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Elevation of Hepatic Glutathione S-Transferase Activities and Protection against Mutagenic Metabolites of Benzo(a)pyrene by Dietary Antioxidants[1]

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ABSTRACT

Addition of either 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) or 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (ethoxyquin) to the diet greatly decreases the levels of mutagenic metabolites of benzo(*a*)pyrene in CD-1 mice. (R. P. Batzinger, S-Y. L. Ou, and E. Bueding, Cancer Res., *38*: 000, 1978). The mutagenic activity of the urinary metabolites of benzo(*a*)pyrene is markedly reduced in the presence of glutathione together with the liver cytosols of rats or mice fed on a diet containing BHA. The liver cytosols of mice and rats maintained on control diets are much less effective in this respect. Dietary BHA causes increases in mouse and rat hepatic glutathione *S*-transferase (EC 2.5.1.18) specific activities with 1,2-dichloro-4-nitrobenzene, 1-chloro-2,4-dinitrobenzene, *p*-nitrobenzylchloride, and Δ^5-androstene-3,17-dione. In the mouse the increases are larger (5- to 10-fold) and are dependent on the dose and duration of administration of BHA. Increases in these glutathione *S*-transferase specific activities were also observed in mouse hepatic cytosols after feeding of ethoxyquin. Direct addition of reduced glutathione and purified glutathione *S*-transferases A and B obtained from rat liver to the mutagenicity assay system mimicked the effect of the rodent cytosols. Since BHA and ethoxyquin are known to reduce the neoplastic effects of a variety of potent carcinogens, we suggest that the protective effects of these antioxidants may be accounted for, at least in part, by their ability to elevate the glutathione *S*-transferases. These enzymes inactivate arene oxides and other hydrophobic electrophiles by catalyzing their conjugation with glutathione.

INTRODUCTION

The induction of experimental tumors in rodents by a variety of chemical carcinogens can be prevented by the administration of certain "antioxidants." (The term antioxidant is used in this study to designate compounds that can act as biological antioxidants, although it is by no means clear as to whether the effects on enzyme activities described in this study can be in fact ascribed to their antioxidant properties as such.) This phenomenon was first explored systematically by Wattenberg *et al.* (41-44), whose observations have been extended by Weisburger *et al.* (45), Grantham *et al.* (14), and Ulland *et al.* (38). Antioxidants reduce the production of tumors at various anatomical sites by a wide variety of chemical carcinogens, including aromatic polycyclic hydrocarbons, urethan, dimethylhydrazine, nitrosamines, azo dyes, and aminofluorenes. The principal compounds used for protection against carcinogens have been BHA,[3] 3,5-di-*tert*-butyl-4-hydroxytoluene, ethoxyquin, and bis(diethylthiocarbamyl)disulfide (disulfiram). BHA and ethoxyquin are of special interest, since they are widely used as preservatives in the diets of humans and domestic animals, respectively. Various theories have been advanced to explain these effects of antioxidants, but as yet no mechanism has been established unequivocally. Potential mechanisms include: (*a*) direct interaction of the carcinogen or of its activated metabolic products with the antioxidant; (*b*) enhanced activities of enzymes that inactivate the proximate or ultimate carcinogens, thereby diverting them from the damaging interactions with critical macromolecular components, thus preventing the initiation of the malignant process; (*c*) blockade of specific metabolic activation processes required to convert procarcinogens to their reactive intermediates; and (*d*) more efficient mechanisms for repair of damage to DNA.

Antioxidant-mediated protection of rodents against the neoplastic effects of carcinogens appears to show little specificity with respect to the chemical nature of the carcinogen, the route of its administration, the site of tumor formation, or the chemical structure or route of administration of the antioxidant. Hence, the antineoplastic effect is likely to depend on some generalized mechanisms, the nature of which has not been hitherto elucidated.

Batzinger *et al.* (3) have reported recently that the urine of mice receiving i.m. injections of BP is highly mutagenic for 2 of the sensitive tester strains of histidine-requiring *Salmonella typhimurium* developed by Ames *et al.* (1) and McCann *et al.* (30). The urine induced reversions of both tester strains TA98 and TA100. The mutagenic activities of these urine specimens were enhanced moderately by the presence of rat liver microsomes and cytosol (the so-called S9 fraction) derived from rats treated with a hydrocarbon inducer of microsomal drug-metabolizing enzymes. The mutagenic activities of the urine samples obtained from BP-treated mice were also increased if these urine samples were subjected to the action of β-glucuronidase, suggesting that at least some of the mutagenic metabolites were present as conjugates. The mutagenic activity of the urinary

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[3] The abbreviations used are: BHA, 2(3)-*tert*-butyl-4-hydroxyanisole (a mixture containing approximately 4% of compound in which the *tert*-butyl group is meta to the phenolic hydroxyl group and containing 96% of compound in which the *tert*-butyl group is ortho to the phenolic hydroxyl group); BP, benzo(*a*)pyrene; GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; NBC, *p*-nitrobenzylchloride.

metabolites of BP was markedly reduced when the mice given injections of this hydrocarbon were fed a diet supplemented with either BHA or ethoxyquin. This reduction was observed when the urine was tested directly, hydrolyzed with β-glucuronidase, activated with the S9 fraction, or subjected to both of these procedures (3).

The mutagenic activity of BP was also detected in the host-mediated assay system (26), in which the *Salmonella* tester strains were exposed for 6 hr to the environment of the peritoneal cavities of mice that had been given i.m. injections of this hydrocarbon. A dramatic reduction in the number of revertants produced was observed when the host mice receiving the injections of BP had been fed for 10 days on a diet supplemented with 0.75% of BHA by weight. Lower levels of mutagenic activity in BHA-fed hosts were also observed with a number of chemotherapeutic agents (3).

This study deals with the analysis of mechanisms of protection by 2 antioxidants against the mutagenic metabolites of BP. We show that BHA and ethoxyquin, which block the neoplastic and mutagenic activities of carcinogens, also raise dramatically the levels of several hepatic GSH *S*-transferases in mice and rats. The mutagenic activity of the urinary metabolites of BP in the mouse can be substantially reduced or virtually eliminated by the addition to the test system of both reduced GSH and rodent liver cytosols containing such elevated levels of GSH *S*-transferases. Furthermore, the addition of GSH and certain highly purified GSH *S*-transferases to the mutagenicity assay system produced a substantial reduction in the mutagenic activity of the urine of BP-treated mice.

A number of studies have dealt with the influence of these antioxidants administered *in vivo* on the activities of other enzymes concerned with metabolic activation and inactivation of carcinogens (8, 13, 17, 21, 29, 37, 44). Studies from our own laboratories (9, 10) have shown that the administration of BHA causes large increases in the activity of hepatic microsomal epoxide hydratase in CD-1 mice. These increases are less pronounced in Sprague-Dawley rats. Changes in other enzymes concerned with the metabolism of carcinogens have also been observed (9).[4]

MATERIALS AND METHODS

Treatment of Animals. Two types of animals were used in all of the experiments described: female CD-1 mice (Charles River Breeding Laboratories, Wilmington, Mass.), 6 to 8 weeks old unless otherwise specified; and male Sprague-Dawley rats (Madison, Wis.), 7 weeks old. These animals were housed in stainless steel hanging cages (4 mice or 1 rat/cage) without bedding and were fed *ad libitum* either food pellets (RMH 1000; Charles River Breeding Laboratories) or Purina laboratory chow (Code 5001; Ralston-Purina, St. Louis, Mo.) in powder form. The additives BHA (Sigma Chemical Co., St. Louis, Mo.) and ethoxyquin (Santoquin; a gift from Monsanto, St. Louis, Mo.) were incorporated into the latter diet by mixing in a Liquid-Solids Blender (Patterson-Kelley Co., East Stroudsburg, Pa.). Alternatively, as specified below, BHA was administered by gavage in Emulphor EL-620 (a gift from GAF Corp., New York, N. Y.). Control animals received the same form of diet (pellet or powder) as did the corresponding experimental animals. Mice and rats were killed by cervical dislocation and decapitation, respectively.

Preparation of Hepatic Cytosol Fractions for Enzyme Studies. All steps were carried out at 0–4°. Fresh mouse livers were homogenized in 19 ml of 0.25 M sucrose per g of tissue. To the supernatant obtained by centrifugation at 9000 × *g* for 15 min was added 0.1 volume of 0.1 M $CaCl_2$ in 0.25 M sucrose. Centrifugation at 37,000 × *g* for 15 min then yielded a clear cytosol fraction. Rat livers were perfused with cold 0.15 M KCl, frozen in liquid nitrogen, and stored at −80° overnight. Each liver was homogenized in 3 volumes of 0.25 M sucrose. To the supernatant fluid obtained by centrifugation at 9000 × *g* for 15 min was added 0.2 volume of 0.1 M $CaCl_2$. Centrifugation at 37,000 × *g* for 15 min then yielded a clear cytosol fraction.

Preparation of Hepatic Cytosol Fractions for Addition to Mutagenicity Assays. The animals received the diets specified in the individual protocols. The livers were removed from mice and rats under aseptic conditions. All subsequent steps were carried out at 0–4° under sterile conditions. The livers were homogenized with 3 volumes of 0.15 M KCl. The homogenates were centrifuged at 9,000 × *g* for 15 min, and the resultant supernatant was further centrifuged at 100,000 × *g* for 60 min to yield the cytosol fractions which were then further diluted with 2 volumes of 0.85% NaCl solution prior to use in the experiments described in Tables 3 to 6. The microsomal pellet was suspended in a volume of 0.15 M KCl equivalent to the volume of the 9,000 × *g* supernatant from which it was derived. This microsome suspension was used directly for the experiments described in Table 5.

Determinations of GSH S-Transferase Activities. CDNB, DCNB, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, and NBC were obtained from Eastman Organic Chemicals (Rochester, N. Y.), and Δ⁵-androstene-3,17-dione was prepared as previously described (24). GSH and dithiothreitol were obtained from Sigma. Initial velocities were measured spectrophotometrically according to published methods (15, 22) for those reactions that yield a GSH conjugate as a final product and for the isomerization of Δ⁵-androstene-3,17-dione by rat liver preparations (5). With mouse liver preparations, GSH and dithiothreitol were omitted from the standard assay system with Δ⁵-androstene-3,17-dione, since substantially higher activities were observed in the absence of added GSH.[5] Specific activities were based on protein

[4] Y-N. Cha and E. Bueding. Effect of 2(3)-*tert*-Butyl-4-hydroxyanisole Administration on the Activities of Several Hepatic Microsomal and Cytoplasmic Enzymes in Mice. Biochem. Pharmacol., in press, 1978.

[5] The GSH-dependent steroid isomerases (EC 5.3.3.1) of rat and human liver have previously been shown to be identical with some of the GSH *S*-transferases (6). A relationship between the cytosol steroid isomerase of mouse liver and the GSH *S*-transferases has not been clearly established. Unlike rat and human liver cytosols, the most prominent activities of crude and partially purified mouse liver cytosols in isomerizing Δ⁵-androstene-3,17-dione are inhibited by added GSH. These facts are consistent with the finding that, with this substrate, the specific enzyme activities in crude extracts fell off with increasing concentrations of cytosol. However, after isoelectric focusing of these preparations, strict proportionality between velocity of steroid isomerization and amount of enzyme was observed.

concentrations determined by the method of Lowry *et al.* (28), with crystalline bovine serum albumin as a standard.

Isoelectric Focusing. Isoelectric focusing (39) was carried out in sucrose gradients at 1–2° in a 110-ml column (Model 8100-1; LKB Instruments, Inc., Rockville, Md.). Ampholine solutions (LKB) of different pI ranges, as specified below, were used in combinations designed primarily to achieve desired separations rather than to maximize linearity of the resulting pH gradients. The absorbance of the column effluent at 280 nm, in a 0.3-cm light path, was recorded automatically. Fractions of approximately 1 ml were collected and assayed for enzyme activities. The pH values of the fractions were measured at 0° immediately after collection.

Purification of Rat Liver GSH *S*-Transferases A and B. The transferase A used in the mutagenicity assays was obtained from the cytosols of livers of Sprague-Dawley rats fed a diet containing 0.75% BHA for 8 days. The enzyme was passed through DEAE-cellulose (Cellex D; Bio-Rad, Richmond, Calif.), precipitated with ammonium sulfate, dialyzed, and subjected to gradient elution from carboxymethylcellulose according to the method of Habig *et al.* (15), followed by isoelectric focusing at pI 8.0 to 9.5. The pI of the enzyme was 9.0. Subsequent gel filtration on a column of Sephadex G-150 yielded an enzyme with a specific activity of 55 μmol/min/mg with CDNB and 5.1 μmol/min/mg with DCNB. The transferase B was prepared similarly (from livers of rats that had not received BHA) and had a specific activity of 1.51 μmol/min/mg with Δ^5-androstene-3,17-dione (6).

Mutagenicity Assays. The assays for mutagenic activity of urine for *S. typhimurium* tester strains TA98 and TA100 were conducted with the use of the methods developed by Ames *et al.* (1), as modified by Batzinger *et al.* (3). Enzyme preparations, GSH, and/or other sulfhydryl compounds were added to the top agar layer (2 ml), as specified in the individual protocols. The GSH (Tables 3 to 6) and other sulfhydryl compounds (Table 3) were added in 0.2 ml of a solvent composed of equal volumes of dimethyl sulfoxide and 0.85% NaCl. This vehicle was added to the control assays. For the experiments with the purified transferases (Table 7), the GSH was added in 0.1 ml of 10 mM potassium phosphate buffer (pH 6.8).

Collection and Treatment of Urine from BP-treated Mice. Pooled urine was collected in metabolism cages, usually from groups of 10 female mice (12 to 14 weeks old for experiments recorded in Tables 3 to 6 and 6 to 8 weeks old for the experiments in Table 7) during a 24-hr control period and for 24 hr following the i.m. injection of 2 mg of BP (approximately 100 mg/kg; Sigma) in 0.1 ml of sesame oil. The urine was collected in containers packed in ice, sterilized by passage through filters of 0.22-μm pore size (type GS; Millipore, Bedford, Mass.), and stored at −80° until used. After adjustment of the pH to 6.0 by addition of a predetermined volume of 1 N HCl, the urine samples were incubated for 3 hr at 37° with 0.1 volume of a preparation of β-glucuronidase and arylsulfatase from *Helix pomatia* (from Boehringer Mannheim, Indianapolis, Ind.). This enzyme mixture was first dialyzed overnight at 4° against 0.1 M sodium acetate buffer, pH 4.5, and after dialysis 1.0 ml liberated 11.7 μmol of phenolphthalein per min at 38° from phenolphthalein mono-β-glucuronide). The arylsulfatase component of the mixture is inactive under these conditions of hydrolysis of urinary metabolites (3).

RESULTS

Hepatic GSH *S*-Transferase Levels in Rodents Receiving Dietary Antioxidants

Effect of Dose and Duration of Treatment with BHA on Mouse Hepatic GSH *S*-Transferases. Administration p.o. of BHA to mice resulted in greatly enhanced GSH *S*-transferase specific activities in the hepatic cytosol. The magnitude of this effect was dependent upon the dosage and the duration of BHA treatment. Daily administration of BHA by gavage in Emulphor EL-620 (5 ml/kg) to groups of 6 mice in doses ranging from 100 to 1000 mg/kg body weight for 4 days raised the GSH *S*-transferase specific activities for several substrates in a dose-dependent manner, at least up to 750 mg/kg. In hepatic cytosols, GSH *S*-transferase activity for CDNB at this dosage was 13.2 ± 0.7 (S.E.) μmol/min/mg (n = 4), as compared with 2.0 ± 0.2 μmol per min per mg (n = 4) for those animals that received the vehicle only. The effect of gavage feeding of 750 mg of BHA per kg body weight on the GSH *S*-transferase activities could be closely approximated by inclusion of 0.75% BHA by weight in a powdered diet. In 4 days this treatment raised the GSH *S*-transferase specific activities for CDNB in hepatic cytosols from 2.4 ± 0.1 (n = 5) to 14.9 ± 0.3 μmol/min/mg (n = 5). During this period of treatment, the wet liver weights also increased by about 50% ($p < 0.01$) over that of the control animals, whereas no difference in body weight was observed between the 2 groups (*cf.* Refs. 8 and 17).

The effects of the duration of BHA feeding (0.75%) on the levels of mouse hepatic GSH *S*-transferase activities with 3 substrates are shown in Chart 1.[6] These activities were elevated 3- to 5-fold in 3 days and continued to rise over the entire 12-day feeding period. The specific activities of GSH *S*-transferases for CDNB, DCNB, and NBC attained levels of 6 to 11 times that of control values under these conditions, and the rate of isomerization of Δ^5-androstene-3,17-dione was also increased severalfold (not shown). Upon return of the animals that had received the BHA diet for 12 days to the control diet, these transferase activities fell to control values with a half-life of about 4 days.

GSH *S*-transferase activities with CDNB, DCNB, and NBC were proportional to the concentration of cytosol fraction in the assay systems for cytosols from BHA-fed mice, ethoxyquin-fed mice, and control mice throughout the concentration ranges used in these studies.[5] Furthermore, assays of mixtures of cytosols from control and BHA-fed mice gave additive activities. Thus, the large differences in enzymatic activities do not appear to result from the presence of readily dissociable modifiers in the cytosol preparations. Kinetic measurements with varying concentrations

[6] Specific activities of mouse liver cytosol fractions were determined with the aralkyl epoxide 1,2-epoxy-3-(*p*-nitrophenoxy)propane in several experiments (data not presented). With this substrate there was no consistent difference in levels of activity between control and BHA-fed mice. The average levels for groups of BHA-fed mice ranged from 80 to 160% of the levels in the corresponding control groups, and these differences did not appear to correlate with any identifiable experimental variables.

of GSH and CDNB indicated that the large differences between the activities of cytosols from control and BHA-fed mice result from differences in V_{max} rather than K_m (data not presented). Although caution must be exercised in interpreting measurements of aggregate activities of several catalytic proteins, the large differences observed are consistent with the interpretation that absolute changes in enzyme levels are being observed, rather than the effects of activators or the removal of inhibitors.

Effects of BHA and Ethoxyquin on Mouse Hepatic GSH S-Transferase Activities. In 12 days of 0.75% BHA in the diet, the GSH *S*-transferase specific activities of mouse hepatic cytosols rose to as high as 11 times those of control mice (Table 1). Elevations of these enzyme activities were also observed when the animals were fed 0.5% ethoxyquin in the diet. A higher concentration of ethoxyquin (1%) induced loss of body weight. Administration of phenobarbital (0.1% in drinking water for 10 days), which is known to elevate rat liver GSH *S*-transferase levels (2, 11, 12, 16, 31), also resulted in elevation of several transferase activities in mouse liver, but in no case was the effect as large as with BHA under the conditions examined (data not shown).

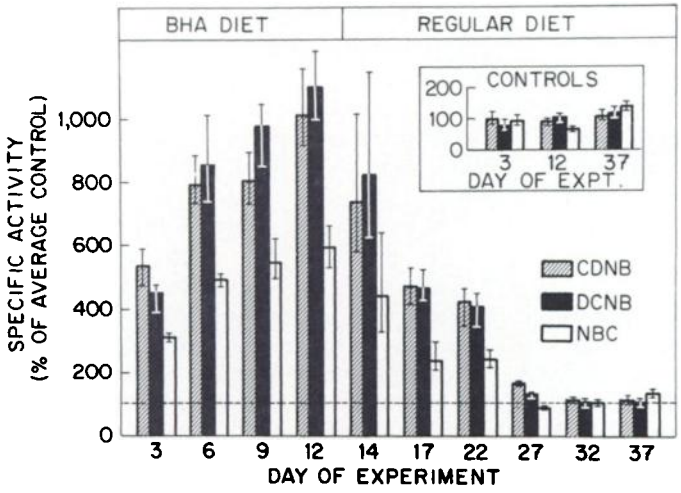

Chart 1. Response of mouse hepatic cytosol GSH *S*-transferase activities to dietary BHA. Cytosols were prepared by the calcium precipitation procedure ("Materials and Methods"). Enzyme measurements were made with CDNB, DCNB, and NBC as substrates. Each *bar* represents the mean transferase specific activity of 3 mouse livers; *vertical lines* bisecting *bars*, extremes. Control values were established on mice maintained on a powdered diet throughout the 37-day experimental period; the transferase specific activities were determined on Days 3, 12, and 37, and the means of all of these measurements were assigned the value of 100% for each substrate. The mean control transferase activities (for Days 3, 12, and 37) were (μmol/min/mg): CDNB, 2.13 ± 0.11; DCNB, 0.0682 ± 0.0047; NBC, 0.217 ± 0.023. Mice in the experimental group were fed for up to 12 days on a powdered diet containing 0.75% BHA and then maintained for an additional 25 days on a control diet. Transferase measurements were made on Days 3, 6, 9, and 12 after initiation of the diet containing 0.75% BHA and on Days 2, 5, 10, 15, 20, and 25 after return of the remaining mice to the control diet. The specific enzyme activities at each time point are expressed as a percentage of the means for all of the controls; *dashed line*, 100% level. *EXPT.*, experiment.

Isoelectric Focusing of Hepatic Cytosol Fractions from Mice Fed on Control and BHA Diets. Comparison of the isolectric focusing patterns of hepatic cytosols from mice fed for 7 days on powdered diets with or without added BHA are presented in Chart 2. The GSH *S*-transferase activities of the fractions are shown for 3 substrates (CDNB, DCNB, and Δ^5-androstene-3,17-dione). Qualitatively, very similar patterns of enzyme activities were obtained with the cytosols from control mice and BHA-fed mice, and the much higher enzymatic activities of the latter, still evident after the electrofocusing, appeared to be associated with correspondingly higher peaks of absorbance at 280 nm. The distribution of enzymatic activities demonstrates that the increased activities due to BHA administration are associated with multiple GSH *S*-transferase species that differ in their isoelectric points.

Effect of Treatment with BHA on Rat Hepatic GSH S-Transferases. In the rat, as in the mouse, the feeding of BHA caused the elevation of the activities of several GSH *S*-transferases. The results obtained with rats fed for 8 days on a diet containing 0.75% BHA are given in Table 2. The wet liver weight increased from 8.3 ± 0.3 to 10.0 ± 0.5 g. In the rat the elevations of the specific activities observed with each of 5 substrates were much more modest (40 to 110% increase) than were those observed in mice. Moreover, the relative activities toward the different substrates were quite different in mice and rats (Tables 1 and 2).

Isoelectric Focusing of Partially Purified Liver Cytosol GSH S-Transferases from Rats Fed on Control and BHA Diets. The GSH *S*-transferases of hepatic cytosols of rats fed for 8 days on a diet containing 0.75% BHA and of rats fed on the control diet were purified partially and were subjected to isoelectric focusing as described in Chart 3.

The patterns of transferase activities in the isoelectric focusing fractions from these 2 preparations are qualitatively very similar. However, in the preparation from BHA-fed rats, significantly higher enzyme activities are observed for each of the transferase peaks, except in the pH 7.7 to 7.8 region. The peak at pH 9.0 to 9.1 may be primarily transferase A (19) on the basis of both its isoelectric point

Table 1

GSH S-transferase activities of liver cytosol fractions of mice treated with BHA and ethoxyquin

The cytosols were prepared by the calcium precipitation procedure ("Materials and Methods").

Treatment	Duration of treatment (days)	Animals/ group	GSH *S*-transferase specific activities (nmol/min/mg)			
			CDNB	DCNB	NBC	Δ^5-Androstene-3,17-dione[b]
Powder diet	12	3	1,940 ± 103[a]	71.3 ± 0.3	141 ± 1	3.3 ± 0.7
BHA (0.75%)	12	3	21,600 ± 1,600	752 ± 39	1,288 ± 84	17.3 ± 0.6
Powder diet	12	5	2,280 ± 120	27.0 ± 7	266 ± 7	4.0 ± 0.5
Ethoxyquin (0.5%)	12	5	11,350 ± 1,400	179 ± 3	865 ± 86	12.3 ± 1.3

[a] Mean ± S.E. of determinations on individual livers.

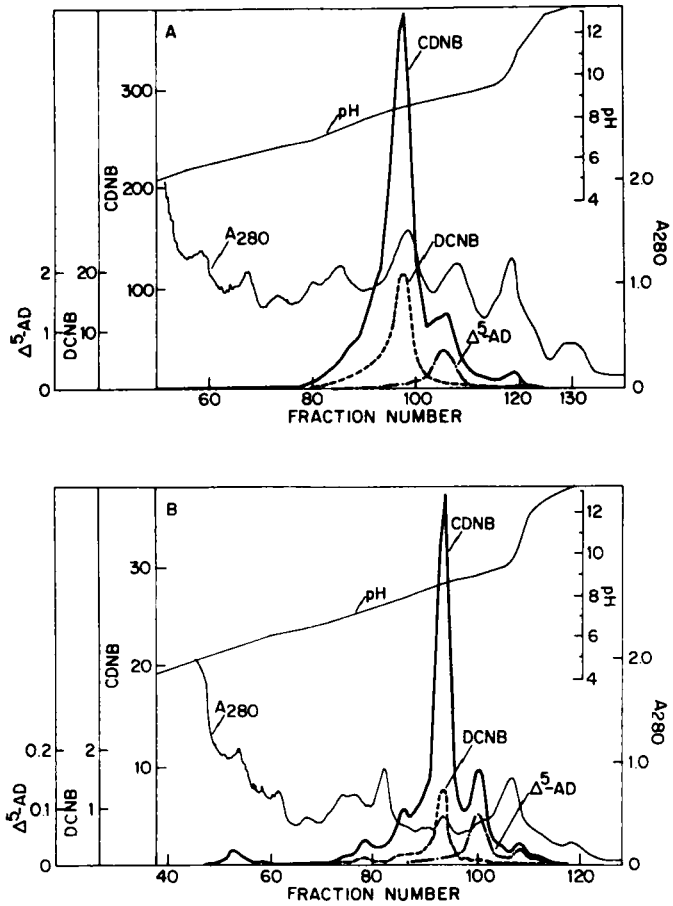

Chart 2. Isoelectric focusing patterns of liver cytosols from mice fed a BHA-containing diet (*A*) and a control diet (*B*). Note the 10-fold scale difference in the enzyme activities for *A* and *B*. Two groups of mice (10 animals in each) were fed for 7 days a powdered diet or the same diet containing 0.75% BHA. The pooled liver weights were 15.9 and 19.9 g, respectively. The livers were homogenized at 0° in 0.25 M sucrose, and the total volume of homogenate was made up to 320 ml. To the supernatant obtained by centrifugation at 9000 × *g* for 15 min was added 0.1 volume of 0.1 M $CaCl_2$ in 0.25 M sucrose. Centrifugation at 37,000 × *g* for 15 min then yielded a clear supernatant (cytosol fraction). The protein concentrations and specific activities (CDNB) were 3.16 mg/ml (1.94 μmol/min/mg) and 3.97 mg/ml (19.8 μmol/min/mg) for the preparations obtained from control diet and BHA-fed mice, respectively. 2-Mercaptoethanol was added to a final concentration of 14.4 mM, and the preparations were stored at −80° until further processing. Portions (51 ml) of each cytosol were subjected to isoelectric focusing by inclusion in the light solution of a sucrose density gradient to which were added 2.4 ml of Ampholine (pI 3.5 to 10) and 0.3 ml of Ampholine (pI 9 to 11). The electrofocusing was carried out at 1° for 69 hr at 900 V. Fractions of 1.05 ml were collected. The absorbance of the effluent was monitored at 280 nm, and the fractions were assayed for GSH *S*-transferase activities with CDNB, DCNB, and Δ5-androstene-3,17-dione (*Δ5-AD*) as substrates. Although the distribution patterns of enzymatic activities are qualitatively quite similar, markedly increased transferase activities are observed at several pI's in the sample (*A*) derived from BHA-fed mice.

and its ratio of activities with CDNB and DCNB. In the region above pH 9.5, the activity with Δ5-androstene-3,17-dione is characteristic of transferase B; transferase AA, which has an isoelectric point of 10 (19), may account for the differences in the ratio of the 2 enzymatic activities shown. The activity with 1,2-epoxy-3-(*p*-nitrophenoxy)propane at pH 7.7 to 7.8 is probably attributable to transferase E. Transferase C has an isoelectric point of 8 (19) and, based on its relative concentration and specific activity with DCNB (19), normally accounts for about 50% of the total activity with this substrate. However, in these preparations, only minor activity with DCNB appears in this pH region. A major peak of activity with CDNB and DCNB at pH 8.4 to 8.5, which does not correspond in isoelectric point to any of the transferases known to be active with these substrates (19), may be related to transferase C.

Effect of Rodent Liver Cytosols and Purified GSH *S*-Transferases on the Mutagenic Activity of the Urine of Mice Treated with BP

Liver Cytosol and Microsome Fractions of Rats and Mice Fed on BHA. Pools of urine collected during 24-hr periods immediately preceding (control urine) and following (BP urine) the injection of BP (100 mg/kg) into CD1 mice were sterilized and hydrolyzed with β-glucuronidase as described. When portions (0.2 ml) of the control urine were added to the mutagenicity assay system, the number of revertants per plate was not significantly or systematically different from that observed when urine was omitted from the assay system. Thus, for tester strain TA100, the number of revertants per plate was 165 ± 3.1 (n = 18) in the presence of 0.2 ml of urine and 169 ± 3.8 (n = 18) in its absence. For tester strain TA98, the corresponding numbers are 34 ± 2.0 (n = 12) and 30 ± 1.6 (n = 12). In contrast, the addition of 0.2 ml of similarly treated urine from mice that had received BP resulted in the production in one series of 303 to 345 revertants/plate in excess of the above control values (Tables 3 to 6) with either tester strain TA98 or TA100. A lower degree of mutagenicity was observed in a second series of experiments (Table 7), in which the BP urine was obtained from younger mice (see "Materials and Methods"). In multiple checks strict proportionality between the number of revertants per plate in excess of controls and the volume of BP urine added was observed.

The addition of liver cytosol (0.2 ml) derived from BHA-fed rats and GSH (5 mg) to the top agar with the 0.2-ml aliquot of BP urine reduced the number of revertants in excess of that of controls to about one-fourth for both tester strains TA98 and TA100 (Tables 3, 4, and 6). This effect was dependent upon the presence of both GSH and the cytosol fraction, since GSH itself was completely ineffective and the rat liver cytosol fractions themselves at the level tested were also essentially inactive. The degree of reduction in mutagenic activity of BP urine was dependent upon the amount of GSH added, up to saturating concentrations (Table 3), and upon the amount of liver cytosol (Table 4). Sulfhydryl compounds other than GSH, such as L-cysteine, cysteamine, and dithiothreitol, at molar levels comparable to 5 mg of GSH per plate were completely ineffective (Table 3). The cytosols obtained from rats fed the control powdered diet were far less effective than were those from the BHA-fed animals in reducing the mutagenicity of BP urine for tester strain TA100 (Table 4). Cytosols from BHA-fed rats and those from control rats were comparable in their effectiveness when tested at 0.1 ml/plate with TA98 (Table 6). However, since this volume appeared to be maximally effective in reducing mutagenicity, at least in the case of the BHA-fed rat cytosol (0.2 ml was not significantly more effective than was 0.1 ml), it is not possible to infer from these data the relative effectiveness of these 2 cytosol preparations with TA98.

Table 2

GSH S-transferase activities of liver cytosol fractions of rats fed control and BHA diets

The cytosols were prepared by the calcium precipitation procedure ("Materials and Methods"). The enzymes were assayed on individual livers of 5 rats fed the diet containing 0.75% BHA and 6 rats fed the control powder diet for 8 days.

Diet	Protein concentration (mg/ml)	GSH S-transferase specific activities (nm/min/mg)				
		CDNB	DCNB	NBC	1,2-Epoxy-3-(*p*-nitrophenoxy)propane	Δ^5-Androstene-3,17-dione
Powder diet	22.0 ± 0.6	918 ± 18	46.0 ± 1.5	193 ± 5	20.5 ± 1.4	34.1 ± 4.0
BHA (0.75%)	21.3 ± 0.8	1800 ± 84[a]	95.3 ± 2.0[a]	346 ± 7[a]	34.4 ± 1.3[a]	47.2 ± 2.1[b]
Ratio of BHA to control		2.0	2.1	1.8	1.7	1.4

[a] Significantly different from controls ($p < 0.001$).
[b] Significantly different from controls ($p < 0.05$).

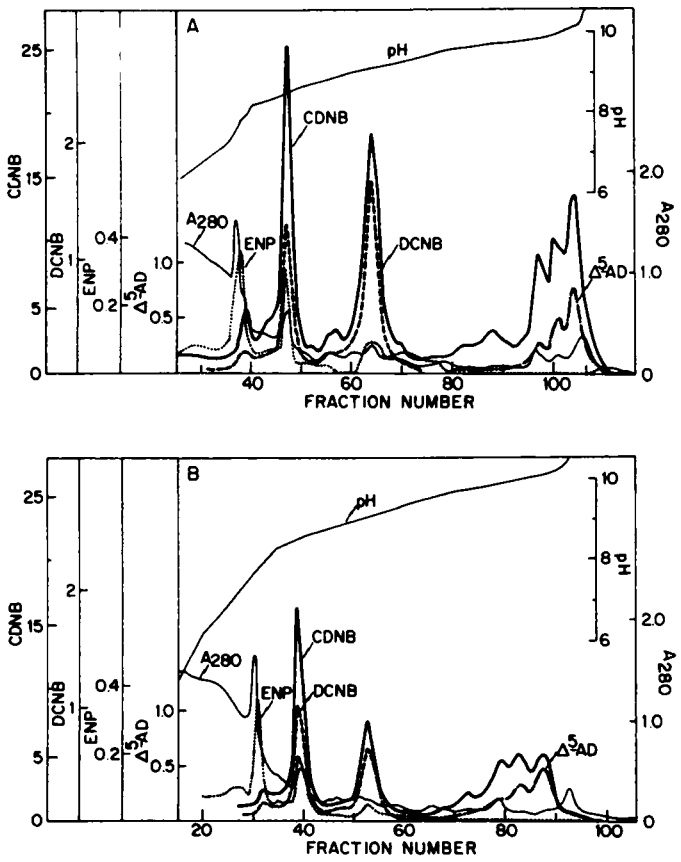

Chart 3. Isoelectric focusing patterns of liver cytosols from rats fed a BHA-containing diet (*A*) and a control diet (*B*). Five rats were fed for 8 days a powdered diet containing 0.75% BHA; 6 rats received the same diet without BHA. Rat liver cytosols were prepared as described under "Materials and Methods." Partial purification of the GSH *S*-transferases was performed essentially according to the initial steps of the procedure described by Habig *et al.* (15), including passage through DEAE-cellulose, precipitation with ammonium sulfate, and dialysis. These procedures increased the specific activities (for CDNB) 3.3- to 3.5-fold. The recoveries of enzyme activities in this fraction in relation to the original cytosols varied from 80 to 105% when measured with each of 5 substrates. A fractional volume of each preparation, equivalent to 10 ml of the corresponding cytosol fraction, was subjected to isoelectric focusing for 4.5 days (900 V) at 1° in a sucrose gradient containing the following Ampholine solutions: pI 9 to 11, 1.4 ml; pI 8 to 9.5, 1.2 ml; and pI 6 to 8, 0.1 ml. Fractions of 1.03 ml were collected. The absorbance of the effluent was monitored at 280 nm, and the fractions were assayed for GSH *S*-transferase activities with CDNB, DCNB, 1,2-epoxy-3-(*p*-nitrophenoxy)propane (*ENP*), and Δ^5-androstene-3,17-dione (Δ^5-*AD*). Although the distribution patterns of enzymatic activities are qualitatively quite similar, increased transferase activities are observed at several pI's in the sample (*A*) derived from the BHA-fed rats.

Table 3

Effect of GSH and other sulfhydryl compounds on the reduction by rat liver cytosol of the mutagenic potency (for TA100) of urines obtained from mice treated with BP

The cytosol was obtained from rats fed for 10 days on a powdered diet containing 0.75% BHA. The cytosol was prepared as described under "Materials and Methods." The volume of urine added to each plate was 0.2 ml.

Additions to mutagenicity assay system	No. of revertants/plate		
	Control urine	BP urine	No. of revertants in excess of controls
None	154 ± 2[a]	468 ± 5	314
Glutathione, 5 mg	151 ± 3	472 ± 1	321
Rat liver cytosol (BHA diet), 0.2 ml	132 ± 3	451 ± 17	319
Rat liver cytosol (BHA diet), 0.2 ml, + GSH, 5 mg	141 ± 2	227 ± 2	86
None[b]	177 ± 2	491 ± 3	314
	151 ± 2	489 ± 4	338
	137 ± 2	482 ± 1	345
Rat liver cytosol (BHA diet), 0.2 ml			
+ Cysteine, 2.59 mg	154 ± 1	488 ± 1	334
+ Cysteamine, 1.81 mg	151 ± 2	493 ± 5	342
+ Dithiothreitol, 2.51 mg	167 ± 5	510 ± 2	343
+ GSH, 0.5 mg	144 ± 6	323 ± 2	179
+ GSH, 1.5 mg	143 ± 1	285 ± 4	142
+ GSH, 5.0 mg	160 ± 9	238 ± 3	78
	156 ± 3	257 ± 6	101
+ GSH, 25 mg	144 ± 1	237 ± 2	93

[a] Mean of duplicate determinations ± one-half of the difference between them.
[b] These control values were obtained in different experiments done on different days.

As shown in Tables 5 and 6, the liver cytosols (0.1 ml, equivalent to approximately 8.33 mg fresh liver weight) obtained from mice fed the BHA-containing diet were highly effective in reducing the mutagenicity of BP urine for tester strain TA100 (to less than 15% of controls) and TA98 (to about 7% of control values), provided that GSH was also present. The liver cytosols obtained from mice fed the control diet were ineffective in reducing the mutagenicity of BP urine for TA100 but quite effective in doing so for TA98 (Tables 5 and 6). Boiled cytosols were not effective (data

Table 4

Effects of liver cytosols from rats maintained on control and BHA diets on the mutagenic potency (for TA100) of urines from mice treated with BP

All plates contained 5 mg GSH. The cytosols were obtained from rats maintained for 10 days on powdered diets with or without 0.75% BHA. Cytosols were prepared as described under "Materials and Methods." The volume of urine added to each plate was 0.2 ml.

Additions to mutagenicity assay system (ml)	No. of revertants/plate		
	Control urine	BP urine	No. of revertants in excess of controls
None[a]	177 ± 2[b]	491 ± 3	314
	151 ± 2	489 ± 4	338
	137 ± 2	482 ± 1	345
Rat liver cytosol (BHA diet)			
0.003	176 ± 4	515 ± 7	339
0.01	172 ± 3	387 ± 2	215
0.03	179 ± 1	305 ± 6	126
0.10	173 ± 2	237 ± 2	64
	154 ± 2	261 ± 2	107
0.2	156 ± 3	257 ± 6	101
	160 ± 9	238 ± 3	78
Rat liver cytosol (control diet)			
0.10	167 ± 2	415 ± 5	248
0.20	166 ± 2	365 ± 1	199
	171 ± 3	346 ± 3	175

[a] These control values were obtained in different experiments on different days.

[b] Mean of duplicate determinations ± one-half of the difference between them.

Table 5

Effects of liver cytosols and microsomes from mice maintained on control and BHA diets and effects of GSH on mutagenic potency (for TA100) of urines obtained from mice treated with BP

All plates contained 5 mg of GSH, except where indicated. The mouse liver cytosols and microsomes were prepared as indicated under "Materials and Methods." The volume of urine added to each plate was 0.2 ml.

Additions to mutagenicity assay system	No. of revertants/plate		
	Control urine	BP urine	No. of revertants in excess of controls
None	150 ± 8[a]	496 ± 1	346
Mouse liver cytosol (BHA diet)			
0.001 ml		391 ± 3	241
0.01 ml		231 ± 2	81
0.1 ml	148 ± 2	197 ± 1	49
0.1 ml (GSH omitted)	142 ± 7	497 ± 1	355
Mouse liver cytosol (control diet)			
0.01 ml		488 ± 5	338
0.1 ml	145 ± 3	463 ± 4	318
Mouse liver microsomes			
BHA diet, 0.1 ml	161 ± 2	465 ± 3	304
Control diet, 0.1 ml	150 ± 3	494 ± 14	344

[a] Mean of duplicate determinations ± one-half of the difference between them.

not presented).

In both mice (Table 5) and rats (not shown), the microsome fraction (from BHA-fed or control animals) was almost completely ineffective in reducing the mutagenicity of BP urine.

Purified Rat Liver GSH *S*-Transferases. The above experiments with mouse and rat liver cytosols strongly suggested that a soluble GSH-dependent enyzme system was responsible for the inactivation of the mutagenic metabolites of BP present in the urine of mice to whom this hydrocarbon had been administered. Consequently, highly purified preparations of rat liver GSH *S*-transferases A and B were tested by direct addition to the assay systems (TA98 and TA100). Each was effective in reducing the mutagenic activity of BP urine (Table 7). The effect was related to the amount of enzyme protein added. There appears to be some specificity with respect to both the tester strain and the type of transferase. At the highest level tested, both transferases were effective in reducing the number of revertants in excess of controls induced in TA100. The reduction produced by transferase A (118 μg/plate) was to one-half that of control values and the reduction produced by transferase B (245 μg/plate) was to one-fifth that of control values. In the case of the frame-shift mutant (TA98), the effectiveness of transferase B appeared to be considerably less than that of transferase A. The preparation of transferase A used in these studies did not retain full activity during the dialysis prior to testing. This enzyme is more stable in the presence of GSH, glycerol, and EDTA (15), and its specific activity with CDNB declined from 55 to 40 μmol/min/mg when the latter 2 stabilizers were removed prior to addition of the enzyme to the mutagenicity assay system. Further loss of activity might well be expected to have occurred during the process of addition to the top agar and when the temperature was raised to above 40°.

DISCUSSION

It has been suggested that antioxidants might exert their anticarcinogenic effects, at least in part, by affecting the activities of enzymes involved in BP metabolism (44). The observations that p.o. administration of BHA to mice yielded a marked reduction in the mutagenic activity of BP metabolites in the host-mediated assay, as well as in urine, and that the latter effect was also elicited by ethoxyquin demonstrated that these antioxidants dramatically alter the metabolism of BP *in vivo* (3).

In this investigation, it was observed that liver cytosols from mice and rats, when supplemented with GSH, were capable of decreasing substantially the mutagenic activity of urine from mice that had been treated with BP and that cytosols from animals that received a BHA-supplemented diet were far more effective than were those from animals on a control diet. The observation that tissue levels of acid-soluble sulfhydryl compounds were greatly elevated by BHA administration (3) is of interest in relation to the specific requirement for GSH, in addition to hepatic cytosol, for expression of this antimutagenic effect. Among the GSH-requiring enzymes of hepatic cytosol are the GSH *S*-transferases, a family of detoxifying enzymes (19) that catalyzes the conjugation of GSH with a number of arene oxides (7,

Table 6

Effect of mouse and rat liver cytosols on mutagenic potency (for TA98) of urine from mice treated with BP

Mouse and rat liver cytosols were prepared as described under "Materials and Methods." The volume of urine added per plate was 0.2 ml.

Additions to mutagenicity assay system		No. of revertants/plate		
Cytosol	GSH (mg)	Control urine	BP urine	No. of revertants in excess of controls
None	0	27 ± 2[a]	341 ± 11	314
Rat liver cytosol (BHA diet), 0.2 ml	0	26 ± 1	325 ± 4	299
Rat liver cytosol (BHA diet), 0.2 ml	5	27 ± 2	104 ± 1	77
None	5	25 ± 2	345 ± 4	320
None	0	30 ± 1	333 ± 3	303
Rat liver cytosol (BHA diet), 0.1 ml	5	27 ± 3	108 ± 1	81
Rat liver cytosol (control diet), 0.1 ml	5	28 ± 3	130 ± 1	102
Mouse liver cytosol (BHA diet), 0.1 ml	5	26 ± 1	47 ± 1	21
Mouse liver cytosol (control diet), 0.1 ml	5	26 ± 1	164 ± 3	138

[a] Means of duplicate determinations ± one-half of the difference between them.

Table 7

Effects of purified rat liver GSH S-transferases A and B on the mutagenicity of urine of mice treated with BP

The indicated quantities of enzyme protein were added in 0.1 ml of 10 mM potassium phosphate buffer (pH 6.8) containing 0.3 mM GSH. The GSH (5 mg, 16.3 μmol) was added to the mutagenicity assay system in 0.1 ml of the same buffer. The amount of GSH (0.03 to 0.06 μmol) added with the enzyme was negligible. The volume of urine added per plate was 0.2 ml.

Additions to mutagenicity assay system		No. of revertants/plate					
		TA100			TA98		
GSH (mg)	Transferase (μg protein)	Control urine	BP urine	No. of revertants in excess of controls	Control urine	BP urine	No. of revertants in excess of controls
		Transferase A					
0	0	164 ± 5[a]	328 ± 7	164	38 ± 3	173 ± 12	135
5	0	160 ± 2	322 ± 3	162	33 ± 1	182 ± 1	149
5	118	159 ± 4	237 ± 7	78	35 ± 3	89 ± 4	54
5	35.4		303 ± 4	144		121 ± 10	86
5	11.8		323 ± 3	164		154 ± 2	119
5	1.18		336 ± 6	177		180 ± 2	145
		Transferase B					
0	0	183 ± 15	437 ± 4	254	33 ± 4	194 ± 8	161
5	245	191 ± 3	238 ± 20	47	40 ± 3	153 ± 2	113
5	73.5		301 ± 10	110		173 ± 2	133
5	24.5		369 ± 11	178		184 ± 1	144
5	2.45		417 ± 6	226		195 ± 2	155

[a] Means of duplicate determinations ± one-half of the difference between them.

18, 32–34, 40) as well as with a variety of other hydrophobic electrophiles (19). Some of the relationships between these enzymes and chemical carcinogenesis have been the subject of a recent review (36). Liver cytosols from BHA-fed mice and rats exhibited much higher levels of GSH *S*-transferase activities than did those of control animals. Isoelectric focusing of these preparations revealed that these BHA-mediated elevations of hepatic transferase activities represented increases in multiple GSH *S*-transferase species. After electrofocusing of mouse liver cytosol in which some of these enzyme activities were enhanced more than 10-fold by BHA pretreatment, it was especially evident that the fractions possessing these elevated transferase activities also exhibited much higher absorbance at 280 nm, suggesting that increased amounts of enzyme protein were present. Further study will be required to elucidate the mechanism by which BHA and ethoxyquin mediate the observed increases in the GSH *S*-transferases. Transferases A and B, purified from rat liver, when added to the mutagenicity assay system with GSH, were both very effective in

reducing the mutagenic activity of urine from mice that had been treated with BP. The nature of these relatively stable mutagenic metabolites was not investigated; since the GSH *S*-transferases have extremely broad ranges of substrate specificity (19), we do not infer that epoxides necessarily comprise all of the urinary mutagens inactivated by these enzymes. Numerous investigations demonstrating the conjugation of arene oxides with GSH by liver homogenates and soluble fractions were summarized by Sims and Grover (34) in 1974. More recently, it has been established that the K-region epoxide of BP can serve as a substrate for some of the GSH *S*-transferases. Pure preparations of transferases A, B, C, and E of rat liver and transferases β and δ of human liver were found to possess substantial activity in the conjugation of benzo(*a*)pyrene 4,5-oxide with GSH (32). This reaction was also catalyzed by a GSH transferase purified from sheep liver on the basis of its activity with naphthalene 1,2-oxide and GSH (18).

Thus, at least some of the mutagenic metabolites of BP are GSH transferase substrates and, consequently, the observed antimutagenic effect of dietary BHA may be accounted for at least partly by the observed increases in the levels of these enzymes.

The role of the GSH *S*-transferases in detoxification of non-K-region epoxides and diol-epoxides of BP is less clear. The relative instability of these metabolites precludes their presence in the urine preparations used in these studies. However, the non-K-region metabolites of BP are of special interest (20, 23) with respect to the anticarcinogenic effects of BHA; thus the possibility that they may serve as GSH *S*-transferase substrates *in vivo* is an important consideration. Several lines of evidence indicate that benzo ring epoxides of BP and bay-region epoxides of BP dihydrodiols, as well as diol-epoxides of several benzo(*a*)anthracene derivatives, may be subject to detoxification by enzymatic conjugation with GSH (18, 33, 40).

The conjugation of arene oxides, as well as of numerous other substrates possessing an electrophilic carbon atom, with GSH yields products containing the exceptionally stable thio ether linkage (19); these products may be excreted in the bile as GSH conjugates or further metabolized into cysteine conjugates or mercapturic acids (7). The ability of BHA and ethoxyquin to increase dramatically the levels of hepatic GSH *S*-transferase activities thus could constitute one of the mechanisms by which these 2 antioxidants exert protective effects against BP-induced carcinogenesis. The enhancement of these hepatic enzyme activities may well have much wider significance with respect to chemical carcinogenesis and toxicity, since the GSH *S*-transferases are capable of detoxifying a wide variety of hydrophobic electrophiles, either by binding them covalently, *e.g.*, metabolites of dimethylaminoazobenzene (25) and 3-methylcholanthrene (27, 35), or by catalyzing their conjugation with GSH (7, 18, 19).

BHA and ethoxyquin each exhibit pronounced effects on at least several of the enzymes involved directly or indirectly in the metabolism of carcinogens (4, 8–10, 13, 17, 21, 29, 37, 44). The relative importance of any specific metabolic pathway is likely to depend upon the nature and route of administration of the carcinogen and may well be expected to vary widely with tissue, strain, and species.

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