Enhancement of O-dealkylation in Mouse Liver by Dietary Administrations of BHA and BHT: Studies with Isolated Perfused Livers and Hepatic Microsomes*

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Effects of feeding 2(3)-tert-butyl 4-hydroxyanisole (BHA) and 3,5-di-tert-butyl 4-hydroxytoluene (BHT) on the rates of mixed function oxidation and conjugation enzyme reactions have been determined using isolated hepatic microsomal fractions and isolated perfused livers of mice. The treatments with either of the antioxidants have increased the rates of O-demethylation for p-nitroanisole and of O-deethylation for 7-ethoxycoumarin up to 2-fold, both in microsomes and in perfused liver. Analysis of the perfusate showed that the increased amounts of p-nitrophenol and 7-hydroxycoumarin produced by the elevated mixed-function oxidase activities were reflected by the increase in the amounts of glucuronide conjugates and not in the increase for the amounts of the sulfate ester conjugates. Comparison of results also indicated that in the perfused liver, the maximal rate of metabolite conjugation is limited by the maximal rates of the initial mixed function oxidase activities.

Key Words: Mixed-function oxidation, conjugation enzyme reactions, antioxidants (BHA, BHT), perfused liver.

It has been well established that the use of food-additive antioxidants, such as 2(3)-tert-butyl 4-hydroxyanisole (BHA) and 3,5 di-tert-butyl 4-hydroxytoluene (BHT) protect a variety of rodent target tissues against the production of tumors and toxicity inducible by a wide variety of chemical carcinogens (reviewed by Wattenberg 1980; Slaga & Bracken 1977; Weisburger et al. 1977). As for the mechanism of this protective effect, recent studies dealing particularly with BHA have demonstrated that mixed-function oxidase activities were either altered (Speier & Wattenberg 1975; Lam & Wattenberg 1977; Cha & Bueding 1979; Dock et al. 1982a) or sensitively inhibited (Cummings et al. 1985), and at the same time, various detoxification and conjugation enzyme activities catalyzing the inactivation of reactive

metabolites generated by the mixed function oxidases were markedly elevated (Benson et al. 1978, 1979, 1980; Cha et al. 1978, 1982a, 1982b, 1983, 1984; Dock et al. 1982a, 1982b; Moldeus et al. 1982). Therefore, the protective effects of BHA against chemical toxicities could be accounted for, at least in part, by combinations of these actions which may lower the intracellular concentrations of reactive metabolic products.

However, since these observations were made with subcellular fractions of liver tissue, little is known about their physiological importance in vivo. Therefore, in this study, we have attempted to correlate the in vitro and in vivo (ex vivo) biochemical effects produced with the dietary administrations of BHA and BHT, by comparing the results obtained with isolated microsomal fractions with those of isolated perfused livers of mice.

MATERIALS AND METHODS

Animals: Groups of 4- to 5-week-old female CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA) were housed in hanging, stainless steel wire cages and provided with periods of 12 hours each of alternating light and darkness and with free access to Purina Laboratory pellet chow (Code 5001: Ralston-Purina Co., St. Louis, MO) and tap water initially for

1 to 2 weeks, for acclimation. Thereafter, pellet diets containing 0.75% BHA or 0.5% BHT were given to experimental animals (4 mice per cage) for 10 to 30 days. Both BHA and BHT were purchased from Sigma Chemical Co. (St. Louis, MO) and sent to the Purina Co. to be made into pellets for diets containing these antioxidants. The body weights of the mice at the time of the experiment were 30 to 40 g for all three groups (i.e., control, BHA, and BHT) and their liver weights, determined after perfusion experiments, were 1.55 ±0.34, 2.45 ± 0.63, and 2.08 ± 0.39 g (mean ± SEM; n=12 to 16) for the control, BHA-, and BHT-treated animals, respectively.

Microsomal Enzyme Assays: Livers obtained from mice sacrificed by cervical dislocation were homogenized individually in 35 ml of ice cold sucrose (0.25 M), using a Teflon-glass homogenizer (6 strokes). All subsequent steps for the isolation of washed microsomes and storage were taken as previously described (Cha & Bueding 1979). Protein concentrations were determined by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin (Sigma) as the standard. The levels of cytochromes P-450 and b-5 were measured by the method of Omura and Sato (1964a, 1964b), utilizing an Aminco DW-2 spectrophotometer (American Instrument Co, Silver Springs, MD) operating in a split beam scanning mode. The activities of the microsomal mixed-function oxidases were measured, using either p-nitroanisole (8 mM) or 7-ethoxycoumarin (147 μM) as substrates. The O-demethylation of p-nitroanisole was carried out in STMA solution (0.25 M Sucrose, 100 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and 0.1% albumin) containing 20 mM semicarbazide, and the production of HCHO was quantitated according to the method described by Nash (1953). The O-deethylation of 7-ethoxycoumarin was measured as described by Ullrich and Weber (1972). The activity of UDP-glucuronyl transferase, prior to detergent activation, was determined, using p-nitrophenol as the substrate according to the methods of Mills and Smith (1963).

Liver Perfusion: Mice were anesthetized with sodium pentobarbital (0.1 gm/kg body weight) injected intraperitoneally. After the abdominal cavity was opened, one ligature was placed around the vena cava, above the renal vein, and two around the portal vein, above the splanchnic vein. A tapered polyethylene cannula connected to a perfusion apparatus (Scholz *et al.* 1973) was inserted into the portal vein and was secured with ligatures. Krebs-Hensleit bicarbonate buffer, equilibrated with oxygen and carbon dioxide mixture (95:5) at 37°C (hereafter referred to as KHB), was flowing through the cannula (3-5 ml/min) at the time of the insertion. In order to prevent the possible swelling of the liver during the time between the insertion of the cannula and the puncturing of the heart, perfusion was halted for about 35 seconds. During this short time, the thoracic cavity was quickly opened, the heart was punctured, and flow reinstituted. Ligatures were then tightened and the liver was carefully freed from the surrounding tissues. After the liver was transferred to a plastic perfusion block holder, the flow rate was increased to 10-13 ml/min and held at this rate for at least 10 min to achieve equilibration before the experiments were begun. The perfusate was not recirculated.

Assays of Mixed-Function Oxidation, and Conjugation: The 7-ethoxycoumarin and p-nitroanisole (Sigma Chemical Co.) were dissolved in KHB and were infused at concentrations of 200 μM. Effluent from the liver was collected every 2 min and assayed for the unconjugated, free 7-hydroxycoumarin or p-nitrophenol as well as their glucuronide and sulfate conjugates, as described in detail elsewhere (Ji *et al.* 1980; Reinke *et al.* 1980). Concentrations of 7-hydroxycoumarin in 1 ml samples were determined fluorometrically prior to and after hydrolysis with purified β-glucuronidase and/or arylsulfatase containing the β-glucuronidase (both from Sigma Chemical Co.), using a commercial quartz light guide (Jena Glaswerk Schott, Mainz, West Germany) coupled to a Johnson Foundation CF-1 fluorometer (U. Pennsylvania, Philadelphia, PA) as was described by Ji *et al.* (1980). Excitation wavelength filters were between 300-400 nm (Corning glass filter 5849) and emission wavelength filters were between 400-500 nm (Kodak Wratten gelatin filters 2E and 47). The fluorescence readings of samples were converted into 7-hydroxycoumarin concentrations employing the appropriate calibration curves obtained with standard solutions containing 7-hydroxycoumarin (Sigma Chemical Co.) dissolved in KHB. Similarly, concentrations of free p-nitrophenol in 1 ml effluents were determined spectrophotometrically prior to and after enzymatic hydrolysis (Reinke *et al.* 1980). The absorbance readings were converted into p-nitrophenol concentrations using the standard curve obtained with the compound dissolved in KHB. The rates of mixed function oxidation of p-nitroanisole and 7-ethoxycoumarin were calculated at first by combining the respective metabolites existing as free, glucuronide and sulfate conjugates in the effluent samples, and then by multiplying the factor obtained from the hourly flow rate divided by the weight of the liver.

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RESULTS

Effects on Microsomal Enzyme Activities and Cytochrome Contents: Results of the effects of feeding the two common food-additive antioxidants, on specific activities and contents of hepatic microsomal enzymes involved in O-dealkylations and glucuronidation are shown in Table 1. The concentration of cytochrome P-450 was markedly increased by the BHT feeding, but it was only minimally increased by the BHA diet. On the other hand, the concentrations of cytochrome b-5 were moderately elevated by both compounds, to similar extents. Similar to the changes that have been observed in the concentration of cytochrome P-450, the O-demethylase activity of p-nitroanisole was increased nearly 2-fold by the BHT diet, but only minimally increased by the BHA diet. However, in the case of the O-deethylase activity of 7-ethoxycoumarin, it was the BHA diet, but not the BHT diet, that produced 2-fold elevation. The UDP-glucuronyl transferase activity, determined by using p-nitrophenol, a product of the O-demethylation of p-nitroanisole, was increased only by the BHA, not by the BHT feeding. In the assay of this enzyme activity, we have intentionally avoided the prior activation with detergents to simulate the in vivo conditions.

Interrelationship of Mixed Function Oxidation and Conjugation in Perfused Mouse Liver: A typical example of results obtained with a non-recirculating infusion of 7-ethoxycoumarin (Fig. 1A) or of p-nitroanisole (Fig. 1B) in control mouse liver is shown. For both compounds, maximal rates of mixed function oxidation (combination of free and conjugated metabolites) were achieved within 6 to 8 min after the initiation of infusion. The maximum rate of O-demethylation (p-nitroanisole) was about twice that of O-deethylation (7-ethoxycoumarin). With both substrates, approximately 50% of the metabolic products coming out in the perfusate were glucuronide conjugates, the remainder being equally distributed between the free O-dealkylated products and their sulfate conjugates. The maximal rate for O-demethylation of p-nitroanisole was maintained for only 4 minutes. Subsequently, the metabolic rate gradually declined to about 60% of the maximal rate in 30 additional min of continuous infusion. In contrast, the maximal rate for the O-deethylation of 7-ethoxycoumarin was maintained at a relatively constant level throughout the perfusion. Upon termination of infusing these compounds, all parameters returned to their respective normal base line values in 15 to 20 minutes.

Table 1. Effects of feeding BHA and BHT on hepatic microsomal mixed-function oxidase, UDP-glucuronyl transferase activities, and cytochrome contents

Measurement	Diets		
	Control	BHA	BHT
Mixed-function oxidases (nmoles/min/mg protein)			
p-Nitroanisole (n=4)	30.3±1.7	39.0±1.0*	63.5±4.2***
7-Ethoxycoumarin (n=4)	0.94±0.08	2.07±0.04***	1.20±0.01***
UDP-Glucuronyl transferase with p-nitrophenol (nmoles/min/mg protein) (n=4)	28±1.2	46±0.8*	30±1.3
Cytochrome content (nmoles/mg protein)			
P-450 (n=4)	0.57±0.01	0.60±0.01*	0.89±0.02***
B_s (n=4)	0.38±0.02	0.58±0.02**	0.52±0.02**

Mean ± S.E.M.

  * Significant different from control, $p < .05$ (student's t-test).
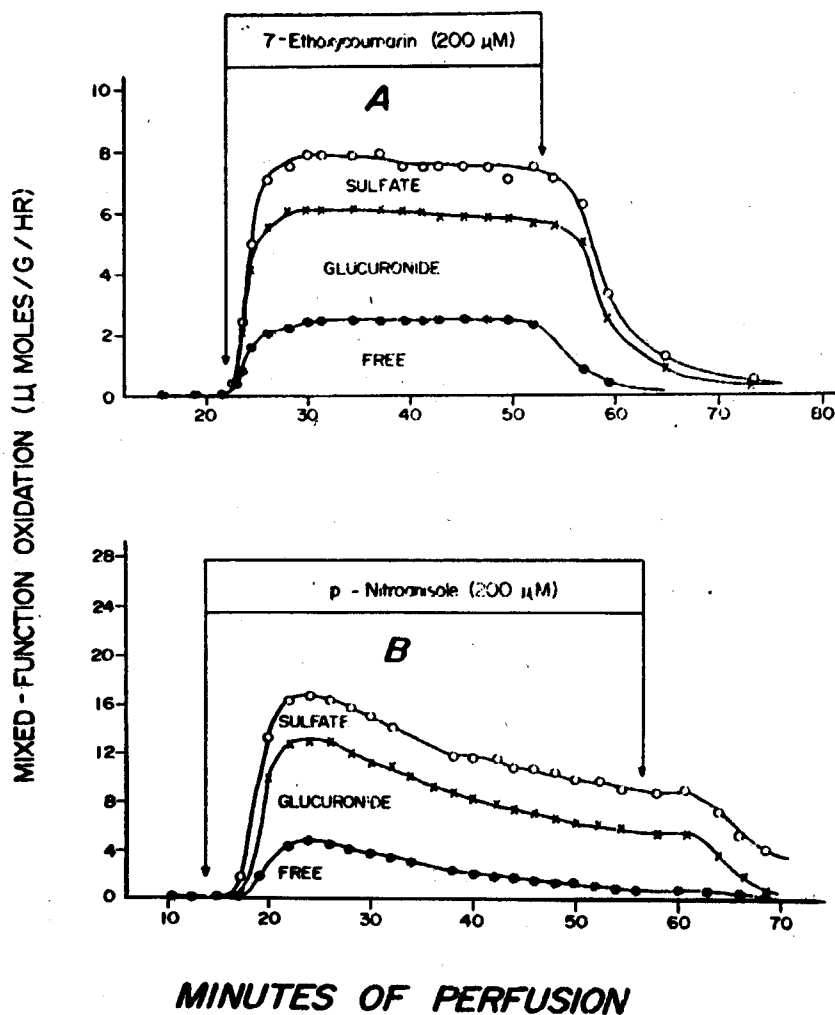
 ** $p < .01$

 *** $p < .001$

Fig. 1. The kinetics of the mixed-function oxidation of 7-ethoxycoumarin (A) and p-nitroanisole (B) in the isolated, perfused mouse liver. Mouse livers were perfused at a flow rate of 12 ml/min at 37°C as described in Methods. The infusion of 7-ethoxycoumarin or p-nitroanisole is indicated by the horizontal bars. Samples of effluent perfusate were collected every 2 minutes for determination of glucuronide and sulfate conjugates as well as for free O-dealkylated products leaving the liver.

Effect of BHA and BHT Treatment on Mixed-Function Oxidation in Perfused Liver: One typical example from among several of results obtained with each experimental group, is compared with that of the control, in fig 2. The values in the figure represent combined levels of metabolites (free, plus conjugates) and are indicative of the overall rate of mixed-function oxidation. Both of the BHA and BHT diets produced sizable increases in the activity of O-deethylation of 7-ethoxycoumarin (Fig. 2A and Table

2) and O-demethylation of p-nitroanisole (Fig. 2B and Table 3). For example, the feeding of BHA resulted in a 45% elevation of both O-dealkylation reactions, and the BHT diet caused 65% and 95% increases of O-deethylation and O-demethylase activities, respectively.

With 7-ethoxycoumarin as the substrate (Table 2), the increased production of 7-hydroxycoumarin resulting from the feeding of the BHA or the BHT has been primarily glucuronidated, and only the amount
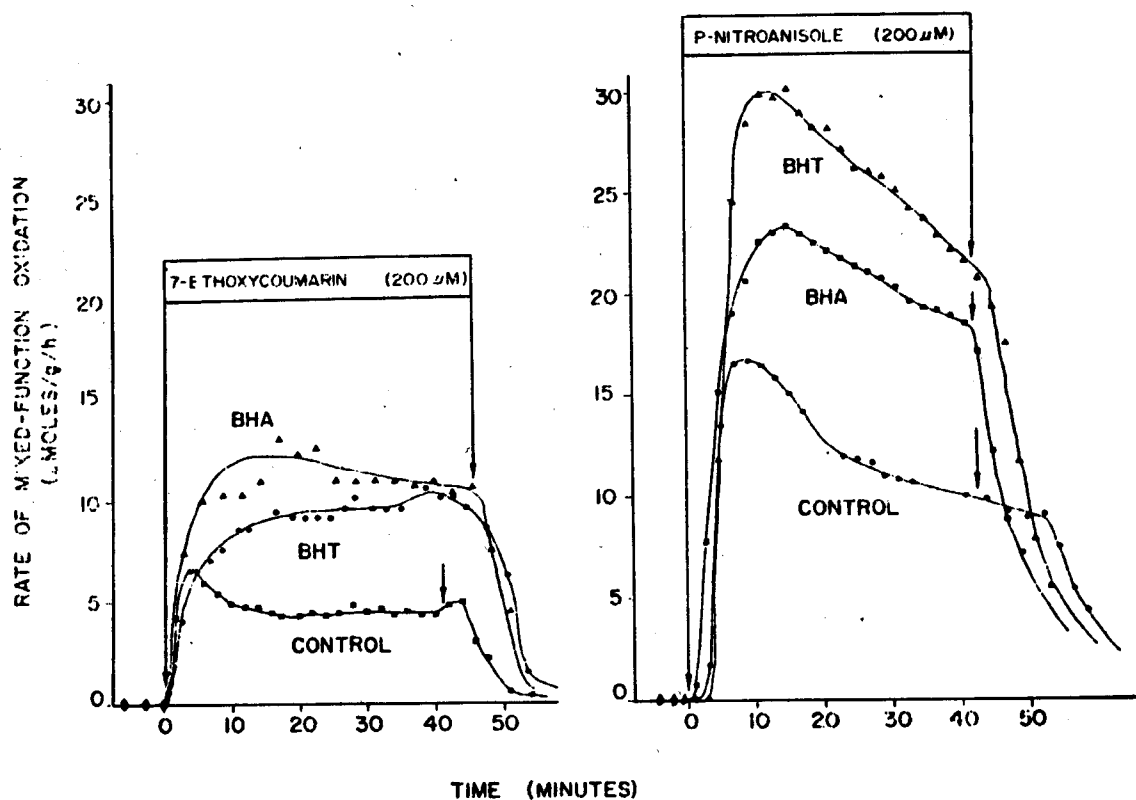
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Fig. 2. Effect of BHA- or BHT- treatment on 7-ethoxycoumarin O-deethylation (A) and p-nitroanisole O-demethylation (B). Condition as in figure 1.

Table 2. Effects of BHA and BHT treatment on the mixed-function oxidation of 7-ethoxycoumarin and conjugation of 7-hydroxycoumarin by the perfused mouse liver

Diet	N	Moles of 7-hydroxycoumarin formed/g of liver/hr			
		Total	Glucuronide	Sulfate	Free
Control	8	5.7±0.9	1.9±0.4	2.4±0.6	1.4±0.3
3,5-di-tert-butyl 4-hydroxytoluene (BHT)	8	8.2±0.5**	5.2±0.9**	1.3±0.4	1.8±0.4
2(3)-tert-butyl 4-hydroxyanisole (BHA)	7	9.4±1.0**	4.0±0.4	4.0±0.9	1.4±0.3

Mean ± S.E.M.

** Significantly different from control, p<.05 (student's t-test). Mice were treated with antioxidants for 10 to 30 days, and the livers were isolated and perfused as described in "Materials and Methods"

Table 3. Effects of antioxidants on the mixed-function oxidation of p-nitroanisole and conjugation of p-nitrophenol in the perfused mouse liver.

Diet	N	Moles of p-nitrophenolate formed/g of liver/hr			
		Total	Glucuronide	Sulfate	Free
Control	5	·14.5±0.8	7.5±0.7	1.6±0.6	5.4±0.5
2(3)-tert-butyl 4-hydroxyanisole (BHA)	5	21.2±2.8*	18.3±2.6**	2.0±0.7	1.3±0.4***
3,5-di-tert-butyl hydroxytoluene (BHT)	5	28.3±3.3**	21.0±3.7**	1.7±0.3	5.6±0.9

Mean ± S.E.M.
  * Significantly different from control, p<.05 (student's t-test)
  ** p<.01
  *** p<.001
Conditions as in Table 1. The rates listed here represent the maximal rates.

of unconjugated metabolites coming out from the perfused liver has remained constant. The amount coming out as sulfate conjugate appeared to be slightly decreased by the BHA treatment and increased by the BHT treatment, respectively. However, these changes were not statistically significant, as indicated by the large values of standard errors of the mean. In cases when p-nitroanisole was used as the substrate (Table 3), again, the major portion of the p-nitrophenol produced by the increased O-demethylase activity resulting from antioxidant treatments came out as elevated levels of glucuronides. With BHA, but not with BHT treatment, the enhanced rate of p-nitrophenol glucuronide formation was also accompanied by a diminished output of free p-nitrophenol. The levels of sulfate ester leaving the liver were not altered. These results obtained with p-nitroanisole and tabulated in Table 3 represent the maximal peak rate values for mixed-function oxidation, in view of the gradual decline in O-demethylation, especially with this substrate.

DISCUSSION

Studies done on the mechanisms of anticarcinogenic effects observed with food additive antioxidants, such as BHA and BHT, have indicated that these antioxidants may directly inhibit the production of reactive electrophilic species of ultimate carcinogens (Cummings et al. 1985), or may indirectly reduce the levels of reactive metabolites by the enhanced neutralization of the reactive metabolites that have been produced (Cha & Heine 1982b). This reasoning is based on several recent biochemical studies focuss-

ed on the balance between the formation of metabolites and the elimination of reactive species. These studies have revealed unusually marked elevations for several of the metabolic detoxification enzyme activities (Benson et al. 1978, 1979, 1980; Cha et al. 1978, 1979, 1982a, 1982b, 1983, 1984; Dock et al. 1982a, 1982b; Lam & Wattenberg 1977; Moldeus et al. 1982; Speier & Wattenberg 1975). However, as these results have been obtained by using subcellular fractions, the in vivo significance of elevated conjugation reactions has not been demonstrated. Toward this goal, the use of intact hepatocytes of perfused liver can offer distinct advantages in elucidating the role of elevated conjugation reactions resulting from treatments with these protective antioxidants. Therefore, we have compared some of these dietary antioxidant-dependent biochemical changes observed in vitro (Table 1) with those occurring ex vivo (Table 2 & 3, and Figs. 1 & 2).

In an intact hepatocyte, the oxidation and conjugation reactions are tightly coupled, and consequently, for compounds requiring oxidation by mixed-function oxidases prior to being conjugated, the rates of their glucuronide formations would depend on the activities of oxidases, but not on the activities of conjugating enzymes (Anderson et al. 1978; Moldeus et al. 1978). Therefore, the greater increase in p-nitroanisole O-demethylation activity induced by the BHT-diet (Table 1) is responsible for the greater amount of total p-nitrophenol produced which came out from the perfused liver obtained from the BHT-treated mouse (Fig. 2B). Similarly, as the O-deethylation of 7-ethoxycoumarin was increased to a higher level (Table 1), the total amount of

7-hydroxycoumarin coming out from the BHA-treated mouse liver was greater (Fig. 2A). These results were observed despite the fact that UDP-glucuronyl transferase activity was not increased by the feeding of BHT (Table 1). This would indicate that, even without any elevations of the glucuronidation or sulfation enzyme activities, the extra amount of p-nitrophenol produced could be conjugated in the BHT treated mouse liver. Analysis of perfusates following digestion with specific hydrolytic enzymes (Table 2 and 3) support this hypothesis further. Therefore, significantly higher amounts of glucuronide conjugates of both substrates came out from either the BHT- or the BHA-treated, perfused mice livers.

The amounts of sulfate ester conjugates for these dealkylated products which came out from the perfused livers were not increased (Table 2 and 3). This finding is consistent with an earlier study in which the isolated hepatocytes obtained from BHA-treated mice did not produce greater amounts of sulfate esters with harmol of paracetamol, even when the hepatocytes were incubated in the presence of excess inorganic sulfate (Moldeus et al. 1982). This was further substantiated by the lack of increase in sulfotransferase activities as well as the lack of synthesis of activated sulfate by the hepatic cytosols obtained from BHA-treated mice (Cha & Heine, unpublished observation). Thus, as was expected, the levels of sulfate ester in perfusates were not changed (Table 2 and 3).

The existence of an extra reserve capacity for glucuronidation, but a limited capacity for sulfation, as has been observed in this study with perfused mouse liver, has supported the conclusions of Andersson et al. (1978) and Moldeus et al. (1978), which were obtained with isolated hepatocytes. The sulfate conjugation pathway is said to have a low capacity but a high affinity, and the glucuronide conjugation pathway, a low affinity but a high capacity (Moldeus 1978). Even under normal conditions, the levels of unconjugated, free 7-hydroxycoumarin in the perfusate were higher than the levels of sulfate esters. This indicates that the capacity for sulfate conjugation is limited and is already saturated (Table 2). Thus, any metabolities resulting from an increase in their production by the enhanced O-dealkylation enzyme activities could all be excreted as glucuronide conjugates without any intensification of the glucuronidation enzyme activities. Therefore, most probably, the large reserve capacity of the glucuronidation pathway would pick up any excess metabolites produced by the enhanced dealkylation reactions that have resulted from treatment with these antioxidants. Under these circumstances, a more realistic test for the functional

significance of elevated glucuronyl transferase activity (Table 1) would have come from experiments in which large amounts of p-nitrophenol or 7-hydroxycoumarin were infused. This type of experiment has been done with harmol and paracetamol, using isolated hepatocytes obtained from the BHA-treated mice, and the observed results have confirmed the supposition made by Moldeus et al. (1982). Based on these observations, the enhanced glucuronide conjugation capacity brought about by the antioxidant treatment would exert its protective role against phenolic chemical carcinogens only when overwhelming amounts are administered or produced.

In the perfused mouse liver, while the peak rate for the production of 7-hydroxycoumarin was maintained, the peak rate for p-nitrophenol production declined with time (Fig. 1 & 2). This decline may be specifically associated with p-nitrophenol which is a structural analog of dinitrophenol, a well known uncoupler of the mitochondrial electron transport chain (Lardy & Wellman 1953). If, indeed, the produced p-nitrophenol also uncouples the mitochondrial function, this observed result would implicate the involvement of intact mitochondrial function in the supporting of the microsomal mixed-function oxidation reactions (Moldeus et al. 1973). Alternatively, if the mitochondria were uncoupled by the p-nitrophenol produced, it would limit the production of ATP which is required for the biosynthesis of cofactors such as activated sulfate and UDP-glucuronic acid. This would bring about suppression of the conjugation reactions. Such a study is under consideration.

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