Biochem. J. (1968) **106**, 783
Printed in Great Britain

The Metabolism of 3,5-Di-*tert.*-butyl-4-hydroxytoluene in the Rat and in Man

By J. W. DANIEL, J. C. GAGE and D. I. JONES
Imperial Chemical Industries Ltd., Industrial Hygiene Research Laboratories, Alderley Park, Cheshire

(*Received* 29 *August* 1967)

1. The major metabolites of 3,5-di-*tert.*-butyl-4-hydroxytoluene (BHT) in the rat are 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid (BHT-acid), both free (9% of the dose) and as a glucuronide (15%), and *S*-(3,5-di-*tert.*-butyl-4-hydroxybenzyl)-*N*-acetylcysteine. 2. The mercapturic acid does not appear to derive from the usually accepted enzyme mechanism, and may involve a non-enzymic reaction between BHT free radical and cysteine. 3. The ester glucuronide and mercapturic acid found in rat urine are also the major metabolites in rat bile and must be responsible for the enterohepatic circulation. 4. Free BHT-acid is the main component in rat faeces. 5. In man, BHT-acid, free and conjugated, is a minor component in urine, and the mercapturic acid is virtually absent. The bulk of the radioactivity is excreted as the ether-insoluble glucuronide of a metabolite in which the ring methyl group and one *tert.*-butyl methyl group are oxidized to carboxyl groups, and a methyl group on the other *tert.*-butyl group is also oxidized, probably to an aldehyde group. 6. These differences in metabolism by the rat and by man are sufficient to account for the difference in excretion by the two species.

The compound BHT* (butylated hydroxytoluene, I) has been the subject of extensive toxicological investigation on account of its use as an antioxidant in foods for human and animal consumption. During these studies, measurements of the excretion of radioactivity after the administration of [^{14}C]BHT to animals (Daniel & Gage, 1965) and to man (Daniel, Gage, Jones & Stevens, 1967) have revealed differences that may be due to a species variation in the metabolic fate of BHT.

Investigations into the metabolism of BHT after oral administration to the rabbit (Dacre, 1961; Akagi & Aoki, 1962; Aoki, 1962) have shown the major metabolite in urine to be BHT-acid, formed by oxidation of the ring methyl group, and which is excreted both free and as a glucuronic acid conjugate. Another glucuronide has been detected that, on the basis of the infrared spectrum, has been identified as the conjugate of an alcohol produced either by oxidation of a *tert.*-butyl group or by oxidation of the ring methyl group. Using [^{14}C]-BHT, Daniel & Gage (1965) demonstrated that the delay in excretion that had been observed by earlier authors after the administration of an oral dose of BHT to rats was due to an enterohepatic circulation, and this was later confirmed by Ladomery, Ryan & Wright (1967*a*) after parenteral administration to rats. Ladomery, Ryan & Wright (1967*b*) claimed that the metabolites in rat bile after parenteral injection were somewhat similar to those in urine; they reported BHT-acid as an unspecified conjugate to be a major component, together with small amounts of the intermediate alcohol and aldehyde oxidation products, while another major metabolite remained unidentified. Daniel *et al.* (1967) produced evidence that the enterohepatic circulation observed in the rat did not occur in man, and they attributed this to a difference in metabolism by the two species. This has been the subject of the present investigation.

* Abbreviations: BHT, 3,5-di-*tert.*-butyl-4-hydroxytoluene; BHT-acid, 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid.

MATERIALS AND METHODS

BHT. Unlabelled BHT (m.p. 70°) was obtained from Imperial Chemical Industries Ltd. (Heavy Organic Chemicals Division), Billingham, Co. Durham. It was stated to be not less than 99·5% pure. [^{14}C]BHT, randomly labelled in the methyl groups of both *tert.*-butyl groups, was obtained from the New England Nuclear Corp. (Boston, Mass., U.S.A.); the sample had a specific activity of 2·46 μc/mg.; when chromatographed on thin-layer plates of silica gel with light petroleum (b.p. 60–80°) as eluent, only a single radioactive component was detected.

3,5-*Di*-tert.-*butyl*-4-*hydroxybenzyl alcohol* (*Ionox* 100).

This was obtained from Shell Chemical Co. Ltd., Shell Centre, London.

3,5-Di-tert.-butyl-4-hydroxybenzaldehyde. This compound, m.p. 186–187°; was prepared by oxidation of BHT with bromine. The aldehyde was converted by a Cannizzaro reaction into very pale-yellow crystals of 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid, m.p. 213° (Found: C, 72·3; H, 9·0. Calc. for $C_{15}H_{22}O_3$: C, 72·0; H, 8·8%).

3,5-Di-tert.-butyl-4-hydroxyhippuric acid. 3,5-Di-*tert.*-butyl-4-hydroxybenzoic acid was heated under reflux for 2 hr. with glycine ethyl ester, dicyclohexylcarbodi-imide and triethylamine in tetrahydrofuran (Sheehan & Hess, 1955). The solution was filtered and the filtrate evaporated to dryness, and the ethyl ester (m.p. 152°) was obtained by crystallization of the residue from benzene (Found: C, 68·1; H, 8·6; N, 4·6. Calc. for $C_{19}H_{29}NO_4$: C, 68·0; H, 8·7; N, 4·2%). The required acid (m.p. 189°) was obtained by hydrolysis of this ester with N-NaOH (Found: C, 65·5; H, 7·9; N, 4·5. Calc. for $C_{17}H_{25}NO_4$: C, 66·5; H, 8·1; N, 4·6%). When chromatographed on paper with the solvent systems C and F of Dacre (1961), R_F values of 0·78 and 0·66 were obtained.

S-(3,5-Di-tert.-butyl-4-hydroxybenzyl)-N-acetyl-L-cysteine. To a solution of *N*-acetyl-L-cysteine (10 m-moles) in methanol (50 ml.) was added sodium methoxide (0·6 g. of Na in 15 ml. of methanol) followed by a solution of 3,5-di-*tert.*-butyl-4-hydroxybenzyl bromide (10 m-moles) in ether (25 ml.). The mixture was refluxed for 2 hr. in an atmosphere of N_2, the solvent then removed under reduced pressure and the residue dissolved in water (25 ml.). The aqueous solution was acidified with HCl, evaporated to dryness and the residue crystallized from ethyl acetate–light petroleum (b.p. 40–60°) to give white crystals, m.p. 135–137° (Found: C, 62·4; H, 7·6; N, 3·7; S, 8·1; $C_{20}H_{31}NO_4S$ requires C, 63·0; H, 7·9; N, 3·7; S, 8·4%).

Collection of metabolites from the rat and from man. Single oral doses (10–400 mg./kg.) of [^{14}C]BHT were administered in olive-oil solution to groups of male Alderley Park strain specific-pathogen-free albino Wistar rats, weight 225–275 g. Urine, faeces and bile were collected, as described by Daniel & Gage (1965). Samples of urine were collected from two human male subjects after a single oral dose (0·5 mg./kg.) of [^{14}C]BHT administered in a gelatin capsule (Daniel *et al.* 1967). Details of the amount and radioactivity of the doses, and of the collection of the samples, are given in Table 2.

Solvent fractionation of the metabolites. The following procedure was used for the fractionation of urine and of bile, and for the further separation of metabolites liberated after hydrolysis of conjugates.

The sample, acidified to pH 3, was continuously extracted with ether for 18 hr. and, after separation of the ether extract, the aqueous layer containing the ether-insoluble metabolites was termed fraction F1. Acidic metabolites were separated by shaking the ether layer with aq. 5% (w/v) Na_2CO_3, and the ether layer containing neutral unconjugated metabolites, or neutral compounds formed by hydrolysis of ester sulphates during the ether extraction, was termed fraction F2. The Na_2CO_3 extract was acidified to pH 6 and extracted with ether to give a fraction, F3, containing weakly acidic substances. The remaining aqueous layer was adjusted to pH 3 and extracted with benzene, to give fraction F4 containing benzene-soluble acids, and then continuously with ether to give fraction F5 containing the remainder of the ether-soluble acids.

Chromatography. Fractions were chromatographed on thin-layer plates of silica gel G or alumina, and eluted with the solvent systems shown in Table 1. Radioactive areas were located by radioautography, with contact periods varying from 1 to 14 days. Non-radioactive reference compounds were detected by spraying with Gibbs reagent. For the purification of metabolites before identification, a solution was applied as a band to a thin-layer plate, and after development the radioactive area was located and removed, and the radioactive material was extracted from the support with methanol. The process was then repeated on another thin-layer plate with a different solvent system if necessary. The R_F values and the colours with Gibbs reagent for the unlabelled reference samples are shown in Table 1.

Amino acids were separated by ascending chromatography on Whatman no. 1 paper, and located by spraying with ninhydrin, or by the azide–iodine reagent specific for sulphur-containing compounds (Chargaff, Levine & Green, 1948).

Acidic metabolites were separated on columns (2 cm. × 15 cm.) of DEAE-cellulose (Whatman DE-11 grade) and developed with methanolic solutions of acetic acid of increasing strength, or by gradient elution, with a mixing flask of 100 ml. capacity.

Measurement of radioactivity. Liquid-scintillation counting of the biological samples and of their subsequent fractions was according to the methods described by Daniel & Gage (1965) and by Gage (1967).

Table 1. *Thin-layer chromatography of reference compounds*

Thin-layer chromatography was carried out on silica gel with the following solvent systems: *A*, butan-1-ol saturated with 2 N-NH_3; *B*, butan-1-ol–ethanol–water (2:1:1, by vol.); *C*, butan-1-ol–acetic acid–water (12:3:5, by vol.); *D*, benzene–ethanol (9:1, v/v); *E*, chloroform; *F*, light petroleum (b.p. 60–80°).

	Gibbs reagent colour	R_F in solvent systems					
		A	*B*	*C*	*D*	*E*	*F*
3,5-Di-*tert.*-butyl-4-hydroxytoluene	Yellow	—	—	—	0·77	0·92	0·43
3,5-Di-*tert.*-butyl-4-hydroxybenzyl alcohol	Magenta	—	—	—	0·54	0·68	—
3,5-Di-*tert.*-butyl-4-hydroxybenzaldehyde	Magenta	—	—	—	0·75	0·58	—
3,5-Di-*tert.*-butyl-4-hydroxybenzoic acid	Magenta	0·5	0·82	0·96	—	—	0
3,5-Di-*tert.*-butyl-4-hydroxyhippuric acid	Magenta	0·36	0·74	0·88	—	—	—
S-(3,5-Di-*tert.*-butyl-4-hydroxybenzyl)-*N*-acetylcysteine	Yellow	0·31	0·73	0·84	—	—	—

Glucuronides. Glucuronides were detected in samples and fractions by the naphtharesorcinol reaction. They were separated by precipitation with basic lead acetate, and the free acids were liberated by treating the precipitate with H_2S (Kamil, Smith & Williams, 1952). The solution was filtered and concentrated under reduced pressure.

Glucuronides were hydrolysed either by heating under reflux with 2N-NaOH for 30min. or by incubation with 500 units of β-glucuronidase (Koch–Light Laboratories Ltd., Colnbrook, Bucks.)/ml. in 0·2M-acetate buffer, pH4·5, for 18–48hr. at 37°. The product after hydrolysis was subjected to the solvent fractionation process described above.

Measurement of spectra. Ultraviolet spectra were measured with a Unicam SP.800 recording spectrophotometer, and infrared spectra with a Perkin–Elmer 21 spectrometer with the sample either dispersed in liquid paraffin or in CCl_4 solution. Nuclear-magnetic-resonance spectra of metabolite solutions in $CDCl_3$ or perfluoroacetic anhydride were determined with a Varian HA-100 spectrometer. Mass spectra were obtained with an MS9 double-focussing spectrometer (Associated Electrical Industries Ltd., Manchester).

RESULTS

The radioactivity in the various fractions of rat urine (four experiments) and bile and of human urine (two experiments), expressed as a percentage of the dose administered, is shown in Table 2.

Separation of metabolites from rat urine fractions

Fraction F1 (Expt. 1). Concentrated hydrochloric acid (0·1vol.) was added to this fraction, which was then boiled under reflux in an atmosphere of nitrogen for 30min. Extraction with ether then removed 80% of the radioactivity, and the extract was shown by the fractionation procedure described in the Materials and Methods section to contain only neutral metabolites. Thin-layer chromatography (silica gel, solvent system *D*) revealed the presence of at least five substances.

Fraction F2 (Expt. 1). Thin-layer chromatography (silica gel, solvent system *D*) of the neutral metabolites in this fraction revealed five substances with R_F values 0, 0·37, 0·41, 0·47 and 0·72.

Fraction F3 (Expt. 2). This fraction contained a single substance with R_F 0·5 (silica gel, solvent system *A*). It was purified on silica-gel plates by using successively solvent systems *A* and *B*. The product (metabolite RU1) was crystallized from aqueous methanol (m.p. 208°).

Fraction F4 (Expt. 1). This fraction gave a strong reaction for amino acids after hydrolysis with 5N-hydrochloric acid, but only a weak reaction for glucuronides. Chromatography (silica gel, solvent system *C*) revealed one major metabolite (R_F 0·87), which gave a yellow colour with Gibbs reagent, and two minor metabolites. The fraction was evaporated to dryness under reduced pressure and the residue dissolved in methanol and applied to a column of DEAE-cellulose. The column was eluted successively with methanol (20ml.), 0·1N-acetic acid in methanol (80ml.) and finally 0·3N-acetic acid in methanol. Fractions (10ml.) were collected at 30min. intervals; two fast-moving minor components containing 6 and 4% of the radioactivity were rejected and most of the radioactivity (87%) was collected in fractions 18–28. These fractions were combined and evaporated to dryness, and the residue was dissolved in ether and purified by thin-layer chromatography (silica gel, solvent systems *A*

Table 2. *Solvent fractionation of radioactivity in rat urine and bile, and human urine*

Radioactivity is presented as percentage of dose administered. Pooled bile from two rats was fractionated. See the text for meaning of fractions F1–F5.

Dose (mg./rat or man)	95·5	103·5	1·06	2·82	110	73	40	40
Radioactivity (μc/rat or man)	4·6	8·1	2·6	6·8	4·3	2·9	98	4·8
No. in group	9	4	5	5	1	1	1	1
Duration of collection (hr.)	72	96	96	72	48	48	24	16

	Radioactivity (% of dose)							
	Rat urine				Rat bile		Human urine	
Expt. no.	1	2	3	4			5	6
Total radioactivity excreted	57·5	58·5	19·1	24·4	25·7	36·0	49·5	46
Radioactivity in fractions								
F1	13·5	6·7	1·8	1·6	11·0		40	33
F2	0·9	1·7	1·0	1·0	0·7		0·5	0
F3	9·0	26·3	10·0	10·5	0·8		1·0	1·0
F4	13·0	10·8	2·4	5·8	2·9		0·5	0·6
F5	19·2	11·1	2·3	5·5	17·5		7·0	10·6

and *B*). The solution obtained contained a single radioactive substance (metabolite RU2) with R_F value 0·31 (silica gel, solvent system *A*).

*Fraction F*5 (*Expt.* 1). This fraction gave a strong positive reaction for glucuronides. Two radioactive metabolites were revealed by chromatography (silica gel, solvent system *C*) with R_F values 0·67 and 0·73; these appear to be identical with the minor metabolites present in fraction F4. The fraction was evaporated to dryness and the residue dissolved in methanol and applied to a column of DEAE-cellulose. After elution with 60ml. of 0·1N-acetic acid in methanol, the column was subjected to gradient elution with 0·1N-acetic acid in methanol in the mixing vessel and 5N-acetic acid in methanol in the reservoir. Two metabolites were found in the 5ml. fractions; 13% of the radioactivity occurred in fractions 52–64, and 70% in fractions 72–96. The compounds in these two fractions were termed metabolite RU3 and metabolite RU4 respectively; both gave a positive reaction for glucuronides.

Separation of metabolites from human urine fractions

*Fraction F*1. After hydrolysis of a portion of this fraction by boiling with 1vol. of 4N-sodium hydroxide for 30min., 85% of the radioactivity was obtained as ether-soluble acids. A similar conversion was obtained by hydrolysis with β-glucuronidase. Chromatography of the products (silica gel, solvent system *B*) showed two radioactive spots with R_F values in the region of 0·5 and 0·65. Glucuronides were precipitated from the remainder of this fraction and hydrolysed with 2N-sodium hydroxide. The solution was adjusted to pH6 and shaken with ether; no radioactivity was extracted. The solution was then adjusted to pH3 and extracted again with ether for 18hr. The residue after evaporation of the ether was dissolved in methanol and fractionated on a column of DEAE-cellulose, with 0·1N-acetic acid in methanol as the eluent, 10ml. fractions being collected. A major component (compound HU1) containing 72% of the radioactivity added to the column was found in fractions 19–27, and a minor component in fractions 9–12 (18% of the radioactivity) and another in fractions 42–48 (8% of the radioactivity). The metabolite HU1 was purified by chromatography (silica gel, solvent system *B*).

*Fractions F*2, *F*3 *and F*4. These fractions from human urine contained only a very small amount of radioactivity and they were not further investigated. The radioactive components in fractions F3 and F4 appeared to be identical with the metabolites RU1 and RU2 in rat urine.

*Fraction F*5. This fraction gave a positive reaction for glucuronides. On hydrolysis with β-glucuronidase and subsequent solvent fractionation, 35% of the radioactivity remained with the acids extractable into ether at pH3, and 65% was in the neutral fraction. Chromatography (silica gel, solvent system *A*) showed that the acid fraction contained one component identical with metabolite RU1 by co-chromatography. The neutral fraction was separated into a number of components on silica gel (solvent system *D*).

Separation of metabolites from rat bile fractions

*Fraction F*3. The small amount of radioactivity in this fraction was associated with one acid, which on co-chromatography (silica gel, solvent system *A*) appeared to be identical with metabolite RU1.

*Fraction F*4. Co-chromatography of this fraction indicated that the radioactive component present was identical with metabolite RU2.

*Fraction F*5. This fraction gave a positive reaction for glucuronic acid. It was purified by chromatography on silica gel (solvent system *A*). A portion of the purified metabolite was hydrolysed with sodium hydroxide and then solvent fractionated; 77% of the radioactivity was associated with an acid that on co-chromatography appeared to be identical with metabolite RU1, 4% appeared in the neutral fraction and 19% appeared to be unhydrolysed. After enzyme hydrolysis the amounts of metabolite RU1, neutral material and unhydrolysed material were 53, 8 and 39% respectively.

Identification of metabolites

*Metabolite RU*1. The ultraviolet spectrum of this metabolite had an absorption maximum at 257mμ and a minimum at 238mμ, and was identical with that of synthesized 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid (III), and the $\epsilon_{max.}$ 11000 was very close to the value 10000 for the authentic material. Co-chromatography (silica gel, solvent system *A*) confirmed the identity and the m.p. of the authentic material was not depressed by the addition of metabolite RU1. The measured specific radioactivity of metabolite RU1 was 0·041μc/mg., compared with the theoretical value of 0·043μc/mg. (Found: C, 72·0; H, 8·9. Calc. for $C_{15}H_{22}O_3$: C, 72·0; H, 8·8%).

*Metabolite RU*2. Although this compound gave a reaction for amino acids on hydrolysis, its R_F value 0·3 (silica gel, solvent system *A*) and the yellow colour produced on the chromatogram with Gibbs reagent clearly indicated that it was not 3,5-di-*tert.*-butyl-4-hydroxyhippuric acid. Boiling with 1vol. of conc. hydrochloric acid for 4hr. in an atmosphere of nitrogen yielded a yellow neutral compound with R_F 0·79 (silica gel, solvent system *A*). Ionox 100 by the same acid treatment also yields a yellow neutral compound with the same R_F value. The acid hydrolysate also contains two sulphur-containing amino acids, detected on paper by

Table 3. *Comparison of nuclear-magnetic-resonance spectrum of metabolite RU 2 with that of* S-(3,5-*di*-tert.-*butyl*-4-*hydroxybenzyl*)-N-*acetylcysteine*

Metabolite RU2			Authentic sample			
τ	Pattern	Relative intensity	τ	Pattern	Relative intensity	Proton assignment
			0·14	Broad singlet	1	Carboxyl
2·96	Singlet	2	2·98	Singlet	2	Aromatic
3·68	Doublet	1	3·60	Doublet	1	Amide
4·4	Broad singlet	1 (+1)				Carboxyl (+ hydroxyl)
			4·90	Broad singlet	1	Hydroxyl
5·35	Multiplet	1	5·40	Multiplet	1	Cysteine methine
6·36	Singlet	2	6·40	Singlet	2	*S*-Benzyl methylene
7·10	Doublet	2	7·12	Doublet	2	Cysteine methylene
8·04	Singlet	3	8·05	Singlet	3	*N*-Acetyl methyl
8·60	Singlet	18	8·62	Singlet	18	*tert.*-Butyl methy

spraying with ninhydrin and iodine–azide reagent; these were identified as cystine and cysteine by co-chromatography. These observations suggested a mercapturic acid derived from BHT. The nuclear - magnetic - resonance measurements are shown in Table 3; the presence of the two-proton aromatic singlet and a singlet of τ6·38 (methylene adjacent to an aromatic ring and a sulphur atom) suggested that metabolite RU 2 was *S*-(3,5-di-*tert.*-butyl-4-hydroxybenzyl)-*N*-acetylcysteine (V). The mass spectrum of metabolite RU 2 exhibited a molecular ion at *m/e* 381, which by accurate mass measurement was found to correspond to the elemental composition $C_{20}H_{31}NO_4S$ required for the proposed mercapturic acid conjugate. Confirmation of this structure was obtained by co-chromatography of the metabolite with an authentic sample, and also by comparison of the nuclear-magnetic-resonance and mass spectra (Table 3). The infrared spectra of the metabolite and of the synthesized material both showed absorption maxima at 3690, 3300, 2500, 1730, 1650, 1530 and 1310 cm.$^{-1}$, and their ultraviolet spectra both showed absorption maxima at 233 and 276 mμ, with a shoulder at 280–283 mμ.

Metabolite RU 3. The solution of this metabolite was evaporated to dryness under reduced pressure, and the residue dissolved in acetate buffer and subjected to hydrolysis with β-glucuronidase. Subsequent solvent fractionation revealed 95% neutral compounds. Chromatography (alumina, solvent system *E*) showed several radioactive compounds, a minor one of which was probably Ionox 100 (II).

Metabolite RU 4. After hydrolysis with β-glucuronidase and solvent fractionation, a single weakly acidic substance was recovered. This gave an ultraviolet spectrum identical with that of metabolite RU 1, and on chromatography (silica gel, solvent system *A*) the two substances migrated together. Metabolite RU 4 was therefore identified as the ester glucuronide of 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid (III).

Metabolite HU 1. The evidence obtained during the separation of this metabolite indicated that it was excreted in urine as an ester glucuronide. On hydrolysis of this glucuronide with 2N-sodium hydroxide an acid was obtained that was extractable into ether at pH 3 but not at pH 6, and was therefore stronger than the acid produced by oxidation of the ring methyl group of BHT. Nevertheless, its ultraviolet spectrum, with a maximum at 254 mμ and a minimum at 231 mμ, was very close to that of BHT-acid, suggesting that metabolite HU 1 was a benzoic acid derivative with a second carboxyl group responsible for the higher acidity. Evidence of two carboxylic groups was also obtained from the infrared spectrum. The product was methylated with diazomethane and further purified on silica gel. The mass spectrum demonstrated a molecular ion at *m/e* 322, and accurate measurement gave the formula $C_{17}H_{22}O_6$. The nuclear-magnetic-resonance spectrum showed two peaks in the aromatic region at τ2·4 and 2·5, two peaks assigned to methoxyl resonances at τ6·10 and 6·36, and two peaks (aliphatic methyl) at τ8·40 and 8·71; the relative intensities of these six peaks were approximately 1:1:3:3:6:6. The absence of a signal of relative intensity 3 at approximately τ8·0 indicated the absence of a ring methyl group, and the presence of two distinct aromatic signals and two peaks of relative intensity 6 indicated that both *tert.*-butyl groups had been attacked but not in the same manner. The two methoxyl groups could be attributed to methylation of carboxyl groups, one deriving from oxidation of a ring methyl group and the other from oxidation of a *tert.*-butyl methyl group. There was no direct evidence for the nature of the change in the second *tert.*-butyl group, but, on the basis of the formula given above, the only

possibility is the oxidation of a methyl group to an aldehyde group. No signal for a formyl proton was observed between τ 0·0 and 1·0, but it is possible that a signal at τ 4·6 is derived from a hydrated aldehyde group. This is not in conflict with an *m/e* value of 322, for a water molecule would be lost under the conditions of measurement. This evidence indicates that metabolite HU 1 is 4 - carboxy - 2 - (1 - carboxy - 1 methylethyl) - 6 - (1 - formyl-1-methylethyl)phenol, possibly as a hydrate.

Neutral material. Thin-layer chromatography (silica gel, solvent system *D*) of neutral fractions, both free and after hydrolysis, revealed the presence of up to five compounds, none of which corresponded to BHT, Ionox 100 or 3,5-di-*tert.*-butyl-4-hydroxybenzaldehyde. An acetylated derivative of an ether glucuronide extracted from rabbit urine was supplied to us by Dr J. C. Dacre, which he claimed (Dacre, 1961) to be methyl [β'-(3-*tert.*-butyl-2-hydroxy - 5 - methylphenyl) - $\beta'\beta'$ - dimethyl 2,3,4 - tri - *O* - acetyl - β - D - glucopyranosid]uronate; this was subjected to acid hydrolysis, but the product did not appear to be identical with any component of the neutral fraction. These neutral products are unstable during isolation; BHT is known to give a variety of compounds on mild oxidation, and this investigation has shown that Ionox 100 is readily degraded when heated in acid in the absence of oxygen. As some of the neutral metabolites detected may therefore be artifacts, and each constitutes only a small proportion of the dose, they were not further investigated.

Rat faeces acetone extract

From chromatographic evidence the major metabolite in this fraction was metabolite RU 1, and there was also a small amount of radioactive material with R_F about 0·43 (silica gel, solvent system *F*) that on co-chromatography appeared to be BHT.

DISCUSSION

The qualitative information on the metabolites in rat and human urine, and in rat bile, is collected in Table 4, together with an estimate of the amounts present. The major metabolite in rat urine is the acid produced by oxidation of the ring methyl group (BHT-acid, III), which is excreted both free and conjugated with glucuronic acid. This is in accord with Dacre's (1961) observations in the rabbit, although the glycine conjugate, which he claimed to be excreted by that species, does not appear in rat urine. Dacre's (1961) own evidence for this metabolite is not entirely satisfactory, as a sample of the authentic glycine conjugate, when subjected to chromatography under the conditions that he described, gives R_F values different from those that he claimed. Doubt about Dacre's (1961) evidence has also been expressed by Wright, Akintonwa, Crowne & Hathway (1965), who failed to find this conjugate in their study of the metabolism of Ionox 100 by the rat and the dog. BHT-acid can be extracted by ether at pH 6; this suggests that it is a very weak acid, and possibly too weak to form the acyl-CoA derivative, which is stated by Schachter & Taggart (1954) to mediate the glycine conjugation of benzoic acid derivatives.

The second major metabolite of BHT in the rat is a mercapturic acid (V), which appears to be formed by a mechanism not previously described. Although aromatic hydrocarbons are known to yield mercapturic acids through some intermediate oxidation product (Boyland & Sims, 1958), this has not been reported for aliphatic hydrocarbons or for alkyl substituents on aromatic rings. Ionox 100 (Wright *et al.* 1965) does not form a mercapturic acid, and though a benzyl mercapturic acid is known to be

Table 4. *Metabolites in rat urine and bile, and in human urine*

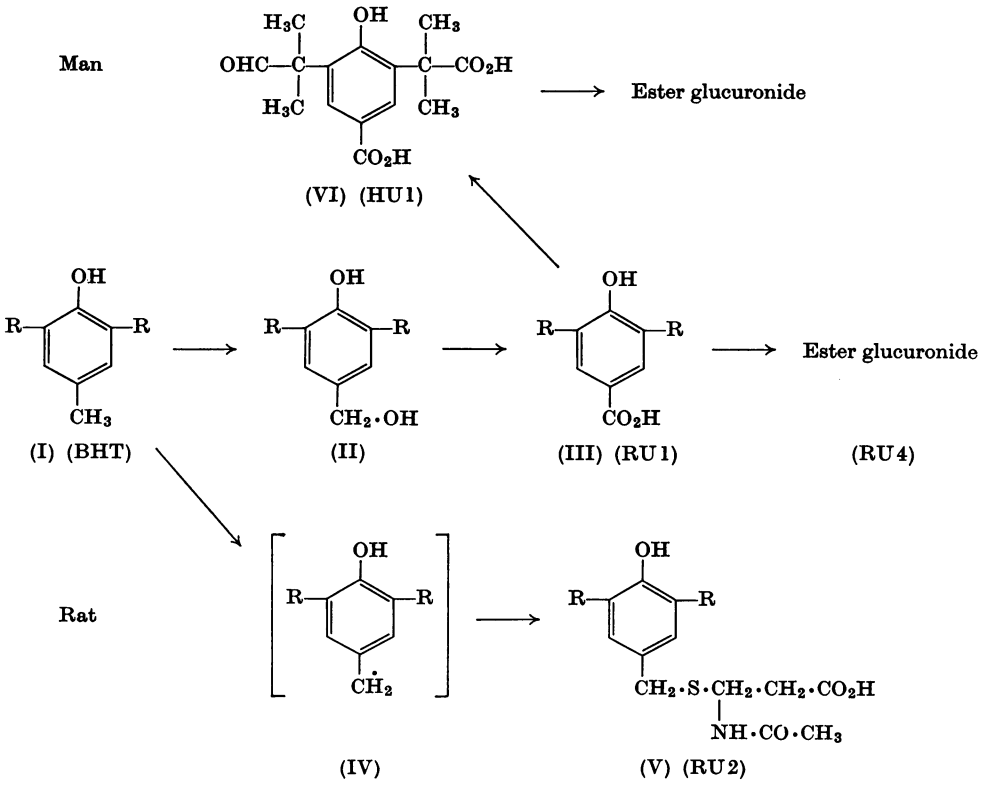
Rat urine results are based on Expt. 1, human urine on Expt. 5 (Table 2). Figures represent percentage conversion of BHT administered.

Metabolite	Conversion of BHT (%)		
	Rat urine	Rat bile	Human urine
3,5-Di-*tert.*-butyl-4-hydroxybenzoic acid (III)	9	0·8	1
(3,5-Di-*tert.*-butyl-4-hydroxybenzoyl β-D-glucopyranosid)uronic acid	15	14	2
S-(3,5-Di-*tert.*-butyl-4-hydroxybenzyl)-*N*-acetylcysteine (V)	11	3	—
4-Carboxy-2-(1-carboxy-1-methylethyl)-6-(1-formyl-1-methylethyl)phenol (? hydrate) (VI) as ester glucuronide	—	—	35
Neutral compounds			
Free	1	0·7	0·5
Glucuronides	4	2	5
Ester sulphates (?)	13·5	—	—

formed from benzyl chloride (Stekol, 1938), there is no precedent for an intermediate product of this nature in the metabolism of substituted phenols. Mercapturic acid formation by glutathione *S*-alkyltransferase (Johnson, 1966) is limited to alkyl halides. The mechanism involving side-chain epoxidation, proposed by James & Jeffery (1964) and James & White (1967), appears to be ruled out on structural grounds. Moreover, the formation of the mercapturic acid is not attended by hydroxylation elsewhere in the molecule. A more likely mechanism involves the free radical (IV), which appears to be formed when BHT accepts a single electron, and which is considered to be involved in the antioxidant action of this compound (Cosgrove & Waters, 1951); this free radical may then react with cysteine by a non-enzymic mechanism and the product acetylated to yield a mercapturic acid.

The amount of neutral metabolites excreted by the rat is small, the total excretion as free and conjugated derivatives being less than 10%. In this context, compounds containing the BHT phenolic group are classed as neutral, as the steric effect of the adjacent *tert.*-butyl groups inhibits phenolic properties. This low excretion is contrary to observations in the rabbit, and there is no evidence in the rat of the excretion of a BHT metabolite hydroxylated at a *tert.*-butyl group (Dacre, 1961), though there is some evidence of hydroxylation at the ring methyl group (Ionox 100, II), presumably an intermediate stage in the formation of BHT-acid. There is little point in undertaking a further investigation of these neutral compounds as they readily undergo change when processed. When Ionox 100 is boiled with dilute acid it is rapidly degraded to a variety of products, and it is possible that this compound may be the major neutral metabolite excreted in urine.

The metabolites found in rat bile consist almost entirely of the conjugates occurring in urine, the bulk of the radioactivity being due to the glucuronide of BHT-acid, but significant amounts of the mercapturic acid and of glucuronides of neutral metabolites are also present. Unconjugated metabolites are almost entirely absent, confirming the view that only water-soluble conjugates are excreted in bile. All of the derivatives found in bile are also lipid-soluble and only weakly acidic, and



Scheme 1. Metabolism of BHT in the rat and in man (R represents *tert.*-butyl).

are therefore capable of being reabsorbed by the gut to give an enterohepatic circulation. The occurrence of unconjugated BHT-acid in faeces does not necessarily indicate that the glucuronide of this metabolite must be broken down in the gut before it can be reabsorbed.

The results in Table 2 suggest that at the lower dose of BHT there is a lower content of metabolites in the urine associated with a lower excretion of conjugates; in these experiments the partition of conjugates between bile and urine appears to have been in favour of the bile. However, an earlier investigation (Daniel & Gage, 1965) has indicated that high urinary excretion in the rat may occur with low BHT dosage.

The metabolism of BHT in man is very different from that which occurs in the rat. The amount of BHT-acid and its glucuronide in urine is very small, and only a trace of the mercapturic acid derivative is present. In place of these ether-soluble derivatives, the major metabolite is an ether-insoluble glucuronide of a BHT oxidation product, in which all three alkyl substituents have been attacked (VI). Although this structure has not been confirmed by independent synthesis, it is very probable that oxidation of the ring methyl group and a *tert.*-butyl group has produced a dicarboxylic acid, and the second *tert.*-butyl group has been oxidized to an aldehyde group, possibly masked by hydration.

On the basis of these observations, the reactions shown in Scheme 1 may be proposed for the metabolism of BHT in the rat and in man. It seems very probable that the enterohepatic circulation in the rat, with attended delayed urinary excretion, derives from the biliary excretion of BHT-acid glucuronide, and possibly also of the BHT mercapturic acid. The rapidity of the first phase of urinary excretion in man, in which about one-half the dose appears within 24hr. (Daniel *et al.* 1967), indicates that there is no considerable enterohepatic circulation in man, and, if the glucuronide of compound (VI) is responsible for the faecal excretion in man, it cannot be readily reabsorbed from the gut. A little BHT-acid glucuronide is found in human urine and it is possible that an enterohepatic circulation occurs to a minor extent. It is conceivable that the second phase of slow urinary excretion in man, attributed by Daniel *et al.* (1967) to a slow release from the tissues, may be due to such a circulation; if so, absorption from the gut and excretion through the liver must be very efficient to lead to the observed half-life in the body.

The results obtained in this investigation are sufficient to account for the observed differences in the kinetics of BHT excretion in the rat and in man, though they give no indication whether the pharmacological effects of BHT in the two species are likely to be different.

We acknowledge the assistance of Dr B. R. Webster and Dr G. R. Bedford of Imperial Chemical Industries Ltd. (Pharmaceuticals Division) in the measurement and interpretation of the spectra. Several of the intermediates were synthesized in these laboratories by Dr M. A. Stevens and Mr G. H. Walker. Technical assistance was provided by Mr P. A. Lefevre.

REFERENCES

Akagi, M. & Aoki, I. (1962). *Chem. pharm. Bull., Tokyo*, **10**, 101.
Aoki, I. (1962). *Chem. pharm. Bull., Tokyo*, **10**, 105.
Boyland, E. & Sims, P. (1958). *Biochem. J.* **68**, 440.
Chargaff, E., Levine, C. & Green, C. (1948). *J. biol. Chem.* **175**, 67.
Cosgrove, S. L. & Waters, W. A. (1951). *J. chem. Soc.* p. 388.
Dacre, J. C. (1961). *Biochem. J.* **78**, 758.
Daniel, J. W. & Gage, J. C. (1965). *Fd Cosmet. Toxicol.* **3**, 405.
Daniel, J. W., Gage, J. C., Jones, D. I. & Stevens, M. A. (1967). *Fd Cosmet. Toxicol.* **5**, 475.
Gage, J. C. (1967). *Fd Cosmet. Toxicol.* **5**, 349.
James, S. P. & Jeffery, D. J. (1964). *Biochem. J.* **93**, 16P.
James, S. P. & White, D. A. (1967). *Biochem. J.* **104**, 914.
Johnson, M. K. (1966). *Biochem. J.* **98**, 44.
Kamil, I. A., Smith, J. N. & Williams, R. T. (1952). *Biochem. J.* **50**, 235.
Ladomery, L. G., Ryan, A. J. & Wright, S. E. (1967*a*). *J. Pharm., Lond.*, **19**, 383.
Ladomery, L. G., Ryan, A. J. & Wright, S. E. (1967*b*). *J. Pharm., Lond.*, **19**, 388.
Schachter, D. & Taggart, J. V. (1954). *J. biol. Chem.* **208**, 263.
Sheehan, J. C. & Hess, G. P. (1955). *J. Amer. chem. Soc.* **77**, 1067.
Stekol, J. A. (1938). *J. biol. Chem.* **124**, 129.
Wright, A. S., Akintonwa, D. A. A., Crowne, R. S. & Hathway, D. E. (1965). *Biochem. J.* **97**, 303.