[Chem. Pharm. Bull. 27(2) 480—485 (1979)]

UDC 547.563.4.04.09 : 615.31.011.5.076.9

Biological Fate of Butylated Hydroxytoluene (BHT); Binding *in Vitro* of BHT to Liver Microsomes

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(Received September 26, 1978)

The binding of ^{14}C-labeled butylated hydroxytoluene (^{14}C-BHT) to the liver microsomes was studied *in vitro*. Binding of ^{14}C-BHT to microsomal macromolecules was linear with time and protein concentration. Oxygen and dihydronicotinamide adenine dinucleotide phosphate (NADPH) were necessary for this binding, while carbon monoxide or SKF-525A inhibited it. It is indicated that a cytochrome P-450-linked monooxygenase system mediated the binding. In addition the binding was more pronounced in the liver microsomes from animals pretreated with inducers (phenobarbital or BHT) of microsomal monooxygenase system. From the effects of pretreatment with these inducers, it is suggested that a cytochrome P-450 with a high BHT oxidase activity is mainly responsible for the metabolic activation of BHT in the liver microsomes. The microsomes in the brain, kidneys and spleen were devoid of BHT-binding capacity, but in the lung microsomes the binding capacity was about 40% of that in the liver microsomes.

Keywords——antioxidant; butylated hydroxytoluene; rat liver; microsomal macromolecules; binding of radioactivity

Butylated hydroxytoluene (3,5-di-*tert*-butyl-4-hydroxytoluene, BHT) is widely used as an antioxidant in porcessed foods and petroleum products. Many toxicological studies on this compound using experimental animals have been reported.[2–5] Study on biological fate of a food additive is very important for evaluation of its safety. Though its *in vivo* metabolism was reviewed by Hathway,[6] intracellular fate of BHT has not been reported. Therefore, we have been studying the behavior of this compound in the liver cell of rat.[7,8] This paper describes a metabolite(s) binding to microsomal macromolecules during the metabolic activation of BHT by hepatic microsomal preparations *in vitro*.

Materials and Methods

Materials——3,5-Di-*tert*-butyl-4-hydroxytoluene[toluene methyl-^{14}C] (specific radioactivity, 2.2 μCi/mg) was purchased from New England Corporation (Boston, Mass.). When ^{14}C-BHT was chromatographed on thin-layer plates of silica gel with light petroleum (bp 60—80°) as a solvent, only a single radioactive component was detected. Chemical compounds were obtained from the following companies: nonradioactive BHT from Wako Pure Chemicals Co. (Osaka); nicotinamide adenine dinucleotide phosphate (NADP) and glucose 6-phosphate from Sigma Chemicals Co. (St. Louis, Mo); glucose 6-phosphate dehydrogenase from Boehringer-Mannheim Co. (W. Germany); SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride) from Smith, Kline and French Laboratories (Philadelphia, Pa); sodium phenobarbital from Fujinaga Pharmaceutical Co. (Tokyo); and all other chemicals used were of the highest obtainable purity.

Pretreatment of Animals——Male Wistar rats (SPF) weighing 140—170 g were used and divided into groups of three rats for each experiment. Separate groups of animals were treated with phenobarbital

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(PB, 80 mg/kg, *i.p.*, daily for 5 days) or BHT (50 or 500 mg/kg, *p.o.*, daily for 3 days), and were used 24 hr after the last treatment. SKF-525A (50 mg/kg, *i.p.*) was given 30 min prior to use. Control rats received equal volumes of vehicles (saline for PB and SKF-525A; olive oil for BHT) for similar periods.

Preparation of Microsomes——The animals were sacrificed by decapitation; the livers and other tissues were removed immediately and as much blood as possible was removed with saline. All subsequent manipulations were carried out at below 4°. The livers and other tissues of three rats were combined and homogenized in 1.15% KCl containing 10 mM phosphate buffer (pH 7.4) and 1 mM EDTA in a Potter-Elvehjem homogenizer. The homogenate was made up to a volume corresponding to a 25% suspension, and centrifuged for 15 min at $10000 \times g$ in a Sorval centrifuge; the $10000 \times g$ supernatant was then centrifuged for 1 hr at $105000 \times g$ in a Beckman L5-75 ultracentrifuge. The supernatant (cytosol fraction) was removed and the microsomal pellet was resuspended, washed in 1.15% KCl, and then recentrifuged for 1 hr at $105000 \times g$. The microsomal pellet washed was suspended in 0.1 M phosphate buffer (pH 7.4) and used within a few hours after preparation.

Binding to Microsomal Macromolecules——Incubations were performed in triplicate, with a 10-ml glass centrifuge tube. Each incubation mixture usually contained 2 mg of microsomal protein, 1.0 mM NADP, 10 mM glucose 6-phosphate, 3.5 units of glucose 6-phosphate dehydrogenase, and 7.5 mM $MgCl_2$ in a total volume of 2 ml of 0.1 M phosphate buffer (pH 7.4). The mixture was preincubated at 37° for 2 min and the reaction was started by the addition of ^{14}C-BHT (150 μg) in 20 μl of 2-methoxyethanol, with vigorous shaking to assure its even distribution. The reaction was carried out at 37° for a suitable period of time with constant shaking in an air and stopped by adding 5 ml of 10% trichloroacetic acid. The resultant precipitate was collected by centrifugation, resuspended in 4 ml of 7.5% trichloroacetic acid, and mixed in a Vortex shaker with a narrow spatula inserted into the tube to facilitate mixing. The tube was centrifuged again. The washed pellet was mixed with the spatula as above, and then extracted successively with 4 ml each of 80% methanol (twice), 80% hot methanol (twice), methanol–ether (1 : 1, v/v, four times), and 80% methanol (twice). After the last extraction, no further radioactivity could be removed from the pellet. The thoroughly extracted precipitate was dissolved in 1 N NaOH for the determination of radioactivity bound to microsomal macromolecules.

Other Methods——Radioactivity was measured by a Beckman Scintillation Spectrometer, Model LS-355, and corrected by external standard methods. The scintillation medium[9] used consisted of 2 volumes of toluene phosphor (4 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene (dimethyl-POPOP) per 1000 ml of toluene) and 1 volume of Triton X-100. Protein was determined by the method of Lowry *et al.*[10] by using bovine serum albumin as standard. The content of cytochrome P-450 was measured as described by Omura and Sato.[11] BHT oxidase activity was measured as described by Gilbert and Golberg.[12] Aminopyrine N-demethylase and aniline hydroxylase activities were measured as described by Mazel.[13]

Results

In order to investigate binding of ^{14}C–BHT to microsomal macromolecules, the linearity of binding assay was established with respect to time and concentration of microsomal protein, as shown in Fig. 1. The binding of radioactivity to microsomal macromolecules is a time dependent process, which is linear up to about 15 min in microsomes of untreated control rats and 5 min in microsomes of phenobarbital-pretreated rats, respectively. The binding was completely abolished when NADPH was absent in the incubation mixture. Moreover, the binding was linear with microsomal protein up to about a concentration of 1.5 mg/ml of incubation mixture. These results indicate that the binding was of enzymatic nature. Therefore, all subsequent experiments were carried out with incubations with about 1 mg protein/ml for 10 min or 5 min (in case of microsomes prepared from phenobarbital or BHT pretreated rats).

Table I shows the effect of varying incubation conditions on the binding of ^{14}C–BHT to microsomal macromolecules. Two inhibitors of microsomal monooxygenase system containing cytochrome P-450, CO and SKF-525A, significantly diminished the binding of ^{14}C–BHT

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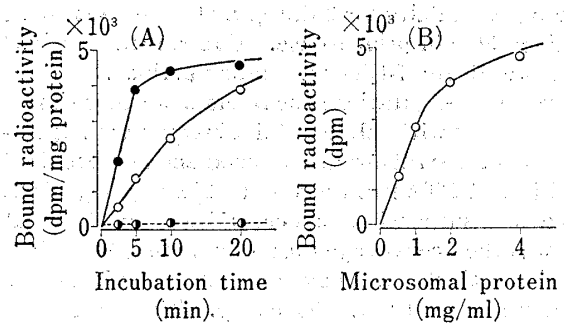

Fig. 1. Binding of ^{14}C–BHT to Liver Microsomes

(A) Time course of binding of ^{14}C-BHT to microsomes. Liver microsomes (microsomal protein 1.0 mg/ml) from untreated control (—○—) and phenobarbital-pretreated (—●—) rats were incubated with ^{14}C-BHT in the presence of an NADPH-generating system, and both microsomes were also incubated with ^{14}C-BHT in the absence of an NADPH-generating system (---◑---), as described in Materials and Methods.
(B) The concentration of microsomal protein from untreated rats in the assay was varied; all incubation were 10 min.
Each point represents means of three determinations.

to microsomal macromolecules. When NADPH was excluded from the incubation mixture or microsomes were boiled for 10 min before incubation, an about 99% inhibition of the binding was observed. The binding was also inhibited almost completely when nitrogen was used instead of air as the atmosphere. In addition SH-compounds, cysteine and reduced glutatione, decreased the binding significantly. These results suggest that cytochrome P-450-linked monooxygenase system was required in the conversion of BHT to metabolically active material(s) capable of binding to macromolecules.

Table II shows the binding of ^{14}C–BHT to various hepatic subcellular fractions in NADPH-generating system. In the microsomal fraction, the amount of radioactivity bound was more than 10-fold of that in the

Table I. Effect of Various Incubation Conditions on Binding *in Vitro* of ^{14}C–BHT to Macromolecules from Liver Microsomes

Incubation condition	Amount bound[b] (dpm/mg protein/10 min)	Inhibition (%)
Complete[a]	2634± 9	—
Microsomes boiled	21± 4	99.2
−NADPH	36± 3	98.6
$-O_2$ (100% N_2 atmosphere)	173±21	93.5
+Carbon monoxide (90% CO, 10% O_2)	241±30	90.9
+Cysteine (1.0 mM)	632±13	76.0
+Glutatione (1.0 mM)	246±15	90.7
+SKF-525A (1.0 mM)	39±10	98.5

a) Microsomes were incubated as described in Materials and Methods, except as noted. Boiling of microsomes proceeded for 10 min.
b) Values are expressed as means±S.D. of three determinations per each incubation condition.

Table II. Binding of ^{14}C–BHT to Different Subcellular Liver Fractions[a]

Fraction[b]	Amount bound[c] (dpm/mg protein/10 min)
Nuclear	315± 19
Heavy mitochondrial	124± 3
Light mitochondrial	342± 22
Microsomal	3163±202
Supernatant	39± 14

a) The liver was homogenized with 0.25 M sucrose in a Potter-Elvehjem homogenizer to make a 10% homogenate, and the homogenate was fractionated by differential centrifugation according to the method of de Duve, *et al.* [C. de Duve, B.C. Pressman. R. Gianeto. R. Wattiaux and F. Applemans, *Biochem. J.*, **60**, 604 (1955)].
b) Added at a protein concentration of 1.0 mg/ml.
c) Values are expressed as means±S.D. of three experiments.

Table III. Binding of ^{14}C–BHT to Microsomes isolated from Various Tissues

Tissue	Amount bound[a] (dpm/mg protein/10 min)	
Liver	2378±71	(100.00)[b]
Brain	15± 2	(0.63)
Lung	944±32	(39.70)
Spleen	16± 6	(0.67)
Kidney	134± 8	(5.63)

a) Values are expressed as means ±S.D. of three experiments.
b) Values in parenthesis represent percentage of liver value.

other fractions. These results indicate that the enzymatic capacity to catalyze binding of ^{14}C–BHT to macromolecules is mainly concentrated on the microsomal fraction.

When the microsomes prepared from various tissues were examined for the binding of ^{14}C–BHT (Table III), it was found that the binding reaction was most pronounced in the liver. The microsomes in the kidneys, brain and spleen were practically devoid of ^{14}C–BHT-binding capacity, whereas a significant amount of radioactivity (about 40% of liver value) was bound to the lung microsomal macromolecules.

Table IV. Effects Pretreatment with Phenobarbital and SKF-525A on Liver Microsomal Content of Cytochrome P-450, Activities of BHT Oixdase, Aminopyrine N-demethylase and Aniline Hydroxylase, and Amount of Metabolite (s) of ^{14}C–BHT bound to Microsomal Macromolecules in Rats

Treatment	Cytochrome P-450 (*n* mol/mg protein)	BHT oxidase	Aminopyrine N-demethylase	Aniline hydroxylase	Amount bound (dpm/mg protein/10 min)
			(*n* mol/mg protein/min)		
Control	0.851±0.008 (100)[a]	0.345±0.022 (100)	3.17±0.06 (100)	0.437±0.009 (100)	2384± 21 (100)
PB	1.645±0.008 (194)	0.977±0.029 (283)	7.54±0.14 (238)	0.743±0.010 (170)	6494±106 (273)
SKF-525A	0.769±0.010 (90)	0.152±0.022 (44)	0.98±0.02 (31)	0.442±0.002 (101)	618± 11 (26)

a) Values in parenthesis represent percentage of controls.
Each value represents the mean±S.D. of three determinations.

Table IV shows the binding of ^{14}C–BHT to microsomes prepared from phenobarbital or SKF-525A pretreated rats. The content of cytochrome P-450 and the activities of drug-metabolizing enzymes significantly increased by pretreatment of rats with phenobarbital. In addition the amount of radioactivity bound to microsomal macromolecules increased about 2.7-fold of the control. On the other hand in the SKF-525A pretreatment, the amount of radioactivity bound to microsomal macromolecules markedly decreased (25% of the control). This decrease was accompanied by a decrement in the content of cytochrome P-450 as well as the activities of drug-metabolizing enzymes except for aniline hydroxylase activity.

BHT is by itself an inducer of microsomal enzymes, leading to enlargement of the liver due to an increase in the synthesis of enzymes.[14,15] Therefore, effects of pretreatment with BHT on binding of ^{14}C–BHT to microsomal macromolecules was investigated, and the results are shown in Table V. The increase in radioactivity bound to microsomal macromolecules closely paralleled with an increase in the content of cytochrome P-450 and in the activity of BHT oxidase. Furthermore, the increase in the amount of bound radioactivity was

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dependent on the dose of BHT given, although no significant changes were found in the other drug-metabolizing enzymes. The results presented in Table IV and V indicate clearly that the binding of ^{14}C–BHT to microsomal macromolecules is strongly influenced by the induction or inhibition of microsomal monooxygenase system.

Table V. Effects of Pretreatment with BHT on Liver Microsomal Content of Cytochrome P-450, Activities of BHT Oxidase, Aminopyrine N-demethylase and Aniline Hydroxylase, and Amount of Metabolite (s) of ^{14}C–BHT bound to Microsomal Macromolecules in Rats

Treatment	Cytochrome P-450 (n mol/mg protein)	BHT oxidase	Aminopyrine N-demethylase	Aniline hydroxylase	Amount bound (dpm/mg protein/10 min)
		(n mol/mg protein/min)			
Control	0.831±0.028 (100)[a]	0.317±0.012 (100)	3.21±0.03 (100)	0.452±0.002 (100)	2632±151 (100)
BHT (50 mg/kg)	0.887±0.030 (107)	0.375±0.012 (118)	2.93±0.02 (91)	0.454±0.004 (100)	3474±128 (132)
BHT (500 mg/kg)	1.014±0.017 (122)	0.590±0.023 (186)	3.44±0.04 (107)	0.409±0.008 (107)	4711± 67 (179)

a) Values in parenthesis represent percentage of controls.
Each value represents the mean±S.D. of three determinations.

Discussion

The results presented above indicate that the binding of BHT to microsomal macromolecules is mediated by a cytochrome P-450-linked monooxygenase system. The evidence for this conclusion can be summarized as follows: (a) the binding requires oxygen and NADPH (Table I), (b) two inhibitors of the microsomal cytochrome P-450-linked monooxygenase system, CO and SKF-525A, significantly diminished the binding (Table I), (c) pretreatment of animals with phenobarbital, an inducer of cytochrome P-450 synthesis, induces an elevation in the BHT-binding capacity of microsomes, though pretreatment of animals with SKF-525A, an inhibitor of the monooxygenase system, induces a decrement in the binding capacity (Table IV). It is likely that the monooxygenase system converts BHT to chemically activated material(s) which subsequently binds to microsomal macromolecules. Furthermore, the bound radioactivity is probably the result of covalent linking between the active material(s) and microsomal macromolecules since this radioactivity was not removable by an exhaustive extraction with methanol-ether (1: 1, v/v) or with ether. The nature of the activated material(s) is to be investigated by future work.

The thiol compounds such as glutathione and cysteine, which are suggested to prevent the cytotoxic effects of activated metabolites of foreign compounds,[16] decreased the amount of radioactive material(s) bound to microsomal macromolecules (Table I). Both compounds might remove the reactive BHT metabolite(s) formed by microsomal oxidation, possibly by establishing a chemically stable thioether binding.

The present study shows that the binding capacity among the hepatic subcellular fractions was most pronounced in the microsomal fraction (Table II), but the microsomes in the brain, spleen and kidney were practically devoid of the capacity of the binding (Table III). In our *in vivo* experiments,[8] we found that some of the radioactivity in hepatic subcellular fractions after administration of ^{14}C–BHT was bound to macromolecules of microsomal fraction, and smaller amounts of bound radioactivity was found in the other fractions. In addition the radioactivity bound to macromolecules was found in various tissues a long time

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after the administration. From these *in vivo* and *in vitro* experiments, it is suggested that the activated BHT metabolite(s) produced by microsomal monooxygenase system in the liver is bound to macromolecules in each subcellular fraction, and that this activated metabolite(s) distributed by blood stream from the liver is also bound to macromolecules in various tissues. Though the amount of cytochrome P-450 in the lung is very low compared with that in the liver,[17] the binding capacity in the lung microsomes was shown to be about 40% of that in liver microsomes. The cause is unclear, but the lung microsomes may play an important part in metabolism of BHT *in vivo*. It is necessary to investigate this problem.

Although both phenobarbital and BHT are inducers of liver microsomal enzymes,[14,18] in the present experiments the effect of phenobarbital differed from that of BHT with which there was no significant increase in the activities of aminopyrine N-demethylase and aniline hydroxylase except for BHT oxidase activity (Table IV, V). After pretreatment of rats with BHT, the increase in binding of ^{14}C–BHT to microsomal macromolecules closely paralleled with that in the content of cytochrome P-450 and BHT oxidase activity. Furthermore, the behaviour of these three components was dependent on the dose of BHT given. It may be concluded that a cytochrome P-450 with BHT oxidase activity is principally, if not solely, responsible for metabolic activation of BHT for the binding reaction.

It has been reported that BHT has an inhibitory effect on the action of certain chemical carcinogens, including some aromatic amine derivatives.[19] The underlying mechanism appears to be the alteration of the microsomal system that metabolizes the carcinogen, leading to a decrease in binding of the carcinogen or its metabolites to DNA.[20,21] In our preliminary experiments, the radioactive material(s) from ^{14}C–BHT administered orally to rats was bound to nucleic acid and protein in the liver. Therefore, it can be presumed that activated BHT metabolite(s) competes with binding of carcinogen or its metabolites to nucleic acid, and that this competition is responsible for a part of inhibitory effect on tumor formation by some carcinogens given.

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