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On the mechanism of binding of ^{14}C-labeled butylated hydroxytoluene to liver ribonucleic 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. In previous *in vivo* and *in vitro* studies [1–3], we found that (a) BHT is converted to chemically activated material(s) by a cytochrome P-450-linked monooxygenase system, and (b) some of the activated material(s), which may be BHT-alcohol* or quinone methide [4], subsequently binds to cellular macromolecules. The binding of radioactive material to liver RNA of rats treated with [^{14}C]BHT increased remarkably after a lag period of about 6 hr; the specific radioactivity was about eight or seventeen times that in protein or DNA after 24 hr respectively. In addition, a considerable amount of radioactivity remained tightly bound to RNA for a long time [3]. On the basis of those results, it was suggested that the affinity of radioactive material to RNA is remarkably higher than that to other macromolecules. The present paper deals, therefore, with the binding site of radioactive material on liver RNA isolated from rats treated with [^{14}C]BHT.

Materials and methods

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 by thin-layer chromatography and found to be more than 99 percent. Chemical compounds were obtained from the following companies: sodium phenobarbital from the Fujinaga Pharmaceutical Co. (Tokyo, Japan); mononucleotides (AMP, CMP, GMP and UMP) from the Sigma Chemical Co. (St. Louis, MO); and ribonuclease T_2 from Sankyo, Ltd. (Tokyo, Japan); all other chemicals used were obtained from commercial suppliers, and of the highest available purity.

Male Wistar rats, weighing 150–160 g, received 80 mg/kg sodium phenobarbital in distilled water by intraperitoneal injection each day for 5 days before treatment with [^{14}C]BHT; the corresponding control animals received physiological saline. Twenty-four hours after the last phenobarbital dose, all animals received orally 50 mg(50 μCi)/kg of [^{14}C]BHT dissolved in olive oil and were killed 24 hr after the administration. The details of the preparation of liver RNA were described previously [3]. The RNA isolated was mainly ribosomal RNA [5] and was free from detectable protein. In addition, the ratio of the RNA absorbance at 260 nm to that at 280 nm was usually from 2.1 to 2.2. The radioactivity of RNA isolated from untreated rats was 615 ± 30 dpm/mg, whereas that isolated from rats pretreated with phenobarbital was 1050 ± 200 dpm/mg (means ± S.D. of three animals). About 8 mg of RNA was hydrolyzed in 1.2 ml of 50 mM acetate buffer (pH 4.5) with 67 units of ribonuclease T_2 for 24 hr at 37°. After the hydrolysis, 0.5 ml of the solution was added to 1.5 ml of 20 mM carbonate buffer (pH 9.7), and the mixture was applied to a column (bed volume: 1 × 40 cm) of QAE-Sephadex A-25 (Pharmacia Fine Chemicals Co., Uppsala, Sweden). The elution was performed with 350 ml of 20 mM carbonate buffer (pH 9.7), followed by a continuous sodium chloride gradient from 0.0 to 0.4 M at room temperature. The flow rate was about 30 ml/hr, and 5 ml fractions were collected. Aliquots of the fraction were used for the determination of (a) radioactivity by the procedure described previously [3], and (b) nucleotide content by absorbance at 260 nm.

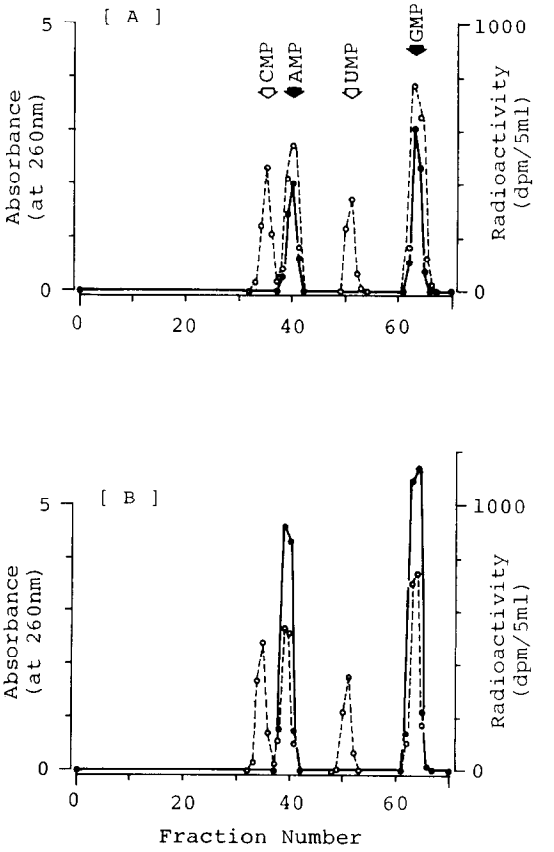

Fig. 1. Ion exchange chromatography of enzymatic hydrolysate of liver RNA isolated from rats ([A], untreated; [B], phenobarbital pretreated) after oral administration of [^{14}C]BHT. Aliquots of each fraction (volume 5 ml) were used for the determination of radioactivity (—●—) and of absorbance at 260 nm (--○--). The arrows show that the position of authentic mononucleotides eluted from the same column.

* Abbreviations: BHT-alcohol, (2,6-di-*tert*-butyl-4-hydroxymethylphenol; quinone methide, 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone; AMP, adenosine 3′-monophosphoric acid; CMP, cytidine 3′-monophosphoric acid; GMP, guanosine 3′-monophosphoric acid; UMP, uridine 3′-monophosphoric acid; ribonuclease T_2, ribonucleate 3′-oligonucleotidohydrolase (EC 3.1.27.1); poly-A, polyadenylic acid; and poly-G, polyguanylic acid.

Results and discussion

Figure 1 shows the ion exchange chromatography of enzymatic hydrolysis of liver RNA isolated from rats 24 hr after the administration of [^{14}C]BHT. QAE-Sephadex A-25 was chosen as the ion exchanger. Four peaks derived from nucleotides were eluted from the QAE-Sephadex column. The elution profiles of these nucleotides were similar to those of authentic mononucleotides—AMP, CMP, GMP and UMP. The radioactivity, however, was detected in the fractions in which AMP and GMP were found to be eluted. Furthermore, the elution profiles of these two radioactive peaks were coincident with the profiles of u.v. absorption corresponding to AMP and GMP fractions. This phenomenon was found in both RNA hydrolysates isolated from untreated rats and rats pretreated with phenobarbital and indicates that the radioactive material found in liver RNA after the administration of [^{14}C]BHT has an especially high affinity for AMP and GMP. Generally, more than 96 percent of the radioactivity applied to the columns was recovered in the total eluate. Of the total radioactivity recovered, 41 percent was found in the AMP fraction of the RNA hydrolysate isolated from untreated rats, whereas 58.1 percent was observed in the GMP fraction. In RNA hydrolysate isolated from rats pretreated with phenobarbital, 44.3 percent was found in the AMP fraction while 55.1 percent was found in the GMP fraction. The total amount of radioactivity in other fractions was negligibly small (0.6 to 0.9 percent of total). It is, therefore, apparent that though the amount of radioactive material in the RNA fraction was increased (>170 percent per mg macromolecules compared with control) by phenobarbital treatment, the distribution of radioactive material between the AMP and GMP fractions was not affected by the treatment.

To investigate the binding of radioactive material to AMP or GMP, the absorption spectrum of the AMP or GMP fraction was recorded in the u.v. region (data are not shown). No noteworthy spectral change was observed in each nucleotide fraction. When an active metabolite formed by microsomal enzymes reacts with nucleic acids or nucleotides, the spectrum of nucleic acids or nucleotides changes [6–8]. The spectra, however, suggest that the radioactive material in RNA was not bound to the nucleotides. Though it is not shown in the figure, paper chromatography of the AMP or GMP fraction revealed only one spot, which was coincident with the R_f value of authentic AMP or GMP, under ultraviolet light.

The results of the present study suggest that the radioactive material found in RNA was incorporated into the molecular structures of AMP and GMP rather than being bound to a moiety of the nucleotides. This speculation is supported by the following experiments or observations. First, when RNA, poly-A or poly-G was incubated with [^{14}C]BHT in an NADPH-generating system containing microsomes of liver or other tissues (lung, kidney or spleen), radioactivity was not detected in each macromolecule isolated from the incubation mixture by known methods ([5]; unpublished data). Second, when either quinone methide or BHT-alcohol, both of which are specifically bound to cysteine *in vitro* [4], was mixed with RNA, poly-A or poly-G in an alcoholic aqueous solution (30 percent ethanol in 50 mM acetate buffer, pH 5.0, or in 0.1 M phosphate buffer, pH 7.4), a noticeable spectral change was not observed in any of the solutions after removing a large excess of the BHT metabolites with ether. In addition, a spectral change was not observed with other BHT metabolites (unpublished data). Third, though the SH compounds, acting as trapping agents for activated metabolites, significantly inhibit the binding of BHT to microsomes in an NADPH-generating system [4], the four mononucleotides used in the present work were not affected by the binding (unpublished data). Fourth, it has been reported that 2,6-di-*tert*-butyl-*p*-benzoquinone and 2,6-di-*tert*-butyl-hydroquinone, which are formed by demethylation of the 4-methyl group in BHT, were found as minor metabolites (about 1.5 per cent of dose) in the feces and urine of rats after BHT treatment [9]. On the basis of the results, it seems likely that a small amount of the ^{14}C-labeled methyl group separated from BHT enters the 1-carbon pool via formaldehyde or formic acid and is incorporated into the purine base in the process of RNA synthesis.

In summary, the binding site of radioactive material on liver RNA in the rat after the oral administration of [^{14}C]BHT was investigated by ion exchange chromatography. The radioactivity in the RNA hydrolysate was detected in the AMP and GMP fractions. The elution profiles of radioactivity were coincident with the profiles of u.v. absorption of the AMP and GMP fractions. Furthermore, the u.v. spectra of the AMP or GMP fraction were coincident with those of authentic AMP or GMP. Our findings suggest that the radioactive material found in RNA was incorporated into the molecular structures of AMP and GMP rather than bound to a moiety of the nucleotides.

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