; BHT) is widely used as an antioxidant in processed foods, cosmetics and petroleum products, this compound has been the subject of extensive toxicological investigation in various animals. The acute toxicity of BHT is low, the LD_{50} in the rat being greater than 2 g/kg body weight [1]. Though generally considered to be safe at the concentrations present in foods (the acceptable daily intake of BHT for man is 0.5 mg/kg [2]), high doses of BHT cause hemorrhagic death accompanied by inhibition of hepatic prothrombin synthesis in rats [3] and pulmonary injury in mice [4, 5]. On the other hand, BHT is considered to be non-hepatotoxic because the hepatic hypertrophy, which is not accompanied by necrosis, is reversible when BHT is removed [6, 7]. However, it seems that BHT can induce some hepatic damage, since the activities of serum marker enzymes indicating hepatic injury are often elevated after the administration of BHT to rats [8, 9].

Our previous studies [10–14] have demonstrated that (a) BHT is converted to highly reactive intermediates by a cytochrome P-450-linked monooxygenase system in hepatic microsomes, and (b) some of the active intermediates specifically bind to the sulfhydryl group of protein, but not to nucleic acids. The results suggest that the irreversible binding of the active intermediates to cellular protein affects the physiological function of hepatic cells.

On the basis of these results and findings, therefore, we investigated whether BHT induces hepatic injury. That is, the objective of this study was to examine the relationship between the activity of serum transaminase and the contents of hepatic glutathione and lipid peroxide after the administration of BHT.

MATERIALS AND METHODS

Materials. The chemical compounds used were obtained from the following companies: BHT and cobaltous chloride ($CoCl_2 \cdot 6H_2O$) from the Wako Chemical Co. (Osaka, Japan), and glutathione (GSH) and biochemical materials from the Sigma Chemical Co. (St. Louis, MO); all other chemicals were of the highest purity available.

Animals and treatment. Male Wistar rats (SPF), weighing 200–220 g, were divided into groups of four rats for each experiment. The animals were starved overnight before use, and all doses were given at the same time of day. A solution of BHT (500 or 1000 mg/kg) dissolved in olive oil was given to each rat by stomach tube; the corresponding control animals received an equivalent volume of olive oil. At definite periods after BHT administration, the rats were decapitated. One group of animals also received 60 mg/kg cobaltous chloride in distilled water by subcutaneous injection each day for 2 days. Twelve hours after the last injection, animals received orally 1000 mg/kg of BHT and were killed 18 hr later; the corresponding control animals received physiological saline and olive oil.

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Preparation of homogenate, microsomes and cytosol. The liver was removed, weighed and perfused with 1.15% KCl containing 10 mM phosphate buffer (pH 7.4). All subsequent manipulations were performed at temperatures near 0°. The liver was minced with scissors and homogenized with KCl-phosphate buffer in a Potter–Elvehjem homogenizer. The homogenate was directly used for the determination of lipid peroxide, GSH and protein. Further, the homogenate was centrifuged at 10,000 *g* for 15 min, and the resulting supernatant fraction was then centrifuged at 105,000 *g* for 60 min. The 105,000 *g* supernatant (cytosol) fraction was employed in the assay of GSH *S*-transferase activity while the microsomal pellet was suspended in 0.1 M phosphate buffer (pH 7.4) for the determination of microsomal cytochrome P-450 content.

Analytical methods. To determine GSH, 10% trichloroacetic acid was added to the homogenate to give a final concentration of 7.5%, and then the supernatant fraction obtained by centrifugation was washed with cold ether. Total GSH was assayed with Ellman's reagent, according to the procedure of Taniguchi *et al.* [15]. GSH *S*-transferase activity was assayed spectrophotometrically with 3,4-dichloro-1-nitrobenzene (DCNB) and 1-chloro-2,4-dinitrobenzene (CDNB) [16]. The content of cytochrome P-450 was measured as described by Omura and Sato [17]. Lipid peroxide was measured as the amount of accumulation of malondialdehyde (MDA) in total incubate with the thiobarbituric reaction; the level of lipid peroxide is expressed in terms of nmoles MDA/g wet weight, which was calculated from the absorbance at 532 nm by using 1,1,3,3-tetramethoxypropane (TMP) as a standard [18]. Protein was determined by the method of Lowry *et al.* [19] with bovine serum albumin as a standard. The activities of serum glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) were determined by u.v. assay (using a Hitachi 705 automatic analyzer) according to the method of Karmen [20].

RESULTS

Figure 1 shows the time-course of changes in the level of hepatic GSH after oral administration of BHT to rats. The level diminished immediately and reached a minimum (low-dose BHT, 9.4% of control; high-dose BHT, 8.7% of control) at 6 hr after BHT administration. Thereafter, the GSH level in the low-dose rats was rapidly restored to the level in control rats. The restoration of GSH level was slower in the high-dose rats than in the low-dose rats, and the level at 12 or 24 hr after administration was 19.1 or 67.9% of the control level respectively. However, the GSH level increased markedly with time and reached about 2-fold of the control level after 48 hr. This tendency was also observed in the low-dose rats. In control animals, the GSH level increased gradually during the early hours (0–12 hr) of the experimental period. This was thought to be a recovery of a GSH level depleted by starvation, rather than a circadian change in hepatic GSH [21]. The results in Fig. 1 indicate that BHT produced remarkable depletion or induction of hepatic GSH with time and that the period of depletion was dependent on the dose.

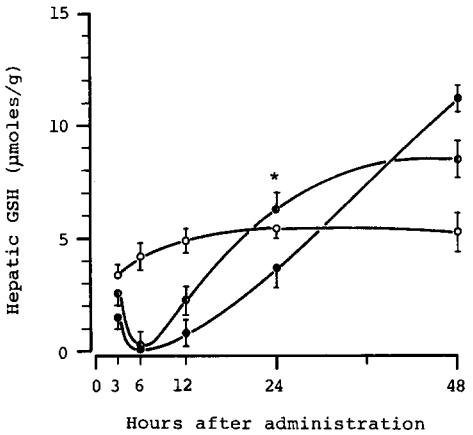

Fig. 1. Time-course of changes in the hepatic GSH level after oral administration of BHT to rats. GSH content was determined at various intervals after BHT (dose: 0 [○], 500 [◐] and 1000 mg/kg [●]), as described in Materials and Methods. Points represent mean ± S.D. of four rats. Key: (*) not significantly different from control group. All other values are significantly different ($P < 0.01$, Student's *t*-test) from the corresponding control values.

GSH *S*-transferase activity was not affected until 24 hr after BHT treatment, reaching about 2.3-fold of the control level at 48 hr (Table 1). Since this response was accompanied by an increase in GSH level, the results in Fig. 1 and Table 1 indicate that the mercapturic acid conjugation system was induced by BHT. BHT is converted to activated intermediates by the microsomal monooxygenase [10], and some of the activated intermediates are conjugated to water-soluble products with GSH [12, 14].

No significant change occurred, after BHT administration, in the content of hepatic lipid peroxide throughout the experimental period (Table 2). In addition, the content of lipid peroxide per mg hepatic protein in BHT-treated rats was similar to that in control rats throughout the experimental period (data are not shown).

Figure 2 shows the time-course of changes in the activities of serum GOT and GPT after the oral administration of BHT. The determination of activities of both enzymes is useful for following the progress of hepatic injury [22]. Though the activities of GOT and GPT were not affected by the low-dose BHT throughout the experimental period, both activities in the high-dose rats increased remarkably after a lag period of about 6 hr following BHT treatment. After 24 hr the activity of GOT or GPT reached a maximum and was about 38- or 65-fold that in control rats respectively. At that time, although the livers of the low-dose rats were histologically normal, the livers of the high-dose rats exhibited centrilobular necrosis and hemorrhage (Fig. 3). Thereafter, the activities of both enzymes decreased quickly toward control level. The results show that hepatic damage was induced by high-dose BHT and that the damage was rapidly reversible, occurring in a short time.

Table 1. Effect of BHT on the activity of GSH *S*-transferase in rat liver*

BHT dose (mg/kg)	GSH *S*-transferase activity				
	Time after administration (hr)				
	3	6	12	24	48
Substrate: CDNB					
0	1.73 ± 0.12 (100)	1.23 ± 0.06 (100)	1.32 ± 0.04 (100)	1.68 ± 0.14 (100)	1.34 ± 0.08 (100)
500	1.74 ± 0.30 (101)	1.24 ± 0.10 (101)	1.48 ± 0.23 (112)	2.39 ± 0.18† (142)	2.88 ± 0.15† (215)
1000	1.59 ± 0.04 (92)	1.27 ± 0.05 (103)	1.44 ± 0.05 (109)	1.96 ± 0.14† (117)	3.04 ± 0.17† (227)
Substrate: DCNB					
0	59.6 ± 6.5 (100)	47.2 ± 2.5 (100)	52.2 ± 2.9 (100)	56.4 ± 3.2 (100)	52.6 ± 2.0 (100)
500	62.9 ± 6.9 (106)	43.7 ± 3.6 (93)	53.9 ± 8.1 (103)	74.5 ± 7.6† (132)	123.0 ± 20.0† (234)
1000	59.5 ± 3.4 (100)	48.3 ± 2.8 (102)	52.2 ± 9.1 (100)	66.5 ± 3.3† (118)	121.3 ± 14.0† (231)

* GSH *S*-transferase activity (CDNB: μmoles conjugate formed/mg protein/min, DCNB: nmoles conjugate formed/mg protein/min) was determined at various intervals after oral administration of BHT (0, 500 and 1000 mg/kg), as described in Materials and Methods. Each value represents the mean ± S.D. (N = 4). Values in parentheses represent percentage of control.

† Significant difference from control group ($P < 0.01$, Student's *t*-test).

To study whether or not BHT-induced hepatic damage is due to the activated metabolites, the relation between the contents of cytochrome P-450 and GSH in the liver and the transaminase activity in the serum was investigated in cobaltous chloride pretreated or untreated rats 18 hr after BHT (1000 mg/kg). The results are shown in Table 3. The body and liver weights, and contents of lipid peroxide and protein, were similar in all animals. However, the content of cytochrome P-450 decreased to about 65% of control rats after pretreatment with cobaltous chloride alone. Although the GSH level was decreased significantly by BHT, the level in cobaltous-chloride-pretreated rats increased about 1.5-fold compared to untreated rats. Further, in cobaltous chloride and BHT-cotreated rats, the depression of GSH level was less than that in the BHT-treated rats. On the other hand, high activities of GOT and GPT were diminished nearly to control level by the pretreatment of rats with cobaltous chloride. Slight elevations of the activities of the transaminases were found in rats treated with cobaltous chloride, but the mechanism is unclear. The results in Table 3 show that pretreatment with cobaltous chloride, an inhibitor of cytochrome P-450 synthesis [23], prevented the BHT-induced release of the transaminases from the liver cells to the blood stream. Further, since a partial hepatic congestion existed in BHT-treated rats but was not found in rats pretreated with cobaltous chloride (data are not shown), it may be concluded that the hepatic damage produced by BHT was prevented by cobaltous chloride.

DISCUSSION

Although typical hepatic damage, except for tissue hypertropy, induced by BHT has not been reported previously in the rat [2, 6, 7], it is apparent that a marked elevation of serum transaminase activity by

Table 2. Effect of BHT on the level of lipid peroxide in rat liver*

BHT dose (mg/kg)	Lipid peroxide content (nmoles MDA/g liver)				
	Time after administration (hr)				
	3	6	12	24	48
0	351 ± 48 (100)	340 ± 38 (100)	356 ± 34 (100)	303 ± 37 (100)	327 ± 37 (100)
500	365 ± 26 (104)	351 ± 32 (103)	337 ± 52 (95)	317 ± 93 (105)	353 ± 41 (108)
1000	333 ± 29 (95)	329 ± 43 (97)	379 ± 31 (106)	307 ± 44 (101)	343 ± 59 (105)

* Lipid peroxide content was determined at various intervals after oral administration of BHT (0, 500 and 1000 mg/kg), as described in Materials and Methods. Each value represents the mean ± S.D. from four individual animals. Values in parentheses represent percentage of control.

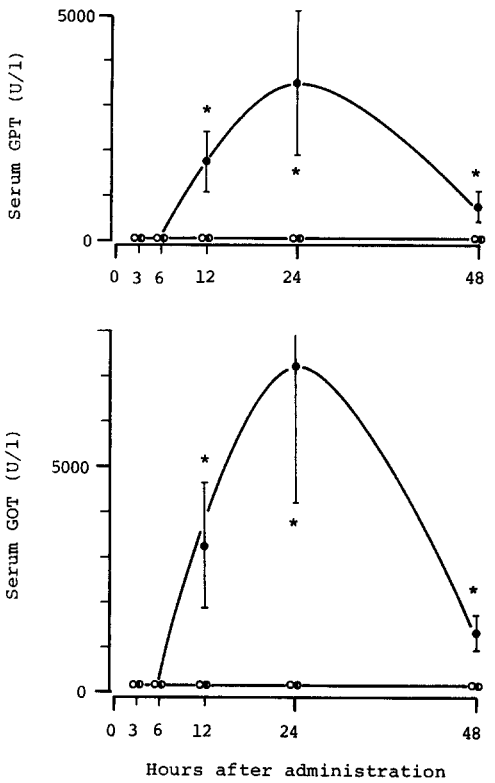

Fig. 2. Time-course of changes in the activities of serum GOT and GPT after oral administration of BHT to rats. Activities of GOT and GPT were determined at various intervals after BHT (dose: 0 [○], 500 [◐] and 1000 mg/kg [●]), as described in Materials and Methods. Points represent mean ± S.D. of four rats. Key: (*) significant difference from control group ($P < 0.01$).

BHT indicates an acute hepatocellular injury. This conclusion was confirmed histologically by centrilobular necrosis. In the present study, it seems likely that the hepatic damage was associated with prolonged depletion of GSH and/or the activated BHT metabolites rather than with lipid peroxidation in the liver. The evidence for GSH depletion can be summarized as follows: (a) after the prolonged depletion of GSH, the activities of GOT and GPT increased rapidly (Figs. 1 and 2), (b) when the activities of the transaminases were increased or when the GSH level was remarkably diminished by BHT, the content of hepatic lipid peroxide did not change (Figs. 1 and 2 and Table 2), and (c) BHT itself is antioxidant: BHT has been shown to abolish NADPH-dependent microsomal lipid peroxidation *in vitro* [24].

The lipid peroxidation of cellular membrane has been proposed as one of the mechanisms by which a number of foreign compounds produce structural tissue injury [25, 26]. However, for certain chemicals such as acetaminophen and bromobenzene, the importance of peroxidative damage is a matter of continued discussion [27–29]. Mitchell *et al.* [30] reported that lipid peroxidation which occurred during chemically induced hepatocellular necrosis was due to the depletion of GSH and had little to do with the initiation of hepatic injury. Lipid peroxidation in liver tissue assayed by thiobarbituric reaction was confirmed by measurement of conjugated diene accumulation, which is a sensitive assay, as well as the measurement of ethane production that can be used for study *in vivo* [31]. Though the data are not shown in Tables 2 and 3, no noticeable change in the formation of conjugated dienes (the ratio of peroxidized lipid [A_λ: 233 nm] to non-peroxidized lipid [A_λ: 217 nm] [32, 33]) in the lipid of microsomal

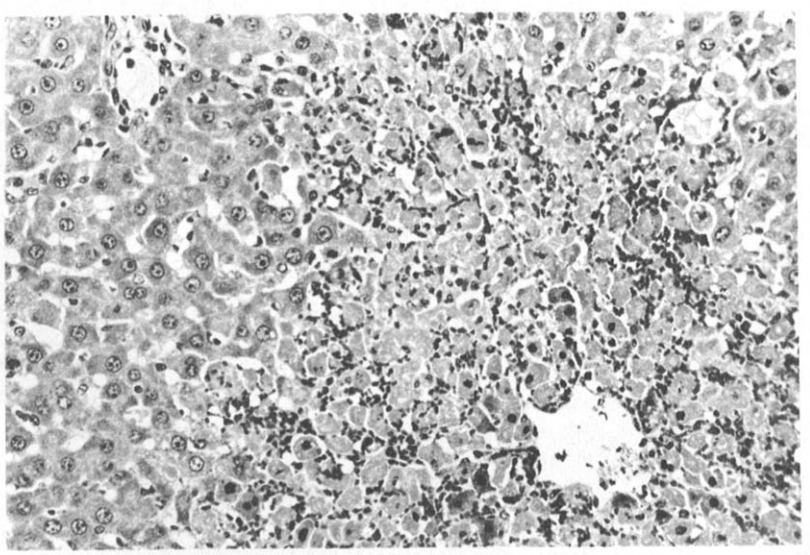

Fig. 3. Histological appearance of liver tissues of rats 24 hr after oral administration of BHT (1000 mg/kg), showing centrilobular necrosis and hemorrhage. Segments of liver were fixed in 10% neutral buffered formalin solution, embedded in paraffin, and stained with hematoxylin and eosin. Magnification, 200×.

Table 3. Effects of pretreatment with cobaltous chloride on the BHT-induced changes in levels of hepatic cytochrome P-450, GSH and lipid peroxide and in activities of serum GOT and GPT*

Treatment	Body wt (g)	Liver wt (g/100 g body wt)	Liver: Protein (mg/g liver)	Liver: Cytochrome P-450 (nmoles/mg protein)	Liver: GSH (μmoles/g)	Liver: Lipid peroxide (nmoles/g)	Serum: GPT (units/l)	Serum: GOT (units/l)
None	178 ± 21	4.70 ± 0.28	164 ± 9	0.746 ± 0.099	5.89 ± 0.44	343 ± 16	57 ± 9	206 ± 43
BHT	173 ± 11	4.30 ± 0.51	173 ± 24	0.799 ± 0.083	1.73 ± 0.24†	356 ± 52	2523 ± 1083†	5920 ± 2273†
$CoCl_2$	162 ± 12	4.85 ± 0.09	160 ± 7	0.487 ± 0.051†	8.54 ± 0.53†	350 ± 47	83 ± 11†	336 ± 62†
$CoCl_2$ + BHT	166 ± 13	4.51 ± 0.25	169 ± 16	0.425 ± 0.016†‡§	3.75 ± 0.68†‡§	333 ± 18	70 ± 17‡	320 ± 60†‡

* Animals pretreated with cobaltous chloride (60 mg/kg; s.c. injection each day for 2 days) were killed 18 hr after oral administration of BHT (1000 mg/kg). Contents of protein, cytochrome P-450, GHS and lipid peroxide in the liver and activities of GOT and GPT in the serum were determined, as described in Materials and Methods. Each value represents the mean ± S.D. from four individual animals.

† Significant difference from control (none) group ($P < 0.01$).

‡ Significant difference between BHT group and $CoCl_2$ + BHT group ($P < 0.01$).

§ Significant difference between $CoCl_2$ group and $CoCl_2$ + BHT group ($P < 0.01$).

fraction was observed in BHT-treated animals. Actually, the ratios in control and treated animals at 18 hr after BHT administration were 0.152 ± 0.024 and 0.153 ± 0.023 respectively. Further, when GSH level was a minimum (at 6 hr), the ratio in BHT-treated rats (0.154 ± 0.024) was similar to that in control rats (0.155 ± 0.024). Takahashi and Hiraga [34] also found that the level of lipid peroxide in a serum decreased after BHT treatment of rats. Therefore, the results suggest that the hepatic damage produced by BHT was associated with a consequence of GSH depletion rather than with lipid peroxidation.

On the other hand, the hepatic damage by BHT could be prevented by pretreatment with cobaltous chloride, which induced the depletion of cytochrome P-450 content and an increase in GSH content (Table 3). This result suggests that the active intermediates of BHT, rather than the parent compound, induced the tissue damage. Recently, several studies showed that many toxic chemicals undergo activation by cytochrome P-450-linked monooxygenase to form reactive intermediates, and that the irreversible binding of some intermediates to cellular macromolecules is responsible for tissue injury [35, 36]. In a previous study [10], we found that carbon monoxide or SKF-525-A, inhibitors of the microsomal monooxygenase system, significantly diminish the covalent binding of BHT to protein. Since the activated metabolites of BHT react with cysteine or GSH and produce water-soluble conjugates, the binding of BHT to protein is inhibited by these sulfhydryl compounds [12, 14]. When the GSH level reached a minimum 6 hr after BHT (Fig. 1), the amount of BHT bound to liver protein was a maximum at that time [11]. The symmetrical relationship suggests that GSH plays an important role in the detoxification of the activated metabolites of BHT. On the basis of the results, it seems reasonable to assume that when the hepatic GSH level is low for a long time, or when excessive quantities of the activated metabolites are produced, the metabolites bind to essential cellular macromolecules causing hepatic damage.

In conclusion, we have demonstrated that a single large dose of BHT to rats induced an acute hepatic injury accompanied by necrosis. The hepatic damage was confirmed by biochemical (assessed by serum GOT and GPT activities) and histological examinations.

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