4,4′-Ethylenebis(2,6-di-*tert*-butylphenol) (Ⅷ) was prepared by oxidation of BHT with alkaline $K_3Fe(CN)_6$ by the method described by Cook, *et al.*[10] and by Fijisaki.[6]

Analytical Method——All spectrophotometric measurements were made by Shimadzu Spectrophotometer Model DU-50.

Free and Total Phenols——a) Folin—Ciocalteu Method[11]: This method was useful for the determination of phenol in normal urine and water-soluble phenols, but not for BHT and its derivatives, probably because of the insolubility of the latter in water.
b) Mahon-Chapman method[12]: The calibration curve for BHT satisfied Beer's law in a concentration range of $12\sim240\,\gamma$/cc. with good reproducibility. A satisfactory result was also obtained in recovery test with rabbit urine mixed with a known amount of BHT.

Glucuronides——Total glucuronic acid was measured by the naphthoresorcinol picrate method modified by Ishidate.[13] Urine was diluted to give a concentration of $10\sim80\,\gamma$/cc. for glucuronide and readings were made at 570 mμ.

Ethereal Sulfates——These were determined by Fiscke's modification[14] of benzidine method.

Paper Chromatography——Ascending development was employed, using Toyo Roshi No. 51 chromatography paper.

Results

Excretion of Normal Metabolites——The mean daily excretion by undosed rabbits was, from 206±26 cc. of urine of pH 7.6±0.3: Free phenols, 5.9±2.1 mg.; combined phenols, 53.1±2.0 mg.; total glucuronic acids, 126±10 mg.; ethereal sulfates, 17±9 mg.

Metabolites of BHT——The analysis of urine after administration of BHT is summarized in Table I and Fig. 1. These results show that about 60% of the administered dose is accounted for in free phenols, ethereal sulfates, and glucuronides.

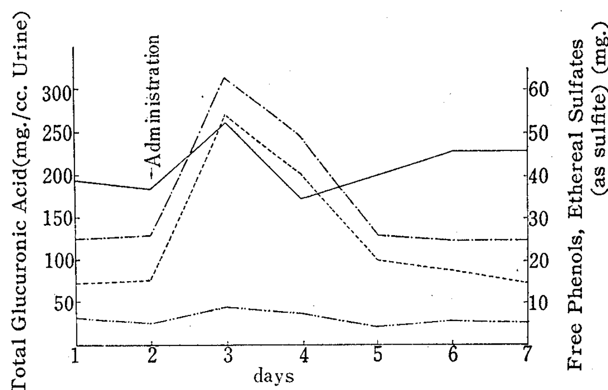

Fig. 1. Quantitative Analysis of the Urine of a Rabbit receiving BHT (1 g. BHT/animal)

——— Urine volume
-·-·-·- Free phenols
— — — Total glucuronic acid
- - - - - Ethereal sulfates

Results expressed as mean value from six rabbits.

Table I. Metabolites excreted in Rabbit Urine 24 and 48 hours after administration of BHT

	24 hr.	48 hr.		24 hr.	48 hr.
Urine	yellow (6)	strong yellow	Glucuronides	24.1±3.5(6)	13.4±3.6(6)
pH	7.6±0.8(6)	7.2±0.3(6)	Ethereal sulfates	10.2±2.3(6)	6.5±3.1(6)
Free phenols	4.7(2)	2.1(2)			

Results expressed as mean percentage of the dose with number of experiments in parentheses

Paper Chromatography of the Derivatives of BHT——The solvent mixtures (by vol.) used were (A) methanol-water (4:1), (B) petr. ether (b.p. 40~60°)-80% formic acid (2:1), and (C) isopropanol-ammonia-water (20:1:2). The detecting reagents used were (1) Gibbs reagent (0.1% ethanol solution of 2,6-dichloroquinone chlorimide), followed by 5% sodium

10) C.D. Cook, N.G. Nash, H.R. Flannagan: J. Am. Chem. Soc., **77**, 1783 (1955).
11) O. Folin, V. Ciocalteu: J. Biol. Chem., **73**, 627 (1927).
12) J.H. Mahon, R.A. Chapman: Anal. Chem., **23**, 1120 (1951).
13) M. Ishidate, T. Nambara: This Bulletin, **5**, 515 (1957).
14) C.H. Fiscke: J. Biol. Chem., **47**, 59 (1921).

NII-Electronic Library Service

barbital solution; (2) phosphomolybdic acid reagent (0.5% phosphomolybdic acid in ethanol), followed by exposure to ammonia fumes; and (3) diazo reagent (0.3% *p*-nitroaniline in 8% (w/v) hydrochloric acid (25 cc.) mixed with 5% (w/v) of sodium nitrite (1.5 cc.), prepared immediately before spraying. This is followed by addition of sodium carbonate (20%)).

The results are shown in Table II. In all cases, the solvent front was run 23 cm.

Table II. Rf Values of BHT Derivatives

Compound	Rf in solvent system A 1	A 2[a]	B	C	Coloration to detecting reagents (1)	(2)	(3)
p-Cresel	0.83	0.80	0.25	0.89	blue	bluish black	red
(IV)	0.84	0.56	0.15	0.89	〃	〃	light yellow
(I)	0.95	0.11	0.95		no color	〃	no color
(III)	0.88	0.77	0.43	0.92	violet	yellowish green	yellow
(V)	0.88	0.87	0.95	0.72	〃	〃	〃
(VI)	0.88	0.80		0.75	〃	〃	〃
(VII)		0.35			no color	bluish black	no color
(VIII)	0.00	0.03		0.96	〃	bluish green	〃

a) Paper (Toyo Roshi No. 51) was sprayed uniformly with camellia oil (10 g.) in Et_2O (100 cc.).

Paper Chromatography of the Metabolites——The solvent used was (C). Compounds were detected on paper chromatograms by spraying (1), (2), or (3) reagent.

The collected 24-hr. urine of rabbit receiving BHT was adjusted to pH 6 with dil. sulfuric acid and continuously extracted with ether for 6 hours. The extract indicated 10 spots (Rf 0.92 (X_1), 0.76 (X_2), 0.55 (X_3), 0.41 (X_4), 0.36 (X_5), 0.24 (X_6), 0.13 (X_7), 0.07 (X_8), 0.03 (X_9), 0.02 (X_{10})). The spots X_1, X_2, X_4, and X_7 gave a positive reaction to phosphomolybdic acid reagent, and X_1, X_2, X_3, X_5, X_6, and X_8 were positive to diazo reagent. Among these spots, X_3, X_5, X_6, X_9, and X_{10} seemed to be normal components of undosed urine. The spots X_1, X_2, and X_7 gave violet color by the Gibbs reagent, yellowish green by phosphomolybdic acid reagent, and yellow by diazo reagent. It is, therefore, suggested that these spots are those of BHT-metabolites.

The spot X_1 was found to be the same as that of α-hydroxy-2,6-di-*tert*-butyl-*p*-cresol (III) by paper chromatogaphy run in a different solvent system and by the ultraviolet absorption spectrum. The spot X_2 seems to be that of 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (V).

In a later work,[15] these two compounds were isolated from the urine of a rabbit receiving BHT.

The spots at Rf 0.41 (X_4), 0.13 (X_7), and 0.07 (X_8) are also assumed to be those of some additional metabolites of BHT, which were not characterized. These unknown spots are being examined.

When the chromatogram using Toyo Roshi No. 51, with the solvent (A), was sprayed uniformly with camellia oil (10 g.) in ether (100 cc.), a new spot appeared at Rf 0.03, which colored yellowish green to phosphomolybdic acid reagent but not to the Gibbs reagent. This was identical with the spot of synthesized 4,4′-ethylenebis(2,6-di-*tert*-butylphenol) (VIII). This compound was later isolated from the BHT-dosed urine.[15]

Unchanged BHT was not detected by paper chromatography in the BHT-dosed urine.

The extract obtained from the feces of rabbit receiving BHT was continuously extracted with ether for 6 hours. By paper chromatography run by the solvent (A, 2), it gave a spot at Rf 0.11 which colored dark blue with phosphomolybdic acid reagent and

15) I. Aoki: This Bulletin, **10**, 105 (1962).

NII-Electronic Library Service

UDC 581.13 : 547.563 : 543·544.42

18. **Masuo Akagi and Isamu Aoki**: Studies on Food Additives. VI.*[1] Metabolism of 2,6-Di-*tert*-butyl-*p*-cresol (BHT) in a Rabbit. (1). Determination and Paper Chromatography of a Metabolite.

(*Faculty of Pharmaceutical Sciences, School of Medicine, Hokkaido University**[2])

The recent increasing use of food additives is of importance in respect to their acute and chronic toxicity which may be expected when these additives are consumed continuously for a long time. In order to know the actual feature of chronic toxicity of these food additives, their metabolic fate should be examined.

In the course of previous studies on the antioxidative effect of sulfur-containing compounds on concentrated solution of methyl oleate and vitamin A,[1] problems on the metabolic fate of antioxidants in animal body seemed of interest.

Among the important commercial antioxidants added in edible fats, the metabolism of 2,6-di-*tert*-butyl-*p*-cresol (BHT) has never been examined, in contrast to those of butylated hydroxyanisole (BHA), propyl gallate, and 2,4,5-trihydroxybutyrophenone, which were studied by Dacre, *et al.*,[2] Astill, *et al.*,[3] Booth, *et al.*,[4] and Astill, *et al.*[5]

The present investigation is concerned with the determination and paper chromatography of BHT as the preliminary work on its metabolism. A discussion on BHT metabolism is also included.

Experimental

Animal, Diet, and Dosage——The rabbits used weighed 1.8~3.0 kg. They were fed with a standard diet (oats 50 g., carrot 100 g., and cabbage 200 g.) and were kept separately in metabolism cages designed to permit separate collection of urine and feces. BHT filled in a capsule was administered orally in a dose of 0.5 g./kg. body wt. Urinary collection was made every 24 hr. after administration of the compound. The decomposition of metabolites was prevented by the addition of toluene to the collection bottles.

Materials——BHT (I) was obtained from a commercial product. 3,5-Di-*tert*-butyl-4-hydroxybenzaldehyde (II), m.p. 189°, was prepared by oxidation of BHT with Br_2 after the method of Fujisaki,[6] and α-hydroxy-2,6-di-*tert*-butyl-*p*-cresol (III), m.p. 137~138°, by reduction of (II) with $NaBH_4$ in 90% yield according to the modified method of Coppinger, *et al.*[7] 2-*tert*-Butyl-*p*-cresol (IV), m.p. 52°, was synthesized by *tert*-butylation of *p*-cresol with *tert*-BuOH according to Chichibabin's procedure[8] and 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (V), m.p. 210~211°, by the Canizzaro reaction of (II) after the method of Yohe, *et al.*[9] 3,5-Di-*tert*-butyl-4-hydroxybenzylacetate (VI), m.p. 98~99°, was prepared by oxidation of BHT with Br_2 in AcOH according to the method of Coppinger, *et al.*[7] α-Methoxy-2,6-di-*tert*-butyl-*p*-cresol (VII), m.p. 100~101°, was synthesized by oxidation of BHT (4 g.) with Br_2 (1.2 cc.) in MeOH (200 cc.) at 60° and recrystallized from MeOH-H_2O to blunt needles. Yield, 3.5 g. *Anal.* Calcd. for $C_{16}H_{26}O_2$: C, 76.75; H, 10.47. Found: C, 76.60; H, 10.82.

This compound showed no depression of melting point on admixture with the authentic compound, prepared by treatment of α-bromo-2,6-di-*tert*-butyl-*p*-cresol with MeOH.[6]

---

*1 Part V. M. Akagi, I. Aoki: Yakugaku Zasshi, **81**, 492 (1961).
*2 Kita-12-jo, Nishi-5-chome, Sapporo, Hokkaido (赤木満洲雄, 青木 勇).

1) M. Akagi, I. Aoki: Yakugaku Zasshi, **77**, 1121, 1341 (1957).
2) J.C. Dacre, F.A. Denz, T.N. Kennedy: Biochem. J., **64**, 777 (1956).
3) B.D. Astill, D.W. Fasset, R.L. Rowndabush: *Ibid.*, **75**, 543 (1960).
4) A.N. Booth, M.S. Masri, D.J. Robbins, O.H. Emerson, F.T. Jones, F. DeEds: J. Biol. Chem., **234**, 3014 (1959).
5) B.D. Astill, D.W. Fassett, R.L. Rowndabush: Biochem. J., **72**, 451 (1959).
6) T. Fujisaki: Nippon Kagaku Zasshi, **77**, 733 (1956).
7) G.M. Coppinger, J.W. Campbell: J. Am. Chem. Soc., **75**, 734 (1953).
8) A. Chichibabin: Compt. rend., **198**, 1239 (1934) (C.A., **28**, 3722 (1934)).
9) G.R. Yohe, J.E. Dunbar, R.L. Pedrotti, F.M. Scheidt, F.G.H. Lee, F.C. Smith: J. Org. Chem., **21**, 1289 (1956).

NII-Electronic Library Service

this was identified with BHT. Further, α-hydroxy-2,6-di-*tert*-butyl-*p*-cresol (III) was detected in the same extract.

Isolation of Unchanged BHT in the Feces of Rabbits receiving BHT——The collected feces were continuously extracted with ether for 10 hours and the extract was evaporated to dryness. The residue was sublimed in a reduced pressure (water pump, 10~15 mm. Hg, bath temp., 80°) and the sublimate was recrystallized twice from methanol-water to 0.01 g. of square crystals, m.p. 69~70°. This compound showed no depression of melting point with authentic sample of BHT and had the same ultraviolet and infrared absorption spectra.

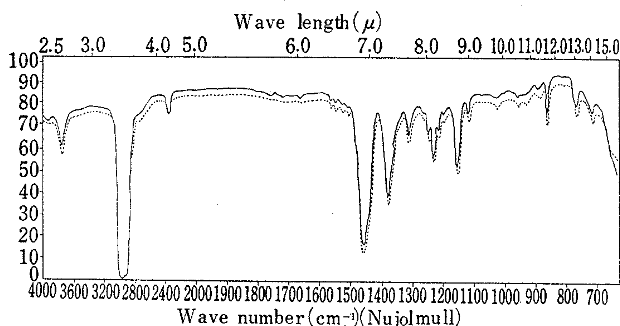

Fig. 2. Infrared Absorption Spectra of Authentic BHT and Isolated BHT

——— authentic BHT
------ isolated BHT

Discussion

In connection with metabolism of BHT analogs, *p*-cresol was studied by Bray, *et al.*[16] and *tert*-butylbenzene by Williams, *et al.*[17] and by Bernhard, *et al.*[18] The metabolism of other various phenols has been studied (Williams[19]) and the principal fate of these compounds (ethereal sulfate and glucuronide conjugation, oxidation of alkyl groups) is well known, but less information is available about the metabolism of hindered phenols. BHT is a typical hindered phenol, insoluble in alkaline media, and its hydroxyl group does not react with diazomethane or acetylating agents. An attempt to synthesize the ethereal sulfate by the methods of Elbs,[20] Baumann,[21] Burkhardt, *et al.*,[22] and of Verley,[23] and on glucuronide by the methods of Helferich, *et al.*[24] and Bollenback, *et al.*[25] were also unsuccessful. Thus, BHT behaved in an anomalous manner in chemical reaction, probably because of steric hindrance resulting from two *tert*-butyl groups *ortho* to the hydroxyl group. However, as was found in the present investigation, it is interesting that BHT was excreted 17% by way of ethereal sulfate and 37% by way of glucuronide conjugation.

When BHT was administered to a rabbit, excretion of metabolites was comparatively slow and this may be due to the difficult absorption of BHT from the intestine. Isolation of unchanged BHT from the feces can be considered on the same basis.

Ordinary phenolic compounds give a blue color with the Gibbs reagent, but *para*-substituted phenols give no color reaction. It is interesting that α-hydroxy-2,6-di-*tert*-

16) H.G. Bray, V. Thorpe, K. White: Biochem. J., **46**, 279 (1950).
17) R.T. Williams, D. Robinson: *Ibid.*, **56**, 159 (1955).
18) K. Bernhard, H. Thommen: Helv. Chim. Acta, **41**, 536 (1958).
19) R.T. Williams: "Detoxication Mechanism," (1959), John Wiley & Sons, Inc., New York.
20) K. Elbs: J. prakt. Chem., **48**, 179 (1893).
21) E. Baumann: Chem. Ber., **9**, 54 (1876).
22) G.N. Burkhardt, A. Lapworth: J. Chem. Soc., **1926**, 684.
23) M.A. Verley: Bull. soc. chim. France, **25**, 46 (1901).
24) B. Helferich, A. Beiger: Chem. Ber., **90**, 2492 (1957).
25) G.H. Bollenback, J.W. Long, D.G. Benjamin, J.A. Lindquist: J. Am. Chem. Soc., **77**, 3310 (1955).

NII-Electronic Library Service

butyl-*p*-cresol, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid, and 3,5-di-*tert*-butyl-4-hydroxybenzylacetate (derivatives of BHT and, *ortho*- and *para*-substituted phenols) were found to give violet color with the Gibbs reagent.

The authors are indebted to Prof. T. Ukita of the University of Tokyo for his kind advice and suggestion, to Mr. Haga of this Faculty for the determination of glucuronic acid, and to Chugai Pharmaceutical Co. Ltd. for their supply of glucuronolactone. Thanks are also due to Mr. Narita of the analysis room of this Faculty for the elementary analysis. This work was supported partly by a Grant-in-Aid for Scientific Research provided by the Prefectural Government of Hokkaido to which the authors' thanks are due.

Summary

Metabolism of the antioxidant, 2,6-di-*tert*-butyl-*p*-cresol (BHT), was studied in a rabbit. After administration of 0.5 g./kg. of BHT by mouth, 37.5% of it was excreted as glucuronides, 16.7% as ethereal sulfates, and 6.8% as free phenols.

α-Hydroxy-2,6-di-*tert*-butyl-*p*-cresol, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid and 4,4′-ethylenebis(2,6-di-*tert*-butylphenol) were identified by paper chromatography from the rabbit urine 24 hours after the administration of 0.5 g./kg. of BHT.

Unchanged BHT was isolated from the feces, but not from the urine.

(Received February 15, 1961)

UDC 581.13 : 547.563

19. Isamu Aoki : Studies on Food Additives. VII.*1 Metabolism of 2,6-Di-*tert*-butyl-*p*-cresol in a Rabbit. (2). Isolation of a Metabolite.

(*Faculty of Pharmaceutical Science, Medical School, Hokkaido University**2)

In the previous work of this series, α-hydroxy-2,6-di-*tert*-butyl-*p*-cresol (BHT-alc, M_1), 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (BHT-acid, M_2), and 4,4′-ethylenebis(2,6-di-*tert*-butylphenol) (BHT-diphenylethane, M_3) were detected in the urine of rabbit receiving 2,6-di-*tert*-butyl-*p*-cresol (BHT) by means of paper chromatography and it was assumed that BHT was metabolized partly by oxidation of its methyl group.

In the present work, further examinations were made on the isolation of the metabolites, BHT-alc, BHT-acid, and BHT-diphenylethane, and on the identification of 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde (BHT-ald) as its 2,4-dinitrophenylhydrazone (M_4), together with a glucuronide (BHTG, M_6) as its methyl acetate derivative from the urine of rabbits receiving BHT.

Experimental*3

Materials——BHT was obtained from a commercial product. BHT-ald was prepared by oxidation of BHT with Br_2 after the method of Fujisaki[1] and its 2,4-dinitrophenylhydrazone was prepared by the method described by Campbell, *et al.*[2] BHT-alc, BHT-acid, and BHT-diphenylethane were

---

*1 Part VI. M. Akagi, I. Aoki : This Bulletin, **10**, 101 (1962).
*2 Kita-12-jo, Nishi-5-chome, Sapporo, Hokkaido (青木 勇).
*3 All melting points are uncorrected.

1) T. Fujisaki : Nippon Kagaku Zasshi, **77**, 731 (1956).
2) J.W. Campbell, G.M. Coppinger : J. Am. Chem. Soc., **74**, 1469 (1952).

NII-Electronic Library Service