[Chem. Pharm. Bull. 27(2) 442—446 (1979)]

UDC 547.563.4.04.09 : 543.52.06

Biological Fate of Butylated Hydroxytoluene (BHT); Binding *in Vivo* of BHT to Macromolecules of Rat Liver[1]

Yoshio Nakagawa, Kogo Hiraga,[2a] and Tetsuya Suga[2b]

Department of Toxicology, Tokyo Metropolitan Research Laboratory of Public Health[2a] *and Department of Clinical Biochemistry, Tokyo College of Pharmacy*[2b]

(Received September 1, 1978)

The binding of radioactive material(s) to macromolecules in the liver and other tissues was investigated in rats, after oral administration of ^{14}C-BHT. The total radioactivity and the radioactivity bound to macromolecules in the liver reached a maximum in 6 hr and in 6—12 hr, respectively. The rate of decrease of bound radioactivity was slower than that of total radioactivity, and, therefore, the binding ratio (bound radioactivity in the term of % of total radioactivity) increased with time. The bound radioactivity in the liver was found in all subcellular fractions, although the radioactivity in the microsomal fraction was higher than that in other fractions. In addition, the pretreatment of animals with phenobarbital further increased the amount of radioactivity bound to the microsomes. The bound radioactivity was also found in other tissues, the binding ratio in the lung and kidneys increased with time and that in the brain and spleen remained at the level of about 50% throughout the experimental period.

Keywords——antioxidant; butylated hydroxytoluene; rat; oral administration; liver microsomes; binding; radioactivity

Butylated hydroxytoluene (BHT; 3, 5-di-*tert*-butyl-4-hydroxytoluene) is widely used as an antioxidant and stabilizer in processed foods and petroleum products. Many toxicological studies on this compound using experimental animals have been reported.[3] Though its *in vivo* metabolism was reviewed by Hathway,[4] intracellular fate of BHT has not been reported.

In our preceding paper,[5] we reported that the radioactivity, which was incorporated into the supernatant fraction in the liver after the administration of ^{14}C-labeled BHT, migrated to the microsomal fraction, and that the affinity of radioactivity with the microsomal fraction is higher than that with other particulate fractions. In the present work we studied by *in vivo* experiments the binding of BHT to macromolecules of the liver and other tissues.

Materials and Methods

Animals——Male Wistar rats (SPF) weighing 170—200 g were used and divided into groups of 3 rats for each experiment.

Materials——3,5-Di-*tert*-butyl-4-hydroxytoluene[toluene methyl-^{14}C] (specific radioactivity, 2.2 μCi/mg) was purchased from New England Nuclear Corporation (Boston, Mass.). The toluene methyl group of BHT is known to be resistant against metabolic conversion in the rat.[3,4] 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. All other chemicals used were of the highest obtainable purity.

Treatment of Animals——The animals were checked for general physical condition and were starved over night before use. The doses were all administered at the same time of the day. A solution of ^{14}C-BHT

1) This work was presented at the 98 th Annual Meeting of Pharmaceutical Society of Japan, Okayama, April 1978.
2) Location: *a) 24-1, Hyakunincho 3 chome, Shinjuku-ku, Tokyo 160, Japan; b) 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan.*
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(5 mg/rat, 11 μCi/ml) dissolved in olive oil was given to each rat by a stomach tube. At definite periods after the administration, rats were sacrificed by decapitation. A group of animals (130—140 g) also received 80 mg/kg of sodium phenobarbital in distilled water by intraperitoneal injection each day for 5 days before BHT treatment; the corresponding control animals received physiological saline. Twenty-four hours after the last phenobarbital dose, all animals orally received 50 mg (50 μCi)/kg of ^{14}C-BHT (original radioactive compound was diluted with cold BHT) and were sacrificed 24 hr after the administration.

Preparation of Tissue Homogenates——The liver and other tissues were removed, and as much blood as possible was removed with saline. All subsequent manipulations were carried out below 4°. The liver and other tissues were minced with scissors and homogenized with 0.25 M sucrose in a Potter–Elvehjem homogenizer. The homogenate was filtered through a layer of nylon cloth and made up to a volume corresponding to a 10% suspension.

Cell Fractionation of Liver——The homogenate was fractionated by differential centrifugation according to the method of de Duve, *et al.*[6] The microsomal fractions from phenobarbital-pretreated rats were isolated by centrifugation of the postmitochondrial ($10000 \times g$) supernatant at $105000 \times g$ for 60 min, using a Beckman L5-75 ultracentrifuge. The microsomal pellets were suspended in 0.25 M sucrose, recentrifuged at $105000 \times g$ for 60 min, and finally resuspended in 0.25 M sucrose. Each fraction obtained by the above procedure was used for the determination of total radioactivity and protein content.

Removal of Unbound Radioactivity from Macromolecules——The washing procedures were carried out in a 10-ml centrifuge tube. To 1 ml portion of the homogenate or subcellular fractions 3 ml of 10% trichloroacetic acid was added to precipitate macromolecules. 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 glass 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, twice), 80% methanol (twice), and other organic solvents (*cf.* Table I). The thoroughly extracted precipitate was dissolved in 1 N NaOH for the determination of bound radioactivity and protein content.

Assay Methods——Radioactivity was measured by a Beckman Scintillation Spectrometer, Model LS-355, and corrected by external standard methods. The scintillation medium used consisted of 2 volumes of toluene phosphor (4 g of PPO and 100 mg of dimethyl-POPOP per 1000 ml of toluene) and 1 volume of Triton X-100.[7] The radioactivity was measured by adding the sample (0.5—1.0 ml) and some distilled water, which was needed to solubilize the tissues, to 10 ml of the scintillator. Protein was determined by the method of Lowry, *et al.*,[8] using bovine serum albumin as a standard.

Results and Discussion

Removal of Unbound Radioactivity from Liver Macromolecules

In order to examine the radioactivity bound to liver macromolecules, the protein fraction precipitated with trichloroacetic acid was washed stepwise with various organic solvents.

Table I. Removal of Unbound Radioactivity from Macromolecules

Step	Washing procedure[a]		Recovery of unbound radioactivity[b]	
			Per cent of total	Cumulative
1	10% TCA	(twice)	52.6	52.6
2	80% methanol	(twice)	39.2	91.8
3	80% hot methanol	(twice)	3.9	95.7
4	Methanol–ether	(1 : 1, v/v, twice)	1.6	97.3
5	80% methanol	(twice)	0.9	98.2
6	Chloroform		1.1	99.3
7	Acetone		0.7	100.0
8	Heptane		—	
9	Residue (radioactivity bound to macromolecules)			

a) See Materials and Methods for experimental details.
b) Values are the means of two determinations.

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Table I shows the degree of removal of unbound radioactivity from the macromolecules, and the value in each step is expressed as percentages of total radioactivity extracted up to step 8.

After step 6, the amount of extracted radioactivity was less than 2 per cent. Therefore, the washing procedure in subsequent experiments was generally carried out until step 5. Radioactivity was no longer removed from the residue (step 9) by extraction with methanol–ether (1: 1, v/v) or other organic solvents for 24 hr. This result suggests that the radioactive material(s) in the residue is bound covalently to macromolecules.

Binding of Radioactivity to Liver Macromolecules

After oral administration of ^{14}C–BHT (5 mg/rat, 11 μCi) to rats, the amount of radioactivity bound to macromolecules and total radioactivity in the liver were determined at various time intervals, as shown in Fig. 1.

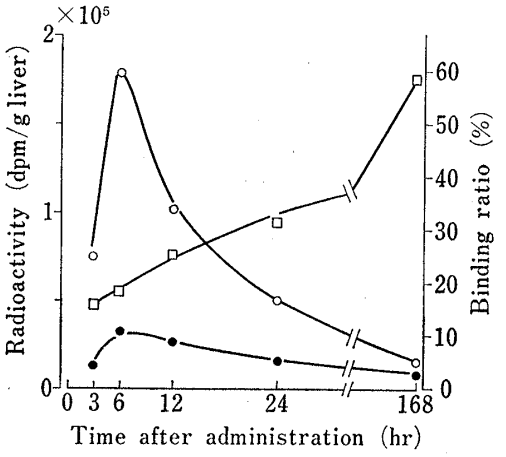

Fig. 1. Time Course of Changes in Radioactivity Bound to Macromolecules of Rat Liver after Oral Administration of ^{14}C–BHT

Total liver radioactivity (○), bound radioactivity (●), and ratio of bound radioactivity to total liver radioactivity (□) were determined at various interval after ^{14}C-BHT(5 mg/rat; 11 μCi) as described in Methods. Points represent means of three animals.

Table II. Subcellular Distribution of Bound Radioactivity in Rat Liver 24 hr after Administration of ^{14}C–BHT

Cell fraction	Bound radioactivity (dpm/mg protein)
Nuclear	151.6±13.1
Heavy mitochondrial	118.0±10.2
Light mitochondrial	208.2± 9.7
Microsomal	301.9±33.3
Supernatant	143.7± 9.2

Three rats received ^{14}C-BHT (5 mg/rat, *p.o.*: 11 μCi) and were sacrificed 24 hr later.

The total radioactivity reached a maximum 6 hr after the administration, the bound radioactivity became the highest 6—12 hr later, and thereafter both radioactivities decreased with time. However, the rate of decrease of bound radioactivity was slower than that of total radioactivity. This result leads to a conclusion that the binding ratio (percentage of bound radioactivity to total radioactivity) increased 17% to 59% with time.

Table II shows the distribution of radioactivity bound to macromolecules in subcellular fractions 24 hr after the administration.

The bound radioactivity was found in all subcellular fractions, although the radioactivity in the microsomal fraction was higher than that in other fractions.

We already reported that the radioactivity, incorporated into the supernatant fraction in the liver after the administration of ^{14}C–BHT, migrated to the microsomal fraction, and the affinity of radioactivity with the microsomal fraction is higher than that with other particulate fractions.[5] Therefore, in order to investigate the binding of radioactive material(s) to the microsomes, the radioactivity bound to microsomes, prepared from phenobarbital-pretreated and untreated control rats 24 hr after the administration of ^{14}C–BHT (50 mg/50 μCi/kg), was determined, as shown in Table III.

Table III. Effect of Phenobarbital (PB) on ^{14}C–BHT binding to Microsomes

Treatment	Body weight (g)	Liver weight (g/100 g B.W.)	Microsomal protein (mg/g liver)
Non	132.5±3.5	5.2±0.4	21.9±0.4
PB	131.5±2.5	6.2±0.1*	26.4±2.6*

Treatment	Total Radioactivity (dpm/g liver)		Bound radioactivity (dpm/mg protein)	
	Liver	Microsomes	Liver	Microsomes
	×10^4	×10^3		
Non	3.99±0.29	7.35±0.86	64.8±10.2	94.4±11.8
PB	6.00±0.35*	11.67±1.44*	94.5± 3.3*	115.9± 3.0*

Differences between Non and PB-treated animals were compared by Student's t-test, and are denoted by* $p<0.05$. Each value is the means±S.D. of three rats.

Table IV. Distribution of Total and Bound Radioactivities in Various Tissues 24 hr (a) or 168 hr (b) after Oral Administration of ^{14}C–BHT

(a) 24 hr

Tissues	Total radioactivity (dpm/g tissue)	Bound radioactivity (dpm/g tissue)	Bound radioactivity (dpm/mg protein)[b]	Binding ratio (%)
	×10^3	×10^3		
Brain	2.7± 0.4	1.3±0.1	15.1± 0.3	49.8± 8.9
Lung	30.7±12.5	18.0±8.3	138.4±21.0	58.0± 3.5
Liver	50.7± 1.6	16.2±1.6	171.3±46.8	31.9± 2.3
Spleen	11.7± 4.6	7.0±2.6	68.7±17.2	60.4± 4.5
Kidney	20.1± 6.0	9.2±3.1	71.7±19.0	45.3± 1.5
Blood	9.3± 1.8[a]			

(b) 168 hr

Tissues	Total radioactivity (dpm/g tissue)	Bound radioactivity (dpm/g tissue)	Bound radioactivity (dpm/mg protein)[b]	Binding ratio (%)
	×10^3	×10^3		
Brain	2.0± 0.3	0.9±0.3	9.3± 3.5	42.2±11.7
Lung	9.3± 0.8	6.6±1.4	66.7±10.8	70.5± 8.4
Liver	18.3± 1.1	10.9±1.7	72.5±13.1	59.4± 5.7
Spleen	4.8± 0.1	2.5±0.3	22.2± 3.6	52.3± 4.8
Kidney	6.5± 0.5	3.6±0.7	30.4± 4.5	55.5± 5.6
Blood	3.9± 0.5[a]			

a) dpm/ml.
b) Protein content was measured by the biuret method with bovine serum albumin as the standard.[13]
Three rats were orally administered with ^{14}C-BHT at a level of 5 mg/rat (11 μCi).
Binding ratio is expressed as the percentage of bound radioactivity to total radioactivity.
Each value is the means±S.D.

The body weight of phenobarbital-pretreated and untreated rats was similar, but the liver weight and the protein content of microsomal fraction were increased significantly in the phenobarbital-treated group. The total radioactivity in the liver and microsomes in the phenobarbital-treated group was higher than that in the untreated group. The radioactivities bound to macromolecules in the microsomes and liver of phenobarbital-treated group were found to be 123% and 145% higher than those in the untreated group, respectively. This result indicates that the binding of radioactive material(s) was more pronounced in the microsomes prepared from animals pretreated with inducer of microsomal drug-metabolizing enzymes.

The *in vivo* experiments described above indicate that a certain amount of radioactive material(s) after the administration of ^{14}C–BHT are tightly bound to macromolecules in hepatocytes. It was found that acetaminophen,[9] halobenzene,[10] and polychlorinated biphenyls,[11] which are chemically inert, can be converted by a cytochrome P-450 enzyme in the liver microsomes to their active metabolites, some of which became covalently bound to macromolecules. Since BHT is a chemically stable compound, it is likely that metabolic activation of BHT is required for the binding reaction.[12]

Binding of Radioactivity to Macromolecules in Various Tissues

Table IV shows the distribution of total and bound radioactivities in various tissues 24 or 168 hr after the administration of ^{14}C–BHT (5 mg/rat, 11 μCi).

The highest total and bound radioactivities were found in the liver throughout the experimental period. Both radioactivities were found much higher in the lung than in other tissues and blood. It was recently found that an enlargement and pathological changes were induced in mouse lung by BHT.[14–16] On the other hand, no such changes have been reported for rats treated with BHT, although we are interested in higher concentration of radioactivity in the lung in this experiment.

The binding ratio in the liver, lung, and kidneys increased with time and that in the brain and spleen remained about 50% throughout experimental period (Table IV).

Excretion of radioactivity from rats receiving ^{14}C–BHT in a single oral dose was slow, because the recovery of radioactivity excreted in urine and feces was 50—60% of the dose until 1 week later.[17] In addition, Ladomery *et al.*,[18] and Daniel and Gage[19] found slow rate of excretion, which was not complete even after 1 week. For this reason, they concluded that the radioactivity associated with ^{14}C–BHT or its metabolites was reabsorbed by a rapid enterohepatic circulation. However, this result suggests that a considerable amount of radioactive material(s) remained tightly bound onto macromolecules in various tissues for a long time.

In the present work, some of the radioactive material(s) from ^{14}C–BHT orally administered to rats was found to be tightly bound to macromolecules in the liver and other tissues, and further examination is necessary for the identification of bound radioactive material(s) and its effect on the function of liver cells.

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