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ON THE MECHANISM OF COVALENT BINDING OF BUTYLATED HYDROXYTOLUENE TO MICROSOMAL PROTEIN

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Abstract—The structures of cysteine conjugates of 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) and the binding sites of BHT metabolites on microsomal protein were investigated by ^{13}C nuclear magnetic resonance (^{13}C-NMR) and gas–liquid chromatography/mass spectrometry. The cysteine conjugates of 2,6-di-*tert*-butyl-4-hydroxymethylphenol (BHT-alcohol) and 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone (quinone methide), which are metabolites of BHT found in rat liver and specifically reacts with thiol compounds, were prepared as alcoholic aqueous solutions. The molecular structure of the cysteine conjugate of BHT-alcohol agreed completely with that of quinone methide in ^{13}C-NMR spectra or mass spectra. These spectra of both conjugates further showed that the conjugates are due to thioether binding between the 4-methyl group of metabolites and the sulfhydryl group of cysteine. When [^{14}C]BHT-bound microsomes prepared *in vitro* were enzymatically hydrolyzed with Pronase E, the major radioactive material that eluted with methanol from a column of Amberlite XAD-2 and gave a positive ninhydrin reaction was identified as a cysteine conjugate of BHT by comparing its R_f values on TLC and mass spectrum. On the basis of the results, it was apparent that the binding site of activated substituents of BHT on protein was mainly the sulfhydryl group of cysteine residue.

Butylated hydroxytoluene (BHT)§ is widely used as an antioxidant in processed foods and petroleum products. Though generally considered to be safe at the concentration present in foods (the acceptable daily intake of BHT for man is 0.5 mg/kg [1]), high doses of this compound cause hemorrhagic death in rats [2] and lung injury in mice [3, 4]. It has been presumed that such damage is due to the interaction between active metabolites of BHT and some cellular components.

We have been studying the effect of this compound on cellular macromolecules in the rat [5–9]. Previous studies have demonstrated that (a) BHT is converted to a highly reactive intermediate(s) by a cytochrome P-450-linked monooxygenase system in the microsomes [5, 6], and (b) some of the activated material(s) specifically binds to proteins, but not to nucleic acids [7, 9]. The fact of (b) supports the observed decrease in carcinogenesis by BHT in rats and mice [1, 10, 11]. Furthermore, in an *in vitro* study [8], we found that BHT-alcohol and quinone methide, which are intermediates of BHT in rat liver, react with cysteine and produce water-soluble cysteine conjugates. In this paper, therefore, the structures of the cysteine conjugates of BHT-alcohol and quinone methide were investigated by ^{13}C-NMR and GC/MS. In addition, we have focused on the binding site of the activated materials of BHT on the protein.

MATERIALS AND METHODS

Materials. 3,5-Di-*tert*-butyl-4-hydroxytoluene (toluene [methyl-^{14}C]) ([^{14}C]BHT, specific radioactivity, 0.485 μCi/μmole) was purchased from the New England Nuclear Corp. (Boston, MA). The radiochemical purity of the compounds was rechecked with TLC, by using petroleum ether or *n*-butanol–acetic acid–water (12:3:5, by vol.) as a solvent system, before use in the experiments and found to be more than 99%. Other chemical compounds were obtained from the following companies: non-radioactive BHT from the Tokyo Chemical Industry Co. (Japan) and BHT-alcohol from Shell Chemicals Inc. (U.K.). In addition, quinone methide was synthesized and purified by known methods [12, 13]. On TLC and GLC, the synthesized compound was shown to be a single component. All other chemicals used were obtained from commercial suppliers and were of the highest purity available.

Preparation of cysteine conjugates. The experimental details of the preparation of cysteine conjugates with BHT-alcohol or quinone methide have been described previously [8]. The reaction mixture consisted of 20 mmoles BHT-alcohol or quinone methide in 100 ml of ethanol and 20 mmoles of L-cysteine in 200 ml of 50 mM acetate buffer (pH 5.0). The reaction was performed at 39° for 18 hr. The mixture was extracted with ether to remove unbound

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§ Abbreviations: BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene; BHT-alcohol, 2,6-di-*tert*-butyl-4-hydroxymethylphenol; BHT-acid, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid; quinone methide, 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone; ^{13}C-NMR, ^{13}C nuclear magnetic resonance; and GC/MS, gas–liquid chromatography/mass spectrometry.

metabolites of BHT. The aqueous phase was passed through a 3 × 40 cm column of Amberlite XAD-2 resin (Rohm & Hass Co., NJ). The column was washed with 2000 ml of water and then eluted with 500 ml of methanol. The eluate was evaporated to dryness *in vacuo*.

Enzymatic hydrolysis of [^{14}C]BHT-bound microsomes. The details of the preparation of rat liver microsomes and of the binding of [^{14}C]BHT to microsomes have been described previously [6, 8]. A sample of about 20 mg of [^{14}C]BHT-bound microsomal pellets, which were isolated from the incubation mixture by centrifugation (for 60 min at 105,000 *g*), was hydrolyzed in 10 ml of 0.1 M Tris–HCl buffer (pH 7.4) with 2000 units of Pronase E (Kaken Chemicals Co., Tokyo) for 24 hr at 40°. After hydrolysis, the mixture was recentrifuged for 60 min at 105,000 *g*. The supernatant fraction was extracted with ether to remove free [^{14}C]BHT. The aqueous phase was then passed through a column of Amberlite XAD-2 resin in a manner similar to that described above, and the methanol fraction was evaporated to dryness *in vacuo*.

Thin-layer chromatography. The residue derived from the methanol fraction was redissolved in a small amount of methanol and then subjected to TLC (Kieselgel 60F_{254} precoated plate, layer thickness of 0.25 mm, E. Merck Co., Darmstadt, West Germany) by using (a) *n*-butanol–acetic acid–water (12:3:5, by vol.), (b) *n*-butanol saturated with 2 N NH_4OH, or (c) chloroform–methanol (50:1, v/v) as a solvent. The chromatograms were visualized by spraying with ninhydrin reagent. Furthermore, the radioactivity on thin-layer plates was determined with a Berthold thin-layer radiochromatography scanner, model II (Berthold Laboratories, Wildbad, Germany).

Determination of spectra. Spectra of ^{13}C-NMR were obtained on a JEOL JMN-FX270 NMR spectrometer (Tokyo, Japan) operating at 67.8 MHz, equipped with a NM-3974 disk data system. Spectra were determined in Fourier transform mode by using 16 K data points and a 15 KHz spectra width. The compound was dissolved in methanol-d_1 (CH_3OD, E. Merck Co.), and tetramethylsilane (TMS) was added as an internal reference. Chemical shifts are reported in ppm downfield from TMS and are accurate to ±0.1 ppm.

Mass spectra of the compound were obtained on GC/MS by use of a Hewlett–Packard 5710A gas chromatograph (Palo Alto, CA) equipped with a 2 mm × 1.2 m glass column packed with 3% OV-1 on Chromosorb W (80–100 mesh) and a JEOL JMS-D300 mass spectrometer. The temperatures of the column oven, injection port and separator were maintained at 200°, 250° and 260° respectively. The carrier gas was helium, and the rate of gas flow was 20 ml/min. The ionizing potential and ionizing current were 70 eV and 300 μA. The spectra were determined in the electron-impact mode, and the data were acquired by using a JEOL JMA-2000 mass data analysis system. The effluent from the chromatograph was monitored by using the total ion current, and the mass spectra were taken in the mass range of 50–600 a.m.u.

RESULTS AND DISCUSSION

Previous studies showed that BHT-alcohol and quinone methide, which are metabolites of BHT in rat liver, react with cysteine and produce water-soluble conjugates [8]. Therefore, the structures of both conjugates were investigated by ^{13}C-NMR and

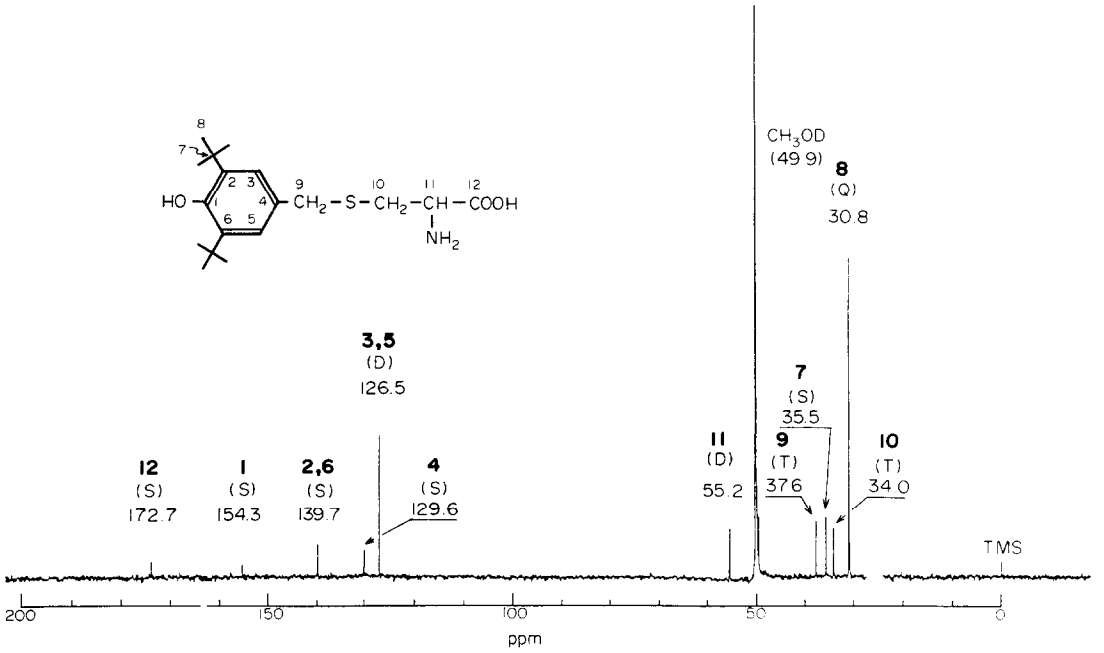

Fig. 1. ^{13}C-NMR spectra (67.8 MHz) of cysteine conjugates of BHT-alcohol and quinone methide. The bold-face numbers above each signal indicate the assignments of each carbon in the structural formula. The spectra of off-resonance proton decoupled ^{13}C-NMR are shown in parentheses: (S) singlet, (D) doublet, (T) triplet and (Q) quartet.

GC/MS. Figure 1 shows the ^{13}C-NMR spectra of the conjugates. The spectrum of the cysteine conjugate of BHT-alcohol was in perfect accord with that of quinone methide. Furthermore, as shown in Fig. 2, the mass spectra of the trimethylsilylated derivatives of both conjugates had the same patterns of fragmentation and molecular ions $[M]^+$. Under the chromatographic conditions used, both trimethylsilylated derivatives had the same retention time of 31.3 min. These results indicate that both cysteine conjugates have essentially the same molecular structure.

On the basis of the chemical shifts (shown under the parentheses in Fig. 1) of each carbon and the spectrum of off-resonance proton decoupled ^{13}C-NMR (shown in parentheses), the molecular structures of the conjugates were presumed as shown in Fig. 1. The sulfur bearing carbon atoms (C-9 and C-10) were especially assigned on the basis of the result (both carbons appeared as triplet) of off-resonance decoupled ^{13}C-NMR and of the comparison with data for benzyl cysteine (unpublished data). Moreover, assignments of each carbon were made from data obtained from BHT [14], cysteine [15] and glutathione and its conjugates [16–19], for the nature of the cysteine conjugate of BHT has not been reported previously.

On the other hand, the molecular structure of the cysteine conjugate is supported by mass spectrum, as shown in Fig. 2. The trimethylsilylated derivative was prepared by heating the cysteine conjugates in acetonitrile containing *N,O*-bis(trimethylsilyl)acetamide (25%, v/v) at 50° for 15 min and was subjected to GC/MS. The molecular weight is given by a molecular ion peak at *m/z* 555 corresponding to the trimethylsilyl derivative of the conjugates and confirmed by the M-15 peak at *m/z* 540, which was generated by the elimination of CH_3. The fragment ions, which are *m/z* 73, 75, 100 and 147, have been generally observed in trimethylsilylated derivatives of amino acids [20] and these derivatives [21, 22]. In addition, the structures of other characteristic ions are interpreted in Fig. 2. Although the source of fragment ions at *m/z* 395, 407 and 480 remains unexplained, these ions seem to be generated from the parent ion or intermediate-fragment ions. The results presented in Figs. 1 and 2 indicate that the cysteine conjugate of BHT was due to a thioether binding between the 4-methyl group of BHT-alcohol or quinone methide and the sulfhydryl group of cysteine.

To investigate the binding site of BHT metabolites on the protein, [^{14}C]BHT-bound microsomes prepared *in vitro* were enzymatically hydrolyzed with protease. Pronase E was chosen as the protease since it has been reported that the substrate specificity of the enzyme is very low and that most peptides of substrate protein are indiscriminately hydrolyzed by the enzyme [23, 24]. Actually, over 90% of the radioactivity bound to microsomal protein was found in the 105,000 *g* supernatant fraction of the hydrolyzate. Figure 3 shows the distribution of radioactive materials derived from [^{14}C]BHT-bound microsomal protein on TLC. In the case of the acidic solvent system (A), about 85% of the total radioactivity on the thin-layer plate was found in the peak at the R_f value of 0.55 and about 8 or 7% of the radioactivity was recovered in the peaks at R_f values of 0.37 or 0.75. Since all of these radioactive materials gave a positive ninhydrin reaction, it indicates that the materials were in a [^{14}C]BHT-bound amino acid com-

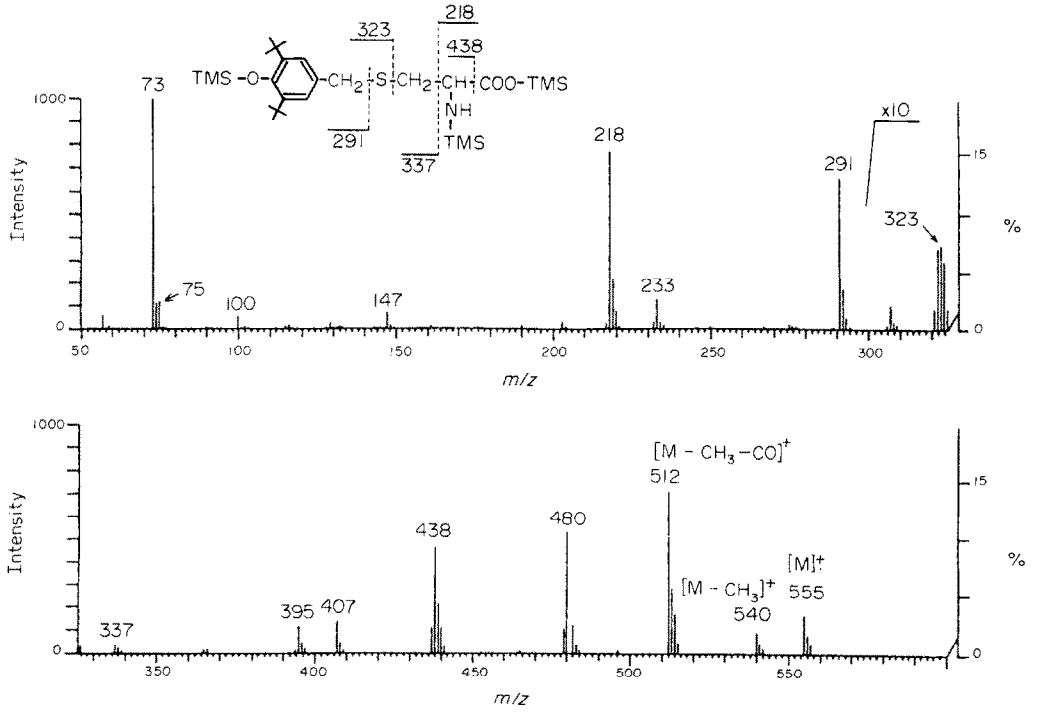

Fig. 2. Mass spectra of trimethylsilyl derivatives of cysteine conjugates derived from BHT-alcohol and quinone methide.

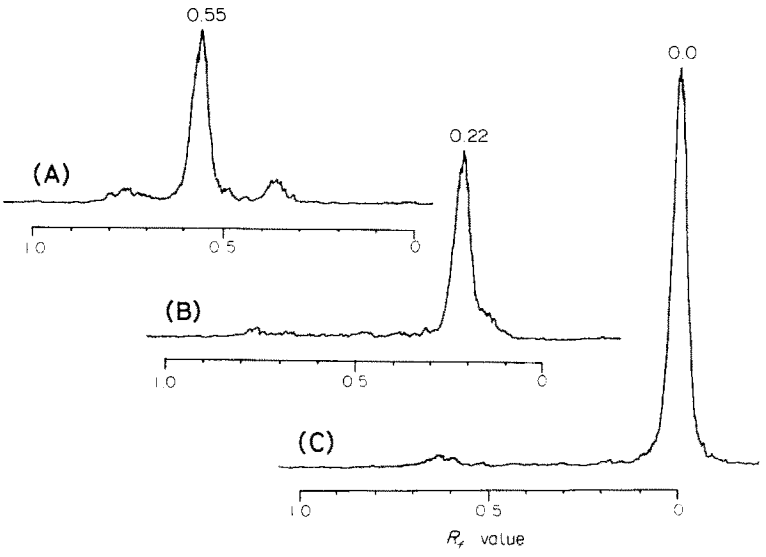

Fig. 3. Radioactivity scan of thin-layer chromatography of enzymatic hydrolyzate of [^{14}C]BHT-bound microsomes. Solvent systems: (A) *n*-butanol–acetic acid–water (12:3:5, by vol.); (B) *n*-butanol saturated with 2 N NH_4OH; and (C) chloroform–methanol (50:1, v/v).

plex. Furthermore, the major radioactive peak was observed alone at R_f values of 0.22 (in the basic solvent B) and 0.0 (in the neutral solvent C). These R_f values of the major peak were in fair agreement with those in the cysteine conjugate of BHT-alcohol or quinone methide as shown in the previous study [8].

The result suggests that the major radioactive material and the cysteine conjugate of BHT are similar compounds. To confirm further this speculation, the mass spectrum of the BHT-bound amino acid complex, which was prepared with non-radioactive BHT and extracted with methanol from the area corresponding to the major radioactive peak on the silica gel plate, was compared with that of the cysteine conjugate. Though it is not shown in the figure, the mass spectrum of the trimethylsilyl derivative of the BHT-bound amino acid complex was essentially the same as that of the cysteine conjugate of BHT, as shown in Fig. 2. Therefore, the results indicate that the binding site of BHT metabolites on microsomal protein is mainly the sulfhydryl group of cysteine residue.

We have already reported that the activated material(s) of BHT specifically binds to protein rather than to nucleic acid [7, 9]. On the basis of previous and present studies, Fig. 4 presents a schematic diagram in which a part of BHT-alcohol or quinone methide produced by the microsomal monooxygenase system is covalently bound to the sulfhydryl groups of protein and cellular thiol compounds. In preliminary experiments, we found that quinone methide reacted with cysteine more rapidly than BHT-alcohol to conjugates. Takahashi and Hiraga [13] identified quinone methide as a major metabolite in liver after the oral administration of BHT to the rat. Tajima *et al.* [25] have shown that BHT-alcohol, like BHT, is converted to quinone methide in the rat. Furthermore, the other quinone methides and semiquinones are highly reactive inter-

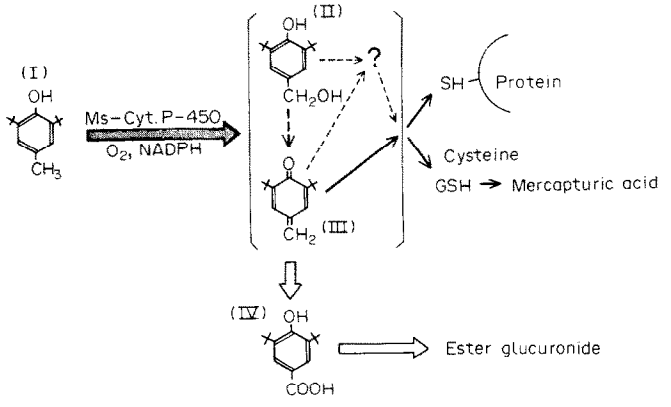

Fig. 4. Irreversible binding of BHT metabolites to cellular protein and thiol compounds in rat liver. White arrows indicate the main metabolic pathway of BHT [30]. BHT and its metabolites: (I) BHT, (II) BHT-alcohol, (III) quinone methide and (IV) BHT-acid.

mediates in chemical reactions, and their possible involvement in toxic damage or biochemical effects has been suggested [26–29]. Consequently, quinone methide itself may be an activated material bound to protein *in vivo*. Daniel *et al.* [30] found that BHT-acid (9% of the administered dose), its glucuronide (15%), and BHT-mercapturic acid [*S*-(3,5-di-*tert*-butyl-4-hydroxybenzyl)-*N*-acetyl-L-cysteine, 11%] are excreted in rat urine as major metabolites. They have described the formation of mercapturic acid by a non-enzymatic reaction between BHT free radicals and cysteine. However, the results of the present study suggest that activated material such as quinone methide also forms BHT-mercapturic acid by conjugation to glutathione or cysteine.

Since the sulfhydryl group plays an important role in the enzymatic action and molecular structure of proteins, it seems likely that the irreversible binding of BHT metabolites to sulfhydryl groups of cellular protein affects the physiological function of cells. For instance, Takahashi and Hiraga [31] reported that the activity of phylloquinone epoxide reductase, which is associated with the activation of prothrombin in liver microsomes [32, 33] and has a sulfhydryl group for active site of the enzyme [34], was inhibited by quinone methide. As a result, it is presumed that the decrease in prothrombin concentration of plasma is responsible for hemorrhagic death in rats. Furthermore, Kehrer and Witschi [35] reported that a reactive metabolite of BHT, rather than the parent compound, produces lung damage in mice. Therefore, it seems that the covalent binding of BHT metabolites to cellular protein is a biochemical clue in the investigation of the unknown latent toxicity of this compound.

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