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The Metabolism of 3:5-Di-*tert.*-butyl-4-hydroxytoluene and 3:5-Di-*tert.*-butyl-4-hydroxybenzoic Acid in the Rabbit

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(Received 27 April 1960)

The compound known commercially as butylated hydroxytoluene (3:5-di-*tert.*-butyl-4-hydroxytoluene) is an antioxidant developed initially for use with petrol, petroleum products and rubber. Dugan, Marx, Weir & Kraybill (1954) found butylated hydroxytoluene to be an effective antioxidant for animal fats at a concentration of 0.01%, and legislative approval for its addition to fats and foods containing fats intended for human consumption has already been given in eight countries including U.S.A., Denmark, Sweden and the United Kingdom. The toxicity of the compound to rabbits, rats, guinea pigs, cats and dogs was investigated by Deichmann, Clemmer, Rakoczy & Bianchine (1955), who found that when it was given orally to rabbits, there was an increased excretion of both glucuronide and ethereal sulphate in the urine. The causes of the toxic and lethal effects in rabbits given large doses of butylated hydroxytoluene were investigated by Denz & Llauro (1957) and some aspects of its chronic toxicity in rats were studied by Brown, Johnson & O'Halloran (1959).

It will be shown in this paper that 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid is a metabolite of

butylated hydroxytoluene in both the free and conjugated forms. The metabolism of this acid and of the antioxidant butylated hydroxytoluene has been investigated in the rabbit and the major metabolites excreted in the urine have been isolated and characterized. A summary of these results has been published (Dacre, 1960).

EXPERIMENTAL

Animals, diet and dosage. The rabbits used were does of the New Zealand white strain, weighing 1.33–2.0 kg. They were maintained on a standard pellet diet (wheat, 40%; pollard, 33%; bran, 27%) and during experiments were kept singly in metabolism cages designed to permit the separate collection of urine and faeces. For the isolation of metabolites in quantity, rabbits were maintained on a modified diet (wheat, 35%; pollard, 20%; meat meal, 15%; bran, 10%; maize meal, 10%; milk powder, 7.5%; bone meal, 1.7%; cod-liver oil, 0.8%). Water and food were supplied *ad lib.* and the urine was collected daily and stored at -10° . In the initial experiments, single doses of 0.8 g. of butylated hydroxytoluene (BHT)/rabbit were dissolved in 7.2 ml. of olive oil and administered by stomach tube. In later experiments, doses of 0.5 and 1.0 g. in 5 ml. of olive oil/rabbit or 0.8 g. of BHT in 4 ml. of olive oil/kg. body wt. of the

animals were given. Three consecutive daily doses of 0.28 and 0.65 g. of 3:5-di-*tert*.-butyl-4-hydroxybenzoic acid in 5 and 10 ml. respectively of olive oil were similarly administered to a rabbit. The animals usually refused food during the 24 hr. after receiving single doses of BHT and they died between 4 and 10 days after the discontinuation of the repeated doses. No obvious ill-effects were observed in the rabbit receiving the three daily doses of the acid, apart from mild anorexia.

Materials. BHT (Ionol) was purchased from the Shell Company of New Zealand and bacterial β -glucuronidase from the Sigma Chemical Co., St Louis, Mo., U.S.A. 2:4-Dihydroxytoluene was given by Dr S. Dagley, University of Leeds; 3:5-di-*tert*.-butyl-4-hydroxybenzoic acid and 3:5-di-*tert*.-butyl-4-hydroxybenzyl alcohol, for mixed m.p. and determination of spectra, were given by Dr G. R. Yohe, Section of Coal Chemistry, Illinois State Geological Survey Division, Urbana.

Preparation of reference compounds

Combustion analyses were carried out by Dr A. D. Campbell, Chemistry Department, University of Otago. All methoxy (OMe) values are corrected (see Campbell & Chettleburgh, 1959). All melting points are uncorrected.

Derivatives of butylated hydroxytoluene. 3:5-Di-*tert*.-butyl-4-hydroxybenzaldehyde was prepared by bromine oxidation of BHT dissolved in *tert*.-butanol according to Coppinger & Campbell (1953); 3:5-di-*tert*.-butyl-4-hydroxybenzoic acid was prepared from the aldehyde by the Cannizzaro reaction (10–15% yield) and 3:5-di-*tert*.-butyl-4-hydroxybenzyl alcohol by lithium aluminium hydride reduction of the aldehyde according to Yohe *et al.* (1956). The acid was also prepared in greater yield from the urine of rabbits which had been given BHT orally.

3:5-Di-*tert*.-butyl-4-hydroxybenzoic acid from rabbit urine. Urine (250 ml., pH 5.65, containing approx. 16 μ moles of total phenols/ml.) from rabbits given BHT, was incubated with β -glucuronidase powder (0.2 g., about 10 000 units) with intermittent shaking in a water bath at 37° for 24–30 hr. The crystalline material which separated was collected, dried and extracted with peroxide-free ether (2 $\times$ 200 ml.) to remove the enzyme residue. Removal of the solvent *in vacuo* yielded 0.54 g. of colourless crystals, m.p. 185–195°. Extraction of the residual urine with peroxide-free ether (2 $\times$ 200 ml.) yielded a further 60 mg. (total recovery 64%). One recrystallization from light petroleum (b.p. 60–80°) or vacuum sublimation (110–120°/0.05 mm. Hg) gave colourless plates of 3:5-di-*tert*.-butyl-4-hydroxybenzoic acid, m.p. 210°, unchanged by admixture with an authentic sample (Found: C, 71.75; H, 8.9; mol. wt. 228. Calc. for $C_{15}H_{22}O_5$: C, 72.0; H, 8.8%; mol. wt. 250). Yohe *et al.* (1956) record m.p. 218° for this acid. When determined in sealed tubes from a cold electrically-heated block, Yohe's sample, the sample described above and a mixture of the two all melted at 209–210°.

Methyl 3:5-di-*tert*.-butyl-4-hydroxybenzoate. 3:5-Di-*tert*.-butyl-4-hydroxybenzoic acid (100 mg.) was dissolved in dry ether (100 ml.) and diazomethane (about 2 g.) in dry ether was slowly added with intermittent shaking. The reaction mixture was kept for 4 hr. and the ether was then removed *in vacuo* to yield dull-yellowish crystalline plates of m.p. 163–165° (82 mg., 78%). Recrystallization from light petroleum (b.p. 60–80°) gave large colourless plates, m.p. 164–165° with sublimation, of methyl 3:5-di-*tert*.-

butyl-4-hydroxybenzoate (Found: C, 72.75; H, 8.9; OMe, 11.2. $C_{16}H_{24}O_5$ requires C, 72.7; H, 9.1; OMe, 11.7%).

3:5-Di-*tert*.-butyl-2:4-dihydroxytoluene. 2:4-Dihydroxytoluene (1.5 g., m.p. 94–95°) was dissolved in dry benzene (50 ml.) and conc. H_2SO_4 (0.1 ml.) added. Dry isobutylene was bubbled through the stirred mixture at 50–60° for 4 hr. The benzene soln. was shaken out with 2N-NaOH (2 $\times$ 25 ml.) and then with water. The benzene was removed *in vacuo* to give almost colourless crystals (1.44 g., 50%) of m.p. 145–146°. Repeated sublimation *in vacuo* below 100° yielded large colourless rods, m.p. 146–147°, of 3:5-di-*tert*.-butyl-2:4-dihydroxytoluene (Found: C, 76.4; H, 10.25. $C_{18}H_{24}O_2$ requires C, 76.2; H, 10.2%).

Analytical methods

All spectrophotometric measurements were made with a model SP. 600 spectrophotometer (Unicam Instruments Ltd., London).

Free and total phenols. Phenols were determined on diluted rabbit urine, both before and after acid hydrolysis, by the method of Folin & Ciocalteu (1927). The final concentration of phenol was adjusted to 0.3–1.5 mm and the developed colour was measured at 700 μ . The calibration curves of extinction against concentration for phenol, quinol, 2:4-dihydroxytoluene, 3:5-di-*tert*.-butyl-2:4-dihydroxytoluene and 3:5-di-*tert*.-butyl-4-hydroxybenzoic acid gave the same line for the range 0.3–1.5 mm. BHT does not give a blue colour with the Folin & Ciocalteu reagent. When 3:5-di-*tert*.-butyl-4-hydroxybenzoyl β -D-glucosiduronic acid (17.5, 35 and 70 μ moles/ml.) was added to urine, the mean recoveries, determined as free phenols, were 101.3, 98.9 and 90.0% respectively. This glucuronide therefore would contribute to free phenol values.

Non-conjugable phenols. These were determined by extracting urine at pH 5.5–6.5 with peroxide-free ether and analysing the evaporated extracts by the Folin & Ciocalteu method.

Glucuronides. (a) Total glucuronides. The naphtharesorcinol method, as modified by Bray, Humphris, Thorpe, White & Wood (1952), was used for the estimation of total glucosiduronic acid. The naphtharesorcinol reagent (aq. 0.4%) was prepared according to Fishman & Green (1955). Urine was diluted to give a glucuronide concentration between 0.5 and 2.0 mm and readings were made at 600 μ . A calibration curve was prepared with 2-*tert*.-butyl-4-methoxyphenyl β -D-glucosiduronic acid (Dacre, Denz & Kennedy, 1956).

(b) Ester glucuronides. The Shaffer & Hartmann (1921) copper reduction method as described by Bray & Thorpe (1954) was used for the estimation of glucosiduronic acid liberated by alkaline hydrolysis of ester-type glucuronides.

Hippuric acids. Glycine conjugates in diluted rabbit urine were estimated by the micro-method of Gaffney, Schreier, DiFerrante & Altman (1954) as modified by El Masry, Smith & Williams (1956). The yellow azlactone formed from the hippuric acid and *p*-dimethylamino benzaldehyde was measured at 460 μ . The calibration curve prepared with hippuric acid was linear over the range 0–0.56 mm.

Mercapturic acids. The method of Stekol (1936) (iodine consumption after hydrolysis by alkali) as modified by Parke & Williams (1951) was used.

Ethereal sulphates. These were determined by Sperber's (1948) modification of the turbidimetric method. The

calibration curve was made with K_2SO_4 and readings were taken in the range 0.1–0.5 mg. of SO_3 .

Paper chromatography

Phenolic compounds and their metabolites were separated on Whatman no. 1 filter-paper sheets by the descending method with the solvent systems described in Tables 1 and 2. The solvent front was run for 30–40 cm. The compounds were detected and identified on paper with the following spray reagents (all prepared on a w/v basis). (1) Gibbs reagent: a solution of 2,6-dichloro-*p*-benzoquinone-4-chloroimine (0.1%) in ethanol (95%) followed by a solution of sodium tetraborate (aq. 2.0%, pH 9.24). (2) Pauly reagent: a freshly prepared diazotized sulphanilic acid soln. (0.3%) in HCl (8%) followed by a solution of Na_2CO_3 (aq. 20%). (3) Molybdophosphoric acid-ammonia reagent: a solution of dodecamolybdophosphoric acid (0.5%) in ethanol (95%) followed immediately by exposure to conc. ammonia fumes (Mitchell, 1957). (4) Naphtharesorcinol-trichloroacetic acid reagent: this was as described by Elliott, Parke & Williams (1959); the reagent does not react on paper with acetylated-methylated glucuronides. (5) Altman reagent: a freshly prepared solution of *p*-dimethylaminobenzaldehyde (4%) in acetic anhydride (containing a few crystals of sodium acetate), followed by heating at 130–150° for 1–2 min. (Gaffney *et al.* 1954).

Glycine. This was identified by the method of Consden, Gordon & Martin (1944). The solvent system used was phenol saturated with water (3% ammonia added in the tank) and the acid was detected with a ninhydrin spray reagent (0.1% in butan-1-ol).

Spectrophotometry

Ultraviolet-absorption spectra were measured with a model DK2 recording spectrophotometer (Beckman Instruments Inc., Calif., U.S.A.). The u.v.-absorption data for the derivatives and metabolites of BHT are set out in Tables 1 and 2 respectively. Infrared spectra of BHT and its metabolites were measured with a model IR2 Beckman recording spectrophotometer in KBr disks.

Metabolites of butylated hydroxytoluene

Urine from rabbits given BHT had pH 5.8–6.3 and reduced Fehling and Benedict reagents. It gave no colour reaction with aq. or ethanolic $FeCl_3$ soln. and did not react with 2,4-dinitrophenylhydrazine. By paper chromatography of the urine, compounds A, B, C and D were detected and some of their properties are shown in Table 2. Compound A was detected in ether extracts of urine as collected and B, C and D in ether extracts of urine at pH 2–3. BHT was not detected, and in some urines free glucuronic acid and glucuronolactone were present.

Isolation of 3:5-di-*tert*-butyl-4-hydroxybenzoic acid. Urine (250 ml., pH 6.0) from a bulked supply collected over 7 days after a rabbit was given the first of five repeated daily doses of BHT (1 g.) was extracted with peroxide-free ether (4 × 100 ml.). The combined extracts were reduced *in vacuo* to yield a brownish-yellow crystalline deposit, m.p. 180–185° (0.32 g., 10.8% of the dose). This material was redissolved in ether (100 ml.) and then shaken out with $NaHCO_3$ soln. (aq. 5%); the acid was liberated from the bicarbonate solution and collected by extraction with ether (2 × 100 ml.). Repeated recrystallization from light

petroleum (b.p. 60–80°) gave large colourless plates, m.p. 208–209° (0.2 g.), which was not depressed when admixed with authentic 3:5-di-*tert*-butyl-4-hydroxybenzoic acid (Found: C, 72.4; H, 8.4%).

Preparation of the ether-soluble glucuronide fractions. Urine (200 ml.) from which the non-conjugated phenols had been removed (see above) was adjusted to pH 1.5–2.0 with dil. H_2SO_4 and extracted again with peroxide-free ether (3 × 200 ml.). The combined extracts were reduced *in vacuo* to a yellow-brown gum (1.0 g.), which gave a strongly positive test for both glucuronic acid and phenol (fraction 1). Paper chromatography showed the presence of glucuronides B and C as well as trace amounts of the glycine conjugate (compound D). The residual urine contained about 10% of the original glucuronide-reacting material. Continuous ether extraction removed this almost completely as well as the glycine conjugate. The solvent was removed *in vacuo* to yield a yellow-brown gum (60 mg.) (fraction 2). Paper chromatography showed the presence of compounds B, C and D.

Enzymic hydrolyses with β -glucuronidase. The β -glucuronidase powder was examined for hippuricase (aminoacylase 1) activity by the methods of Ellis & Walker (1942), hippuric acid being used as the substrate. At pH 3.1, 4.6 and 6.1 no free glycine was found after incubation for 2, 24 and 48 hr. with β -glucuronidase at 37°. The manufacturers stated that the enzyme preparation had no sulphatase activity on *p*-nitrophenyl sulphate.

(a) Rabbit urine. Urine (150 ml., pH 6.45), penicillin (10 000 units, Glaxo Crystapen) and β -glucuronidase powder (0.1 g., 5000 units) were incubated at 37° in a slow stream of N_2 . After 6 hr. more β -glucuronidase powder (0.1 g.) was added and the incubation continued for a further 24 hr. with intermittent shaking. The crystalline ppt. was collected, dried and extracted with peroxide-free ether (2 × 100 ml.). Removal of the ether *in vacuo* gave an almost colourless crystalline product (0.22 g.), m.p. 170°, which was shown by paper chromatography with solvent system A (Table 1) to contain 3:5-di-*tert*-butyl-4-hydroxybenzoic acid (R_F 0.45) and an unidentified substance (R_F 0.6). This was later shown to be identical with β -(3-*tert*-butyl-2-hydroxy-5-methylphenyl)- $\beta\beta$ -dimethylethanol, the aglycone of glucuronide C. The same compounds were identified in ether extracts of the residual urine.

(b) Ether-soluble glucuronide extract (fraction 1). Glucuronide gum (110 mg.) dissolved in 0.2M-sodium acetate buffer (25 ml., pH 4.5) was incubated with β -glucuronidase (13 mg., 660 units) at 37°. Samples were removed and the free glucuronic acid was determined by the cerimetric method of Levvy (1946). After 1 hr. 72% of the glucuronides were hydrolysed and 77% after 7 hr. In buffer of pH 5.5, 90% of the material was hydrolysed after 20 hr.

Glucuronide gum (0.5 g.) was incubated in acetate buffer (100 ml., pH 5.6) with β -glucuronidase powder (0.1 g., 5000 units) for 24 hr. at 37°. The crystals which separated (185 mg.) had m.p. 170–175°. Paper chromatography with solvent system A (Table 1) showed the same two compounds with R_F 0.45 and 0.6 as described in (a) above. Recrystallization from light petroleum (b.p. 60–80°) gave 3:5-di-*tert*-butyl-4-hydroxybenzoic acid, m.p. 210° unchanged by admixture with the authentic acid, and having the same u.v. spectrum in 95% (w/v) ethanol (Found: C, 72.5; H, 8.6%).

Table 1. R_f values, colour reactions and absorption spectra of butylated hydroxytoluene and its derivatives

Solvent mixtures (by vol., upper phases): A, cyclohexane-methanol (2:1); B, cyclohexane-benzene-methanol (90:10:1); C, ethyl acetate-pyridine-water (2:1:2). Colours of spots: B, blue; G, green; Gy, grey; M, magenta; O, orange; Y, yellow; d, deep; p, pale. Colour on keeping is given in parentheses.

	R_F value in solvent system			Colour with reagent			Light-absorption in 95 % ethanol	
	A	B	C	Gibbs	Pauly	Molybdo-phosphate	λ_{max} (m μ)	ϵ_{max} .
3:5-Di- <i>tert</i> -.butyl-4-hydroxytoluene (BHT)	0.85	0.9	0.9-1.0	pY*	None	pGB	208, 278, 284 ~†	8030, 2200
3:5-Di- <i>tert</i> -.butyl-4-hydroxybenzoic acid	0.45-0.5	0.8	0.9	M	YO	YG	214, 260	26560, 13145
3:5-Di- <i>tert</i> -.butyl-4-hydroxybenzaldehyde	0.65	0.8	—	pM	None	pYG	227.5, 290	18720, 16615
3:5-Di- <i>tert</i> -.butyl-4-hydroxybenzyl alcohol	0.4	0.8	0.9-1.0	M	Y	YG	249, 254.5, 260.5, 281 ~	—
3:5-Di- <i>tert</i> -.butyl-2:4-dihydroxytoluene	0.7	0.9	0.9-1.0	None (pY)	—	pCyB	224, 282.5, 286 ~	7180, 3260
Methyl 3:5-di- <i>tert</i> -.butyl-4-hydroxybenzoate	0.8	0.85	—	None (dB)	None	None	261	13440
β -(3- <i>tert</i> -.Butyl-2-hydroxy-5-methylphenyl)- $\beta\beta$ -dimethylethanol	0.6	0.8-0.85	0.9-1.0	pY	None	pB	283	2790

* Colour is intensified on heating at 100° for 5 min.
† See also Tollenaar & Vos (1956); Phillips & Hinkel (1957).

* Colour is intensified on heating at 100° for 5 min.
† See also Tollenaar & Vos (1956); Phillips & Hinkel (1957).

Table 2. R_f values, colour reactions and light-absorption of metabolites of butylated hydroxytoluene

Solvent mixtures (by vol., upper phases): C, ethyl acetate-pyridine-water (2:1:2); D, lower phase of ethylene dichloride-*tert*-.butanol-acetic acid-water (75:25:30:70); E, butan-1-ol-water (1:1); F, butan-1-ol-2N-ammonia (1:1). Colours of spots: B, blue; M, magenta; Y, yellow; d, deep; p, pale. Colour on keeping is given in parentheses.

	R_f value in solvent system				Colour with reagent		Light-absorption in methanol $\lambda_{\max}$ (m μ)
	C	D	E	F	Gibbs	Naphtha- resorcinol	
Compound A (unconjugated phenol)	0.9	—	0.95-1.0	0.8	pM	None	212, 258
Compound B (ester glucuronide)	0.6	0.75-0.8	0.8	0.5-0.55	Y(M)	dB	210, 266.5
Compound C (ether glucuronide)	0.45-0.5	0.6-0.65	0.7	0.2	B	dB	210, 277
Compound D* (glycine conjugate)	0.3-0.35	0.25	0.5-0.55	0.1	B(dB)	None	211, 261.5, 266~

* Orange-yellow with the Altman reagent.

* Orange-yellow with the Altman reagent.

Isolation of methyl [β -(3-tert.-butyl-2-hydroxy-5-methylphenyl)- β -dimethylethyl 2:3:4-tri-O-acetyl- β -D-glucopyranosid]uronate. From urine (500 ml.) of rabbits given BHT, the glucuronide gum from the basic lead acetate ppt. at pH 8 was prepared and methylated with diazomethane and then acetylated as described by Kamil, Smith & Williams (1951). Trituration of the dark gummy product with absolute ethanol produced large colourless plates (0.66 g.), m.p. 160–162°. Recrystallization from ethanol gave sheaths of long, fine colourless plates of methyl [β -(3-tert.-butyl-2-hydroxy-5-methylphenyl)- β -dimethylethyl 2:3:4-tri-O-acetyl- β -D-glucopyranosid]uronate, m.p. 167.5° (decomp.), $[\alpha]_D^{25} = -45.0^\circ$ in CHCl_3 (c, 1.0); light-absorption max., 208 and 279 m μ (ϵ 10 490 and 2900 respectively) in 95% ethanol (Found: C, 60.7, 61.0; H, 7.2, 7.2; OMe, 5.7, 5.2; $\text{CH}_3\cdot\text{CO}$, 24.2, 25.0; mol.wt., 541. $\text{C}_{28}\text{H}_{40}\text{O}_{11}$ requires C, 60.9; H, 7.3; OMe, 5.6; $\text{CH}_3\cdot\text{CO}$, 23.4%; mol.wt., 552).

Ether-soluble glucuronide gum (fraction 1) (0.5 g.), when methylated, acetylated and triturated as described above, yielded large colourless plates, m.p. 160° (0.2 g.). Recrystallization from tert.-butanol gave long flat colourless plates, or flat colourless prisms from methanol, m.p. 165.5° (decomp.) which was not depressed on admixture with the acetylated-methylated glucuronide from the lead acetate ppt. (Found: C, 60.6, 60.9; H, 7.0, 7.2; OMe, 6.3, 6.1%). This glucuronide was stable to boiling 0.5N-NaOH for 10 min.

Acid hydrolysis of methyl [β -(3-tert.-butyl-2-hydroxy-5-methylphenyl)- β -dimethylethyl 2:3:4-tri-O-acetyl- β -D-glucopyranosid]uronate. The crystalline glucuronide (280 mg.) in methanol (50 ml.) and 2N-HCl (25 ml.) was boiled under reflux for 3 hr. in a slow stream of N_2 . On cooling, the mixture was extracted with light petroleum (b.p. 60–80°) (2 $\times$ 50 ml.). Removal of the solvent *in vacuo* gave long colourless crystalline plates (104 mg., 83%), m.p. 95–96°. Recrystallization from ethanol or sublimation *in vacuo* yielded colourless plates of β -(3-tert.-butyl-2-hydroxy-5-methylphenyl)- β -dimethylethanol, m.p. 110° (Found: C, 76.5, 76.9; H, 10.6, 10.5; OMe, nil. $\text{C}_{18}\text{H}_{24}\text{O}_2$ requires C, 76.2; H, 10.25%).

Acid and enzymic hydrolysis of the crude ether glucuronide (compound C). Ether-soluble glucuronide gum (fraction 1) (200 mg.) was applied to Whatman no. 17 paper and run with solvent system C (Table 2). The band of compound C was cut out and eluted with methanol. Removal of the methanol *in vacuo* yielded a slightly yellowish gum which failed to crystallize from numerous solvents. After hydrolysis of the gum (about 20 mg.) by both methanolic HCl and β -glucuronidase (as described above), followed by paper chromatography of an ether extract, a compound with the same R_F in two solvent systems (A and B, Table 1) as β -(3-tert.-butyl-2-hydroxy-5-methylphenyl)- β -dimethylethanol was identified.

Isolation of methyl (3:5-di-tert.-butyl-4-hydroxybenzoyl 2:3:4-tri-O-acetyl- β -D-glucopyranosid]uronate. The mother liquors remaining from the lead and ether-soluble glucuronide preparations after trituration with absolute ethanol and separation of the acetylated-methylated glucuronide of compound C, should contain the corresponding derivative of glucuronide B. Purifications and fractional separations in numerous solvents failed to effect crystallization of this compound. 3:5-Di-tert.-butyl-4-hydroxybenzoic acid was isolated, however, after acid hydrolysis of this residual gum.

Ether-soluble glucuronide gum (fraction 1) (3.28 g.) from 670 ml. of fresh urine from rabbits given BHT (5 g.), was methylated with diazomethane and then acetylated in acetic anhydride (25 ml.) with perchloric acid (1 drop of aq. 60%) as described by Mead, Smith & Williams (1958). Trituration of the dried precipitated gum with absolute ethanol formed long colourless plates (0.84 g.) of methyl [β -(3-tert.-butyl-2-hydroxy-5-methylphenyl)- β -dimethylethyl 2:3:4-tri-O-acetyl- β -D-glucopyranosid]uronate, m.p. 160° (decomp.); its identity was confirmed by a mixed-m.p. determination and u.v.-absorption spectrum. The residual gum (2.0 g.) was extracted with light petroleum (b.p. 60–80°) to remove any free 3:5-di-tert.-butyl-4-hydroxybenzoic acid and after dissolving in methanol (40 ml.) was remethylated and reacylated as described above. The dried gum, which contained microcrystals, m.p. 118–120° (micro-hot-stage), was dissolved in ethanol (10 ml.) and added to a column of acid-washed alumina. Three equal fractions were collected on elution with ethanol-acetic acid (1:1, v/v). The first fraction, after removal of the solvent *in vacuo*, yielded a slightly coloured amorphous mass (0.38 g.), which on trituration with absolute ethanol gave a mixture of colourless crystals and a glass-like gum. The crystals were separated and quickly washed with ethanol (42 mg., m.p. 130–131°). Recrystallization from ethanol yielded fine colourless needle-like plates of methyl (3:5-di-tert.-butyl-4-hydroxybenzoyl 2:3:4-tri-O-acetyl- β -D-glucopyranosid]uronate, m.p. 136–137° (decomp.), $[\alpha]_D^{25} = -35.0^\circ$ in methanol (c, 1.0); light-absorption max., 217 and 267.5 m μ (ϵ 24 200 and 17 970 respectively) in 95% ethanol [Found: C, 59.8; H, 6.9; OMe, 5.8; $\text{CH}_3\cdot\text{CO}$, 22.8 (corrected value: a blank determination with 3:5-di-tert.-butyl-4-hydroxybenzoic acid gave an acetyl value of 6.4). $\text{C}_{28}\text{H}_{38}\text{O}_{12}$ requires C, 59.4; H, 6.8; OMe, 5.5; $\text{CH}_3\cdot\text{CO}$, 22.8%]. A mixed-m.p. determination with the acetylated-methylated glucuronide from the urine of rabbits given 3:5-di-tert.-butyl-4-hydroxybenzoic acid was not depressed, but a mixed m.p. with the acetylated-methylated glucuronide of compound C (m.p. 167°) was lowered to 121–125°.

Acid hydrolysis of methyl (3:5-di-tert.-butyl-4-hydroxybenzoyl 2:3:4-tri-O-acetyl- β -D-glucopyranosid]uronate. The crystalline glucuronide (100 mg.) in methanol (25 ml.) and 2N-HCl (25 ml.) were boiled under reflux for 4 hr. in a slow stream of N_2 . On cooling, water (25 ml.) was added and the mixture extracted with peroxide-free ether (3 $\times$ 50 ml.). Removal of the ether *in vacuo* after drying over anhydrous MgSO_4 gave yellowish crystals (38 mg., 86.4%), m.p. 185–187°. Paper chromatography with solvent system A (Table 1) showed the presence of 3:5-di-tert.-butyl-4-hydroxybenzoic acid and a trace of methyl 3:5-di-tert.-butyl-4-hydroxybenzoate. Vacuum sublimation of the crystals gave pure 3:5-di-tert.-butyl-4-hydroxybenzoic acid, m.p. 206–207° which was not depressed on admixture with the authentic acid.

Acid and enzymic hydrolysis of the crude ester glucuronide (compound B). Ether-soluble glucuronide gum (fraction 1) (200 mg.) was applied to Whatman no. 17 paper and run with solvent system C (Table 2). The band of compound B was separated and eluted with methanol. Removal of the methanol *in vacuo* yielded a slightly yellow gum which failed to crystallize from numerous solvents, even after rechromatography. After hydrolysis of the gum (about 20 mg.) by both methanolic HCl and β -glucuronidase, followed by paper chromatography of an ether extract and

of the residual mother-liquor concentrates, the only compound identified was 3:5-di-*tert*.-butyl-4-hydroxybenzoic acid.

Acid hydrolysis of the glycine conjugate (compound D). Ether-soluble gum (fraction 2) (40 mg.) was applied to paper and developed with solvent system C (Table 2). The band of the glycine conjugate was cut out and eluted with water (10 ml.). After hydrolysis with HCl, both glycine and 3:5-di-*tert*.-butyl-4-hydroxybenzoic acid were identified by chromatographic analysis.

Metabolites of 3:5-di-tert.-butyl-4-hydroxybenzoic acid

Isolation of unconjugated 3:5-di-tert.-butyl-4-hydroxybenzoic acid. Urine (400 ml., pH 5.85) from a rabbit given 3:5-di-*tert*.-butyl-4-hydroxybenzoic acid (1.95 g.) in three consecutive daily doses, was extracted by shaking with peroxide-free ether (2 × 400 ml.). The extract was dried over anhydrous MgSO₄ and the solvent taken off *in vacuo* to yield a slightly yellow crystalline product. Vacuum sublimation (100–110°/0.05 mm. Hg) gave colourless crystalline plates (38 mg., 2.0% of the dose), m.p. 210° which was undepressed on admixture with authentic 3:5-di-*tert*.-butyl-4-hydroxybenzoic acid. No other compound was detected in the ether extract by paper chromatography in solvent system A (Table 1).

Isolation of methyl (3:5-di-tert.-butyl-4-hydroxybenzoyl 2:3:4-tri-O-acetyl-β-D-glucopyranosid)uronate. Urine (400 ml.) after removal of the non-conjugated phenols (see above) was adjusted to pH 2.2 with dil. HCl and extracted by shaking with peroxide-free ether (3 × 400 ml.). The extract was dried over anhydrous MgSO₄ and the solvent removed *in vacuo* to yield a white friable powder (2.75 g., 93.9%). Chromatographic examination of this material with solvent systems C, E and F (Table 2) gave two spots with *R_F* values and colour reactions identical with those of compounds B and D (Table 2). No other glucuronide-reacting compounds nor any other metabolites were detected in the residual urine, which gave negative Fehling and Benedict tests. The ether-soluble powder (1.6 g.) was methylated with diazomethane and acetylated with perchloric acid as described above. The precipitated gum was washed, dried and then triturated with absolute ethanol. The completely crystalline product (1.8 g.), m.p. 127–128°, was repeatedly recrystallized from ethanol to yield long colourless needles or prisms of methyl (3:5-di-*tert*.-butyl-4-hydroxybenzoyl 2:3:4-tri-O-acetyl-β-D-glucopyranosid)uronate, m.p. 137.5–138° (decomp.) undepressed on admixture with the methylated-acetylated glucuronide of compound B from BHT urine, $[\alpha]_D^{20}$ – 35.0° in methanol (c, 1.0) (Found: C, 59.4, 59.9; H, 6.8, 7.0; OMe, 6.3%).

Acid hydrolysis of the glycine conjugate. The ether-soluble powder (150 mg.) was applied to paper and run with solvent system C (Table 2). The band of the glycine conjugate was cut out and eluted with water (15 ml.). After hydrolysis, both glycine and 3:5-di-*tert*.-butyl-4-hydroxybenzoic acid were identified.

RESULTS

Excretion of normal metabolites. The mean daily excretions (± S.D.), by undosed rabbits were: free phenols, 0.544 ± 0.135; total phenols, 0.667 ± 0.177; total glucuronides, 0.366 ± 0.184; ester glucuron-

ides, 0.230 ± 0.058; ethereal sulphates, 0.164 ± 0.080; glycine conjugates, 0.323 ± 0.103 m-mole.

Metabolites of butylated hydroxytoluene

The results of the analytical determinations of metabolites excreted in rabbit urine after the administration of single and repeated daily doses of butylated hydroxytoluene are set out in Table 3. Excretion of all the metabolites is complete only after 3–4 days after dosing of the animals. About 54% of the administered dose is accounted for, and the recovery of metabolites after four and five repeated doses is not very different from that of single doses of butylated hydroxytoluene.

All the metabolites of butylated hydroxytoluene as indicated by the initial analyses, with the exception of the ethereal sulphates, were satisfactorily accounted for by the paper-chromatographic examination of urine. Methods for the location and identification on paper chromatograms of the ethereal sulphates were not successful.

Non-conjugated phenols. When urines were ether-extracted at pH 5.5–6.5, only one compound was present in the extract. This was shown to be identical with 3:5-di-*tert*.-butyl-4-hydroxybenzoic acid. No unequivocal evidence for butylated hydroxytoluene by chromatographic examinations of solvent extracts or steam-distillates of urine was found and if any is excreted the amount is likely to be very small.

Glucuronide conjugates. The excretion of glucuronides accounts for almost 36% of doses of butylated hydroxytoluene (Table 3) and 16% could be attributed to an ester-type glucuronide (compound B). Paper-chromatographic analysis also confirmed the excretion of two different glucuronides (Table 2). These two metabolites were isolated from urine as the crystalline methylated-acetylated derivatives. The ester glucuronide (compound B) was shown to be identical by m.p., mixed m.p., chromatographic behaviour and u.v. spectrum with the fully acetylated-methylated glucuronide isolated from the urine of rabbits given 3:5-di-*tert*.-butyl-4-hydroxybenzoic acid (see below). The compound is thus formulated as 3:5-di-*tert*.-butyl-4-hydroxybenzoyl β-D-glucosiduronic acid. Elementary analysis of the acetylated-methylated ether glucuronide (compound C) and its crystalline aglycone indicated oxidation of the butylated hydroxytoluene molecule with one atom only of oxygen. The i.r. spectrum of both the aglycone and glucuronide showed the presence of strong bands at 2.90 μ (the sterically hindered hydroxyl group), at 9.45 μ (a primary alcohol side chain) and at 12.90 μ (a phenolic C-methyl group). The spectra support the contention that oxidation of a *tert*.-butyl group to a (1-hydroxy-2:2-dimethyl)ethyl group has taken place. On this evidence the aglycone is

Table 3. *Metabolites excreted in rabbit urine after the administration of single or repeated daily doses of butylated hydroxytoluene*

Results are expressed as a percentage of the dose (single animals). Free phenols: ester glucuronide + non-conjugated phenols. Values obtained by difference are in parentheses.

No. of doses	Daily dose (m-moles/kg.)	Non-conjugated phenols	Free phenols	Total phenols	Total glucuronides	Ester glucuronide	Ether glucuronide	Ethereal sulphate	Glycine conjugate
1	2.28	—	26.2	31.7	35.5	—	—	5.3	—
1	2.43	—	22.4	24.4	38.7	—	—	9.0	—
1	3.64	4.1	19.9	22.0	35.4	18.4	(17.0)	9.9	—
4	1.82	—	—	—	—	—	—	—	—
4	3.65	—	—	—	33.2	14.9	(18.3)	—	—
5	3.63	—	21.2	—	36.1	16.0	(20.1)	7.6	—
5	3.64	8.0	—	—	—	—	—	10.3	1.8
5	3.64	—	30.9	30.7	36.6	14.5	(22.1)	6.0	—
		8.4	24.1	27.2	35.9	16.0	(19.4)	8.0	1.8
	Mean								

* Mean of four separate experiments where only the non-conjugated phenols were determined.

identified as β -(3-*tert*.-butyl-2-hydroxy-5-methyl-phenyl)- β -dimethylethanol and the glucuronide is formulated as β -(3-*tert*.-butyl-2-hydroxy-5-methyl-phenyl)- β -dimethylethyl β -D-glucosiduronic acid. The aglycone has not yet been synthesized.

Glycine conjugate. This metabolite (compound D) was shown to be chromatographically identical with the glycine conjugate that is present in urine after the administration of 3:5-di-*tert*.-butyl-4-hydroxybenzoic acid to a rabbit. Hydrolysis of the conjugate eluted from paper chromatograms confirmed that its structure is 3:5-di-*tert*.-butyl-4-hydroxyhippuric acid.

Ethereal sulphates. About 8% (± 1.9) of doses of butylated hydroxytoluene were excreted as ethereal sulphates (see Table 3).

Mercapturic acids. No consistent indication of the excretion of butylated hydroxytoluene in the form of mercapturic acid conjugates was found.

Metabolites of 3:5-di-tert.-butyl-4-hydroxybenzoic acid

After the oral administration of 3:5-di-*tert*.-butyl-4-hydroxybenzoic acid in three consecutive daily doses to a rabbit, more than 80% was accounted for as ester-type glucuronide and about 9% as a glycine conjugate. Excretion of metabolites was normal on the fifth day after dosing commenced. The recovery of the metabolites is set out in Table 4. The glucuronide was 3:5-di-*tert*.-butyl-4-hydroxybenzoyl β -D-glucosiduronic acid and the glycine conjugate was 3:5-di-*tert*.-butyl-4-hydroxyhippuric acid.

Table 4. *Metabolites excreted in rabbit urine after the administration of 3:5-di-tert.-butyl-4-hydroxybenzoic acid daily over three consecutive days*

Results are expressed as a percentage of the dose (single animals).

	Total dose (m-moles/kg.)	
	2.25	4.70
Non-conjugated phenols	5.2	2.0
Free phenols (non-conjugated phenol + ester glucuronide)	90.5	—
Total phenols	96.2	—
Total glucuronides	84.0	87.5
Ester-type glucuronide	83.5	—
Glycine conjugate	8.8	—

DISCUSSION

The usual methods employed for the estimation of phenols in biological materials (Bray & Thorpe, 1954) cannot be used for butylated hydroxytoluene because of the sterically hindered hydroxyl group. Although the compound reacts with ferric chloride-

2:2'-dipyridyl (Anglin, Mahon & Chapman, 1956; Eastman Chemical Products Inc., 1957), in our hands the method was not sufficiently accurate nor applicable to the routine analysis of butylated hydroxytoluene in urine samples. No free butylated hydroxytoluene was found by the ferric chloride-2:2'-dipyridyl method, nor was it seen in any of the paper chromatograms. Similarly, there was no evidence of conjugates hydrolysable to butylated hydroxytoluene itself. Furthermore, no evidence was found for the conjugation of the metabolites of butylated hydroxytoluene in the normal manner through the hindered hydroxyl group. Confirmation of a free unconjugated hydroxyl group was obtained from measurements of the infrared spectra of these metabolites (cf. Bohn & Campbell, 1957).

From the metabolites isolated it is clear that butylated hydroxytoluene is oxidized either by conversion of the methyl group in position 1 into a carboxyl group or by ω -oxidation of one of the *tert.*-butyl groups to the primary alcohol stage, and that the conjugates are formed through one of these two new groups. As the phenolic hydroxyl of butylated hydroxytoluene is sterically hindered, the pathway by ω -oxidation is probably the one followed for ethereal sulphate synthesis. The conjugation of certain types of primary alcohols with sulphuric acid is known to occur (Williams, 1959). Both types of oxidation have been reported previously: about 10% of an oral dose of *p*-cresol is excreted as free and conjugated *p*-hydroxybenzoic acid (Bray, Thorpe & White, 1950) and up to 90% of a dose of *tert.*-butylbenzene is oxidized by rabbits to 2:2-dimethyl-2-phenylethanol and excreted as a glucuronide (Robinson & Williams, 1955). As far as we are aware, this is the first example of a single compound following both oxidative routes and involving three different conjugating agents.

The study has shown that rabbits can cope with relatively large single doses of butylated hydroxytoluene (Table 3) and up to three daily doses of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid (Table 4). That four and five consecutive daily doses of butylated hydroxytoluene have cumulative and lethal effects is, however, not very reassuring. The metabolites described account for 54.1% ($\pm 7.2\%$) of the dose. The fate of the remainder is unknown.

SUMMARY

1. The metabolism of the antioxidant 3:5-di-*tert.*-butyl-4-hydroxytoluene (BHT) and its oxidation product, 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid, has been studied in the rabbit.

2. Rabbits excreted BHT mainly as glucuronides, some 16.0% as an ester glucuronide and 19.4% as an ether glucuronide. Unconjugated phenol

(8.4%), ethereal sulphate (8.0%) and a glycine conjugate (1.8%) were also excreted. The distribution and recovery of metabolites when four and five daily doses were given was not significantly different from that of single doses of BHT.

3. After three consecutive daily doses of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid, a rabbit excreted 84.0% of the total dose as ester glucuronide, 8.8% as a glycine conjugate and 5.2% was not conjugated.

4. The ester glucuronide, isolated from the urine after both BHT and the acid as the triacetyl methyl ester, is 3:5-di-*tert.*-butyl-4-hydroxybenzoyl β -D-glucosiduronic acid.

5. The ether glucuronide, isolated from BHT urine as the triacetyl methyl ester, is β -(3-*tert.*-butyl-2-hydroxy-5-methylphenyl)- β -D-dimethyl-ethyl β -D-glucosiduronic acid.

6. The glycine conjugate of both BHT and the acid is 3:5-di-*tert.*-butyl-4-hydroxyhippuric acid.

The author is greatly indebted to Professor F. A. Denz, Pathology Department, for many valuable discussions and encouragement during the course of this investigation, and to Professor L. H. Briggs, Chemistry Department, University of Auckland, for the measurements of the infrared spectra.

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Metabolic Effects of α -Oxobutyric Acid: Effect on Respiration of Rat-Liver Homogenates

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(Received 3 May 1960)

The observation that the plasma level of α -amino-*n*-butyric acid is elevated in several pathological states (Iyer, 1956) stimulated interest in the metabolism of the amino acid. This amino acid has been shown to participate in transamination reactions (Iyer & Sukumaran, 1959). It was felt that a study of the metabolism of the corresponding keto acid, α -oxobutyric acid, would help to explain some of the observations.

α -Oxobutyric acid arises chiefly from methionine (Greenberg, 1954) and, probably, also from threonine (Lien & Greenberg, 1952). Long (1942, 1943) has demonstrated that pyruvic-oxidase preparations from brain oxidize α -oxobutyric acid at the same rate as pyruvate. Kinnory, Takeda & Greenberg (1955) have shown that the first step in the catabolism of α -oxobutyric acid is its oxidative decarboxylation to propionic acid. One would therefore expect α -oxobutyric acid to cause an increase in oxygen uptake of liver tissue, which would be a measure of the keto acid catabolized. On this premise we made a manometric study of the metabolism of α -oxobutyric acid. Contrary to expectation, inhibition rather than stimulation of oxygen consumption occurred on the addition of α -oxobutyric acid to rat-liver homogenate.

Further, α -oxobutyric acid causes an appreciable depression in oxygen uptake of rat liver when α -oxoglutarate, succinate, fumarate, malate, oxalo-

acetate, pyruvate, glutamate or aspartate is present as a substrate. α -Oxobutyric acid has been shown to inhibit the formation of citrate from added oxaloacetate and also the utilization of α -oxoglutarate.

MATERIALS AND METHODS

Adult rats were killed by a blow on the head and the liver was dissected out. The liver was rinsed free of blood in ice-cold Krebs-Ringer phosphate solution (Krebs & Eggleston, 1940) and then homogenized in ice-cold Krebs-Ringer phosphate solution containing cytochrome *c* and diphosphopyridine nucleotide (DPN), or in 1.15% (w/v) potassium chloride solution, in a Potter-Elvehjem grinder. Preliminary observations on the depression of endogenous respiration were made with a 30% homogenate in potassium chloride medium. A 10% homogenate in the other medium was subsequently used for studying the effect of α -oxobutyric acid on substrate-stimulated respiration, since such a preparation had the advantage of a relatively low endogenous respiration and a fairly high substrate-stimulated respiration.

Solutions of the potassium salts of citric acid, α -oxoglutaric acid, succinic acid, fumaric acid, L-malic acid, L-glutamic acid, L-aspartic acid, pyruvic acid and α -oxobutyric acid (L. Light and Co. Ltd.) were prepared by neutralizing their aqueous solutions and adjusting the pH to 7.3 with *n*-potassium hydroxide in the cold. The final concentrations used are given in the tables.

The oxygen consumption was measured by conventional Warburg manometry at 38° after allowing 3 min. for