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.

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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.

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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

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*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).

prepared as described in the previous paper.*1

Isolation of BHT-diphenylethane (M_3)——The animals used were rabbits weighing 2.0~2.9 kg. They were housed in metabolism cages and fed daily with a mixture of 50 g. of oats, 100 g. of carrots, and 200 g. of cabbage. BHT filled in capsule was administered orally and urine was collected daily, the decomposition of metabolites was prevented by addition of toluene.

Method A——A total dose of 30 g. of BHT filled in a capsule was administered orally 3 times a day for 3 days to 10 rabbits. The collected 24-hr. urine (5 L.) was filtered through cotton, the filtrate was adjusted to pH 2 with 5*N* H_2SO_4, and was continuously extracted with Et_2O for 60 hr. The extract was carefully warmed to evaporate Et_2O. The residue was dissolved in a mixture of 100 cc. of Et_2O and 10 cc. of MeOH, and poured into 1 L. of petr. ether (b.p. 40~60°) to precipitate glucuronide. The brown-colored syrup (A) that precipitated was used for separation of the derivative of BHT-glucuronide (BHTG, M_5). The clear yellowish brown petr. ether supernatant was extracted 3 times with 200 cc. each of saturated $NaHCO_3$ solution. The combined extract (B) was used for separation of BHT-acid. Then the remaining petr. ether solution was further extracted successively with three 100 cc-portions of 4% Na_2CO_3 solution (extract C) and three 50-cc. portions of 10% KOH solution (extract D). The extract (D) was used for separation of BHT-alc (M_1) and derivatives of BHT-ald (M_4). The residual petr. ether fraction, after washing with H_2O, was evaporated to dryness *in vacuo* at 30°. The residual gummy substance was dried *in vacuo* over silica gel for 24 hr., 2 cc. of EtOH was added to the dried residue, and allowed to stand overnight in a refrigerator.

Yellowish crystalline material that separated from EtOH solution was collected and sublimed in a reduced pressure (water pump 10~15 mm. Hg, bath temp. 170°). The sublimed product was crystallized from EtOH. Yield, 0.1 g. of pure yellowish needles, which melted at 169~170°. This compound showed no depression of melting point with the authentic 4,4'-ethylenebis(2,6-di-*tert*-butylphenol). *Anal.* Calcd. for $C_{30}H_{46}O_2$: C, 82.13; H, 10.57. Found: C, 82.24; H, 10.68.

This compound showed the same ultraviolet and infrared absorption spectra as those of the authentic 4,4'-ethylenebis(2,6-di-*tert*-butylphenol). These results are described in a later section.

Method B——A total dose of 30 g. of BHT was fed to 8 rabbits and the urine was collected for 24 hr. The collected urine (5.8 L.) was acidified to pH 2 with 5*N* H_2SO_4 and extracted 6 times with 500 cc. each of petr. ether. By processing the petr. ether extract as above, 1.1 g. of BHT-diphenylethane (M_3) was obtained.

Chromatographic Separation of BHT-diphenylethane——The residue of petr. ether extract evaporated to dryness was dissolved in 500 cc. of petr. ether and chromatographed through an alumina column by the method described by Frickson, *et al.*[3] The yellow zone of the chromatogram was eluted with petr. ether and its residue gave yellowish orange crystals. This product was purified as above and 0.2 g. of BHT-diphenylethane (M_3) was obtained.

Isolation of BHT-acid (M_2) and Benzoic Acid——The saturated $NaHCO_3$ extract (B) was acidified with 5*N* H_2SO_4. The white crystalline material that separated was collected and sublimed in a reduced pressure (water pump, 10~15 mm. Hg, bath temp., 130°). The sublimed compound was recrystallized 3 times from ligroine (b.p. 80~120°) to 0.15 g. of white needles, m.p. 119~121°, which showed no depression of melting point with BzOH. After separation of the sublimate, the residue was redissolved in $NaHCO_3$ solution, filtered, reprecipitated, and the product was recrystallized from 50% *tert*-BuOH to 0.3 g. of pure white scaly crystals, m.p. 210~211°. This compound showed no depression of melting point with the synthetic 3,5-di-*tert*-butyl-4-hydroxybenzoic acid.[4] *Anal.* Calcd. for $C_{15}H_{22}O_3$: C, 71.97; H, 8.86. Found: C, 72.07; H, 9.12.

Isolation of BHT-alc (M_1) and Derivative of BHT-ald (M_4)——The 10% KOH extract (D) mentioned above was acidified with 5*N* H_2SO_4 and immediately separated yellowish white crystals. The crystals collected by filtration were washed with a small amount of MeOH. A sparingly soluble solid remained on the filter. MeOH solution was evaporated *in vacuo* at 30° and the residue was sublimed in a reduced pressure (water pump, 10~15 mm. Hg, bath temp. 150°). The sublimate was recrystallized once from ligroine (b.p. 80~120°) to 0.05 g. of white needles, m.p. 136~137°, which showed no depression of m.p. with authentic α-hydroxy-2,6-di-*tert*-butyl-*p*-cresol. *Anal.* Calcd. for $C_{15}H_{24}O_2$: C, 76.22; H, 10.24. Found: C, 76.17; H, 10.38.

The MeOH-insoluble solid (about 100 mg.) was dissolved in 25 cc. of EtOH and 100 mg. of 2,4-dinitrophenylhydrazine and two drops of conc. HCl were added to the solution. The mixture was refluxed for 10 min. and allowed to stand at room temperature for 2 days. Reddish brown needles that separated were collected and recrystallized from AcOEt-EtOH. The yield of pure reddish violet needles which melted at 235°, was only 0.01 g. This compound showed no depression of melting point with the 2,4-dinitrophenylhydrazone of systhesized 3,5-di-*tert*-butyl-4-hydroxy-benzaldehyde.

3) J. M. Frickson, E. R. Johnson: Proc. S. Dakota Acad. Sci., **32**, 130 (1953) (C. A., **46**, 6166 (1955)).
4) G. R. Yohe, *et al.* (J. Org. Chem., **21**, 1289 (1956)) gave 217~218° as the melting point of this compound.

The four compounds isolated as above showed similar ultraviolet and infrared absorption spectra as those of the synthetic compounds as will be described in later sections.

Isolation and Characterization of Glucuronide (BHTG, M_5)——The brown syrup (A) (about 20 g.) which precipitated from petr. ether fraction was dissolved in 100 cc. of Et_2O, after removal of insoluble material by filtration, Et_2O solution of CH_2N_2 (obtained from 30 g. of nitrosomethylurea) was added to the filtrate with cooling in an ice bath and the reaction mixture was allowed to stand overnight in a refrigerator. The solvent was removed by evaporation *in vacuo* and the residue was dissolved in 100 cc. of pyridine and 100 cc. of Ac_2O. After the mixture was allowed to stand overnight at room temperature, it was poured into ice-water (1 L.) with stirring. The curde powder which separated was dissolved in 200 cc. of $CHCl_3$ and the solution was washed with dil. HCl and H_2O, dried over anhyd. $CaCl_2$, and the $CHCl_3$ solution was evaporated to dryness *in vacuo*. The residue was recrystallized 3 times from EtOH to 3.5 g. of fine long needles, m.p. 162~163°. $[\alpha]_D^{10}$ −45.5° (c=1.005, $CHCl_3$). *Anal.* Calcd, for $C_{28}H_{40}O_{11}$: C, 60.86; H, 7.30. Found: C, 61.02; H, 7.02.

This compound showed an intensive naphthoresorcinol reaction and carbazole reaction for glucuronic acid, and an infrared absorption at 3395 cm^{-1} for hydroxyl group, and an ultraviolet absorption at 278 mμ for BHT-alc. From the result of analysis, together with the result of color reaction and spectral absorptions, it was considered that this compound might be methyl [(3,5-di-*tert*-butyl-4-hydroxy-benzyl)-2,3,4-tri-O-acetyl-β-D-glucopyranoside]uronate.*4 Attempts to synthesize this compound was yet unsuccessful. The systematic separation of the metabolites is shown in Chart 1.

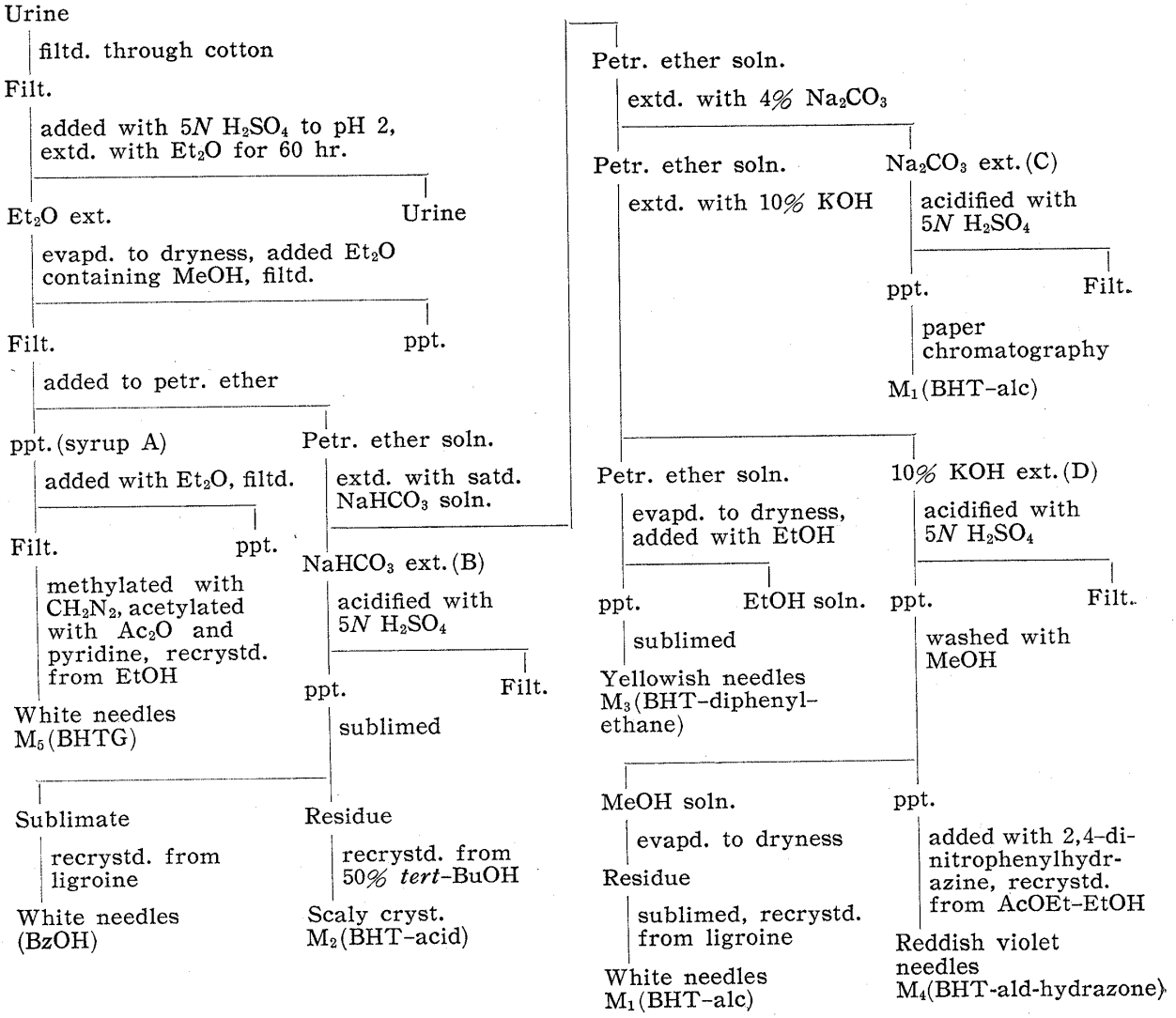


Chart 1. Separation of BHT Metabolites

*4 The structure of this compound is assumed to be methyl[β-(3-*tert*-butyl-2-hydroxy-5-methylphenyl)-β,β-dimethylethyl-2,3,4-tri-O-acetyl-β-D-glucopyranosid]uronate by Dacre (Biochem. J., 78, 758(1961)).

Ultraviolet and Infrared Absorption Spectra of Metabolites——The ultraviolet absorption spectra were determined by the Beckman DK-2 spectrophotometer. Since the absorption spectra of all metabolites indicated the closest resemblance with known samples, these spectra were omitted from Fig. 1 to avoid complexity. Those results are summarized in Table I and Fig. 1.

Table I. Ultraviolet Absorption Spectra (in EtOH) of Metabolites and Derivatives of BHT

Compound	λ_{max} (mμ)	Log ε	Compound	λ_{max} (mμ)	Log ε
p-Cresol	280	4.24	BHT-acid	258	4.70
2-*tert*-Butyl-*p*-cresol	230	4.35	BHT-diphenylethane	276	3.94
	280	4.17	BHTG (M_5)	278	3.60
BHT	276	3.94	M_1	274	3.82
BHT-alc	274	3.82		279	3.81
	280	3.81	M_2	258	4.70
BHT-ald	227	3.85	M_3	276	3.94
	288	3.77	M_4	227	4.64
BHT-ald-hydrazone	227	4.65		303	4.25
	303	4.25		395	4.82
	395	4.82			

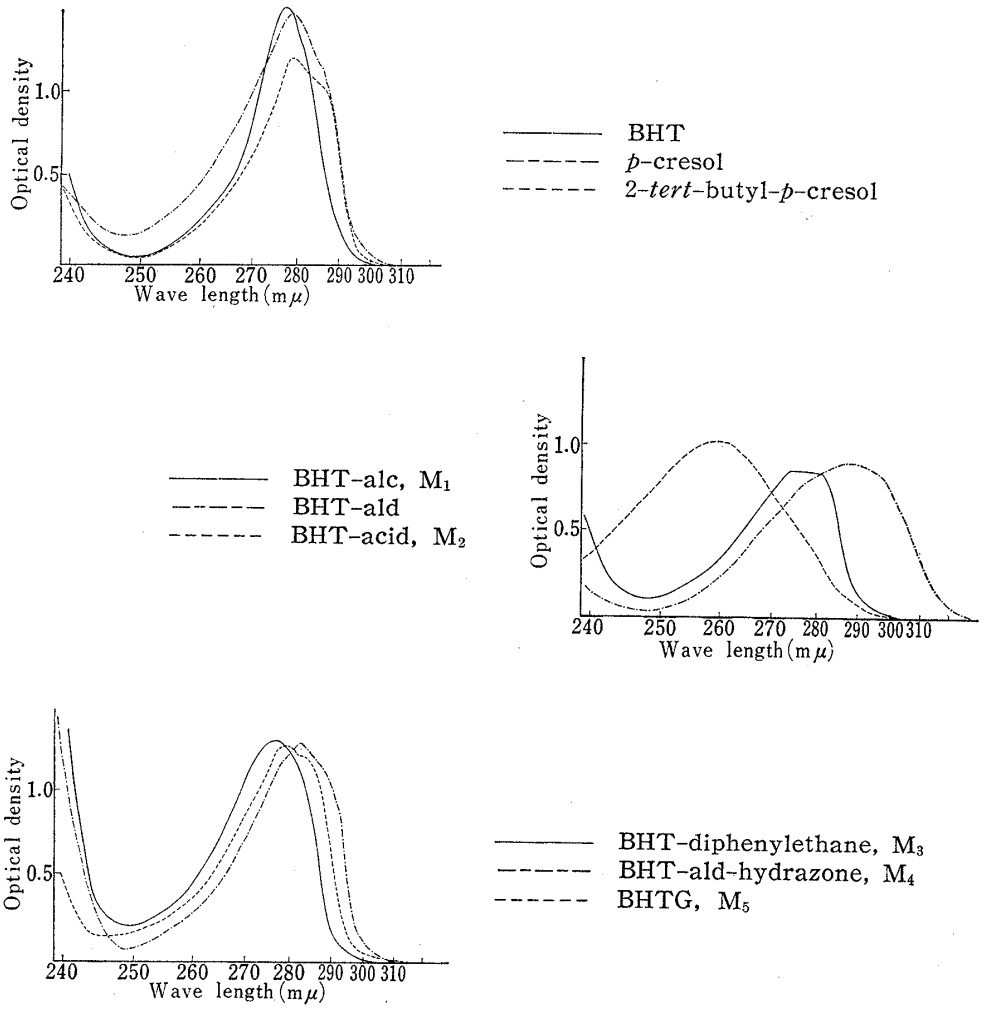


Fig. 1. Ultraviolet Absorption Spectra of Metabolites and Derivatives of BHT

The infrared absorption spectra were determined by Shimadzu Recording Infrared Spectrophotometer Model IR 27A, and the results are shown in Fig. 2. BHT-alc (M_1), BHT-acid (M_2), BHT-ald-hydrazone (M_4), and BHT-diphenylethane (M_3) isolated from the urine of BHT-dosed rabbit indicated complete agreement with authentic samples.

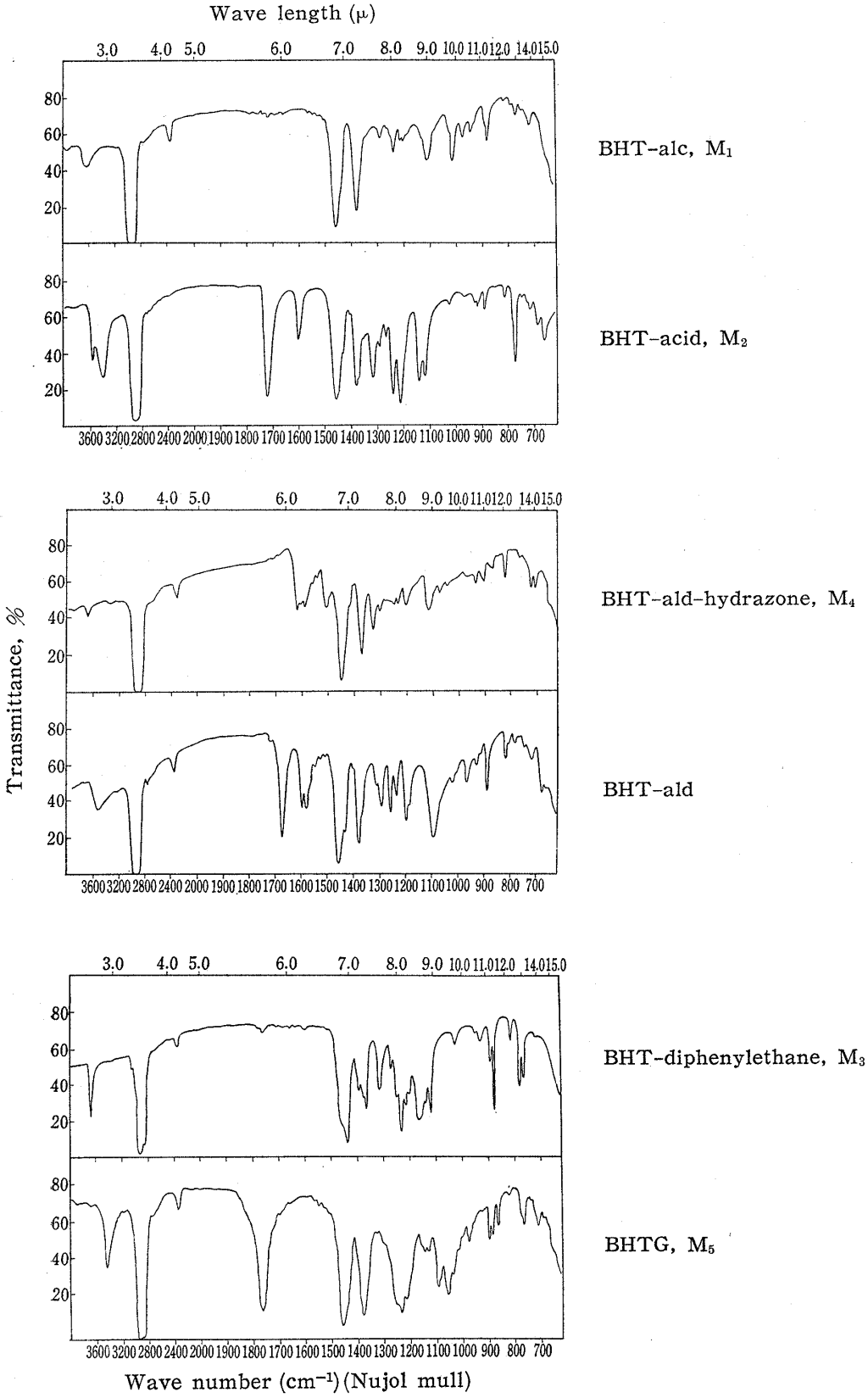
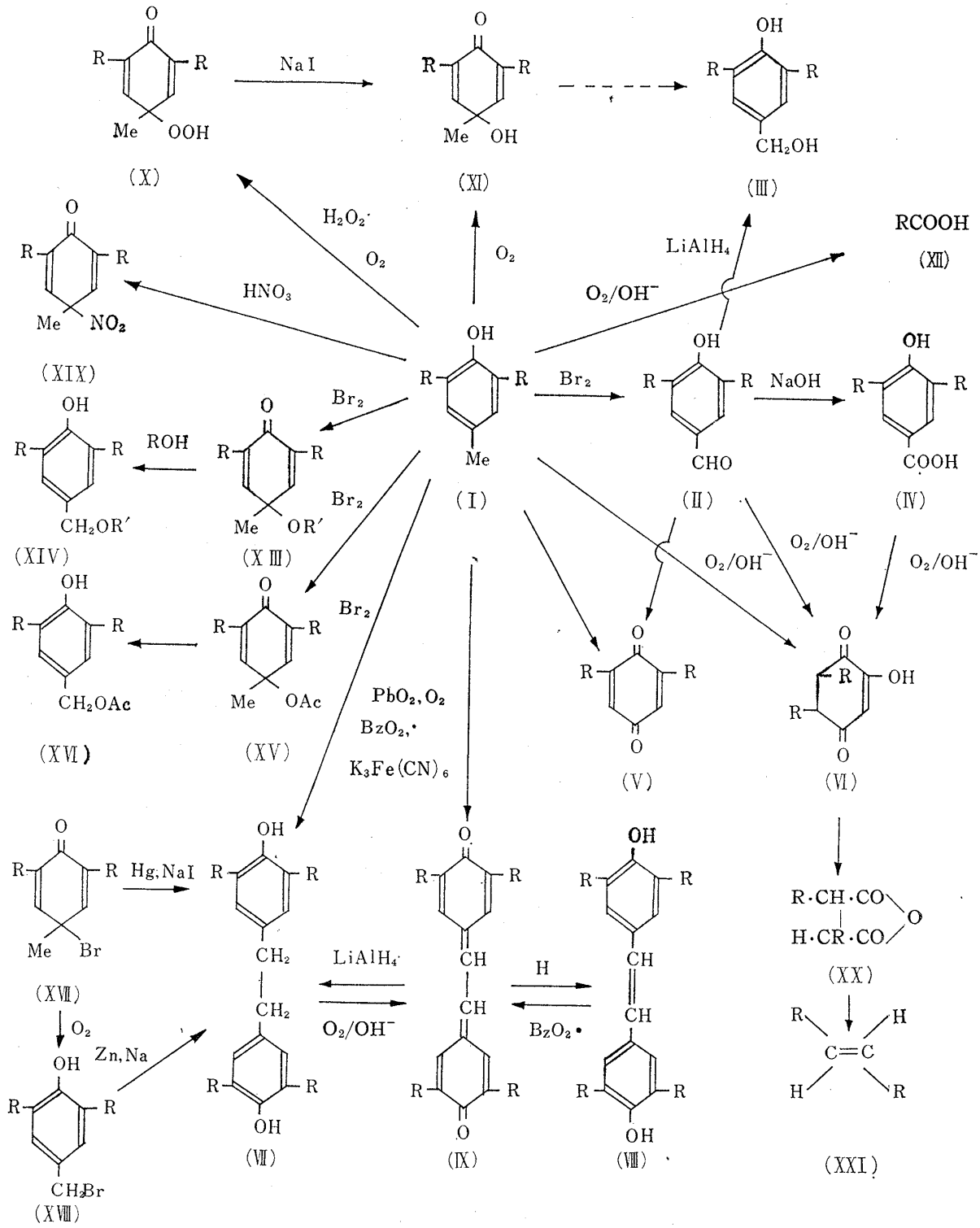


Fig. 2. Infrared Absorption Spectra of Metabolites and Derivatives of BHT

Discussion

In connection with the widespread interest in the use of BHT as an antioxidant in edible fats, numerous investigations concerning the chemical oxidation of this compound have appeared.[1~21]



R=*tert*-Butyl R′=Alkyl

Chart 2. Oxidation of BHT with Several Reagents

The products formed by the chemical oxidation of BHT with various oxidants are summarized in Chart 2.

These investigations clearly indicated that BHT is attacked by the oxidative reagents in its methyl group at the *para*-position of hydroxyl group with the exception of (VI) and (XII).

Among these derivatives, the compounds given in Chart 3 were isolated from the urine of rabbits receiving BHT. The fact that BHT-alc was isolated from the urine indicates that a biotransformation of (XI) produced by initial oxidation of BHT did form *in vivo*, a similar type of reaction as in (XIII) to (XIV) or (XVII) to (XVIII) occurring *in vitro*. Furthermore, the oxidation seemed to proceed stepwise to BHT-ald and then to BHT-acid (M_2).

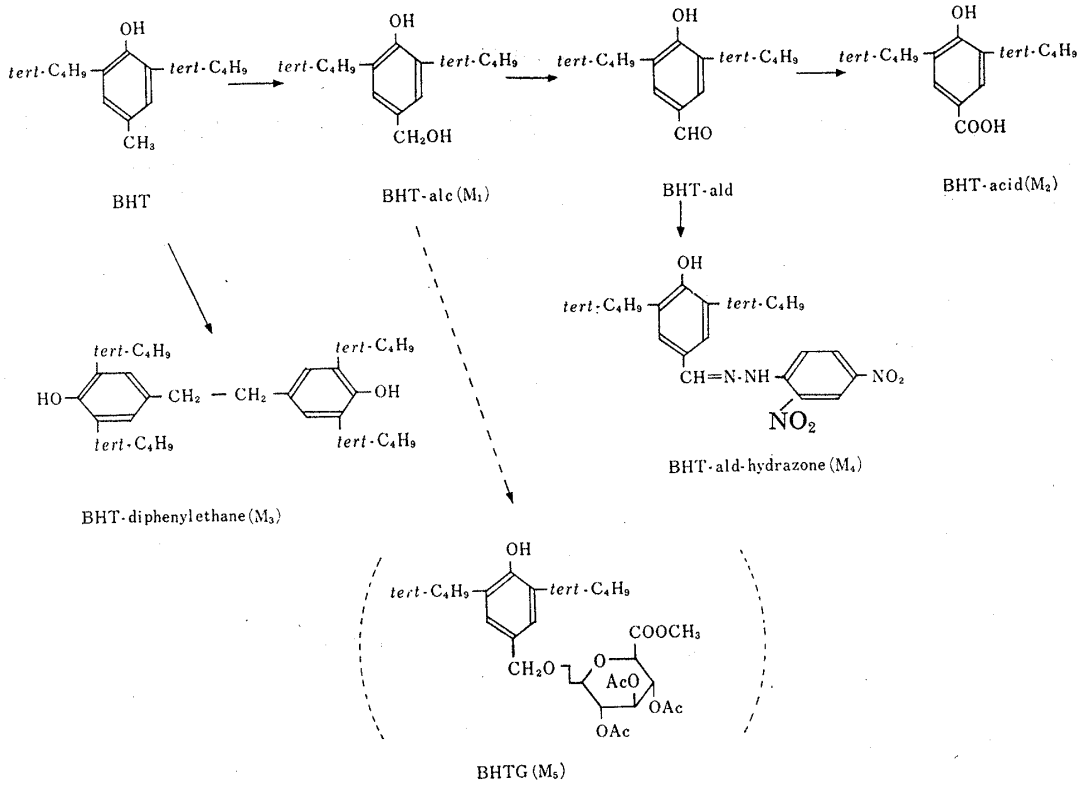


Chart 3.

5) J. H. Coffield, A. H. Fibbey, G. G. Ecke, A. J. Kolka : J. Am. Chem. Soc., **79**, 5019 (1957).
6) G. M. Coppinger, J. W. Campbell : *Ibid.*, **75**, 734 (1953).
7) C. D. Cook : J. Org. Chem., **18**, 261 (1953).
8) C. D. Cook, R. C. Woodworth : J. Am. Chem. Soc., **75**, 6242 (1953).
9) C. D. Cook, N. G. Nash, H. R. Flanagan : *Ibid.*, **77**, 1783 (1955).
10) C. D. Cook. D. A. Kuhn, P. Fianu : *Ibid.*, **78**, 2002 (1956).
11) C. D. Cook, E. S. English, B. J. Wilson : J. Org. Chem., **23**, 755 (1958).
12) G. R. Yohe, J. E. Dunber, M. W. Lansford, R. L. Pedrotti, F. M. Scheidt, F. G. H. Lee, E. C. Smith: *Ibid.*, **24**, 1251 (1959).
13) T. Fujisaki : Nippon Kagaku Zasshi, **77**, 723, 733, 869 (1956).
14) G. M. Coppinger : J. Am. Chem. Soc., **79**, 2758 (1957).
15) S. L. Cosgrove, W. A. Waters : J. Chem. Soc., **1949**, 3189; **1951**, 388.
16) M. S. Kharasch, B. S. Joshi : J. Org. Chem., **22**, 1439 (1957).
17) D. K. Ley : Angew. Chem., **70**, 74 (1958).
18) C. F. H. Allen, D. M. Burness : U. S. Pat. 2,657,222 (C. A., **48**, 12806 (1954)).
19) W. R. Hatchard : J. Am. Chem. Soc., **80**, 3640 (1958).
20) A. C. Witaker : *Ibid.*, **69**, 2414 (1947).
21) G. R. Yohe, D. R. Hill, J. E. Dunber, F. M. Scheidt : *Ibid.*, **75**, 2688 (1953).

The question of whether the BHT-diphenylethane (M_3) is a true metabolite of BHT or not may arise, because the product could be formed by the air oxidation of BHT in alkaline medium.[21] However, this question was denied by the fact that BHT was not identified in the fresh urine of BHT-dosed rabbit and its urine colored strongly yellow in comparison with normal urine.

The author expresses his deep gratitude to Prof. M. Akagi of the University of Hokkaido for his kind guidance throughout the course of this work. Thanks are also due to Prof. T. Ukita of the University of Tokyo for his kind advice and suggestion, and to Mr. Narita of the analysis room of this Faculty, and Mr. Urushibata of the Research Laboratories, Kowa Co., Ltd., for the elementary analyses, and to Chugai Pharmaceutical Co., Ltd. for their supply of glucuronolactone. This work was supported in part by a Grant-in-Aid for Fundamental Scientific Research from the Ministry of Education, to which the author is indebted.

Summary

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 isolated from the urine of rabbits receiving 2,6-di-*tert*-butyl-*p*-cresol, and 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde was identified as its 2,4-dinitrophenylhydrazone.

The glucuronide was isolated also as its methyl acetate derivative from the urine of rabbits receiving 2,6-di-*tert*-butyl-*p*-cresol.

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UDC 615.32[582.949.2]-011

20. Kiyoshi Takiura und Kyōko Koizumi: Über die Bestandteile der chinesischen Droge Tanshin. IV.*1 Zur Kenntnis der Konstitution des Tanshinons II-A.

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Die aus der chinesischen Droge Tanshin, nämlich aus den getrockneten Wurzeln der *Salvia miltiorrhiza*, zuerst von Nakao und Fukushima[1] gewonnene, rote Kristallmasse besteht aus einem Gemenge sehr nahe verwandter *o*-Chinone der Phenanthrenreihe. Die genannten Autoren zerlegten sie durch fraktionierte Umkristallisation in drei Teile und stellten das in Aceton schwer lösliche, braunschwarze Tanshinon-I, $C_{18}H_{12}O_2$, das mäßig lösliche, tief rote Tanshinon-II, $C_{19}H_{18}O_3$, und das ziemlich leicht lösliche, orangerote Tanshinon-III, $C_{19}H_{20}O_3$, dar.

Die chemische Konstitution dieser Chinone hatten von Wessely, *et al.*[2] und gleichzeitig auch Takiura[3] erforscht, und sie hatten für das Tanshinon-I ein- und dieselbe Formel angegeben.

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*1 Der Inhalt dieser Mitteilung wurde 1955 bei ber 75. Jahresversammlung der Japanischen pharmazeutischen Gesellschaft mündlich veröffentlicht.

*2 Toneyama, Toyonaka, Osaka-fu (滝浦 潔, 小泉京子).

1) M. Nakao, M. Fukushima: Reports of Shanghai Sci. Inst., **4**, 103 (1934).
2) F. v. Wessely, S. Wang: Ber., **73**, 19 (1940); F. v. Wessely, A. Bauer: *Ibid.*, **75**, 617 (1942); F. v. Wessely, T. Lauterbach: *ibid.*, **75**, 958 (1942).
3) K. Takiura: Yakugaku Zasshi, **63**, 40 (1943).