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Biological fate of butylated hydroxytoluene (BHT)—Binding of BHT to nucleic acid *in vivo*

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Butylated hydroxytoluene (3,5-di-*tert*-butyl-4-hydroxytoluene; BHT) is widely used as an antioxidant in processed foods and petroleum products. Though toxicological, metabolic and carcinogenic studies on BHT in experimental animals have been reported [1–4], the biological fate of this compound has not been reported. Therefore, we have been studying the behavior of this compound in the rat liver cell. In previous *in vivo* and *in vitro* studies [5, 6], we have found that (a) BHT is converted to chemically activated materials(s) by a cytochrome P-450-linked mono-oxygenase system, (b) a cytochrome P-450 with a high BHT oxidase is mainly involved in the activation of this compound, (c) some of the activated material(s) subsequently binds to cellular macromolecules, and (d) the binding is more pronounced in the microsomes from animals pretreated with phenobarbital or BHT. In this paper, we show that some of the radioactive material(s) from [^{14}C]BHT, orally administered to rats, is bound to nucleic acid and protein in the liver.

3,5-Di-*tert*-butyl-4-hydroxytoluene (toluene [methyl-^{14}C]) (specific radioactivity, 0.485 μCi/μmole) was purchased from the New England Nuclear Corp. (Boston, MA). Radiochemical purity was rechecked with thin-layer chromotography and found to be more than 99 per cent. Nonradioactive BHT was purchased from the Wako Pure Chemicals Co. (Osaka, Japan), and sodium phenobarbital was a gift from the Fujinaga Pharmaceutical Co. (Tokyo, Japan); all the other chemicals used were of the highest obtainable purity.

Male Wistar rats (SPF), weighing 150–160 g, were used and divided into groups of three rats for each experiment. The animals were starved overnight before use, and all doses were given at the same time of the day. A solution of [^{14}C]BHT (5 mg/rat, 11 μCi) disolved in olive oil was given to each rat by a stomach tube. At definite periods after the administration, rats were decapitated. A group of animals also received 80 mg/kg sodium phenobarbital in distilled water by intraperitoneal injection each day for 5 days before treatment with BHT; the corresponding control animals received physiological saline. Twenty-four hr after the last phenobarbital dose, all animals received orally 50 mg (50 μCi)/kg of [^{14}C]BHT (the original radioactive compound was diluted with cold BHT) and were killed 24 hr after the administration.

Isolation of cellular components

Method A. The liver was removed and perfused with saline. All subsequent manipulations were performed at temperatures near 0°. The liver was 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. The washing or extracting procedures were carried out in a 10-ml centrifuge tube. To a 1-ml portion of the homogenate, 3 ml of 10% trichloracetic acid (TCA) were added to precipitate macromolecules. The resultant precipitate was collected by centrifugation, resuspended in 4 ml of 7.5% TCA, and mixed in a Vortex shaker with a narrow glass spatula inserted into the tube. The tube was centrifuged again. The washed pellet was mixed with the spatula as above, and then extracted successively with 80% methanol (four times) and methanol–ether (1:1, v/v, six times). After removal of unbound radioactivity from the macromolecules, the washed pellet was boiled twice with 5% TCA for 15 min. The extracted pellet and TCA supernatant fraction were used as protein fraction and nucleic acid fraction respectively.

Method B. The liver was homogenized with 6% sodium *p*-aminosalicylate in a Potter–Elvehjem homogenizer, and made up to a volume of 6.7% suspension. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were isolated and purified by the procedure of Irving and Veazey [7]. Furthermore, the protein was isolated by the addition of equal volumes of methanol to the phenol extracts in the procedure used above [8], and the protein was freed of nucleic acids by extracting twice with hot TCA. The isolated DNA, RNA and protein fractions were washed repeatedly with methanol–ether (1:1, v/v) or with ether until no further radioactivity could be removed from each macromolecule. The DNA and RNA were dissolved in 0.015 M NaCl–0.0015 M sodium citrate, pH 7.4, and the protein was dissolved in 1 N NaOH, for the determination of bound radioactivity and amount of each macromolecule. The RNA isolated was mainly ribosomal RNA and was free from DNA and soluble RNA [7].

Radioactivity was measured by a Beckman scintillation spectrometer, model LS-355, and corrected by the external standard method. The scintillation medium used consisted of 2 vol. toluene phosphar (4 g PPO and 100 mg dimethyl-POPOP per 1000 ml of toluene)* and 1 vol. Triton X-100 [9]. Protein was determined by the method of Lowry *et al.*

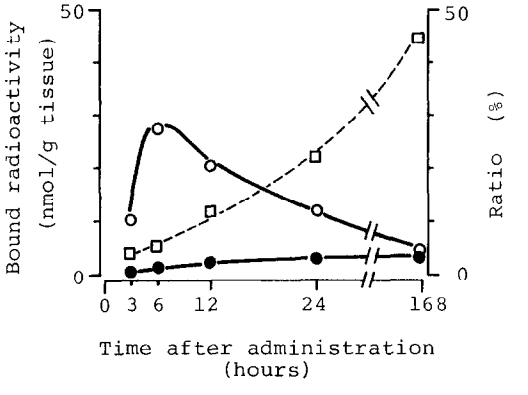

Fig. 1. Time course of changes in radioactivity bound to macromolecules of rat liver after oral administration of [^{14}C]BHT. Radioactivity in the protein fraction (○) and in the nucleic acid fraction (●) was determined at various intervals after [^{14}C]BHT (5 mg/rat; 11 μCi) as described in Method A. The ratio (□) is expressed as the percentage of radioactivity in the nucleic acid fraction over that of the nucleic acid and protein fractions combined. Points represent means of three animals.

* PPO = 2,5-diphenyloxazole; and POPOP = 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene.

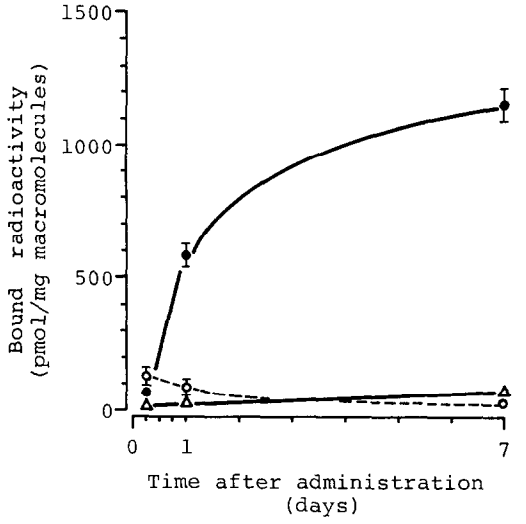

Fig. 2. Time course of binding of [^{14}C]BHT to DNA, RNA and protein of rat liver. Radioactivities of DNA (△), RNA (●) and protein (○) were determined at various intervals after [^{14}C]BHT (5 mg/rat; 11 μCi), as described in Method B. Points represent means ± S.D. of three (6-hr) or two (1- and 7-day) rats.

[10], with bovine serum albumin as a standard. The quantities of DNA and RNA were determined by absorbance at 260 nm. The ratio of absorbance at 260 nm to that at 280 nm was usually 1.9–2.0 for DNA and 2.1–2.2 for RNA. The DNA and RNA were free from detectable protein.

After oral administration of [^{14}C]BHT (5 mg/rat, 11 μCi) to rats, the amounts of radioactivity incorporated into the protein fraction and the nucleic acid fraction of the liver were determined at various intervals, as shown in Fig. 1. The total radioactivity in the protein fraction reached a maximum at 6 hr after administration, and thereafter the radioactivity decreased with time. However, the radioactivity incorporated into the nucleic acid fraction increased with time, and the level remained constant for 1 week. Therefore, the ratio of the percentage of radioactivity in the nucleic acid fraction to that of the nucleic acid and protein fractions combined, increased from about 5 to 50 per cent with time. This result indicates that the kinetic behavior of the radioactive material(s) incorporated into the nucleic acid fraction differed from that of the protein fraction.

Figure 2 shows the time course of specific radioactivities in DNA, RNA and protein fractions. The time course of radioactivity in RNA was quite different from that of DNA or protein. The radioactivity in RNA increased remarkably with time, and after 1 week the level of radioactivity in RNA was about eighteen or thirty-five times that in DNA or protein, respectively. In contrast to this result, the radioactivity in protein reached a maximum at 6 hr after administration, and decreased gradually with time, presumably because of the turnover of protein itself. Although the radioactivity in DNA was lower than that in RNA throughout the experimental period, the radioactivity increased gradually with time and was approximately twice that in protein at 1 week after administration. These results suggest that the radioactive material(s) was incorporated into DNA and, especially, into RNA with time. It seems that the level of radioactivity in the nucleic acid fraction (Fig. 1) is due mainly to the amount of radioactive material(s) incorporated into RNA (Fig. 2). Furthermore, the results presented above suggest that a considerable amount of activated BHT material(s) remained tightly bound to RNA and DNA for a long time.

We have already reported that the radioactive material(s) bound to macromolecules in the liver of the phenobarbital-pretreated rat was higher than that in the treated rats after oral administration of [^{14}C]BHT [5]. Therefore, to investigate the effect of pretreatment with phenobarbital on [^{14}C]BHT binding to DNA, RNA and protein, the radioactivity bound to each macromolecule, separated from phenobarbital-pretreated and untreated rats 24 hr after the administration of 50 mg (50 μCi)/kg of [^{14}C]BHT, was determined, as shown in Table 1. The body weights of the phenobarbital-treated and untreated rats were similar, but the liver weights increased significantly in the phenobarbital-treated group. In addition, the total radioactivity in the livers of the phenobarbital-treated group was higher than in the untreated group. The specific radioactivities in DNA, RNA and protein of the phenobarbital-treated group were found to be 153, 170 and 154 per cent higher than those in the untreated group. An increase in binding of radioactive material(s) to macromolecules in phenobarbital-treated animals suggests that the induction increased the amount of activated BHT metabolite(s) formed during metabolism by liver mixed-function oxidase [6]. Based on the results presented in Fig. 2 and Table 1, it is apparent that the affinity of activated BHT metabolite(s) to RNA is remarkably higher than to other macromolecules.

This incorporation of activated BHT metabolite(s) into the macromolecules is probably the result of covalent binding, since the radioactive material(s) of the purified macromolecules could not be removed by exhaustive extraction with methanol–ether (1:1, v/v) or ether. The activated material(s) may be formed by metabolic activation at the 4-methyl group of BHT, since the increase in binding of [^{14}C]BHT to macromolecules closely parallels the increase in BHT oxidase [6], which is known to oxidize the methyl group of the parent compound [11]. The nature

Table 1. Effect of pretreatment with phenobarbital (PB) on [^{14}C]BHT binding to liver macromolecules*

Treatment	Body wt (g)	Liver wt	Total radioactivity† (nmoles/g)	Total radioactivity† (nmoles/liver)	Bound radioactivity Protein (pmoles/mg macromolecules)	Bound radioactivity DNA (pmoles/mg macromolecules)	Bound radioactivity RNA (pmoles/mg macromolecules)
Control	154 ± 4	8.2 ± 0.3	52.3 ± 4.0	426 ± 38	73.3 ± 6.2	34.4 ± 6.4	577 ± 29
PB	152 ±5	9.5 ± 0.4	69.7 ± 3.3	661 ± 30	112.7 ± 3.8	52.6 ± 8.8	981 ± 197
% of Control	99	116‡	133‡	155‡	154‡	153‡	170‡

* Animals pretreated with PB were killed 24 hr after oral administration of 50 mg (50 μCi)/kg of [^{14}C]BHT. Each value is the mean ± S.D. of three rats.

‡ Differences between control and PB-treated animals were compared by Student's t-test, $P < 0.01$.

† Total radioactivity was measured by adding the homogenate to the scintillator.

of the activated material(s) is to be investigated in future work.

Though BHT has various biological effects [1–4, 12–15], the biological significance of the observed interaction of activated material(s) with RNA and DNA *in vivo* is unclear. It has been reported that the carcinogenicity of several chemicals was inhibited by dietary BHT [16, 17]. The underlying mechanism appears to be an alteration of the microsomal enzyme system that metabolizes the carcinogen, leading to a decrease in binding of the carcinogen or its metabolites to DNA [18, 19]. However, it has been reported that chemical carcinogenesis can also be inhibited by competition of other chemicals for active binding sites in cellular macromolecules [20, 21]. Therefore, it may be presumed that the activated BHT metabolite(s) competes with the binding of a carcinogen or its metabolites to nucleic acid, and that this competition is responsible for part of the inhibitory effect on tumor formation by certain carcinogens.

In summary, the binding of radioactive material(s) to DNA, RNA and protein in rat liver after the oral administration of [^{14}C]BHT was investigated. The total radioactivity in the protein fraction reached a maximum in 6 hr and decreased gradually, but that in the nucleic acid fraction increased with time and the level of radioactivity remained constant for 1 week. The specific radioactivity in RNA increased remarkably with time, and this radioactivity was about eighteen or thirty-five times that in DNA or protein after 1 week. Furthermore, the pretreatment of animals with phenobarbital increased the specific radioactivities in RNA (170 per cent), DNA (153 per cent) and protein (154 per cent).

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