JOURNAL OF THE TENNESSEE ACADEMY OF SCIENCE
VOLUME 62, NUMBER 3, JULY 1987

COVALENT BONDING OF RADIOACTIVITY DERIVED FROM [^{14}C] BUTYLATED HYDROXYTOLUENE TO LIVER RIBONUCLEIC ACID

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ABSTRACT

Butylated hydroxytoluene (BHT) is a free radical scavenger widely used as an antioxidant. When mice were administered [*tert*-butyl-^{14}C] BHT the liver RNA bound significant amounts of radioactivity. Although the majority of the BHT-derived radioactivity was incorporated into the molecular structures of the purine nucleotides, a small portion (approximately 15%) apparently formed an as yet unidentified covalent adduct with a nucleotide. The untraviolet absorbance of the adduct revealed an altered spectrum with an absorption maximum at 275 nm.

INTRODUCTION

Butylated hydroxytoluene (3,5-di-*tert*-butyl-4-hydroxytoluene; BHT), a widely used antioxidant, presumably functions as a scavenger of free radicals to prevent the autoxidation of unsaturated lipids (Hathaway 1966; Babich 1982). It was recently reported that BHT was metabolized to chemical species which apparently bound cellular macro-molecules (Nakagawa et al. 1979a; Nakagawa et al. 1979b; Nakagawa et al. 1980; Nakagawa et al. 1981a; Kehrer and Witschi 1980; Daugherty 1984). The binding of radioactive material to rat liver RNA following administration of [toluene (methyl-^{14}C)] BHT was reported to be increased 400 percent from six to 120 hours after administration of the antioxidant (Nakagawa et al. 1980). The apparent increased binding of BHT to RNA was later shown to result from incorporation of the ^{14}C-labeled methyl group into purine bases and not from the binding of BHT to the nucleotide moieties (Nakagawa et al. 1981b). Similar studies with [^{14}C] BHT administered to mice have also demonstrated macromolecular binding to various tissues and macromolecules (Daugherty 1984). However, although the majority of the radioactive label was associated with the RNA species in the mouse, the amount of BHT-derived radioactivity bound to RNA actually decreased during the time interval from eight hours to four days (Daugherty 1984). Since these later studies in mice were conducted with BHT randomly labeled in the tertiary butyl groups, the nature of the binding of radioactivity derived from [*tert*-butyl-^{14}C] BHT to mouse liver RNA was investigated.

MATERIALS AND METHODS

Eight-week–old male BALB/c mice, 22–28 g, were housed in non-barrier conditions with limited accessibility, and were permitted free access to food and water during the experiments. [^{14}C] BHT, randomly labeled in the tertiary butyl moieties, was purchased from New England Nuclear Corp. The specific activity was 7.96 mCi/mol, and the radiochemical purity was greater than 98.5% as determined by thin layer chromatography on silica gel G plates in *n*-heptane:benzene (4:1). Nonradioactive BHT was purchased from Eastman Kodak Chemical Co. Other chemicals were of reagent grade or better and were purchased from Sigma Chemical Co., Fisher Scientific Co., and New England Nuclear Corp.

The animals received a single intragastric intubation of radioactive BHT (55 mg/kg; 0.020 mCi/animal) at 11 weeks of age. The radioactive BHT was dissolved in olive oil and each animal received 0.05 ml. Five animals were killed by cervical dislocation eight hours after administration of radioactive BHT. Later time intervals were not studied since previous studies had shown that the maximum amount of binding occurred eight hours following administration of BHT randomly labeled in the tertiary butyl groups (Daugherty 1984). The liver was removed, weighed and minced with dissecting scissors and homogenized in 10 volumes of ice-cold 50 mM Tris buffer (pH 7.5) containing 0.25 M sucrose. The liver RNA was prepared as described previously

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(Daugherty 1984). Briefly, the homogenate was precipitated by the addition of trichloroacetic acid to a final concentration of 5% and the lipid components and BHT were extracted twice with 70% ethanol, twice with ethanol-diethyl ether (1:1; v/v) and once with diethyl ether (Nakagawa et al. 1979a). The residue was resuspended with a glass homogenizer in five volumes of a solution prepared by adding 6 ml of 2-butanol to 1 g sodium triisopropylnaphthalene sulphonate, followed by 100 ml water, 6 g sodium p-aminosalicylate and 1 g NaCl. The resuspended material was then extracted by shaking with an equal volume of a solution consisting of phenol:*m*-cresol:8-hydroxyquinoline:water (100 : 14 : 0.1 : 11, by vol.). Following a 20 min period of shaking, the aqueous and phenolic layers were separated by centrifugation at 10,000 g for 15 min. The upper aqueous layer, containing DNA and RNA, was removed and extracted twice with two volumes of chloroform-isoamyl alcohol (20:1; v/v). The RNA was precipitated from the aqueous layer by the addition of four volumes of methanol. The RNA was washed five times with five volumes of methanol, air dried and dissolved in 0.01 M sodium acetate (pH 5.5) buffer. The RNA solution was treated for twenty minutes with DNase (100 µg/ml; free of RNase activity) and the RNA was then precipitated by the addition of 10 volumes of methanol and the pellets were collected by centrifugation and rinsed three times with two ml 1N HCl. Radioactivity associated with the RNA was determined by hydrolysing with 20% $HClO_4$ for 10 min at 90°C.

The ion exchange chromatography was conducted as described previously (Nakagawa et al. 1981b). The radioactivity of the isolated RNA was 3374 ± 288 dpm/mg RNA. Approximately 6 mg of RNA was hydrolyzed in 1.2 ml of 50 mM acetate buffer (pH 4.5) with 67 units of ribonuclease T2 (ribonucleate 3'-oligonucleotide-hydrolase [E. C. 3.1.27.1]) for 24 hours at 37°C. Following the enzymatic hydrolysis, 0.5 ml of the hydrolysate was added to 1.5 ml of 20 mM carbonate buffer (pH 9.7), and the solution was applied to a QAE-Sephadex A-25 column (1 × 40 cm). The hydrolysate was eluted with 375 ml of 20 mM carbonate buffer (pH 9.7), with a continuous sodium chloride gradient from 0.0 to 0.40 M at room temperature. The column was eluted at a rate of 30 ml/hr, fractions of five ml were collected, and aliquots were assayed for radioactivity and absorbance at 260 nm. The fractions containing the unknown radioactivity compound were pooled, evaporated to reduced volume and analyzed by ultraviolet absorption spectrum.

RESULTS AND DISCUSSION

The ion exchange chromatrography profile of an enzymatic hydrolysis of mouse liver RNA eight hours after the administration of radioactive BHT is shown in Figure 1. The elution location of authentic mononucleotides from the same column is indicated by arrows.

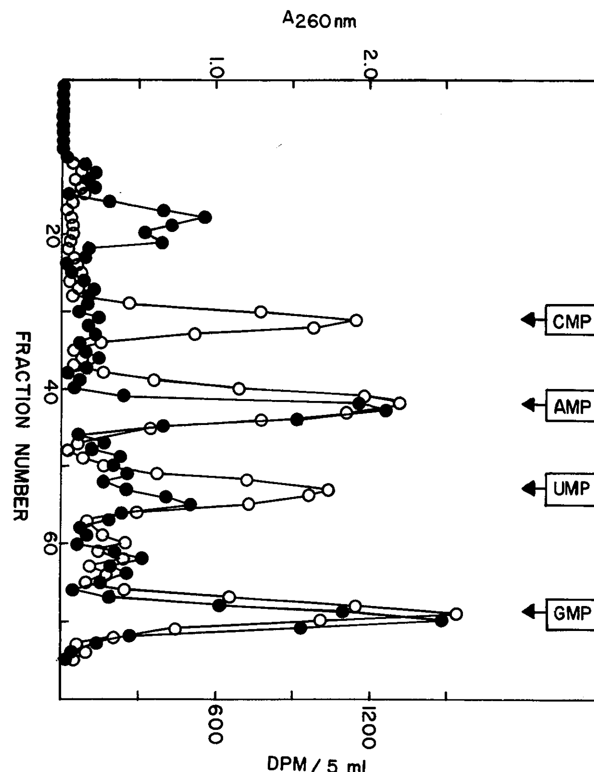

Figure 1. Column chromatography of an enzymatic hydrolysate of mouse liver RNA after oral administration of [^{14}C] BHT. Aliquots of each fraction were used for the determination of radioactivity (black circles) and absorbance (open circles). The arrows indicate the elution position of known mononucleotides: AMP, adenosine 3'-monophosphate; CMP,cytidine 3'-monophosphate; GMP, guanosine 3'-monosphosphate; UMP, uridine 3'-monophosphate.

Although the majority of the radioactivity coeluted with AMP and GMP peaks, a small portion of the radioactivity (approximately 15%) did not elute coincident with any of the remaining nucleotides. Authentic BHT was not eluted under the conditions as described. The ultraviolet absorption spectrum of the unknown compound revealed a decreased absorbance at 260 nm (Figure 2) with a shift in absorbance maximum from 260 to about 275 nm with a shoulder at 310 nm.

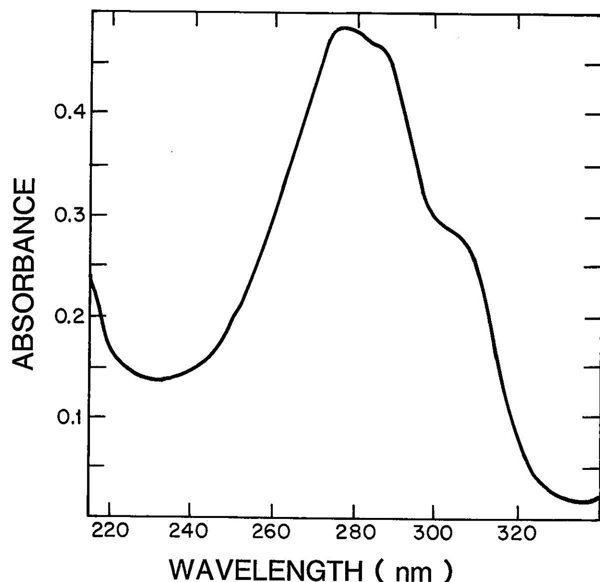

Figure 2. Ultraviolet absorption spectrum of the BHT-nucleotide adduct. Fractions containing the unknown radioactive compound were pooled, evaporated to reduced volume and the spectrum recorded.

The previous studies have shown that the apparent increased binding of BHT to RNA following administration of [toluene (methyl-^{14}C)] BHT resulted from the incorporation of the ^{14}C-labeled methyl group into purine bases and not from the binding of BHT to RNA molecules. However, as shown in Figure 1, similar studies with [*tert*-butyl-^{14}C] BHT demonstrated that a portion of the ^{14}C-label was bound to RNA molecules. The different binding patterns between our studies and those of others are probably due to the different positions of the C-label in the BHT molecule. For example, it is well known that a BHT oxidase system exists which modifies the 4-methyl group of BHT, and results in the 4-methyl carbon entering the one carbon pool (Nakagawa et al. 1981b). Similar *in vitro* studies using 4-methyl labeled butylated hydroxyanisole have also demonstrated the oxidation of this phenolic antioxidant predominantly via a one-electron oxidation process (Rahimtula 1983). In addition, a peroxidase system has been described in which BHT free radicals are produced (Sgaragli et al. 1980).

In summary, we have demonstrated binding of BHT-derived radioactivity to liver RNA following administration of [*tert*-butyl-^{14}C] BHT to mice. Our findings suggest that, although the majority of the BHT-derived radioactivity was incorporated into the molecular structures of the purine nucleotides, a small portion bound covalently to a nucleotide (s). Butylated hydroxytoluene may undergo oxidation to form a BHT free radical which then combines with cellular macromolecules, such as RNA.

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