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Biological fate of butylated hydroxytoluene (BHT): binding of BHT metabolites to cysteine *in vitro*

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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 its *in vivo* metabolism was reviewed by Hathway [1], the intracellular fate of BHT has not been reported. Therefore, we have been studying the behaviour of this compound in the liver cells of the rat. In previous studies [2–4], we found that (a) BHT is converted to activated material(s) by a cytochrome P-450-linked monooxygenase system in the microsomes, and (b) some of the activated material(s) subsequently binds to cellular macromolecules. In an *in vitro* study [3], we found that cysteine and reduced glutathione decreased significantly the binding of BHT to microsomes. This result suggested that the activated material(s) of BHT reacts with the thiol compound, and that the binding sites of protein for such material(s) may be the sulfhydryl group. Therefore, in this paper, the cysteine conjugate of BHT was investigated in a microsomal monooxygenase system. In addition, the conjugation products formed by the enzyme system were isolated from the reaction mixture, and their structures were investigated.

Materials and Methods

Materials. 3,5-Di-*tert*-butyl-4-hydroxytoluene (toluene-[methyl-^{14}C]) (specific radioactivity, 0.485 μCi/μmole) and L-cysteine hydrochloride ([U-^{14}C]) (specific radioactivity, 25.1 μCi/μmole) were purchased from the New England Nuclear Corp. (Boston, MA) and from the Radiochemical Center (Amersham, U.K.) respectively. The radiochemical purities of both chemicals were rechecked by thin-layer chromatography and found to be more than 99 (BHT) and 96 (cysteine) per cent respectively. Other chemical compounds were obtained from the following companies: non-radioactive BHT from the Wako Pure Chemicals Co. (Osaka, Japan), 2,6-di-*tert*-butyl-4-hydroxymethylphenol (BHT-alcohol) from Shell Chemicals Inc. (U.K.), and 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde (BHT-aldehyde) and 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (BHT-acid) from the Aldrich Chemical Co. (Milwaukee, WI). All other chemicals used were of the highest obtainable purity. In addition, 4-hydroperoxy-4-methyl-2,6-di-*tert*-butylcyclohexa-2,5-dienone (BHT-OOH) [5], 4-hydroxy-4-methyl-2,6-di-*tert*-butylcyclohexa-2,5-dienone (BHT-3°OH) [5], and 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone (quinone methide) [6, 7] were synthesized and purified by the known methods. On t.l.c. and g.l.c., these synthesized compounds appeared to be pure and showed up as single components. Furthermore, the melting points of BHT-OOH and BHT-3°OH agreed with literature citations.

Binding of [^{14}C]*BHT to microsomes.* Details of the preparation of liver microsomes and of the binding of [^{14}C]BHT to the microsomes have been described previously [3]. The incubation mixture usually contained 2 mg of microsomal protein, 1.0 mM NADP, 10 mM glucose-6-phosphate, 3.5 units of glucose-6-phosphate dehydrogenase, 7.5 mM $MgCl_2$, and 1 mM L-amino acid or SH-compound in a total volume of 2.0 ml of 0.1 M phosphate buffer (pH 7.4). The mixture was preincubated at 37° for 2 min, and the reaction was started by the addition of [^{14}C]BHT (150 μg) in 20 μl of 2-methoxyethanol. The reaction was carried out for 10 min with constant shaking in air and was stopped by adding 5 ml of 10% trichloroacetic acid. The resultant precipitate was collected by centrifugation and repeatedly washed with 80% methanol or with methanol–ether (1:1, v/v) until no further radioactivity could be removed from the precipitate. The thoroughly extracted precipitate was dissolved in 1 N NaOH for the determination of radioactivity bound to microsomes.

Examination of cysteine conjugate. The composition of the incubation mixture was the same as that of the reaction mixture described above except for the content of [^{14}C]BHT

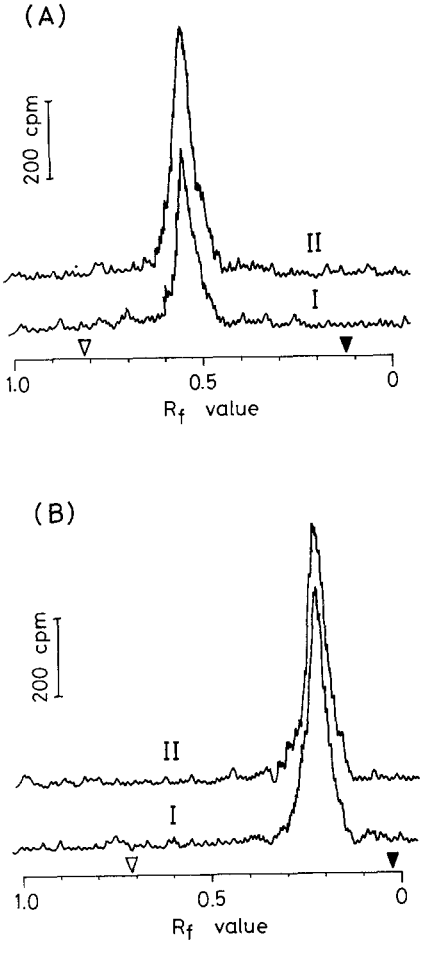

Fig. 1. Radioactivity scan of thin-layer chromatography on methanol eluates (I: [^{14}C]BHT–cysteine system, II: [^{14}C]cysteine–BHT system) from an Amberlite column. Solvent systems: (A) *n*-butanol–acetic acid–water (12:3:5, by vol.), and (B) *n*-butanol saturated with 2 N NH_4OH. R_f values of radioactive authentic compounds: [^{14}C]BHT (▽), and [^{14}C]cysteine (▼).

Table 1. Influence of amino acids on binding of [^{14}C]BHT to microsomes in an NADPH-generating system*

Amino acids	Amount bound† [pmoles·(mg protein)$^{-1}$·10 min^{-1}]	% of control
None (control)	1845 ± 58	100
Alanine	1920 ± 64	104
Arginine	1807 ± 81	98
Aspartic acid	1864 ± 82	101
Cystine	1785 ± 90	97
Cysteic acid	1885 ± 16	102
Cysteine	460 ± 16	25
Glutamic acid	2017 ± 40	109
Histidine	1962 ± 26	106
Hydroxyproline	1909 ± 40	103
Isoleucine	1861 ± 34	101
Leucine	1755 ± 48	95
Lysine	1780 ± 49	96
Methionine	1906 ± 61	103
Phenylalanine	1721 ± 68	93
Proline	1864 ± 50	101
Serine	1780 ± 69	96
Threonine	1804 ± 10	98
Tryptophan	1630 ± 11	89
Tyrosine	1861 ± 62	101
Valine	1862 ± 12	101

* Microsomes were incubated as described in Materials and Methods.
† Values are means ± S.D. of three separate assays per each incubation condition.

Table 2. Influence of thiol compounds and ascorbic acid on the binding of [^{14}C]BHT to microsomes in an NADPH-generating system

Treatment	Amount bound* [pmoles·(mg protein)$^{-1}$·10 min^{-1}]	% of control
Control	1945 ± 34	100
Ascorbic acid	1881 ± 67	97
Glutathione	214 ± 16	11
Cysteamine	260 ± 17	13
Dithiothreitol	641 ± 30	33
Mercaptoethanol	723 ± 23	37

* Values are means ± S.D. of three separate assays per each incubation condition.

and [^{14}C]cysteine; the total volume of the mixture was 20 ml. The reaction was started by the addition of [^{14}C]BHT (0.5 mg, 1.1 μCi) in 0.2 ml of 2-methoxyethanol, and the mixture was incubated at 37° for 30 min. In another experiment, [^{14}C]BHT and cysteine (1 mM) in the mixture were replaced by unlabeled BHT (0.5 mg) and [^{14}C]cysteine (1 mM, 1.25 μCi) respectively. After incubation, the mixture was centrifuged for 1 hr at 105,000 *g*. The supernatant fraction was extracted ten times with 2 vol. of ice-cold ether, and the aqueous phase was bubbled with nitrogen gas to remove the residual ether. The aqueous solution was passed through a column of Amberlite resin.

Reaction of cysteine with BHT metabolites. The reaction mixture consisted of 70 μmoles of BHT metabolite in 5 ml of ethanol and 70 μmoles of [^{14}C]cysteine (1.0 μCi) in 10 ml of 50 mM acetate buffer (pH 5.0). The reaction was performed at 39° for 18 hr. The mixture was extracted five times with ether to remove unbound BHT metabolite. The aqueous phase was bubbled with nitrogen to remove the residual ether and passed through a column of Amberlite resin.

Isolation of cysteine conjugate. The aqueous solution, prepared as described above, was passed through a 2 × 20 cm column of Amberlite XAD-2 resin (Rohm & Haas Co., NJ). The column was washed with 500 ml of water and then eluted with 400 ml of methanol. Generally, more than 92 per cent of the total radioactivity applied to the columns was recovered in the water and methanol eluted. The eluate was evaporated to dryness *in vacuo*, and the residue was redissolved in a small amount of methanol. The methanolic solution was subjected to thin-layer chromatography (kieselgel 60F_{254} precoated plate, layer thickness of 0.25 mm, E. Merck & Co., 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 system.

Other methods. Radioactivity was measured with a Beckman scintillation spectrometer, model LS-355, and was corrected by the external standard method. The scintillation medium used consisted of 2 vol. of toluene phosphar (4 g PPO and 100 mg dimethyl-POPOP per 1000 ml of toluene)* and 1 vol. of Triton X-100 [8]. The radioactivity on thin-layer plates was determined with a Berthold thin-layer

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

Table 3. Thin-layer chromatography of the reaction product of [^{14}C]cysteine with BHT metabolites in ethanol–acetate buffer solution

Reaction mixture		R_f value of product		
		Solvent system*		
	Metabolites of BHT	A	B	C
[^{14}C]Cysteine†	+ none	—‡	—	
	+ BHT	—	—	
	+ BHT-alcohol	0.55	0.22	0.0
	+ BHT-aldehyde	—	—	
	+ BHT-acid	—	—	
	+ BHT-OOH	—	—	
	+ BHT-3°OH	—	—	
	+ Quinone methide	0.55	0.22	0.0

* Developing solvent: (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).

† R_f values on t.l.c. of authentic [^{14}C]cysteine were 0.12 (solvent A) and 0.02 (solvent B).

‡ Not detected.

radiochromatography scanner, model II (Berthold Lab., Wildbad, Germany). Protein was determined by the method of Lowry *et al.* [9] with bovine serum albumin as a standard.

Results and Discussion

Table 1 shows the influence of L-amino acids on the binding of [^{14}C]BHT to microsomes in an NADPH-generating system. Addition of cysteine to the incubation mixture markedly decreased the binding. The binding, however, was not affected by other amino acids. This result indicates that the binding was specifically inhibited by the addition of cysteine to the reaction mixture. In addition, the influence of other thiol compounds or of ascorbic acid on this binding was investigated, and the results are shown in Table 2. All thiol compounds inhibited the binding reaction significantly, whereas ascorbic acid as a reductant did not affect the binding. These results, presented in Tables 1 and 2, suggest that the activate material(s) of BHT formed by the microsomal enzyme system reacts with thiol compounds such as cysteine or glutathione.

To confirm the formation of the activated BHT–cysteine conjugate, [^{14}C]BHT and cysteine or BHT and [^{14}C]cysteine were incubated in a microsomal monooxygenase system. After incubation, the reaction mixture was extracted with ether, and the remaining aqueous solution was passed through a column of Amberlite resin and eluted with methanol as described in Materials and Methods. About 4 per cent of the radioactivity added to the reaction mixture was found in the methanol eluate. Figure 1 shows the distribution of radioactivity on t.l.c. of the methanol eluate. The radioactivities derived from the [^{14}C]BHT–cysteine and [^{14}C]cysteine–BHT systems both showed only one peak at the same R_f values of (A) 0.55 and (B) 0.22 in the two developing solvents used. Though it is not shown in Fig. 1, in the case of the solvent system of chloroform–methanol (50:1, v/v), the radioactive peaks were observed at the same R_f value of 0.0. When either BHT or cysteine was omitted in each reaction system of the above experiments, the radioactive peaks with R_f values of 0.55, 0.22, and 0.0 were not observable. This result indicates that the conjugate of activated material(s) of BHT and cysteine was formed in the incubation system.

In preliminary experiments, the radioactivity was not found in the ether extract from the incubation mixture containing [^{14}C]cysteine and BHT. On the other hand, according to t.l.c., [^{14}C]BHT, and [^{14}C]BHT-alcohol and a trace amount of [^{14}C]BHT-aldehyde, which are formed by the microsomal enzyme system, were found in the ether extract from the incubation mixture containing [^{14}C]BHT and cysteine. Therefore, it is recognized that the conjugate of activated material(s) and cysteine could not be extracted from the mixture with ether, and that the conjugate is a water-soluble product.

To investigate further the formation of the activated BHT metabolite(s)–cysteine conjugate, [^{14}C]cysteine and metabolites of BHT were reacted in ethanolic aqueous solution. The reaction was performed under slightly acidic conditions because the [^{14}C]cysteine conjugate is formed more rapidly in the acidic solution than in the neutral one. The metabolites of BHT used were (a) products of the main metabolic pathway *in vivo* [10]: BHT-alcohol, BHT-aldehyde and BHT-acid, (b) products of the cyclic metabolic pathway *in vitro* [11]: BHT-OOH and BHT-3°OH, and (c) a new metabolite found in the rat liver *in vivo* [7]: quinone methide. After the reaction, the mixture was extracted with ether, and the aqueous solution was passed through a column of Amberlite and eluted with methanol as described in Materials and Methods. Table 3 shows the R_f values of radioactivities derived from the reaction product of [^{14}C]cysteine on t.l.c. of the methanol eluate. From the reaction mixture containing BHT-alcohol or quinone methide, a new radioactive peak differing from [^{14}C]cysteine in R_f value was observed at the R_f values of 0.55, 0.22, and 0.0 in these developing solvents. On the other hand, the new radioactive peak was not found in the case of the other metabolites. Though it is not shown in Table 3, in the case of the neutral solution system, the other metabolites, except for quinone methide and BHT-alcohol, did not react with [^{14}C]cysteine. When BHT metabolites were omitted from the reaction system, most of the radioactivity in the reaction mixture was recovered in washing water from the Amberlite column, and the residual radioactivity eluted with methanol was not observed at the same R_f values in these developing solvents. These R_f values shown in Table 3 were the same as those in Fig. 1. The results presented in Fig. 1 and Table 3 indicate that the activated material(s) of BHT conjugated with cysteine was closely related to BHT-alcohol and quinone methide. Furthermore, since the R_f values of the cysteine conjugate of BHT-alcohol were the same as those of quinone methide, it seems likely that both conjugates are essentially the same in nature.

It has been reported that BHT-mercapturic acid (11% of dose) was excreted in the urine of rats given BHT, and that the formation of this material may be due to the reaction between BHT free radical and cysteine [10]. The present study, however, suggests that quinone methide and BHT-alcohol, formed in the liver, were trapped by non-protein free sulfhydryl compounds such as cysteine and glutathione, that the trapping reaction was due to a thioether binding between the 4-methyl group of both

metabolites and the sulfhydryl group, and that the water-soluble conjugates formed were excreted in the urine. We have already reported that the activated material(s) of BHT is bound to the protein and nucleic acid [2–4]. Therefore, it is suggested that a part of quinone methide or BHT-alcohol is also covalently bound to the sulfhydryl group in protein *in vivo*.

The present study, carried out with the methods of organic solvent-extraction and Amberlite chromatography-t.l.c., supports our earlier speculation [3]: the activated material(s) of BHT reacts with the thiol compound, and the binding sites of protein for such material(s) may be the sulfhydryl group. The structure of the cysteine conjugate of quinone methide or BHT-alcohol is to be investigated in future work.

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Lack of uptake or degradation of adenosine in the termination of its action in the beating carp atrium

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Adenosine and some of its phosphorylated derivatives are known to reduce the force and rate of contraction of atrial muscle in various species [1–4]. This effect has been attributed to a direct action on atrial muscle and also on the sino-atrial pacemaker. More recently [5], adenosine was reported to cause a slow, long-lasting hyperpolarization of cells in the sinus venosus of the frog, which is the primary pacemaker of the region. In most tissues, however, the response to adenosine is brief: isolated heart preparations exposed to adenosine recover spontaneously their original rate and force of contraction. This fade or decay of adenosine effect, observed also in smooth muscle, has been ascribed to the disappearance of adenosine from the bathing fluid through uptake into the tissue, or deamination to inactive inosine [6–8]. Proof of this rested on a demonstration that radiolabeled adenosine added to the bathing medium was gradually taken-up by the tissue where it underwent partial metabolism, and that dipyridamole, a known coronary vasodilator agent, effectively inhibited this uptake [6, 9]. Thus, a given concentration of adenosine seems to be more effective in the presence of dipyridamole, presumably because its effective bath concentration persists longer. Our recent experience with the spontaneously beating carp atrium, however, suggests that other mechanisms may be operative in the fade or potentiation of adenosine action. In this preparation, adenosine induces a dose-dependent, transient loss of inotropicity, followed by recovery. We now show evidence that (1) spontaneous recovery of force of contraction by the muscle occurs in the presence of a persisting bath concentration of adenosine, (2) the rate of recovery is inversely related to adenosine concentration, and (3) dipyridamole still exerts potentiation of the adenosine effect even though blockade of adenosine transport may not be its major contribution.

The spontaneously beating carp atrium preparation, reported earlier [10], lends itself remarkably well to prolonged time–course studies. It delivers about 30,000 contractions over a period of 10 hr without significant change in base-line and responds only to acetylcholine and adenosine and their congeners, but not to noradrenaline, adren-