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EFFECT OF BUTYLATED HYDROXYTOLUENE ON GLUTATHIONE *S*-TRANSFERASE AND GLUTATHIONE PEROXIDASE ACTIVITIES IN RAT LIVER

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Abstract—When rats were fed a diet containing 0.4% (w/w) butylated hydroxytoluene (BHT), glutathione (GSH) *S*-transferase activity towards 1-chloro-2,4-dinitrobenzene (CDNB) increased approximately 3-fold in the liver. Immunotitration studies using the antibodies raised against rat liver GSH *S*-transferase B and GSH *S*-transferase A and C indicated that the increase in GSH *S*-transferase activity was probably due to *de novo* protein synthesis. Since some forms of rat liver GSH *S*-transferases express GSH peroxidase II activity, a concomitant increase in GSH peroxidase II was expected. However, GSH perodixase II activity in the liver of BHT-treated rats remained unchanged. Gel filtration of supernatant fractions from livers of control and BHT-treated rats, followed by isoelectric focusing, indicated that BHT induced the activity of hepatic GSH *S*-transferases, without any apparent effect on GSH peroxidase II activity.

The GSH *S*-transferases catalyze the conjugation of hydrophobic substrates bearing electrophilic groups to glutathione (GSH), providing a means for the detoxication and removal of xenobiotics from the organism [1, 2]. These enzymes are often present in multiple forms in the tissues of many organisms, and they may have other functions such as the binding of bilirubin [3] and the degradation of lipid hydroperoxides through glutathione peroxidase II activity [4, 5]. Rat liver contains several forms of these enzymes, which differ in pI and substrate specificity [6]. Some rat liver GSH *S*-transferase isoenzymes express glutathione peroxidase II activity which reduces cumene hydroperoxide to the corresponding alcohol [4]. However, in rat liver about 65% of glutathione peroxidase activity is catalyzed by glutathione peroxidase I, a selenoenzyme of 85,000-dalton molecular weight which reduces both cumene hydroperoxide and hydrogen peroxide [7]. Therefore, in rat liver, glutathione *S*-transferase contributes only a part of the total glutathione peroxidase activity with cumene hydroperoxide.

The activities of GSH *S*-transferases in rat liver have been observed to increase almost 2-fold upon dietary treatment with the phenolic antioxidant butylated hydroxyanisole (BHA) [8]. However, the effect of BHA on the activities of glutathione peroxidase I and II is not known. In the present studies, the effects of dietary administration of another phenolic antioxidant, butylated hydroxytoluene (BHT), on the activities of glutathione *S*-transferase and glutathione peroxidase II in rat liver were examined. When rats were treated with BHT, GSH *S*-transferase activity in the liver increased several-fold, while both glutathione peroxidase I and II activities remained unaffected.

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MATERIALS AND METHODS

2,6-Ditertiarybutyl-4-hydroxytoluene (BHT) was purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. The purity of this preparation was ascertained through thin-layer chromatography in several solvent systems. NADPH, glutathione reductase (yeast type III), and Sephadex G-200 were purchased from the Sigma Chemical Co. Cumene hydroperoxide was purchased from Koch-Light Laboratories Ltd., U.K., and the actual concentration of hydroperoxide in the stock solution was determined by iodometric titration according to the method of Kokatnur and Jelling [9]. Column isoelectric focusing was performed on an LKB-column 8100, and ampholines were purchased from LKB Produktor, Bromma, Sweden. Carboxymethyl cellulose (CM-52) was purchased from Whatman Ltd., U.K. Other reagents were of analytical grade.

Animal experiments. Eight-week-old male Sprague–Dawley rats from Timco, Houston, TX, were used in these studies. Since regular rat chow contains antioxidants, the rats were kept on AIN 76 semipurified diet supplied by Nutritional Biochemicals, ICN, Plainview, NY. The rats were divided into groups of four and housed in wire cages without bedding on a fixed light and darkness cycle. Rats belonging to the experimental group were given 0.4% (w/w) BHT mixed into their diet. Animals were killed by cardiac puncture at intervals of 1, 7 and 14 days after the beginning of treatment, and their livers, kidneys, and lungs were excised. The livers were perfused *in situ* with ice-cold normal saline. Homogenates (10%, w/v) of the tissues were made in 10 mM potassium phosphate buffer, pH 7.0, in a Potter–Elvehjem glass homogenizer at 4000 rpm. Homogenates were centrifuged at 27,000 *g* for 20 min. Enzyme activities were assayed in the supernatant fraction. In some of the experiments, the

Table 1. Effect of BHT on GSH *S*-transferase and GSH peroxidase activities in rat liver

	GSH *S*-transferase		GSH peroxidase	
Days†	Control	BHT-treated	Control	BHT-treated
1	26.7 ± 3.7	44.2 ± 10.9	82.20 ± 16.2	68.0 ± 18.5
7	24.76 ± 3.2	78.9 ± 19.6	79.17 ± 14.2	80.4 ± 14.62
14	20.73 ± 3.3	63.35 ± 15.7	89.13 ± 15.2	84.41 ± 16.6

* Enzyme activities are expressed as units/g wet weight of liver; one unit of enzyme utilizes 1 μmole substrate per min at 25°. Each set of values represents the mean ± S.D (N = 4).

† Days after the beginning of BHT treatment.

enzyme activities were also determined in the cytosol (105,000 *g* supernatant fraction). Since no significant differences were observed between the enzyme activities in the 27,000 *g* and 105,000 *g* supernatant fractions, throughout these investigations the activities in the 27,000 *g* supernatant fraction were studied. The gel filtration profiles of 105,000 *g* and 27,000 *g* supernatant fractions were also similar.

Enzyme assays. GSH *S*-transferase activity was assayed using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate [6]. GSH peroxidase activity was assayed using 0.5 mM cumene hydroperoxide as described earlier [10]. Antisera raised against GSH *S*-transferase B and GSH *S*-transferase A and C of rat liver were provided by W. B. Jakoby, Institute of Arthritis, Digestion, and Metabolic Diseases, National Institutes of Health, Bethesda, MD, and immunotitrations were performed according to the method described previously [11].

Separation of GSH peroxidase I and II activities. The 27,000 *g* supernatant fractions made from 1 g liver tissue of control and BHT-fed rats were applied to a 100 × 5 cm column of Sephadex G-200 equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 1.4 mM β-mercaptoethanol and 100 mM ammonium sulfate, at a flow rate of 30 ml/hr. Fractions (4.8 ml) were collected and assayed for GSH *S*-transferase and glutathione peroxidase I and II activities. The GSH *S*-transferase and GSH peroxidase II activities eluted into the same fractions, and these fractions were pooled separately from those containing GSH peroxidase I activity (Fig. 1).

Separation of GSH S-transferase isoenzymes. The GSH *S*-transferase isoenzymes were separated by isoelectric focusing and by CM-52 column chromatography. The GSH *S*-transferase and GSH peroxidase II activities pooled from Sephadex G-200 gel filtration of control and BHT-fed rat liver supernatant fractions were dialyzed against distilled water in the cold. They were applied to column isoelectric focusing in the pH range of 3.5 to 10 at 1600 V for 14 h. Fractions (1.5 ml) were collected and assayed for GSH *S*-transferase and GSH peroxidase II activities as described above. For CM-52 column chromatography the pools from Sephadex G-200 gel filtration were dialyzed against 10 mM potassium phosphate buffer, pH 6.7, with 1.4 mM β-mercaptoethanol. The dialyzed enzymes were then applied to 2.5 × 50 cm columns of CM-52 equilibrated with the same buffer at 26 ml/hr. The columns were eluted with a 0–100 mM KCl gradient in the same buffer. Fractions (4.2 ml) were collected and assayed for GSH *S*-transferase activity.

RESULTS

After one day of dietary BHT administration, the total GSH *S*-transferase activity with CDNB as substrate increased roughly 2-fold in the liver of rats (Table 1). After 7 days, the maximum induction of about 3-fold was reached, and this level of activity was maintained until the end of the experiment at 14 days. However, the activities of GSH *S*-transferase in supernatant fractions from lung and kidneys were not elevated by BHT administration. To determine if the increase in hepatic GSH *S*-transferase activities was due to *de novo* protein synthesis or to the modification of existing GSH *S*-transferase proteins, immunotitrations were performed with the antibodies raised against rat liver GSH *S*-transferases B, A and C. Fixed amounts of anti GSH *S*-transferase B or anti A and C antisera precipitated equal amounts of GSH *S*-transferase activity from the BHT-fed and control rat liver supernatant fractions. Total glutathione peroxidase activity in the supernatant fractions from the livers of treated rats was not observed to be affected by BHT treatment when assayed with either 0.1 mM hydrogen peroxide or with 0.5 mM cumene hydroperoxide.

To rule out the possibility that GSH peroxidase I or II might have been inhibited by BHT or any of its metabolites, mixing experiments were performed in which the total glutathione *S*-transferase and glutathione peroxidase activities of mixtures of control and BHT-fed rat liver supernatant fractions were found to be the mean of the added activities. Finally, *in vitro* incubation of the supernatant fractions with 2 mM BHT did not have any inhibitory effect on either GSH *S*-transferase or GSH peroxidase activity.

Since glutathione peroxidase II activity constitutes only about 35% of the total hepatic glutathione peroxidase activity specific for cumene hydroperoxide [7], an increase in glutathione peroxidase II activity might be difficult to detect. Therefore, the activities of GSH peroxidase I and II in the liver supernatant fractions from control and BHT-fed rats were separated by Sephadex G-200 gel filtration. During the gel filtration, GSH peroxidase I and II activities could easily be separated from each other (Fig. 1). The peak of GSH peroxidase II activity was

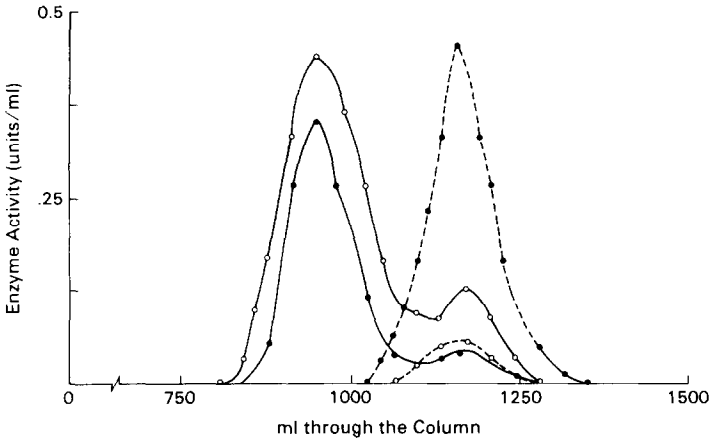

Fig. 1. Sephadex G-200 gel filtration of rat liver GSH *S*-transferase and GSH peroxidases. Key: (○——○) GSH peroxidase activity in control rats; (●——●) GSH peroxidase activity in BHT-fed rats; (○-----○) GSH *S*-transferase activity in control rats; and (●-----●) GSH *S*-transferase activity in BHT-fed rats.

coincident with that of GSH *S*-transferase. While the GSH *S*-transferase activity increased several-fold in BHT-fed rat liver, no proportionate change in GSH peroxidase II activity was observed. The Sephadex G-200 fractions containing GSH peroxidase I and GSH peroxidase II activities were pooled separately. The ratios of GSH peroxidase I and II activities in both the pools from control as well as BHT-fed rat liver supernatant fraction were also similar.

Isoelectric focusing of the GSH *S*-transferase activity pooled from Sephadex G-200 was performed to separate the isoenzymes. In the liver of BHT-fed rats all the GSH *S*-transferase isoenzymes were found to have increased activity (Fig. 2). However, the activity of GSH peroxidase II did not undergo

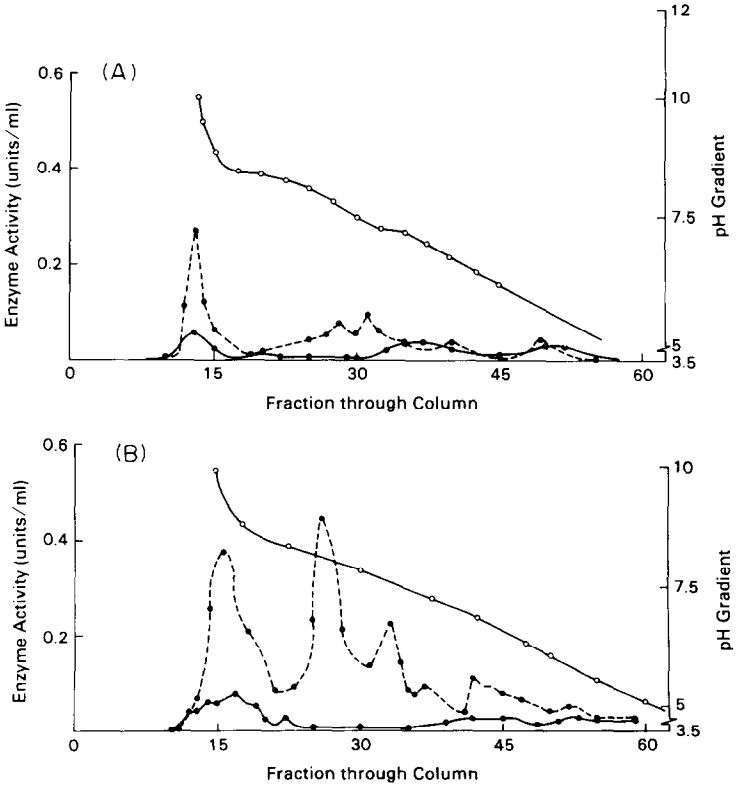

Fig. 2. Isoelectric focusing of GSH *S*-transferase and GSH peroxidase II fraction obtained by Sephadex G-200 gel filtration of liver supernatant fraction from control rats (A) and from BHT-treated rats (B). Key: (○——○) pH gradient; (●——●) GSH peroxidase activity; and (●-----●) GSH *S*-transferase activity.

a proportionate increase. The separation of GSH *S*-transferase isoenzymes by CM-52 column chromatography also indicated that all the forms of GSH *S*-transferase were induced by BHT.

DISCUSSION

The present study indicates that dietary administration of BHT causes a several-fold induction in the GSH *S*-transferase activity towards CDNB in rat liver. Immunotitration studies indicate that the observed increase in GSH *S*-transferase activity was probably due to the *de novo* synthesis of enzymes and not the modification of existing GSH *S*-transferases to a more active form. Apparently homogeneous preparations of rat liver GSH *S*-transferases [5, 12] have been shown to express GSH peroxidase II activity. Similarly, some forms of GSH *S*-transferases of human liver [11], bovine cornea [13], and bovine liver [14] have been purified to homogeneity and shown to express GSH peroxidase II activity. Thus, a concomitant increase in GSH *S*-transferase and GSH peroxidase II activity could be expected during BHT administration. However, the results presented in Table 1 show no significant change in total GSH peroxidase activity in the liver upon BHT administration. Since glutathione peroxidase II activity constitutes a smaller part of the total hepatic glutathione peroxidase activity specific for cumene hydroperoxide [7], an increase in glutathione peroxidase II activity might be difficult to detect. It is also possible that an increase in glutathione peroxidase II activity was accompanied by a compensatory decrease in the activity of GSH peroxidase I. However, the gel filtration studies ruled out these possibilities and indicate that the total GSH peroxidase II activity in the BHT-treated rats was unaffected despite a several-fold increase in the GSH *S*-transferase activity (Fig. 1).

As evidenced by the isoelectric focusing profile of rat liver GSH *S*-transferases, the dietary administration of BHT induced most of the isozymes of GSH *S*-transferase without a corresponding increase in the GSH peroxidase II activity (Fig. 2). Selective induction of rat liver GSH *S*-transferase activity by BHT without any apparent effect on GSH peroxidase activity raises several interesting possibilities. It is possible that BHT selectively induces only those isoenzymes of GSH *S*-transferase which either do not express GSH peroxidase II activity or have insignificant levels of this activity. However, the results of isoelectric focusing and CM-52 column chromatography indicate that most of the forms of rat liver GSH *S*-transferase were induced by BHT. It can also be suggested that, in order to express GSH peroxidase II activity, GSH *S*-transferase proteins may require some type of post-translational modification, such as those suggested by several investigators [14, 15]. That the GSH *S*-transferase and GSH peroxidase activities may differ in their susceptibilities to inactivation during the preparation of rat liver samples for determination of the enzyme activities cannot be ignored. Finally, the possibility of these two activities being expressed by non-identical proteins having similar structures cannot be ruled out. Further studies into the mechanisms by which GSH *S*-transferase proteins express dual catalytic activities are needed to resolve this apparent contradiction.

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