*J. Pharm. Pharmac.*, 1967, **19**, 388–394 Received January 2, 1967

# The biliary metabolites of butylated hydroxytoluene in the rat

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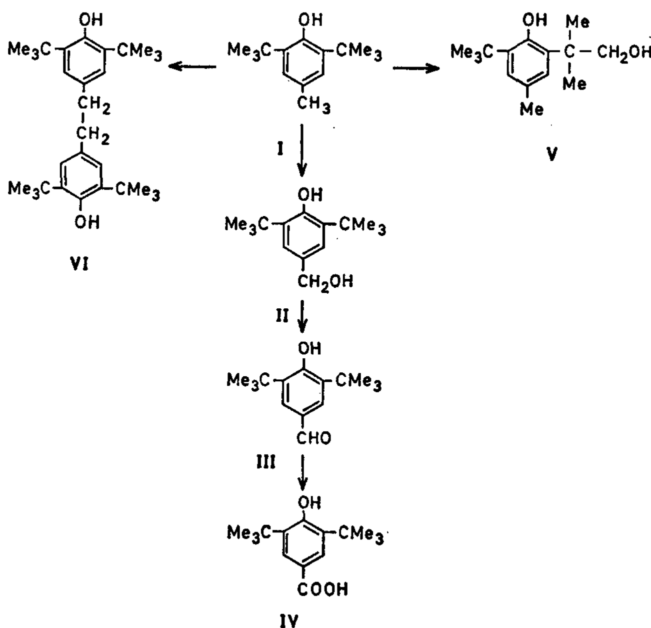
The biliary metabolites from intravenous and intraperitoneal doses of small amounts of [14C]butylated hydroxytoluene have been separated and estimated. The metabolites recognized were the alcohol (II), aldehyde (III) and acid (IV) together with small amounts of the diphenyl ethane (VI). Three other metabolites appear to be present in relatively large amounts. The pattern of metabolites in bile and urine has been compared. It is suggested that the relatively low excretion of the acid (IV) in the urine compared to bile is due to a selective reabsorption of this compound after biliary excretion.

IN a previous paper, Ladomery, Ryan & Wright (1967) described the excretion of small doses (100 $\mu$g) of butylated hydroxytoluene (BHT; I) in the rat. They found that BHT was excreted slowly in urine and faeces because of a rapid enterohepatic circulation. This indicated that BHT was being rapidly metabolized since BHT itself is a non-polar molecule which would not be expected to be excreted by the liver unchanged. This paper describes the detection and estimation of the biliary metabolites from rats given small doses of [14C]BHT. Previous metabolic work on BHT (Dacre, 1961; Akagi & Aoki, 1962) is summarized in Fig. 1.

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## Experimental

### MATERIALS

[$^{14}$*C*]3,5-*Di-t-butyl-4-hydroxytoluene* (2,6-di-t-butyl-*p*-cresol; [$^{14}$C]BHT) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. It was used as 0·02% solution in 50% aqueous ethanol.

3,5-*Di-t-butyl-4-hydroxybenzoic acid* (IV) was prepared by passing isobutylene into a well-stirred mixture of methyl *p*-hydroxybenzoate (6·16 g) and concentrated sulphuric acid (12·0 g) at 100°. When the theoretical quantity of gas (4·5 g) had been taken up (about 36 hr), the mixture was extracted with chloroform-light petroleum (b.p. 60–80°) (1 : 1, 150 ml) and the solution washed with 10% aqueous sodium hydroxide (4 × 50 ml). The organic phase was evaporated to give methyl 3,5-di-t-butyl-4-hydroxybenzoate (2·0 g), m.p. 167–168° from methanol. Dacre (1961) reports m.p. 165°.

The ester was hydrolysed by refluxing with an excess of 0·5N methanolic sodium hydroxide under $N_2$ for 4 hr. Dilution with water and acidification precipitated the acid, m.p. 219–220° from light petroleum (b.p. 60–80°). Dacre (1961) reports 210°; Yoke, Dunbar & others (1956) report m.p. 218°.

3,5-*Di-t-butyl-4-hydroxybenzaldehyde* (III) was prepared by the method of Campbell & Coppinger (1953), m.p. 189° (lit., m.p. 189°). The semicarbazone had m.p. 227° from ethanol. Found: C, 65·8; H, 8·3; N, 14·2%. $C_{16}H_{25}N_3O_2$ requires C, 66·0; H, 8·6; N, 14·4%.

3,5-*Di-t-butyl-4-hydroxybenzyl alcohol* (II) was prepared by reduction of the aldehyde with sodium borohydride, m.p. 145–146° from methanol or light petroleum (b.p. 60–80°) (Bolle & Tomaszewski, 1963, report 140°). Treatment of the alcohol with pyridine and acetic anhydride gave 3,5-di-t-butyl-4-hydroxybenzyl acetate, m.p. 99–100° from light petroleum (b.p. 60–80°). Akagi & Aoki (1962) report m.p. 98–99°.

1,2-*Bis*(3,5-*di-t-butyl-4-hydroxyphenyl*)*ethane* (VI) was prepared by hydrogenating 3,5,3′,5′-tetra-t-butylstilbene-4,4′-quinone (Cook, Nash & Flanagan, 1955) in ethanol at 35° and 200 lb/in$^2$ with Raney nickel. Evaporation of the ethanol gave the diphenylethane, m.p. 173–174° from aqueous acetone. (Cook, 1953, reports m.p. 174–175°).

### CHROMATOGRAPHY

Bile samples were spotted on Whatman No. 1 paper and chromatographed in n-butanol–water (1 : 1) (solvent A). Spots were visualized with Gibbs reagent. Radioactive areas were detected by scanning the paper chromatogram with a Vanguard model 880 Autoscanner. Substances eluted from the paper were hydrolysed with acid and the ether soluble fraction chromatographed on Whatman No. 1 paper using cyclohexane–methanol (2 : 1) (solvent B). Spots were detected as before. These extracts were also chromatographed on thin-layers of silica gel with ether–light petroleum (1 : 1) (solvent C). Radioactive areas on thin-layer plates were detected by exposure to X-ray film.

METABOLIC EXPERIMENTS

Urine and bile were collected as described by Ladomery & others (1967). Hydrolyses were made by refluxing bile with 2N hydrochloric acid under nitrogen for 3 hr. Metabolites were extracted continuously using peroxide-free ether.

REVERSE ISOTOPE DILUTIONS

These were made by adding the required compound to bile. Determinations on unhydrolysed bile were made by adding ether (10 ml) and shaking well. The mixture was left standing at 3° for 48 hr with frequent shaking. The compound was re-isolated from the ether and purified to constant count. Hydrolysis experiments were made by refluxing the bile and reference compound with an equal volume of 4N hydrochloric acid. The hydrolysate was extracted with ether, the compound isolated and purified to constant count.

RADIOACTIVITY

This was assayed by liquid scintillation counting. Biological fluids and chromatogram eluates were accurately pipetted into scintillation fluid. Pure compounds were accurately weighed into vials and dissolved in the scintillator. Radioactive areas from thin-layer plates were removed and suspended in the scintillator. They were kept in the dark for 24 hr before counting.

## Results

Paper chromatography of rat bile containing the metabolites from an intravenous injection of [$^{14}$C]BHT is summarized in Tables 1 and 2. Six

TABLE 1. PAPER CHROMATOGRAPHY OF UNHYDROLYSED RAT BILE AFTER INTRAVENOUS INJECTION OF [$^{14}$C]BHT

| | Unhydrolysed bile; solvent system A | | |
|---|---|---|---|
| Spot | Rf value | % Radioactivity excreted | Compound Rf value reported* |
| A | 0·01 | 3·9 | |
| B | 0·08 | 4·3 | |
| C | 0·54 } | 47·5 | BHT-hippuric acid 0·5–0·55 |
| D | 0·60 } | | |
| E | 0·83 | 28·0 | BHT-ester glucuronide 0·8 |
| F | 0·96 | 16·1 | conjugated phenols 0·95–1·0 |
| Total | | 99·8 | 95% of dose excreted |

* Dacre (1961).

radioactive areas were detected. Spot C, which overlapped with D, corresponded to the glycine conjugate* of 3,5-di-t-butyl-4-hydroxybenzoic

* Wright, Akintonwa & others have been unable to detect this compound as a metabolite of (II) and have questioned Dacre's (1961) findings. We do not wish to imply a positive identification of any biliary conjugate in this present paper.

TABLE 2. CHROMATOGRAPHY OF ELUTED AND ACID HYDROLYSED BILIARY METABOLITES IN SYSTEM B

| Spot hydrolysed | Rf value of hydrolysate | Reference compounds (Fig. 1) | Rf value |
|---|---|---|---|
| A | —* | IV | 0·49 |
| B | 0·06 | III | 0·67 |
| C + D | 0·13, 0·52, 0·81 | I | 0·85 |
| | | VI | 0·88 |
| E | 0·47 | | |
| F | 0·43, 0·70, 0·89 | | |

* Not detected after hydrolysis.

acid (IV) (47·5% of total activity) and spot E to its ester glucuronide (28%) (Dacre, 1961). Spot F was probably unconjugated phenols which amounted to about 16% of the total activity. This was confirmed by reverse isotope dilution (Table 3) which showed that the acid (IV),

TABLE 3. ESTIMATION OF [$^{14}$C]BHT METABOLITES IN RAT BILE AFTER INTRAVENOUS INJECTION OF [$^{14}$C]BHT

| Compound | % Dose in unhydrolysed bile | % Dose in hydrolysed bile |
|---|---|---|
| I | 0·5 | 0·5 |
| VI | 0·4 | 0·4 |
| II | 1·0 | 1·6 |
| III | 5·5 | 5·5 |
| IV | 4·4 | 39·5 |
| Total | 11·8 | 47·5 |

* 95% of administered radioactivity was excreted.

aldehyde (III) and alcohol (II), BHT dimer (VI) and BHT (I) together accounted for 12% of the biliary radioactivity before hydrolysis. Spots A, B and D were unidentified.

Elution of the radioactive areas from the paper chromatograms, acid hydrolysis and chromatography of the hydrolysates gave the results summarized in Table 2. Spot A was lost, spot B appeared to be unaltered. Spots C and D gave the acid (IV) and two unidentified spots. Spot E gave only the acid, while spot F gave the expected acid (IV), aldehyde (III) and dimer (VI). The alcohol (II) was probably in too low a concentration to be detected. Reverse isotope dilution studies (Table 3) of the bile hydrolysate showed that the acid (IV) was a major constituent, being nearly 40% of the total. The aldehyde (III), alcohol (II), BHT (I) and the dimer (VI) were minor constituents. Exactly half (47·5%) of the total activity was accounted for as these substances.

Because of a lack of reference conjugates it was decided to work with the bile hydrolysates. At this time it was found that better resolution of the biliary metabolites was achieved by thin-layer rather than paper chromatography. The chromatography of hydrolysed bile extracts after an intravenous injection of [$^{14}$C]BHT is shown in Table 4. Six radioactive areas were detected with X-ray film. Spot B did not appear in this bile, but was detected in "late" (72 hr) bile (see below) The relation between the unidentified compounds detected on thin-layer plates and on the paper

TABLE 4. THIN-LAYER CHROMATOGRAPHY OF ACID HYDROLYSED BILE AFTER AN INTRAVENOUS INJECTION OF [$^{14}$C]BHT (SOLVENT C)

| Radioactive spot | Rf value | % Dose excreted | Reference compound (see Fig. 1) | Rf value |
|---|---|---|---|---|
| A | 0·0 | 35·0 | | |
| B* | 0·1 | — | | |
| C | 0·25 | 37·1 | IV | 0·23 |
| D | 0·52 | 1·5 | II | 0·52 |
| E | 0·65 | 11·0 | | |
| F | 0·71 | 4·5 | III | 0·71 |
| G | 0·88 | 1·1 | VI } | 0·92 |
| | | | I } | 1·0 |
| Radioactivity residual | | 4·0 | | |
| Total† | | 94·2 | | |

* Metabolite B was found only in bile collected 72 hr after dosing.
† 95% of dose excreted in bile.

chromatograms is unknown. Spot C was the acid (IV), D the alcohol (II), F the aldehyde (III) and G was either BHT (I) or the dimer (VI). Spots A and E were unidentified.

This thin-layer technique was then applied to separate and estimate the metabolites appearing in bile collected for 6 hr immediately after an intraperitoneal injection ("early" bile) and also in the bile collected 72 hr after injection ("late" bile). The results are summarized in Table 5.

TABLE 5. ELUTION AND ESTIMATION OF [$^{14}$C]BHT METABOLITES ON THIN-LAYER CHROMATOGRAMS OF HYDROLYSED BILE AFTER INTRAPERITONEAL ADMINISTRATION

| Compound | % Radioactivity excreted | % Dose excreted |
|---|---|---|
| 0–6 hr bile (51·9% of dose excreted) | | |
| A | 33·8 | 17·5 |
| B | — | — |
| IV | 36·2 | 18·8 |
| II | 2·8 | 1·4 |
| E | 1·8 | 0·9 |
| III | 3·2 | 1·7 |
| VI } I } | 4·3 | 2·2 |
| Residual activity* | 13·4 | 7·0 |
| Total | 95·5 | 49·5 |
| 72–78 bile (11·6% of dose excreted) | | |
| A | 15·0 | 1·7 |
| B | 15·6 | 1·8 |
| IV | 52·6 | 6·1 |
| VI | 4·0 | 0·5 |
| Residual activity* | 11·1 | 1·3 |
| Total | 98·3 | 11·4 |

* Radioactivity remaining on plate after removal of radioactive areas.

After being revealed by exposure to X-ray film, the radioactive areas were scraped off the plates and counted.

In "early" bile the acid (IV) was the major component identified. The aldehyde (III), alcohol (II), BHT (I) and dimer (VI) accounted for only about 10% of the excreted radioactivity. Spot E was also a minor

component. Spot A, however, was present to the same extent as the acid (IV).

In "late" bile a simpler metabolic pattern appeared. Spot A was accompanied by an equal amount of a new spot, B. The acid (IV), was found and also a spot which, in view of the time elapsed since dosing, must have been the dimer (VI).

A complicating factor in this work was that not all the radioactivity spotted on to the plates was accounted for by the areas detected on X-ray film. A count of the activity of the residual silica gel showed this to contain the remaining radioactivity. The reason for this is not known. However, other workers in these laboratories (R. E. Thomas, private communication) have observed similar effects with tritiated cardiac glycosides and genins. The errors introduced by this retention of activity by the gel may be relatively small since there was good agreement between the reverse isotope dilution studies and elution from the plate. It is possible that part of the streaking of radioactivity is due to traces of decomposition products.

Vigorous acid hydrolysis caused loss of metabolite B from "late" bile (Table 6), but did not appreciably alter the amounts of the remaining metabolites. Table 6 also contains a comparison of pooled 4-day urine metabolites and "late" bile metabolites. It is interesting to note that the acid (IV) is not a major urinary constituent as it is in bile.

TABLE 6. ESTIMATION OF [$^{14}$C]BHT METABOLITES IN POOLED URINE AND LATE BILE HYDROLYSED WITH 6N HYDROCHLORIC ACID

| Compound (see Fig. 1) | 1–4 Day urine* | | 72–78 hr bile† | |
|---|---|---|---|---|
| | % Excreted radioactivity | % Dosed radioactivity | % Excreted radioactivity | % Dosed radioactivity |
| A | 20·2 | 6·3 | 15·9 | 1·7 |
| B | — | — | — | — |
| IV | 16·2 | 5·1 | 52·5 | 6·1 |
| II | 24·6 | 7·7 | — | — |
| E | — | — | — | — |
| III | 10·4 | 3·3 | — | — |
| I } VI | — | — | 6·0 | 0·5 |
| Residual activity | 24·0 | 7·2 | 20·1 | 2·3 |
| Total | 95·4 | 29·6 | 94·4 | 10·6 |

* 31·2% of dose excreted in urine.
† 11·6% of dose excreted in 72–78 hr bile.

## Discussion

The differing proportions of metabolites in "early" and "late" bile as well as in pooled 4-day urine suggest a selective process of biliary and urinary excretion. However, as yet, we have little information on the nature of the conjugates excreted in bile, and none on the nature of the compounds re-absorbed from the gut. In addition, nothing is known of the faecal metabolites. Yet it is interesting to speculate about the high proportion of the acid (IV) which appears in the bile. "Early" bile contains 34% and "late" bile 53% of the total excreted activity as (IV),

while pooled 4-day urine contains only 16%. The acid (IV) is mainly excreted as the ester glucuronide by rats (Wright & others, 1965). It would be hydrolysed by plasma esterases and therefore preferentially excreted by the liver. Ether glucuronides would probably be more stable to esterase action and would therefore be excreted more efficiently by the kidney. This assumption agrees with the minor amounts of the alcohol (II) found in bile and the high proportion (25%) in urine. More aldehyde (III) is found in "early" bile than pooled urine. It is possible that there is an equilibrium between the alcohol and aldehyde effected by the reversible alcohol dehydrogenase systems of liver during enterohepatic circulation.

It is interesting that Wright & others (1965) found a slow excretion of label from doses of 3,5-di-t-butyl-4-hydroxybenzyl alcohol[$^{14}$C] (II). Rats and dogs both excreted this compound in urine and faeces over several days. The major metabolite was the acid (IV) together with minor amounts of unidentified metabolites. In the light of the present work and its distribution studies, it is clear that the metabolites from (II) also undergo extensive enterohepatic circulation. Wright & others did not detect any unchanged (II) in the excreta.

Because of the agreement between the urinary excretion of tritiated BHT (Golder, Ryan & Wright, 1962) and [$^{14}$C]BHT (Ladomery, Ryan & Wright, 1963) the latter workers suggested that there might be little attack at the methyl group of BHT. It was known that 50% of the label in tritiated BHT was in the methyl group. It is now clear from the present work that, although the proportion of the acid (IV) is low in the urine, this is due to a process of selective excretion rather than selective oxidation.

Among the metabolites detected by Dacre (1961) was the alcohol, β-(3-t-butyl-2-hydroxy-5-methylphenyl)-ββ-dimethylethanol, formed by oxidation of the t-butyl group. This compound does not seem to have been detected in the present work. It could not be isolated from rabbit urine using Dacre's (1961) method.

*Acknowledgements.* This work forms part of a research programme supported by the National and Medical Research Council of Australia and Grant EF-258 of the U.S. National Institutes of Health.

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