Cyclic Metabolic Pathway of a Butylated Hydroxytoluene by Rat Liver Microsomal Fractions

By CHIADAO CHEN and YON-SHONG SHAW

Department of Biochemistry, Northwestern University, Medical and Dental Schools,
 Chicago, Ill. 60611, U.S.A.

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A cyclic metabolic pathway was obtained when 3,5-di-*t*-butyl-4-hydroxytoluene (BHT) was incubated with a rat liver microsomal preparation. The pathway is as follows: BHT → 4-hydroperoxy-4-methyl-2,6-di-*t*-butylcyclohexa-2,5-dienone (BHT-OOH) → 4-hydroxy-4-methyl-2,6-di-*t*-butylcyclohexa-2,5-dienone (BHT-3°OH) → BHT. This metabolic pathway suggests that antioxidants such as BHT owe their high efficacy, at least in part, to their metabolic regeneration.

BHT (3,5-di-*t*-butyl-4-hydroxytoluene) is widely used as an antioxidant in processed foods, cosmetics, pharmaceuticals and petroleum products. Its metabolism *in vivo*, a subject of investigation in many laboratories, was reviewed by Hathway (1966). The results of these studies are summarized in Scheme 1, which indicates that both types of methyl groups are hydroxylated during metabolism. In a study *in vitro* Gilbert & Goldberg (1967) showed that the enzyme responsible for the oxygenation of BHT to BHT-1°OH (2,6-di-*t*-butyl-4-hydroxymethylphenol) was located in the microsomal fraction.

In our earlier paper on the mechanism of aromatic hydroxylation catalysed by rat liver microsomal preparations with BHT as a representative substrate, we succeeded in identifying BHT-OOH (4-hydro-

peroxy-4-methyl-2,6-di-*t*-butylcyclohexa-2,5-dienone), BHT-3°OH (4-hydroxy-4-methyl-2,6-di-*t*-butylcyclohexa-2,5-dienone) and BHT-1°OH as the products (Shaw & Chen, 1972). It seems plausible that BHT is first oxygenated to BHT-OOH, which is then converted into BHT-3°OH by either (a) the cytochrome *P*-450 system, (b) a GSH peroxidase, (c) a reducing enzyme system present or (d) a chemical reducing agent in the enzyme preparation. Alternatively, BHT-OOH could undergo spontaneous homolytic and/or heterolytic fission to BHT-3°O• radical or a BHT-3°O⁻ anionic species that acquires a hydrogen atom or ion from the environment to yield BHT-3°OH. The oxygenation of this aromatic compound is similar to our work on the mechanism of aliphatic hydroxylation by rat liver 10000g

Scheme 1. Metabolism of BHT as reviewed by Hathway (1966)

Scheme 2. *Proposed cyclic metabolic pathway of BHT*

supernatant with representative substrates, tetralin (Chen & Lin, 1968) and fluorene (Chen & Lin, 1969; Chen & Lefers, 1973).

In the present paper we report that BHT can be metabolized by the hepatic microsomal fraction in a cyclic pathway as depicted in Scheme 2.

Materials and Methods

Male Charles River rats weighing 120–180 g were used. The microsomal fraction was obtained by differential centrifugation as described previously (Shaw & Chen, 1972). Except where indicated, the basic reaction mixture contained NADP⁺, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, microsomal enzyme suspension and sodium phosphate buffer, pH 7.4 (19 ml of 0.2 M-monosodium phosphate and 81 ml of 0.2 M-disodium phosphate to make 100 ml of buffer) in a total of 5 ml. Anaerobic experiments were carried out under N₂. Protein content was measured by the biuret method (Robinson & Hogden, 1940) with crystalline serum albumin as the standard.

On completion of the incubation, 0.2 ml of concn. HCl was added before immediate extraction with peroxide-free ether (Gilbert & Goldberg, 1967). Methyl palmitate was added to serve as an internal standard. The ether extract (60 ml) was dried over Na₂SO₄ and evaporated to dryness under N₂. The residue was redissolved in chloroform and promptly subjected to g.l.c. analysis.

The gas-liquid chromatograph used was equipped with a flame-ionization detector. The U-shaped borosilicate column (150 cm × 6.5 cm outer diam.)

was packed with 3% SE-30 methylsilicone-coated Var-A-Port 30 diatomite (100–120 mesh). N₂ was used as the carrier gas at a flow rate of 25–30 ml/min. The temperature was kept at 110°C until the emergence of BHT-OOH, which took 18–19 min. It was then programmed for a 20°C/min increase to 170°C, at which temperature BHT-1°OH emerged. A column of the same dimension packed with 2% XE-60 silicone nitrile on Gas Chrom Q diatomite (80–100 mesh) has also been used with similar results at temperatures of 100° and 150°C. With the SE-30 column, BHT-3°OH emerged before BHT. The order of emergence of these two compounds was reversed when the XE-60 column was used.

Glucose 6-phosphate, NADP⁺ and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Compound SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride) was a gift from Smith, Kline and French Laboratories, Philadelphia, Pa., U.S.A. BHT was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. BHT-OOH and BHT-3°OH were synthesized by the method of Kharash & Joshi (1957). BHT-1°OH was prepared by the method of Nystrom & Brown (1947). All of these compounds were kept at –10°C before use. On t.l.c. and g.l.c., BHT and its oxygenated derivatives all appeared to be pure and showed up as single components. Their melting points also agreed with literature citations. The gas chromatograph and column packings were from Varian, Walnut Creek, Calif., U.S.A.

More detailed description of the materials and methods may be found in our previous publication (Shaw & Chen, 1972).

Because of many variables, all experiments were performed three or more times to assess reproducibility. There were differences in the percentage yield relative to the control from day to day and from one batch of enzyme to another, but the trend was the same. The data reported here for EDTA (Table 1) are averages of four values from two sets of experiments. All other data are representative of a single typical experiment.

Results and Discussion

Time-course studies (Fig. 1) with BHT as the substrate showed that BHT-OOH, BHT-3°OH and BHT-1°OH were formed as reported previously (Shaw & Chen, 1972). The reactions proceeded rapidly. At 18°C the yield of oxygenated products reached maximum within 5–10 min. When BHT-OOH was used as the substrate (Fig. 2), BHT-3°OH, BHT and BHT-1°OH were obtained. When BHT-3°OH was the substrate (Fig. 3), BHT, BHT-OOH and BHT-1°OH resulted. In other words, the same products were rapidly recovered, regardless of which of the three substrates was used. It should be noted that all four compounds under discussion were stable enough to be extracted from 2M-HCl and chromatography as described without change.

It was reported earlier that with BHT, as in typical enzymic reactions, increase in either the microsomal proteins or substrate concentration led to increases

in product formation (Shaw & Chen, 1972). A similar relationship was found when BHT-OOH was used as the substrate (Figs. 4 and 5). Since the reactions were complex, no detailed kinetic study was possible. Very little BHT and BHT-1°OH were obtained when relatively little BHT-3°OH was used (Fig. 6). As the substrate was increased to 4 μmol per flask, there was a dramatic increase in these two products. At the same time, the initial large production of BHT-OOH levelled off. This indicated that the ring oxygenation was highly efficient. The initial lag of BHT-1°OH production could be due in part to its further oxidation or to oxygenation to its carboxylic acid.

Fig. 2. Effect of incubation time on the formation of BHT-3°OH, BHT-1°OH and BHT from BHT-OOH

The conditions were the same as in Fig. 1; 3.96 μmol of BHT-OOH was used. Δ , BHT-3°OH; $\bullet$, BHT-1°OH; $\circ$, BHT.

Fig. 1. Effect of incubation time on the formation of BHT-OOH, BHT-3°OH and BHT-1°OH from BHT

The incubation mixture (5.0 ml) containing BHT (2.3 μmol) NADPH-generating system composed of glucose 6-phosphate (26.9 μmol), NADP⁺ (2.7 μmol) and glucose 6-phosphate dehydrogenase (5 $\mu\text{mol}/\text{min}$ at 25°C), microsomal proteins (18 mg) and sodium phosphate buffer, pH 7.4, (500 μmol) was incubated at 18°C for the time indicated. $\square$, BHT-OOH; Δ , BHT-3°OH; $\bullet$, BHT-1°OH.

Fig. 3. Effect of incubation time on the formation of BHT, BHT-OOH and BHT-1°OH from BHT-3°OH

The conditions were the same as in Fig. 1; 4.23 μmol of BHT-3°OH was used. $\square$, BHT-OOH; $\bullet$, BHT-1°OH; $\circ$, BHT.

Fig. 4. Effect of BHT-OOH concentration on product formation

The conditions were the same as in Fig. 1. Incubation time was 10 min. Δ , BHT-3°OH; $\bullet$, BHT-1°OH; $\circ$, BHT.

Fig. 5. Effect of protein concentration on BHT-OOH transformation

The conditions were the same as in Fig. 1; 3.96 μ mol of BHT-OOH was used. Incubation time was 10 min. $\bullet$, BHT-1°OH; Δ , BHT-3°OH; $\circ$, BHT.

Table 1 summarizes the findings when BHT, BHT-OOH and BHT-3°OH were incubated under various conditions. All incubations were performed with the same amount of protein, at 18°C for 10 min, to obtain relative values. No activity was observed when the microsomal fraction was omitted or added after boiling, except in the conversion of BHT-OOH into BHT-3°OH. As hydroperoxides are usually highly reactive, metal ions and metal chelates could undoubtedly hasten their conversion into alcohols. The addition of GSH also greatly increased the reduction of BHT-OOH to BHT-3°OH. The significance of the

Fig. 6. Effect of BHT-3°OH concentration on product formation

The conditions were the same as in Fig. 1. Incubation time was 10 min. $\square$, BHT-OOH; $\bullet$, BHT-1°OH; $\circ$, BHT.

effect of GSH on BHT-1°OH production is not clear. There could be a trace amount of NADP⁺ present in the microsomal preparation that was reduced by GSH to NADPH (which was the cofactor for oxygenase) by GSH reductase. One explanation for the increased production of BHT-3°OH would be that BHT-OOH underwent homolytic fission catalytically and/or spontaneously to its oxy radical, BHT-O $\cdot$, which abstracts a hydrogen atom from the environment. Thiols, such as GSH, tend to yield a hydrogen radical and this favours the reduction. Consequently, the reduction of BHT-OOH, though efficiently brought about by the microsomal fraction, proceeded twice as rapidly with the cytosol fraction. The cytosol fraction contained an appreciable amount of GSSG which would reduce a hydroperoxide non-enzymically or act as a cofactor for GSH peroxidase. GSH peroxidase has been shown to be responsible for the reduction of lipid hydroperoxides, cumene hydroperoxide and steroid hydroperoxides (Hrycry & O'Brien, 1972, 1973).

As expected, there was more BHT recovered when BHT-OOH or BHT-3°OH were incubated anaerobically. The resultant lower production of oxygenated compounds was in agreement with the accumulation of BHT. Apparently, only a trace of oxygen was sufficient for the reaction. Repeated evacuation and flushing or gentle bubbling through the solution with either N₂ or helium failed to decrease the oxygen content to the value where no oxygenation products were detectable. As was expected, compound

Table 1. *Factors affecting the cyclic metabolic pathway of BHT*

The reaction mixture containing BHT (2.3 μ mol), BHT-OOH (3.96 μ mol) or BHT-3°OH (4.23 μ mol) was incubated with an NADPH-generating system and microsomal proteins as described in Fig. 1, and under conditions as indicated. Incubation time was 10 min at 18°C under air or as indicated. The same amount of protein, as determined by the biuret method, was used for each cellular fraction.

Substrate	Incubation mixture	Relative amounts of products (%)			
		BHT	BHT-OOH	BHT-3°OH	BHT-1°OH
BHT	Microsomal protein + NADPH	—	100	100	100
	Microsomal protein + NADPH (N ₂)	—	9	3	5
	Microsomal protein + NADPH (CO + O ₂ , 4 : 1)	—	94	46	70
	Microsomal protein	—	26	14	20
	Boiled microsomal protein + NADPH	—	0	<2	0
	Microsomal protein + NADPH + 0.4 mM-EDTA	—	98	103	118
	Microsomal protein + NADPH + 0.196 mM-SKF-525A	—	0	75	46
	10800g supernatant + NADPH	—	25	22	15
	Cytosol + NADPH	—	5	4	3
BHT-OOH	Microsomal protein + NADPH	100	—	100	100
	Microsomal protein + NADPH (N ₂)	610	—	100	28
	Microsomal protein	0	—	82	0
	Boiled microsomal protein + NADPH	0	—	24	0
	Microsomal protein + NADPH + 0.196 mM-SKF-525A	130	—	125	4
	Microsomal protein + 8.1 μ mol of GSH	10	—	178	53
	10800g supernatant + NADPH	42	—	140	23
	Cytosol + NADPH	17	—	222	3
BHT-3°OH	Microsomal protein + NADPH	100	100	—	100
	Microsomal protein + NADPH (N ₂)	314	3	—	49
	Microsomal protein	<2	0	—	0
	Boiled microsomal protein + NADPH	<2	0	—	0
	Microsomal protein + NADPH + 0.4 mM-EDTA	100	100	—	109
	Microsomal protein + NADPH + 0.196 mM-SKF-525A	38	40	—	25
	Microsomal protein + NADPH + 4 mM-MgCl ₂	210	91	—	192

SKF-525A, a common oxygenase inhibitor, lowered the yield of oxygenation products. Judging from the experiments with BHT-3°OH as the substrate, compound SKF-525A also inhibited its dehydration to BHT. The multiplicity of the reactions catalysed by the microsomal preparation did not permit clarification of the mechanism of this reaction. The lack of effect of EDTA suggested that the hydroperoxidation of BHT was mediated by cytochrome P-450, and was not identical with non-enzymic oxygenation of unsaturated lipids (Wills, 1968).

It was noteworthy that Mg²⁺ was able to promote dehydration of BHT-3°OH and formation of BHT-1°OH. As the products were rapidly metabolized and interconverted, it was not possible to ascertain whether Mg²⁺ favoured dehydration or whether it inhibited or promoted further reaction of the products. The understanding of the dehydration reaction was complicated by the finding that the dehydration reaction was not affected by EDTA, was strongly inhibited by compound SKF-525A and required NADPH. Only the requirement for NADPH seemed explainable; for the conversion of BHT-3°OH into BHT was, overall, a reduction reaction.

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