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Biological Fate of Butylated Hydroxytoluene (BHT): Subcellular Distribution of BHT in Rat Liver

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Biological fate of ^{14}C-labeled butylated hydroxytoluene (^{14}C-BHT) in rat liver was investigated. After a single oral dose of ^{14}C-BHT, the radioactivity was reached a maximum concentration in the liver after 6 hr and in the serum after 6—12 hr.

A bulk of radioactivity was found in the supernatant fraction, although the amount of radioactivity and specific radioactivity increased in the microsomal fraction with time. The radioactivity which was incorporated into the microsomal fraction was mainly located in the smooth ER 3—6 hr after the administration.

These results suggest that the radioactivity, which was incorporated into the supernatant fraction, migrated to the microsomal fraction.

Keywords——antioxidant; butylated hydroxytoluene; oral administration; rat liver; subcellular distribution; submicrosomal distribution

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.[2] Though its *in vivo* metabolism was reviewed by Hathway,[3] intracellular distribution of BHT has not been reported.

In the present work, intracellular distribution of BHT in rat liver was examined by differential and density-gradient centrifugation using ^{14}C-labeled BHT in order to clarify the relationship between the metabolic fate and intracellular localization of the compound.

Materials and Methods

Animals——Male Wistar rats (SPF) weighing 170—200 g were used and divided into groups of 3 to 4 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, which is known to be resistant against metabolic conversion in the rat,[2,3] was labeled with ^{14}C. 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.

Treatment——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 (5 mg/rat, 11 μCi/ml; in the experiment on cell fractionation, 15 mg/rat, 33 μCi/ml; in the experiment on submicrosomal fractionation) dissolved in olive oil was given to each rat by a stomach tube.

Cell Fractionation of Rat Liver——At definite time after the administration of ^{14}C-BHT, rats were sacrificed by decapitation and the liver was removed and washed with saline to remove blood. All subsequent manipulations were carried out at below 4°. The liver was homogenized with 0.25 M sucrose in a Potter-Elvehjem homogenizer to make a 10% homogenate. The homogenate was fractionated by differential centrifugation according to the method of de Duve, *et al.*[4]

Each fraction was used for the determination of radioactivity, enzyme activity, and protein.

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Preparation of Microsomal Subfractions——The rough and smooth endoplasmic reticulum (ER) were isolated by using a slight modification of the method described by Imai and Sato.[5] The procedure is shown in Fig. 1.

Both rough and smooth fractions were collected, diluted with distilled water in order to make a concentration of 0.25 M sucrose, and then recentrifuged at $105000 \times g$ for 90 min. The pellets were dissolved in 1.15% KCl, recentrifuged at $105000 \times g$ for 90 min, and finally resuspended in 0.25 M sucrose for the determination of radioactivity, RNA, and protein contents. Both kinds of ER fractions were identified by using electron microscopy.

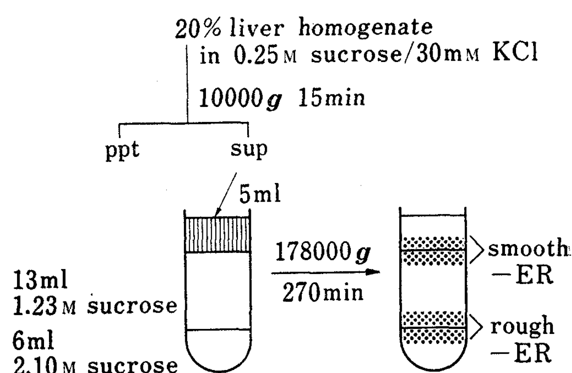

Fig. 1. A Schematic Representation of the Subfraction of $10000 \times g$ Supernatant on a Discontinuous Sucrose Gradient

Assay Methods——Radioactivity was measured by a Beckman Scintillation Spectrometer, Model LS-355, and corrected by external standard methods. The scintillation medium[6] 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. The radioactivity was measured by adding the sample (0.5—1.5 ml) and some distilled water, which was needed to solubilize the tissues, to 10 ml of the scintillator. Recovery of radioactivity was more than 90% in the experiment on cell fractionation and approximately 70% in the experiment on microsomal subfractionation.

Acid phosphatase activity was determined by using 4-nitrophenyl phosphate as a substrate according to the method of Bessey, *et al.*,[7] with some modification. Enzyme preparations were treated with 0.5% final concentration of Triton X-100 before incubation.

Glucose-6-phosphatase activity was determined by using glucose-6-phosphate as a substrate, and the liberated inorganic phosphate was measured according to the method of Lindberg and Ernster,[8] with some modification.

RNA in the microsomes was extracted as described by Schneider[9] and determined by the orcinol reaction.[10]

Protein was determined according to Lowry, *et al.*,[11] using bovine serum albumin as a standard.

Results and Discussion

Changes in Liver and Serum Level of Radioactivity after Administration of ^{14}C-BHT

Since BHT is used as an antioxidant and is taken daily into the human body with food, this compound was given to experimental animals by oral administration.

Male rats were given 5 mg of ^{14}C–BHT (11 μCi/rat) and radioactivity in the liver and serum was measured, as shown in Table I.

Radioactivity in the liver reached a maximum 6 hr after the administration and then decreased with time. At 6 hr after the administration, 5.1% of the dose was found in the liver. Also radioactivity in the serum became the highest within 6—12 hr after the administration and decreased with time. However, a considerable amount of radioactivity was observed in the liver and serum after 48 hr. These findings may support the report of Ladomery, *et al.*[12] that this compound is excreted slowly into urine and feces because of the rapid enterohepatic circulation in the rat.

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Table I. Changes in Tissue Level of Radioactivity after Oral Administration of ^{14}C–BHT in Rat

Time after administration (hr)	Liver dpm/liver ($\times 10^5$)	Liver dpm/g. liver ($\times 10^4$)	Serum dpm/ml ($\times 10^3$)
1	1.84±0.37	3.23±0.69	5.2±0.7
3	5.19±0.38	7.23±0.67	12.2±1.0
6	12.29±1.57	17.42±1.34	30.4±2.5
12	8.94±0.60	11.48±1.33	29.0±1.5
24	5.60±0.45	5.61±0.61	14.0±1.0
48	4.69±0.21	5.56±0.61	8.0±0.9

A single dose (5 mg/rat, 11 μCi) of BHT which was dissolved in olive oil was orally administered to male rats. Each value is the means±S.D. of three or four animals.

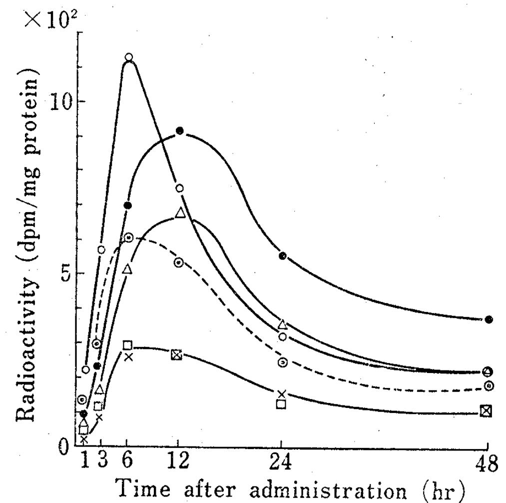

Fig. 2. Time-Course of Changes in Specific Radioactivity in Subcellular Fractions of Rat Liver after Oral Administration of ^{14}C–BHT

Values (means of three or four animals) are expressed as the radioactivity per milligram of protein of each fraction.
---⊙---; homogenate, —□—; nuclear,
—×—; heavy mitochondrial,
—△—; light mitochondrial,
—●—; microsomal, —○—; supernatant.

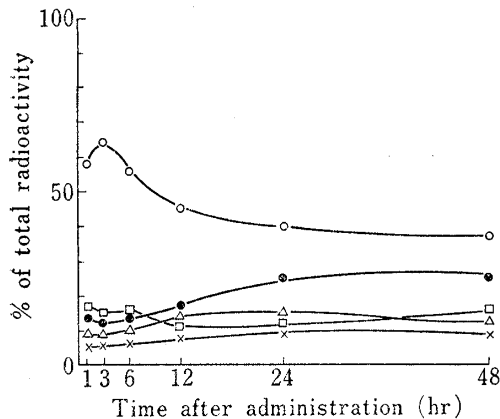

Fig. 3. Changes in Subcellular Distribution of Radioactivity after Oral Administration of ^{14}C-BHT

Values are the means of three or four animals.
—□—; nuclear,
—×—; heavy mitochondrial,
—△—; light mitochondrial,
—●—; microsomal,
—○—; supernatant.

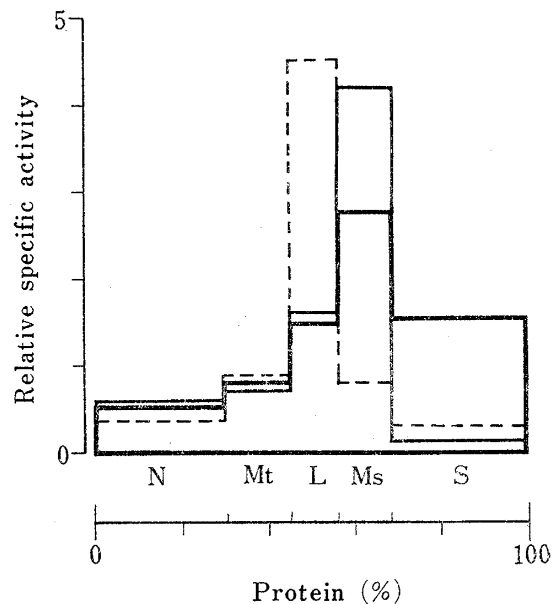

Fig. 4. Distribution Patterns of Radioactivity and Enzyme Activities in Subcellular Fractions at 24 hr after Administration

——; radioactivity,
——; glucose-6-phosphatase activity,
----; acid phosphatase activity.
N, Mt, L, Ms and S are represented as nuclear, heavy mitochondrial, light mitochondrial, microsomal and supernatant fractions respectively. Relative specific activity is percentage of radioactivity or enzymes activity in fraction/ percentage of protein in fraction. Values are the means of four animals.

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Subcellular Distribution of Radioactivity after Administration of ^{14}C-BHT

^{14}C–BHT was given to rats (5 mg, 11 μCi/rat), their liver was removed, and subjected to cell fractionation at timed intervals.

Figure 2 shows the time-course of specific radioactivity in each subcellular fraction. Specific radioactivity in each of the nuclear, heavy mitochondrial, and especially supernatant fraction reached a maximum 6 hr after the administration. On the other hand, radioactivity in the microsomal and light mitochondrial fractions reached a maximum after 12 hr. The rate of decrease of specific radioactivity in the microsomal and light mitochondrial fractions was slower than that in the supernatant fraction.

Figure 3 shows the percentage of radioactivity in each subcellular fraction to total subcellular radioactivity at intervals.

The radioactivity incorporated into the liver cell was found mainly (about 60% of the total) in the supernatant fraction in an earlier stage. However, radioactivity in the microsomal fraction increased about twice with decrease of radioactivity in the supernatant fraction. A considerable amount of radioactivity (25% of the total) was observed in the microsomal fraction after 48 hr but significant changes in radioactivity were not observed in other subcellular fractions. Distribution of radioactivity and enzymes in subcellular fractions 24 hr after the administration is shown Fig. 4. In the particulate fractions, an apparent similarity in the distribution patterns was found between the radioactivity and glucose-6-phosphatase activity, which is a marker enzyme of microsomes.

The results presented in Fig. 3 and 4 indicate that the affinity of radioactivity with the microsomal fraction is higher than that with other particulate fractions. In addition, the results shown in Fig. 2 suggest that radioactivity in the supernatant fraction migrated into the microsomal fraction with time.

Submicrosomal Distribution of Radioactivity after Administration of ^{14}C-BHT

Since radioactivity in the microsomal fraction increased with time, the supernatant fraction, designated as the "10000×***g*** supernatant," was subfractionated by discontinous sucrose gradient cetrifugation; the radioactivity, concentration of RNA, and protein in the rough and smooth ER were determined, as shown in Table II.

Table II. Submicrosomal Distribution of Radioactivity of Rat Liver after Administration of ^{14}C-BHT

Time after administration (hr)	Protein (mg/g. liver)		RNA (μg/mg. protein)			Radioactivity (dpm/mg. protein)		
	Smooth	Rough	Smooth	Rough	Ratio (R/S)	Smooth	Rough	Ratio (S/R)
3	8.80	8.50	28.1	196.7	7.0	700	500	1.40
6	8.40	8.11	27.5	202.4	7.4	1690	1040	1.64
12	8.36	8.05	25.6	178.1	6.9	1440	1460	0.99
24	7.20	8.55	29.7	186.9	6.3	1070	1220	0.88

Rats were orally administered with ^{14}C-BHT at a level of 15 mg/rat (33 μCi). Values are the means of two animals.

The concentration of RNA in the rough ER was six to seven times more than that of the smooth ER. Electron micrographs of the microsomal subfractions revealed that the rough and smooth ER are essentially completely separated, as shown in Fig. 5.

The specific radioactivity in the smooth ER was found to be higher (140—160%) than that in the rough ER until 6 hr after the administration. However, after 12 hr, the specific

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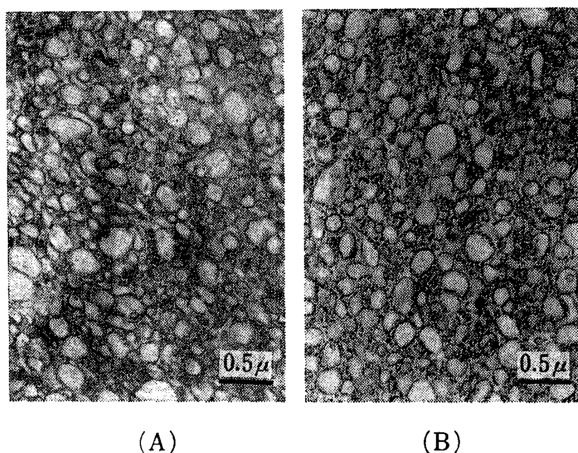

(A) (B)

Fig. 5. Electron Micrographs of Liver Microsomal Subfraction obtained by Centrifugation on a Discontinuous Sucrose Gradient

(A) smooth ER fraction.
(B) rough ER fraction.

radioactivity in both ER was roughly the same. Also, the specific radioactivity in the smooth ER reached a maximum 6 hr after the administration, but the maximum was reached after 12 hr in the rough ER. Therefore, radioactivity in the smooth ER seemed to migrate into the rough ER.

However, the present data are insufficient to determine how the radioactivity in the smooth ER migrated into the rough ER.

In the present experiment, we were interested in what effect the higher affinity of the radioactivity (^{14}C–BHT and/or its metabolites) with the microsomes will have on the function of liver cells, and it is necessary to investigate this problem in more detail.

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