

Enhancements of Mouse Hepatic Cytosol Enzyme Activities Involved in UDP-Glucuronic Acid Synthesis, Glutathione Reduction and Conjugation with Butylated Hydroxyanisole (BHA) and Its Structural Analogs*

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Activities of hepatic cytosol enzymes involved in UDP-glucuronic acid synthesis as well as in glutathione reduction and conjugation systems were determined following administrations of butylated hydroxyanisole (approximately 5 mmol/kg body weight/day) and of equimolar intake doses of its structural analogs. These compounds included the multi-functional group side chain compounds (t-butyl hydroquinone, 4-hydroxyanisole, hydroquinone, benzoquinone) and the mono-functional side chain compounds (t-butyl benzene, anisole, phenol). They were administered to mice for 10 days either by mixing them in the diet or by oral intubations. Results showed that glutathione S-transferase activities were markedly increased by all tested compounds except for the t-butyl benzene. Activities of glutathione reductase and glucose 6-phosphate dehydrogenase were increased together only by BHA and t-butyl hydroquinone. UDP-glucose dehydrogenase and NADH:quinone reductase activities were significantly elevated by the multi-functional side chain compounds, but not by the mono-functional analogs. The relations between chemical structures of tested BHA analogs and elevations of the measured hepatic cytosol conjugation (detoxification) system enzyme activities for the metabolism and excretion of BHA analogs are discussed.

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The phenolic antioxidants such as 2(3)-tert-butyl-4-hydroxyanisole (BHA) and 3,5-di-tert-butyl-4-hydroxytoluene (BHT) are widely used as additives to protect processed food products from free-radical induced damage (Chipault, 1966; Hathway, 1966). Furthermore, dietary administrations of BHA, BHT and of some other phenolic antioxidants have been demonstrated to protect against tumor induction caused by chemical carcinogens of diverse structures in a variety of rodent tissues (Slaga et al., 1977; Wattenberg, 1980; Weisburger et al, 1977). Studies on biochemical mechanisms for the observed anticarcinogenic effects indicated that BHA not only enhances the activity of a variety of conjugation and detoxification enzymes (Anderson et al., 1981; Benson et al., 1978;1980; Cha et al., 1979; 1982; Dock et al., 1982; Miranda et al., 1981; Moldeus et al., 1982), but also shifts the metabolic profile of benzo (a) pyrene, a well known carcinogen (Cha et al., 1979; Ioannou et al., 1982; Lám et al., 1977). Both of these processes may have lowered the intracellular concentrations of reactive carcinogens (Cha et al., 1979; Anderson et al., 1981; Ioannou et al., 1982).

Associated with their uses as synthetic food preservatives, there have been several studies dealing with their metabolism (reviewed by Branen, 1975). They showed that BHA is rapidly metabolized and excreted primarily as glucuronide conjugates of BHA and t-butyl hydroquinone (TBHQ). Furthermore, when rats were pretreated with BHA, the rate and proportion for excretion of a given dose of labelled BHA, administered at a later time, as glucuronides of TBHQ were accelerated (Daniel et al., 1973). This suggested that the administered BHA may enhance not only the rate of its own metabolism to produce TBHQ, but also the rate of its subsequent glucuronidation. This further suggested that the metabolically

produced TBHQ also may have been responsible for the elevation of the enzyme activity involved in its glucuronidation.

Except for the presence of t-butyl side chain, the chemical structures of BHA and its O-demethylated metabolite TBHQ are identical to those of p-hydroxyanisole (HA) and p-hydroquinone (HQ), respectively (Fig. 1). These hydroquinones serve as excellent substrates of glucuronidation and are readily excreted as glucuronides (Fig.2). Alternatively, these hydroquinones are easily auto-oxidized to quinones such as t-butyl quinone(TBQ) and p-benzoquinone (BQ), presumably going through the unstable and reactive semiquinone steps (i.e., TBSQ and SQ in Fig. 1). The highly reactive semiquinones are inactivated by the cellular nucleophilic "buffers" such as the reduced glutathione (GSH) and the glutathione S-transferase proteins (ligandins), individually or cooperatively, by enzymatic catalysis resulting in thioester conjugates. Such formation of thioester conjugates represents the first step in biosynthesis of mercapturic acids which are readily eliminated from the body.

Thus, we have selected structural analogs of BHA which contain either one or more of the following chemical moieties attached to the benzene ring: t-butyl, methoxyl, or hydroxyl groups (all are components of BHA). Compounds like TBHQ, HA, HQ, and BQ contain at least 2 of the 3 functional groups and thus are named as multi-functional compounds. Additionally, we have included t-butyl benzene, anisole, and phenol which are the mono-functional compounds. The effects produced by administering these compounds on the activities of several hepatic cytosol enzymes that have been observed to be markedly enhanced by the BHA treatment were determined and were compared with those produced by BHA. These enzymes include the NADH:

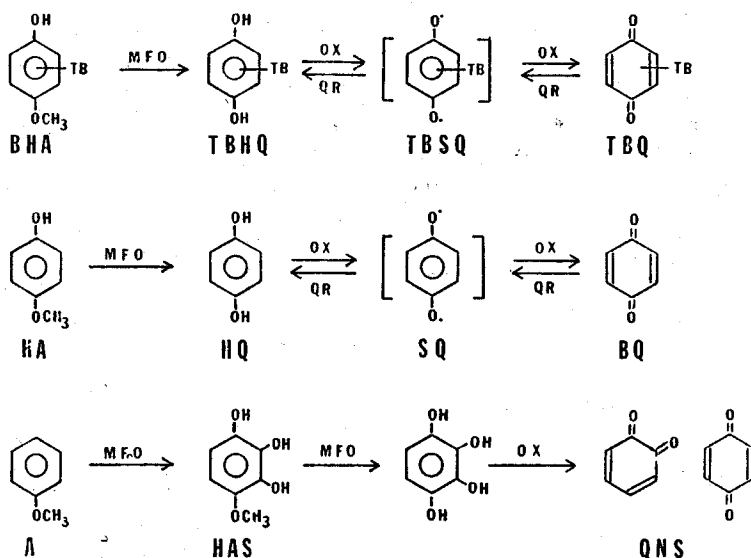


Fig. 1. Chemical structures of butylated hydroxyanisole and hydroxyanisole, as well as their metabolic scheme.

Abbreviations: BHA=2(3)-tert-butyl-4-hydroxyanisole, TBHQ=tert-butylhydroquinone, TBSQ=tert-butylsemiquinone, TBQ=tert-butylquinone, HA=p-hydroxyanisole, HQ=p-hydroquinone, SQ=semiquinone, AS=anisole, HAS=isomers of hydroxylated anisoles, QNS=isomers of quinones, MFO=mixed function oxidation, OX=auto-oxidation, and QR=NAD(P)H:quinone reductase

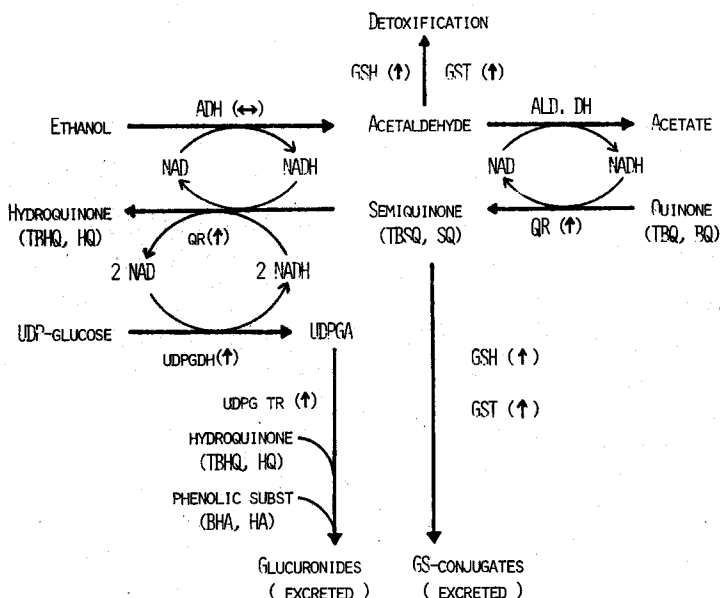


Fig. 2. Schematic diagram for possible involvement of elevated NADH:quinone reductase (QR) activity in the accelerated synthesis of UDP-glucuronic acid (UDPGA) via the enhanced UDP-glucose dehydrogenase (UDPGDH) in the liver of BHA fed mice.

Especially in the presence of TBQ, a known metabolite of BHA, both TBQ and NADH will be converted to TBHQ and NAD, respectively. This will lower the intracellular ratio of NADH: NAD, and will permit accelerated synthesis of UDPGA. This, together with the elevated microsomal UDP-glucuronyl transferase (UDPGA-Tr) activity, would readily conjugate and eliminate the phenols (i.e., BHA, HA, TBHQ, and HQ) and alcohols. The diagram further illustrates the possible detoxification mechanisms for aldehydes (the acetaldehyde arising from ethanol oxidation and the formaldehyde from O-demethylation of BHA and HA) and semiquinones (e.g., TBSQ and SQ). In addition to the increases in QR, UDPGA-Tr and UDPGDH activities, the BHA feeding also elevated the GSH levels and GST activities (indicated by upward arrow). Therefore, the reactive aldehydes and semiquinones could be also detoxified by accelerated conjugations with GSH and easily excreted. Alternatively, the reactive acetaldehyde could be converted to innocuous acetate by the efficiently operating aldehyde dehydrogenase (Ald. DH), and the reactive semiquinones would be effectively glucuronidated following the initial conversion to hydroquinones.

quinone reductase (EC 1.6.99.2) (NADH:QR) and UDP-glucose dehydrogenase (EC 1.1.1.22.) (UDPGDH) involved in the synthesis of UDP-glucuronic acid, which is the co-substrate required for glucuronidation. Also, we have determined the activities of glucose 6-phosphate dehydrogenase (EC 1.1.1.49.) (G6PDH) and glutathione reductase (EC 1.6.4.2.) (GR) which are involved in the regeneration of reduced glutathione required in the conjugation reaction catalyzed by the glutathione S-transferase (EC 2.5.1.18.) (GST).

Therefore, by comparing the effects produced by each of these structural analog compounds of BHA, we have attempted to examine which of the particular chemical moieties attached to the BHA is causing the elevations of several cytosolic enzyme activities thought to be involved in the anticarcinogenic mechanisms of BHA.

MATERIALS AND METHODS

A. Treatment of Animals

Female CD-1 mice (4 to 5 weeks old), purchased from Charles River Breeding Laboratories (Wilmington, MA), were housed in hanging stainless steel wire cages (4-5 mice per cage) with a 12 hour light-dark cycle (7 AM-7

PM) and with free access to tap water and powdered Purina Laboratory Chow (code 5001; Ralston Purina Co., St. Louis, MO) for 1 to 2 weeks of acclimation. Animals were fasted for 24 hours prior to initiation of administering the various experimental compounds for 10 days. These compounds were either mixed with the powdered diet (for solid compounds) or suspended in corn oil (for volatile compounds) at concentrations shown in Table 1.

As a preliminary screen to determine the maximally tolerated doses, the powdered diet and corn oil containing various amounts of the compounds to be studied were given to mice for 7 days. The weight gain and food consumed were monitored daily. The highest tolerated doses without any apparent toxicity were found to be much greater than the dose (5 mmoles/kg body weight/day) employed in this study.

The basis for arriving at this particular dose (5 mmol/kg/day) of various compounds used in this study is as follows: each mouse weighting 25gm consumes 3.0 to 3.5 gm of 0.75% BHA diet per day. This is equivalent to 5.0 to 5.8 mmol BHA/kg/day, respectively. Considering that BHA was mixed with food, which may have prevented its complete absorption, we have chosen the experimental dose of

Table I. Administered dialy doses of BHA and its structural analogs

Compounds	Route of Administration	N	Daily Doses
Control	Diet	5	Powdered Purina Diet (code 5001)
	Gavage	4	Corn Oil (5 ml/kg body wt.)
BHA ¹	Diet	10	7.5mg/gm diet
TBHQ	Diet	5	4.6mg/gm diet
TBB	Gavage	5	670mg/kg body wt.
HA	Diet	5	3.5mg/gm diet
HQ	Diet	5	3.1mg/gm diet
BQ	Diet	5	3.0mg/gm diet
AS	Gavage	4	540mg/kg body wt.
PH	Gavage	5	470mg/kg body wt.

¹ BHA=2(3)-tert-butyl-4-hydroxyanisole

TBHQ=tert-butyl-hydroquinone

TBB=tert-butyl-benzene

HA=4-hydroxyanisole

HQ=p-hydroquinone

BQ=1,4-benzoquinone

AS=anisole

PH=phenol

5.0 mmol/kg/day for all tested compounds, whether mixed in the powdered diet or suspended in corn oil (given by oral injections).

B. Enzyme Assays

Animals were sacrificed by cervical dislocation and their gross body and liver weights were determined. After removing erythrocytes, the livers were homogenized individually in 35 ml of ice cold sucrose (0.25M) using a Teflon-glass homogenizer (6 strokes). All subsequent steps for isolation of hepatic cytosols and washed microsomes were as previously described (Cha et al., 1979). Protein concentrations were determined by the Markwell modification (Markwell et al., 1978) of the Lowry method using crystalline bovine serum albumin (Sigma,

St. Louis, MO) as the standard.

Activities of several cytosolic enzymes such as glucose 6-phosphate dehydrogenase (EC1.1.1.49.;G6PDH) (Lohr and Waller, 1963), oxidized glutathione (GSSG) reductase (EC 1.6.4.2.;GR) (Racker, 1955), glutathione S-transferase (EC 2.5.1.18.; GST) (Habig et al., 1974), UDP-glucose dehydrogenase (EC1.1.1.22; UDPGDH) (Mills and Smith, 1963), and NADH: quinone reductase (EC1.6.99.2.; NADH:QR) (Benson et al., 1980) were all measured using an Aminco DW 2-a spectrophotometer (American Instrument Co., Silver Spring, MD) operating in double-beam and time-base mode. For assays of G6PDH, GR and UDPGDH activity, the changes in concentrations of reduced pyridine nucleotides (i.e., NADH, NADPH) with time were determined from the differences of absorbances at 340 nm and 400 nm, sample and reference wave lengths, respectively ($AM=6.3 \times 10^3 M^{-1} Cm^{-1}$). Similarly, the dicoumarol sensitive NADH:QR activity was measured by determining the differential initial velocity of reduction of 2,6-di-chloro-indophenol (Sigma) at 600 nm-700 nm ($AM=2.1 \times 10^3 M^{-1} Cm^{-1}$) in the absence and presence of 10 μM dicoumarol. The GST activities were measured using 1-chloro-2,4-di-nitrobenzene (CDNB) or 1,2-di-chloro-4-nitrobenzene (DCNB) (both from Eastman Organic Chemicals, Rochester, NY) and reduced glutathione (GSH) as substrates.

C. Materials

Both the 2(3)-t-butyl-4-hydroxyanisole (BHA) and p-hydroxyanisole were purchased from Sigma Chemical Co. (St. Louis, MO) and the t-butyl-hydroquinone (TBHQ) and the t-butyl benzene, p-hydroquinone, p-benzoquinone, anisole, and phenol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Various other substrates for enzyme assays were obtained either from Sigma (e.g., glucose 6-phosphate, 2,6-dichloro indophenol, dicoumarol, etc.) or

from Boehringer-Mannheim Biochemicals (Indianapolis, IN) (e.g., NAD, NADH, NADP, NADPH, UDP-glucose, etc.) and were not purified further.

RESULTS

Enzymes Involved in the Synthesis of DUP-Glucuronic Acid: The activity of dicoumarol sensitive NADH: quinone reductase (NADH:QR), a cytosolic enzyme involved in the production of both NAD and hydroquinones, was increased markedly by all of the tested compounds except for *t*-butyl benzene which was given at equimolar doses. The observed increases of this enzyme activity, however, were graded in such a manner that

Table II. Effects of administering and its structural analogs on the activity of UDP-glucuronic acid synthesis enzyme system¹

Compounds	N	UDPGDH ²	NADH:QR ³
Control ⁴	10	37.6±4.6	162±24
BHA ⁵	10	121.7±7.0*	913±58*
TBHQ	5	69.9±4.5*	539±47 ⁴
TBB	5	41.3±2.9	189±30
HA	5	46.6±4.3**	429±49*
HQ	5	48.1±6.7**	489±42*
BQ	5	47.4±2.0**	427±52*
AS	5	36.3±2.4	290±40*
PH	5	37.5±4.4	393±51*

¹ Values are mean S.D. and *indicates significant differences at $p < 0.01$ and ** indicates significant differences at $p < 0.05$.

² UDPGDH=UDP-glucose dehydrogenase, (n moles NADH/min/mg)

³ NADH:QR=NADH: (quinone acceptor) oxido-reductase, (n mol 2,6-dichloroindophenol reduced/min/mg)

⁴ There were no differences in all of the determined enzyme activities between the two control groups; diet (N=5) and gavage(N=5). Thus the control values were obtained using N=10

⁵ For abbreviations, refer to Table I.

the more the number of attached side chain functional groups, the greater the increases (Table II).

The activity of UDP-glucose dehydrogenase (UDPGDH), a cytosolic enzyme responsible for the production of UDP-glucuronic acid (UDPGA) by reducing NAD to NADH, was increased moderately (up to 3.2 fold) by BHA and TBHQ (Table II). However, the enzyme activity was increased only minimally by hydroquinone, hydroxyanisole, and *p*-benzoquinone. These particular experimental compounds possess two or three side chain functional groups. Interestingly, the enzyme activity was not increased by anisole, phenol and *t*-butyl benzene, the compounds with only one functional group.

The interdependent linkages between these two cytosolic enzymes and also the possible role played by elevating these two enzyme activities simultaneously for the accelerated synthesis of UDPGA will be discussed.

Enzymes involved in the reduction of oxidize glutathione: Glucose 6-phosphate dehydrogenase is one of the principal enzymes involved in the generation of NADPH within hepatic cytosol. Administrations of the multi (two or three) functional group compounds increased this enzyme activity. However, the observed increases produced by TBHQ, hydroxyanisole, and hydroquinone were minimal and barely significant. The mono-functional compounds did not appear to increase the enzyme activity (Table III).

The activity of glutathione reductase, a cytosolic enzyme responsible for the regeneration of reduced glutathione (GSH) from oxidized glutathione (GSSG) by oxidizing NADPH, was significantly increased by BHA and TBHQ. However, the enzyme activity was not increased by hydroxyanisole, hydroquinone, and benzoquinone, the compounds with two functional groups attached to benzene. While the enzyme

Table III. Effects of administering BHA and its structural analogs on glucose 6-phosphate dehydrogenase and glutathione reductase activities¹

Compounds	N	G6PDH ²	GR ³
Control	10	12.0±1.3	59.2± 6.7
BHA ⁴	10	39.9±5.3*	109.5±12.0
TBHQ	5	16.9±2.1**	97.7±11.9*
TBB	5	10.5±2.9	72.6± 3.9*
HA	5	16.3±3.9**	59.5± 6.5
HQ	5	16.7±2.9**	66.3±5.1
BQ	5	25.8±1.4**	64.9± 6.2
AS	5	9.1±1.4**	64.9± 6.2
PH	5	14.9±2.8	73.8± 7.6**

¹ Values are mean S.D. and* indicates significant differences at $p<0.01$ and ** indicates significant differences at $P<0.05$

² G6PDH=glucose 6-phosphate dehydrogenase, (n mol NADPH formed/min/mg).

³ GR=glutathione reductase, (n mol NADPH oxidized/min/mg).

⁴ For abbreviations, refer to Table I.

Table IV. Effects of administering BHA and its structural analogs on glutathione S-transferase activities¹

Compounds	N	GST(CONB) ²	GST(DCNB)
Control	10	654± 89	17.9±2.4
BHA ³	10	15282±1280*	150.5±6.8*
TBHQ	5	5352± 852*	95.8±10.8*
TBB	5	1291± 303*	27.5±3.2*
HA	5	2341± 494*	69.6±9.6*
HQ	5	2301± 381*	75.8±9.7*
BQ	5	3902±409*	73.9±8.4*
AS	5	1687± 268*	34.1±4.7*
PH	5	2143± 374*	53.8±8.9*

¹ Values are mean S.D. and * indicates significant differences from respective controls at $p<0.01$

² GST=glutathione S-transferase activities assayed with chlorodinitrobenzene (CDNB) or di-chloro-nitrobenzene (DCNB), (n mol conjugate formed/min/mg).

³ For abbreviations, refer to Table I.

activity was increased by phenol and t-butyl benzene, the observed increases were minimal and hardly significant ($P<0.05$).

Glutathione S-transferase: All of the tested compounds produced significant elevations in the specific activities of glutathione S-transferases (Table IV). The increases produced by t-butyl benzene, anisole, and phenol (the mono-functional compounds) were, however, much smaller than those resulting with multi-functional compounds.

DISCUSSION

Except for the presence of t-butyl side chain, the chemical structures of BHA and its O-demethylated metabolite TBHQ are identical to those of p-hydroxyanisole (HA) and hydroquinone (HQ), respectively (Fig. 1). Studies on the metabolism of BHA have indicated that it was rapidly metabolized to TBHQ and excreted primarily as the glucuronide conjugates of TBHQ (Daniel et al., 1973). Preliminary results obtained in our laboratory support this. The administered hydroxyanisole is also being O-demethylated to hydroquinone and is excreted in the urine as the glucuronide conjugates of hydroquinone (unpublished observation). Therefore, both of the chronically administered BHA and hydroxyanisole are being converted primarily to TBHQ and hydroquinone, respectively, by the O-demethylation reaction (presumably, also producing large quantities of formaldehyde). Once produced, these hydroquinones serve as excellent substrates of glucuronidation and are readily excreted as glucuronides (Fig. 2).

Alternatively, these hydroquinones are easily auto-oxidized to quinones such as t-butyl quinone (TBQ) and benzoquinone (BQ), presumably going through the unstable and reactive

semiquinone (i.e., TBSQ, SQ) step (Fig. 1). In hepatic cytosol, the oxidized quinones (TBQ, BQ) are converted directly to hydroquinones (TBHQ, HQ) through the divalent reduction catalyzed by NADH:quinone reductase. As demonstrated by the results obtained with the feeding of benzoquinone, these quinones may have been responsible for the marked elevation of the cytosolic NADH:quinone reductase activity, perhaps by the mechanism of substrate induction (Table II).

Results in Table II also show that the UDP-glucose dehydrogenase (UDPGDH) activity is moderately elevated by the feedings of BHA and TBHQ. This enzyme catalyzes the production of UDP-glucuronic acid (UDPGA) and reduces NAD to NADH. The synthesis of UDPGA, a cofactor required for glucuronidation, is maintained at a low level during normal conditions and is under the control of intracellular NAD/NADH concentration ratio (Anderson et al., 1978; Moldeus et al., 1978). Thus, when the exogenous quinone substrates could be provided, the action of NADH:quinone reductase within hepatic cytosol would increase the NAD/NADH concentration ratio, thus permitting continued and accelerated synthesis of UDPGA. In the present experimental situation, perhaps the *t*-butyl quinone or benzoquinone, continuously being generated from the administered BHA and TBHQ, or hydroxyanisole and hydroquinone, respectively, would serve as the required quinone substrates for the cytosolic NADH:quinone reductase and maintain the NADH at a low level. Under these circumstances, the elevated UDPGDH and NADH:quinone reductase activities, as well as the provision of quinone substrates, would increase the NAD/NADH concentration ratio accelerating the UDPGA synthesis. This, together with the elevated UDP-glucuronyl transferase activity of microsomes (Cha et al.,

1979; 1982), would accelerate the overall rate of glucuronide formation (Moldeus et al., 1982) and also the elimination of these chronically administered phenolic compounds (Daniel et al., 1973; Ulland et al., 1973; Lake et al., 1976) (Fig. 2). In fact, in a recent study using isolated hepatocytes obtained from the BHA fed mice, we have demonstrated 5-fold increases in the formation of glucuronides of such phenolic drugs like harmol and paracetamol (Moldeus et al., 1982). Therefore, although it remains to be proven, the metabolic provision of quinone substrates (i.e., TBQ, BQ) arising from the administered phenolic antioxidants may have led to an increased synthesis of UDPGA via the complementary catalytic actions of NADH:quinone reductase and the UDPGDH. By doing so, the rates for elimination of the administered phenolic antioxidants and their metabolites, as well as the phenolic metabolites of chemical carcinogens (i.e., hydroxylated metabolites of benzo(a)pyrene) may be accelerated (Dock et al., 1982; Anderson et al., 1981; Ioannou et al., 1982).

Results shown in Tables II, III, and IV indicate that activities of all hepatic cytosol enzymes which we have measured are elevated by administering the BHA analogs which contain at least two of the three chemical moieties attached to the benzene ring. For example, although the administered doses of the mono-functional compounds were equal to those of the multi-functional compounds, the ability of the former compounds to elevate the measured enzyme activities was much lower than those produced by the latter compounds. This suggests the importance of not only the number of attached chemical moieties, but also perhaps their intra-molecular interactions for the additional increases of these cytotoxic activities.

Cellular GSH, by itself or together with

glutathione S-transferases (GST), can act as non-critical nucleophiles for additional reactions and play a major role in the deactivation of electrophilic compounds such as semiquinones, formaldehydes and reactive carcinogenic metabolites (Smith et al., 1977).

Such formation of thioester conjugates represents the first step in biosynthesis of mercapturic acids which are biologically inactive and are more readily eliminated from the body. Furthermore, the binding actions of GST proteins (ligandin) represent an equally, if not more, important function to serve as a "buffer" protein for the deactivation of reactive metabolites (Gregus and Klassen, 1982; Jakoby, 1977). In these contexts, the concentration of GSH (5-10 mM) and of GST (3-10% of cytosolic proteins) in the normal mammalian liver are extraordinarily high, yet, their levels were further elevated by administering BHA and several other phenolic compounds used in this study (Table IV). Although the underlying mechanisms for these increases are not known, it is plausible that large quantities of the highly reactive formaldehyde (produced by O-demethylation) and semiquinone free-radicals (produced by hydrogen abstraction) arising from these phenolic compounds could have provided the necessary chronic inductive pressures leading to elevations of both GSH and GST.

Results in Table III show that only BHA and TBHQ administrations produced the simultaneous increases of two related enzyme activities involved in the regeneration of reduced glutathione (GSH) from its oxidized dimer (GSSG). These results would lead to an increased concentration ratio between GSH and GSSG within liver cells, as was specifically observed with the BHA treatment (Moldeus et al., 1982). Similarly, it is suspected that the ratio would also be increased by treatments with TBHQ, but perhaps not by others.

In summary, the marked enhancements of several hepatic cytosol enzyme activities involved in the detoxification pathways produced by treatments of BHA and its structural analogs (containing at least two functional groups) may accelerate the elimination of either the administered or the metabolically produced phenolic compounds. In turn, the elevations of these conjugation (detoxification) enzyme systems inherent in living cells by administering phenolic food additives (i.e., BHA, BHT) may confer some serendipitous protection against various environmental chemical carcinogens (Fig. 2).

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