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EFFECTS OF BUTYLATED HYDROXYANISOLE ON GLUTATHIONE *S*-TRANSFERASE AND CATECHOL *O*-METHYLTRANSFERASE ACTIVITIES IN SYRIAN GOLDEN HAMSTERS*

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Abstract—The effects of dietary butylated hydroxyanisole (BHA) on the enzyme activities of glutathione (GSH) *S*-transferase and catechol *O*-methyltransferase (COMT) in the forestomach, small intestinal mucosa, and liver of Syrian golden hamsters and ICR/Ha mice were examined. GSH *S*-transferase activity in the hamster tissues was not enhanced appreciably after 1 or 4 weeks of feeding diets containing various concentrations of BHA. In general, short term (1 week) feeding of diets containing BHA did not differ from longer term (4 weeks) feeding of the same diets. In the forestomach of hamsters, a positive dose response on the activity of GSH *S*-transferase was obtained with increasing concentration of BHA in the diet for 1 or 4 weeks. The maximum effect of dietary BHA in hamsters was observed in the forestomach after 1 week of feeding, which induced an increase in GSH *S*-transferase activity to twice that of the control level. The same induction effect, however, was not apparent in the liver or in the small intestinal mucosa. Dietary BHA, at all concentrations studied, did not elicit any significant change in the activity of the GSH *S*-transferase enzyme in these two tissues. While the increase of enzyme activity in the forestomach of ICR/Ha mice was similar to that observed in the forestomach of hamsters, the induction of GSH *S*-transferase activity in the liver and in the small intestinal mucosa of the two animal species was drastically different. In contrast to the lack of response to dietary BHA in the hamster tissues, the induction of increased enzyme activity in the liver and intestinal mucosa of ICR/Ha mice, after 1 week of 2% BHA feeding, was greater than 7 and 11 times that of control respectively. The ineffectiveness of BHA as an enzyme inducer in the hamster tissues was similar for the activity of COMT. The enzyme activity in all three hamster tissues examined did not change significantly as a result of BHA incorporation into the diet for 1 week. In contrast, the COMT activity in the forestomach and small intestinal mucosa of the mouse was increased with increasing concentration of dietary BHA. At 2% BHA, the enzyme activity in the two tissues was 3 and 2 times that of the control level, respectively, whereas the enzyme activity in the liver remained at control level. These findings suggest that the overall unresponsiveness of detoxifying enzyme systems in the Syrian golden hamsters may be critical in the formation of forestomach tumors caused by BHA.

The phenolic antioxidant, BHA†, is a widely used food additive which has been shown to inhibit chemically induced neoplasia in laboratory animals. Incorporation of 0.5% BHA into commercial diets inhibits neoplasia of the lung, forestomach, and other target tissues induced by a variety of carcinogens [1–3]. The inhibitory action of BHA has been attributed to its ability to enhance the activities of a number of metabolic and detoxifying enzymes. The multisubstrate monooxygenase system is altered significantly by dietary BHA. The alteration was detected by the detailed examination of the metabolism of BP in microsomal preparations from BHA-treated and control mice [4]. The conjugating enzymes, GSH *S*-transferase and UDP-glucuronosyl transferase, are enhanced in hepatic and extrahepatic tissues [5–7]. The end result of the alteration of these enzyme systems is the reduction of reactive metabolites of carcinogens that bind to critical macromolecules. Thus, the binding of BP metabolites to the *in vitro* added DNA in microsomal incubations is reduced by 50% [8, 9]. The BP diol epoxide bound DNA isolated from the forestomach and lung tissues of mice is reduced by more than 50% when the animals are fed BHA [10]. The reduction of BP–DNA binding appears to correlate with the inhibition of tumorigenesis by BHA.

Recently, in contrast to its inhibitory effects, BHA, at a 2.0% addition to the diet, was found to induce neoplasia in the forestomach of male Syrian golden hamsters and F344 rats [11–13]. The different experimental consequences in the hamsters, rats, and mice may be attributed to the different animal species employed in these experiments. The high dose of BHA employed in the carcinogenicity studies, however, suggests the possibility that minor components that are present in commercial BHA or a minor metabolite(s) of BHA may be responsible for the neoplastic effect of this antioxidant. Alterna-

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† Abbreviations: BHA, butylated hydroxyanisole; BP, benzo[*a*]pyrene; GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; SAM, *S*-adenosyl-L-methionine; and COMT, catechol *O*-methyltransferase.

tively, the high dose condition may change the metabolizing and/or detoxifying enzyme systems in susceptible animals in a different manner than the lower dose that was administered to mice in the protection experiments. Such change in enzyme activity may allow reactive metabolites of BHA to accumulate in the forestomach of the animals.

Carcinogens which possess the phenolic moiety have been postulated to undergo metabolic conversion to catechols [14]. These catechol metabolites are then oxidized via the semiquinones to the corresponding ortho quinones. The semiquinones and/or the ortho quinones may be the reactive metabolites that are the carcinogenic species. The enzyme system, COMT, catalyzes the methylation of catechols to their methyl ethers which are not further oxidized to reactive compounds. Thus, COMT may be critical in the detoxification of catechol which appears to be one of the metabolites of BHA [15]. We report, in this study, the effects of BHA on the activities of the two detoxifying enzymes, GSH *S*-transferase and COMT, in the forestomach, small intestinal mucosa, and liver of Syrian golden hamsters and ICR/Ha mice.

MATERIALS AND METHODS

Chemicals. Pure 3-BHA (>99.6% by HPLC) was obtained by fractional crystallization from commercial BHA (Sigma Chemical Co., St. Louis, MO). GSH and SAM were purchased from the Sigma Chemical Co. CDNB was obtained from the Aldrich Chemical Co., Milwaukee, WI. [^{3}H]SAM was purchased from ICN Biochemicals, Cleveland, OH.

Animals. Male Syrian golden hamsters (LVG-VAF) were obtained from Charles River Laboratories (Wilmington, MA). Thirty hamsters, 6 weeks old, were fed a semipurified diet (Nutritional Biochemicals, Clevelend, OH) for 1 week before they were divided into five groups. The experimental groups were fed semipurified diets containing 2.0, 1.0, 0.5, and 0.25% 3-BHA. The control group was fed a semipurified diet without the addition of BHA. All the diets were in the form of 0.5 inch pellets custom made by Nutritional Biochemicals. At the end of 1 week of feeding, three hamsters from each group were killed by decapitation, and the forestomach, small intestinal mucosa, and liver were removed for enzyme preparation. The tissues were homogenized in phosphate buffer, pH 6.5, by means of a Virtis homogenizer. The cytosol, after 100,000 *g* centrifugation for 1 hr, was obtained and was frozen at −65° until used. Three hamsters per group were killed at the end of 4 weeks, and the cytosolic fractions from each tissue were prepared similarly. Each sample represents one tissue from each individual animal.

Female ICR/Ha mice were obtained from Harlan Sprague–Dawley Laboratory (Indianapolis, IN). Fifteen mice, 8 weeks old, were fed a semipurified diet for 1 week before they were divided into five groups. The experimental groups were fed the same diets as those in the hamster experiment. The control group was fed a semipurified diet. At the end of 1 week the mice were killed by decapitation and the tissues were removed. The cytosolic fractions from each tissue were prepared as described above. Each sample represents one tissue from each individual animal.

GSH S-transferase assay. The activity of cytosolic GSH *S*-transferase was assayed according to the method of Habig *et al.* [16] using CDNB as the substrate. The reaction was monitored at 340 nm in a Beckman dual-beam UV-VIS model 25 spectrophotometer equipped with a temperature-controlled cell compartment. Assays were performed at 30° in 0.1 M phosphate buffer, pH 6.5, in the presence of 5 mM GSH and 1 mM CDNB. Complete assay mixture without enzyme was used as the control.

COMT assay. The reactions were performed in standard polyethylene scintillation vials according to the method of Zürcher and Da Prada [17]. One hundred microliters (in some cases 100–300 μl) of cytosol (frozen for less than 2 weeks) was mixed with 500 μl of freshly prepared buffer–substrate mixture, composed of 400 μl potassium phosphate buffer (0.1 M, pH 7.6), 20 μl magnesium chloride (0.1 M), 30 μl catechol (1,2-dihydroxybenzene, 0.05 M), 20 μl dithiothreitol (0.065 M), 10 μl adenosine deaminase (470 units/ml), and 20 μl [^{3}H]SAM (5.5 mM, sp. act. 3.64 Ci/mol). Blanks were prepared in the same way, but the cytosol was replaced with an equal volume of buffer solution. The reaction was started by placing the vials in a shaking water-bath at 37°. At the end of 20 min, the incubation was stopped by immersing the vials in an ice-bath. To each sample, 500 μl of HCl solution (1 M) containing guaiacol (0.1 g/L) and 10 ml of scintillation fluid [5 g of 2-(4′-*tert*-butylphenyl)-5-(4″-diphenyl)-1,3,4-oxadiazole dissolved in 200 ml toluene, made up to 1 L with *n*-hexane) was added. The vials were capped and more than 98% of the [^{3}H]guaiacol formed was extracted into the upper phase by vigorous shaking for 3 min. The samples were allowed to settle before they were counted in a Packard Tricarb B2425 liquid scintillation counter.

RESULTS

Glutathione S-transferase. The activity of GSH *S*-transferase in the hamster tissues was not enhanced appreciably after 1 or 4 weeks of feeding diets containing various concentrations of BHA (Fig. 1). In general, short term (1 week) feeding of diets containing BHA did not differ from longer term (4 weeks) feeding of the same diet. In the forestomach cytosol of hamsters, a positive dose response on the activity of GSH *S*-transferase was obtained with increasing concentration of BHA in the diet for 1 or 4 weeks. The maximum effect of dietary BHA in hamsters was observed in the forestomach after 1 week of feeding, which induced an increase of GSH *S*-transferase activity to twice that of the control level. At 4 weeks, the enzyme activity was found to increase less than 1.5 times that of the control in the forestomach at 2.0% BHA (Table 1). The same observation, however, was not apparent in the liver or in the small intestinal mucosa. Dietary BHA, at all concentrations studied, did not elicit any significant change in the activity of the GSH *S*-transferase enzyme in these two tissues.

While the increase of enzyme activity in the fore-

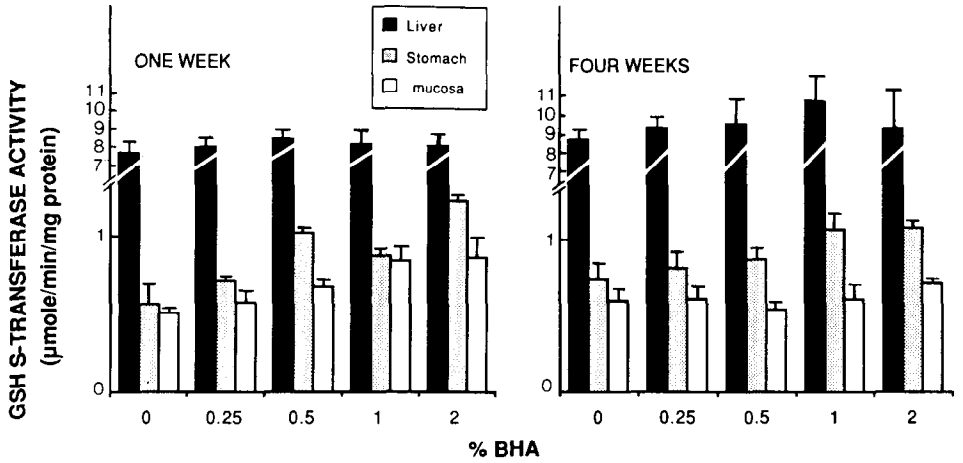

Fig. 1. Effects of various concentrations of dietary BHA on GSH *S*-transferase activity in the cytosols of the liver, forestomach, and small intestinal mucosa of Syrian golden hamsters. Experimental diets containing BHA were fed to hamsters for 1 or 4 weeks. Values are means ± SD, N = 3.

Table 1. Effects of dietary BHA on the activity of glutathione *S*-transferase in cytosols prepared from tissues of Syrian golden hamsters

	Glutathione *S*-transferase activity* (μmol/min/mg protein)					
	Liver		Forestomach		Small intestinal mucosa	
Group	1 Week	4 Weeks	1 Week	4 Weeks	1 Week	4 Weeks
2% BHA†	8.07 ± 0.91‡	9.68 ± 1.73‡	1.23 ± 0.13§	1.08 ± 0.03‖	0.86 ± 0.21‖	0.71 ± 0.03‡
Control	7.73 ± 0.46	8.62 ± 0.80	0.56 ± 0.17	0.73 ± 0.12	0.51 ± 0.03	0.60 ± 0.08

* Values are means ± SD, N = 3.

† BHA (2%) was added to semipurified diet from ICN Nutritional Biochemicals. The diet was given in pellet form for 1 or 4 weeks.

‡–‖ P values were obtained by the two-tailed Student's *t*-test with respect to control: ‡ $P > 0.05$, § $P < 0.01$, and ‖ $P < 0.05$.

stomach of ICR/Ha mice was similar to that observed in the forestomach of hamsters, the induction of GSH *S*-transferase activity in the liver and in the small intestinal mucosa of the two animal species was drastically different. In contrast to the lack of response to dietary BHA in the hamster tissues, the induction of increased enzyme activity in the liver and intestinal mucosa of ICR/Ha mice, after 1 week of 2% BHA feeding, was greater than 7 and 11 times that of control respectively (Table 2). An increase of enzyme activity greater than 5 times that of the control was observed with the lowest concentration (0.25%) of dietary BHA in this tissue (Fig. 2).

Catechol O-methyltransferase. The COMT enzyme activity was determined using catechol as the substrate. In hamsters, the basal level of enzyme activity was highest in the cytosol of the small intestinal mucosa (Fig. 3). The liver and forestomach cytosols had very similar levels of enzyme activity which was approximately one-third that of the small

Table 2. Effects of dietary BHA on the activity of glutathione *S*-transferase in cytosols prepared from tissues of ICR/Ha mice

	Glutathione *S*-transferase activity* (μmol/min/mg protein)		
Group	Liver	Forestomach	Small intestinal mucosa
2% BHA†	11.89 ± 1.69‡	1.97 ± 0.47§	7.81 ± 0.77‡
Control	1.65 ± 0.19	1.45 ± 0.25	0.70 ± 0.10

* Values are means ± SD, N = 3.

† BHA (2%) was added to semipurified diet from ICN Nutritional Biochemicals. The diet was given in pellet form for 1 week.

‡,§ P values were obtained by the two-tailed Student's *t*-test with respect to control: ‡ $P < 0.001$, and § $P > 0.05$.

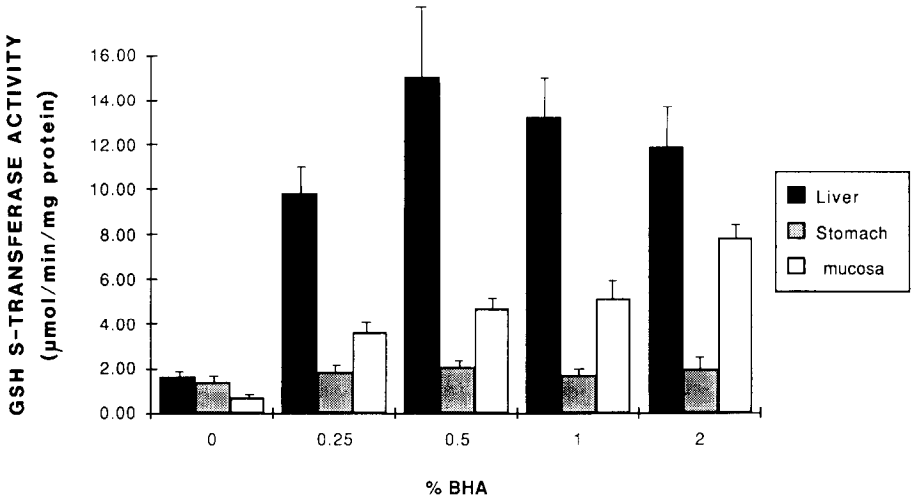

Fig. 2. Effects of various concentrations of dietary BHA on GSH *S*-transferase activity in the cytosols of the liver, forestomach, and small intestinal mucosa of ICR/Ha mice. Experimental diets containing BHA were fed to mice for 1 week. Values are means ± SD, N = 3.

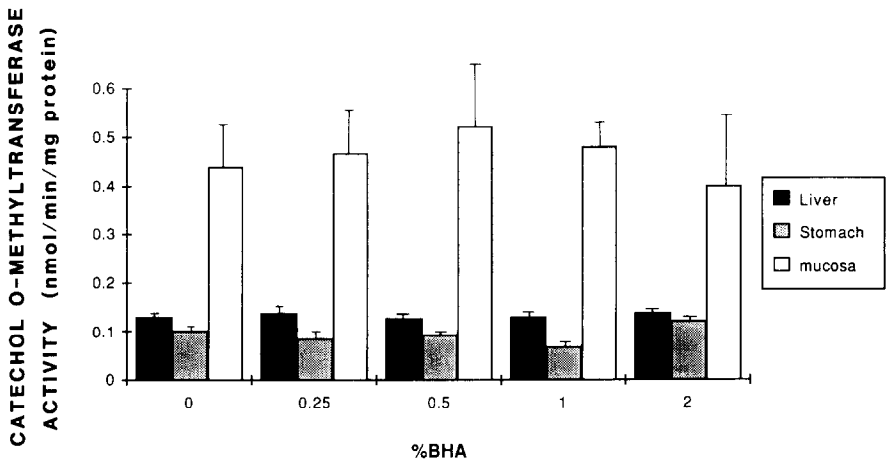

Fig. 3. Effects of various concentrations of dietary BHA on catechol *O*-methyltransferase activity in the cytosols of the liver, forestomach, and small intestinal mucosa of Syrian golden hamsters. Experimental diets containing BHA were fed to hamsters for 1 week. Values are means ± SD, N = 3.

Table 3. Effects of dietary BHA on the activity of catechol *O*-methyltransferase in cytosols prepared from tissues of Syrian golden hamsters

	Catechol *O*-methyltransferase activity* (nmol/min/mg protein)		
Group	Liver	Forestomach	Small intestinal mucosa
2% BHA†	0.14 ± 0.006‡	0.10 ± 0.03‡	0.40 ± 0.15‡
Control	0.13 ± 0.04	0.09 ± 0.02	0.44 ± 0.09

* Values are means ± SD, N = 3.

† BHA (2%) was added to semipurified diet from ICN Nutritional Biochemicals. The diet was given in pellet form for 1 week.

‡ P values were obtained by the two-tailed Student's *t*-test with respect to control: $P > 0.05$.

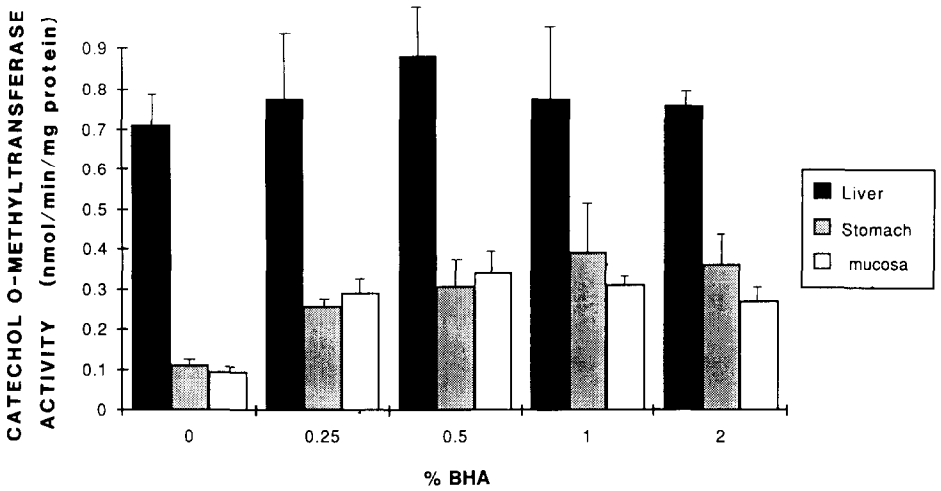

Fig. 4. Effects of various concentrations of dietary BHA on catechol *O*-methyltransferase activity in the cytosols of the liver, forestomach, and small intestinal mucosa of ICR/Ha mice. Experimental diets containing BHA were fed to mice for 1 week. Values are means ± SD, N = 3.

intestinal mucosa. The enzyme activity in all three hamster tissues examined did not change significantly as a result of BHA incorporation into the diet for 1 week (Table 3).

In mice, the basal level of COMT activity was highest in the cytosol of the liver instead of the small intestinal mucosa as noted above (Fig. 4). Addition of BHA in the diet did not change the enzyme level in the liver of the mouse (Table 4). In the forestomach and small intestinal mucosa, however, the COMT activity was increased with increasing concentration of dietary BHA. At 2% BHA, the enzyme activities in the two tissues were 3 and 2 times that of the control level respectively (Table 4).

DISCUSSION

The inhibitory effects of BHA in mice and other laboratory animals have been attributed, at least in part, to its ability to induce increased activity of detoxifying enzymes such as GSH *S*-transferase and UDP-glucuronosyl transferase [18]. The induction of a substantial increase in GSH *S*-transferase activity in the liver and small intestinal mucosa of mice at a low concentration (0.25%) of dietary BHA suggests that this mechanism of action may be crucial in the detoxification of reactive carcinogenic species. In Syrian golden hamsters, however, the activity of the detoxifying enzyme, GSH *S*-transferase, was not induced by BHA to a level that was substantially higher than that of the control. This unresponsiveness towards the inducing effects of dietary BHA was particularly evident in the liver and the small intestinal mucosa. While the enzyme level in these tissues in the mouse was elevated by dietary BHA to greater than 7 and 11 times that of the control, respectively, the same enzyme activity was not stimulated higher than 1.5 times the basal activity in the hamster. Although the control activity in the hamster liver was relatively high compared to that of the ICR/Ha liver, the activity in the small intestinal mucosa was similar to that in the mouse tissue. Thus, the high basal level in the hamster liver does not appear to be the reason for the lack of inducibility by BHA.

Long term feeding of diets containing BHA appeared to induce a less pronounced increase of enzyme activity, as shown in Fig. 1. The enzyme level of control animals after 4 weeks of feeding was consistently higher than that of control animals after 1 week of feeding. This difference may be attributed to the age of the animals. The response of these

Table 4. Effects of dietary BHA on the activity of catechol *O*-methyltransferase in cytosols prepared from tissues of ICR/Ha mice

	Catechol *O*-methyltransferase activity* (nmol/min/mg protein)		
Group	Liver	Forestomach	Small intestinal mucosa
2% BHA†	0.76 ± 0.04‡	0.36 ± 0.08§	0.27 ± 0.03§
Control	0.71 ± 0.07	0.11 ± 0.01	0.12 ± 0.05

* Values are means ± SD, N = 3.

† BHA (2%) was added to semipurified diet from ICN Nutritional Biochemicals. The diet was given in pellet form for 1 week.

‡,§ P values were obtained by the two-tailed Student's *t*-test with respect to control: ‡ $P > 0.3$, and § $P < 0.01$.

enzymes to BHA may not be as sensitive when the hamsters get older. Four weeks of feeding diets containing BHA, on the other hand, may render the animals less sensitive to the induction of BHA. This phenomenon appears to apply to all three tissues examined. If this trend continues throughout the long term feeding experiments that were carried out in the carcinogenicity studies, then it is conceivable that the detoxifying enzyme, GSH *S*-transferase, may not be able to conjugate the activated metabolites of BHA effectively.

Catechols are potential proximate carcinogenic species of phenols. One of the metabolites of BHA has been found to be 3-*tert*-butyl-4-methoxy catechol, which may be one of the activated metabolites of BHA responsible for its carcinogenicity in the hamster forestomach [15]. The enzyme, COMT, catalyzes the methylation of catechols to their corresponding methyl esters which are not further oxidized to reactive intermediates [19]. While there is no direct evidence indicating that BHA catechol is a substrate of COMT, the dependency on the enzyme activity of COMT in the formation of methyl ethers of catechols has been well documented [19]. An increase of COMT activity may contribute to the overall detoxification of BHA. In isolated hepatocytes BHA-glutathione is a major polar metabolite which suggests that BHA is a substrate of GSH *S*-transferase [20]. The observation that the enzyme activities of COMT and GSH *S*-transferase in the hamsters were not affected by dietary BHA at concentrations as high as 2% suggests the possibility that other detoxifying enzymes, such as UDP-glucuronosyl transferase which is responsible for the major detoxification of BHA as the glucuronide [21], may not be altered either. The overall unresponsiveness of enzyme systems in the detoxifying cascade may be critical in the ultimate expression of carcinogenicity of BHA in the hamsters.

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