J. Pharm. Pharmac., 1968, **20**, 610–618 Received January 9, 1968

The role of the tissues and gut micro-organisms in the metabolism of [^{14}C]protocatechuic acid in the rat. Aromatic dehydroxylation

J. C. DACRE* AND R. T. WILLIAMS

The fate of oral doses (0·1 g/kg) of [*carboxy*-^{14}C]3,4-dihydroxybenzoic (protocatechuic) acid in normal and neomycin-treated rats has been studied. In normal rats about 72% of the dose of ^{14}C is excreted in 7 days (about 64% in urine and 8% in faeces) whereas in neomycin-treated rats 99% is excreted (87% in urine and 12% in faeces). In normal rats, the radioactive metabolites found in the urine excreted in 24 hr after dosing were protocatechuic acid (36%), vanillic acid (21%), *m*-hydroxybenzoic acid (2·1%), *p*-hydroxybenzoic acid (1·2%) and *m*-methoxybenzoic acid (2%). After neomycin-treatment protocatechuic acid (55%) and vanillic acid (22%) were found, but the other three were virtually absent. Incubation of [^{14}C]protocatechuic acid with rat intestinal contents yielded $^{14}CO_2$ and small amounts of *m*- and *p*-hydroxybenzoic acid. When [^{14}C]protocatechuic acid was injected intraperitoneally into biliary cannulated rats about 13% appeared in the bile mainly as the 4-*O*-glucuronide of vanillic acid. It is concluded that, whilst the conjugation and methylation of protocatechuic acid occurs in the tissues of the rat, the dehydroxylation and decarboxylation of the acid is carried out by the gut micro-organisms.

THE *p*-dehydroxylation *in vivo* of certain catechol acids to *m*-hydroxy acids was first observed by Booth & others (1955; 1956) and the literature on this reaction has been briefly summarized by Scheline, Williams & Witt (1960). Although 3,4-dihydroxyphenylacetic (homoprotocatechuic) acid is dehydroxylated in rabbits (Booth & others, 1955), it was reported that 3,4-dihydroxybenzoic (protocatechuic) acid was not (DeEds, Booth & Jones, 1957). The fate of protocatechuic acid has now been examined again in rats with the ^{14}C-labelled acid. Previous work on protocatechuic acid had shown that it was excreted by rabbits partly unchanged, partly conjugated with glucuronic acid and sulphate (Dodgson & Williams, 1949) and partly methylated to vanillic acid (DeEds, Booth & Jones, 1955). Tompsett (1959) claimed that in man it was converted to *m*-hydroxybenzoic acid. It is now shown that orally administered protocatechuic acid is dehydroxylated to a minor extent to *m*- and *p*-hydroxybenzoic acid in rats and that *m*-methoxybenzoic acid is also a minor urinary metabolite. Evidence is presented suggesting that dehydroxylation is carried out by gut micro-organisms (see also Shaw, Gutenstein & Jepson, 1961). This work has been briefly reported (Dacre & Williams, 1962).

Experimental

MATERIALS

Protocatechuic acid, m.p. 200° (decomp.), vanillic acid, m.p. 210–211°, veratric acid, m.p. 179°, *m*-methoxybenzoic acid, m.p. 109–110°, catechol, m.p. 105° (Koch-Light Laboratories Ltd., Colnbrook), *m*-hydroxybenzoic

From the Department of Biochemistry, St. Mary's Hospital Medical School, Paddington, London, W.2, England.

* On leave from the New Zealand Medical Research Council, Pathology Research Group, Pathology Department, University of Otago Medical School, Dunedin, N.Z.

acid, m.p. 200°, *p*-hydroxybenzoic acid, m.p. 213–214°, anisic acid, m.p. 184° and benzoic acid, m.p. 120° (British Drug Houses Ltd., Poole) were purchased and purified. Isovanillic acid, m.p. 250°, was prepared according to Perkin & Stoyle (1923) and we are grateful to Dr. L. Reio (Wenner-Gren Institute, Stockholm), for a sample of 3,4-dihydroxyhippuric acid, m.p. 221°. The methyl esters of protocatechuic (m.p. 134·5°), vanillic (m.p. 62–63°), *m*-hydroxybenzoic (m.p. 69°) and *p*-hydroxybenzoic (m.p. 131°) acids were prepared with diazomethane in dry ether. The benzylamine salt of *m*-methoxybenzoic acid (m.p. 112–113°, from ethyl acetate) was also prepared. All these known compounds were purified, and checked for purity by paper chromatography after recrystallization from suitable solvents to constant m.p., which agreed with values given in the literature. Barium [^{14}C]carbonate, specific activity 23·6 mc/mmole, was purchased (Radiochemical Centre, Amersham).

[*carboxy*-^{14}C]Protocatechuic acid was prepared from 3,4-dibenzyloxybromobenzene and $^{14}CO_2$ *via* 3,4-dibenzyloxy[*carboxy*-^{14}C]benzoic acid according to Neish (1959a,b). It had m.p. 200° after recrystallization from water, and specific activity, 1·4 mc/g. The radiochemical yield was 70% based on the $Ba^{14}CO_3$ used.

ANIMALS, DIET AND DOSAGE

Wistar albino rats (females 160–200 g) were maintained throughout on a standard diet (No. 41B of Bruce & Parkes, 1956) and water *ad lib.* During experiments, the animals were kept singly in Perspex cages designed to permit separate collection of urine and faeces. Food was not allowed during the first 12 hr after dosing. The [^{14}C]protocatechuic acid (100 mg/kg) was administered by stomach tube in water whilst the animals were under light ether anaesthesia. Urine and faeces were collected daily and kept at −10° until used. Except in some experiments, urine was collected in the presence of 0·1 ml of 1% aqueous $HgCl_2$ solution.

To reduce gut flora, the rats were given an oral dose of neomycin sulphate (25 mg in 1 ml sterile solution) daily for three days before the administration of [^{14}C]protocatechuic acid on the fourth day.

Biliary cannulation of rats was as described by Abou-El-Makarem, & others (1967). [^{14}C]Protocatechuic acid (100 mg/kg in 1 ml water) was injected intraperitoneally and bile collected for 24 hr. Urine and faeces were also collected and, with the bile, kept at −10°.

EXAMINATION OF EXCRETA

Urine was adjusted to pH 2·0–2·4 with 2N HCl and continuously extracted for 3 hr with peroxide-free ether. The residual urine was freed of ether, treated with an equal volume of 10N HCl and refluxed for 2 hr on an air-bath at 130–140° to hydrolyse conjugates. The hydrolysed urine was extracted with ether as before. Both ether extracts were reduced to 5 ml *in vacuo* for paper chromatographic examination.

The 24 hr biles (pH 8·4) were treated in the same way as urine, but the ether extracts were taken to dryness *in vacuo* and the residue dissolved in ethanol (2–3 ml) for chromatography.

EXTENT OF DEMETHYLATION BY 5N HCl OF METHOXY ACIDS

A solution of vanillic acid (0·25 g) in 5N HCl (50 ml) was boiled under reflux for 2 hr. The cooled solution was continuously extracted with ether for 3 hr and then the dried (anhydr. Na_2SO_4) extract evaporated. The crystalline residue showed a methoxyl value of 16·8% whilst that required for vanillic acid, $C_8H_8O_4$, is 18·5%. Chromatography of this residue in solvent A (Table 1) showed the presence of protocatechuic acid. From the methoxyl value, it can be calculated that 8·8% of the vanillic acid was demethylated under the above conditions. *m*-Methoxybenzoic acid was treated similarly, and demethylation to *m*-hydroxybenzoic acid, which was detected chromatographically, occurred to the extent of 7·9%. (Found on residue: OMe, 18·8%. Calc. for methoxybenzoic acid, $C_8H_8O_3$, OMe 20·4%.)

CHROMATOGRAPHY

The Rf values and colour reactions of the compounds are shown in Table 1.

TABLE 1. Rf VALUES AND COLOUR REACTIONS OF PROTOCATECHUIC ACID AND RELATED COMPOUNDS. Descending chromatography on Whatman No. 1 paper was used. Solvents: A, chloroform–acetic acid–water, 2 : 1 : 1 by vol.; B, isopropanol–ammonia (sp.gr. 0·88)–water, 8 : 1 : 1 by vol. upper phase. Sprays used were Gibbs reagent, diazotized sulphanilic acid (DSA) (Elliott, Parke & Williams, 1959), ammoniacal $AgNO_3$ (Bray & Thorpe, 1954), methyl red and bromophenol blue for the detection of acids and naphthoresorcinol for glucuronides. Fluorescent compounds were detected with a Hanovia Chromatolite lamp (254 mμ)

Compound	Rf value in solvent × 100		Colour reactions			
	A	B	Gibbs	DSA	$AgNO_3$	Fluorescence
3,4-Dihydroxybenzoic acid (protocatechuic)	15–20	5–10	pale blue	pink	black	purple
4-Hydroxy-3-methoxybenzoic acid (vanillic)	75–80	20	blue	orange	none	purple
3-Hydroxy-4-methoxybenzoic acid (isovanillic)	70	30	blue	orange	none	purple
4-Hydroxybenzoic acid	45–50	20–25	blue	yellow	none	quenches ultraviolet
3-Hydroxybenzoic acid	45–50	35–40	blue	yellow	none	purple
3-Methoxybenzoic acid*	90	45	none	none	none	pale purple
4-Methoxybenzoic acid* (anisic)	90–100	50	none	none	none	purple
3,4-Dimethoxybenzoic acid* (veratric)	95–100	—	none	none	none	—
3,4-Dihydroxyhippuric acid†	60–65	55–60	pale blue	—	black	pale purple
Benzoic acid*	90–100	50	none	none	none	pale purple
Catechol	60	75–80	pale blue	purple	black	dark purple

* Detected with indicators as acid spots.
† Gives pale orange-yellow colour with Altman's reagent (Gaffney & others, 1954).

MEASUREMENT OF RADIOACTIVITY

Radiochromatogram strips (1 inch) were passed manually through a Panax lead castle containing an end window Geiger-counter tube (Type MX 123) with a background of 9–15 counts/min. Radioactivity on the strip was counted every cm for times appropriate to the activity encountered.

Liquid or solid samples were counted at "infinite thickness" on nickel-plated iron planchets (2·5 cm) using an end window Geiger-tube (background 9–17 counts/min) coupled to a Panax scaler (Type D657). For samples of low activity, a Panax anti-coincidence unit (Type AU460) coupled to a Panax Dekatron Scaler (Type SA102) was used (background 2·1–3·5 counts/min). Bile and urine were counted as such or after evaporation on the planchet under an infrared lamp. Faeces were counted after homogenizing with suitable volumes of water. Sufficient counts were taken to give a standard error of $\pm 2\%$. Specific activities were determined by comparison with a stable poly[^{14}C-methyl]methacrylate reference of specific activity 1 μc/g in a 2·5 cm diameter planchet (Radiochemical Centre, Amersham).

ISOTOPE DILUTION PROCEDURES

The urine collected over 7 days from a rat was brought to pH 2·0–2·5 with 2N HCl and divided into 5 equal volumes (about 20 ml). To each aliquot there was added 0·25 g of carrier (protocatechuic, vanillic, *m*-hydroxybenzoic, *p*-hydroxybenzoic or *m*-methoxybenzoic acid) and the solution was warmed to dissolve it. Each urine was then continuously extracted for 3 hr with ether and the extract dried (anhydr. Na_2SO_4) and evaporated to dryness and the crystalline residue dissolved in acetone (2 ml).

The residual urine from the above extraction was freed of ether *in vacuo*. and 0·25 g of the same carrier and an equal volume of 10N HCl added. The whole was then boiled under reflux (air-bath at 130–140°) for 2 hr to hydrolyse the conjugates. Ether extraction and evaporation to dryness was repeated as above and the residue taken up in acetone (2 ml).

Each acetone solution was applied as a band on several paper sheets (Whatman 3MM, 18·25 by 22·5 inches) and each sheet developed (descending) with solvent system A (Table 1). Separated bands were located by ultraviolet light, cut out and extracted in a Soxhlet apparatus with ether. The extract was dried (anhydr. Na_2SO_4), the solvent removed and, if necessary, the residue dissolved in acetone (2 ml) for rechromatographing. Each compound was examined as follows.

Protocatechuic acid. Usually the residue did not need further chromatography and it was recrystallized to constant activity from water, then converted to the methyl ester (m.p. 134°) and again recrystallized from water to constant activity.

m- *and* p-*Hydroxybenzoic acids.* These acids were not separated from each other by solvent A. The residue containing them was rechromatographed on Whatman 3MM paper using solvent B (Table 1). The acids were recrystallized from water, and further chromatographed in solvent B, if necessary, to constant specific activity. They were then converted into their methyl esters, methyl *m*-hydroxybenzoate, m.p. 69°, being recrystallized to constant activity from benzene and methyl *p*-hydroxybenzoate, m.p. 131°, from aqueous ethanol.

m-*Methoxybenzoic and vanillic acids.* These were not separated by solvent A, so the band containing them was extracted as before and the

residue chromatographed in solvent B. The chromatography in solvent B was repeated. Vanillic acid was converted into the methyl ester, m.p. 62°, (from aqueous ethanol) and *m*-methoxybenzoic acid into its benzylamine salt, m.p. 112–113° (from ethyl acetate). These were recrystallized to constant activity.

3,4-*Dihydroxyhippuric acid.* An approximate estimate of the amount of this conjugate (Rf 0·55–0·6) was obtained from a radiochromatogram strip developed in solvent system B and counted as above. The authentic compound was not available in sufficient amounts for isotope dilution.

Incubation of protocatechuic acid with gut contents. The contents of the intestinal canal from the duodenum to the anus of two rats were washed into 25 ml of sterile isotonic saline. After mixing, the suspension was divided into two halves and 200 mg of [^{14}C]protocatechuic acid in 2 ml water (specific activity, 7·8 μc/ml) were added to each in sterile flasks. Each flask was connected by a short glass tube to another flask containing N $Ba(OH)_2$, flushed with N_2, and incubated under sterile anaerobic conditions at 37° for 6 or 20 hr. The $BaCO_3$ formed was collected and counted. The incubation mixtures were brought to pH 2·5 with 2N HCl and extracted continuously with ether for 4 hr. The ether was removed and the residue taken up in acetone for paper chromatography in solvent A initially, and then in solvent B (see Table 1) to separate *m*- and *p*-hydroxybenzoic acid. The amounts of the metabolites were estimated from chromatogram strips.

Results

Table 2 shows the distribution of ^{14}C in the excreta of normal and neomycin-treated rats following oral doses of [*carboxy*-^{14}C]protocatechuic acid. In the normal rats some 60–70% of the dose is excreted in the urine and about 8% in the faeces in 7 days. Most of the radioactivity in the urine is, in fact, excreted in the first 12 hr. In the neomycin-treated rats, however, the excretion of ^{14}C is almost quantitative, 87% appearing in the urine and 12% in the faeces.

TABLE 2. EXCRETION OF ^{14}C BY RATS RECEIVING [^{14}C]PROTOCATECHUIC ACID AND THE EFFECT OF PRETREATMENT WITH NEOMYCIN. Female rats (160–210 g) were dosed orally with [^{14}C]protocatechuic acid (0·1 g/kg; 14 μc/kg). The neomycin-treated rats received orally 25 mg of neomycin sulphate daily for 3 days before the protocatechuic acid dose. The results, in % of dose of ^{14}C, are given as averages with ranges in parentheses.

		% of dose excreted by		
		Normal rats		Pretreated rats
Days		Group 1 (6 animals/group)	Group 2 (3 animals/group)	(3 animals/group)
In urine	0–0·5 ..	61 (54–72)	—	60 (52–64)
	0·5–1 ..	4 (0·3–6·0)	—	23 (17–29)
	0 – 1 ..	—	58 (54–65)	—
	2 – 7 ..	6 (3–12)	6 (4·7–7·1)	4 (3·3–4·1)
	0 – 7 ..	71	64	87
In faeces	0 – 7 ..	—*	7·8 (7·6–7·9)	12 (11·3–12)
Total	0 – 7 ..	71 (67–79)	72 (69–77)	99 (96–100)

* Faeces were not examined in this group.

TABLE 3. METABOLITES IN THE URINE OF RATS RECEIVING [^{14}C]PROTOCATECHUIC ACID BEFORE AND AFTER TREATMENT WITH NEOMYCIN. Animals and dosage are similar to those given in Table 2. Average values for 3 rats are given.

Labelled metabolite	% of dose excreted in 24 hr by					
	Normal rats			Pretreated rats		
	Free	Conjugated	Total	Free	Conjugated	Total
3,4-Dihydroxybenzoic acid	21 (18–23)	15 (13–16)*	36	31 (29–32)	24 (21–25)*	55
4-Hydroxy-3-methoxybenzoic acid†	3·7 (2·2–4·3)	17 (15–17)	21	3·6 (3·0–3·7)	18 (17–20)	22
3-Hydroxybenzoic acid ..	1·2 (0·8–1·9)	0·9 (0·6–1·4)	2·1	0·05 (0·01–0·11)	0·04 (0·0–0·1)	0·09
4-Hydroxybenzoic acid ..	0·7 (0·4–1·0)	0·5 (0·3–0·8)	1·2	0·04 (0·0–0·11)	0·04 (0·01–0·07)	0·08
3-Methoxybenzoic acid†	2·0 (1·3–2·8)	0	2·0	trace (0·0–0·1)	0	trace
Sum of above	28·6	33·4	62	34·7	42·1	77

* One of the conjugates, 3,4-hydroxyhippuric acid, amounted roughly to 7·6% of the dose in the normal rats and 8% in the pretreated rats, the estimate being made by counting radiochromatograms.
† The figures for the methoxy acids are corrected for the small demethylation which occurs during acid hydrolysis (see text).

The quantitative aspects of the excretion, in the first 24 hr after dosing, of the various urinary metabolites of protocatechuic acid are shown in Table 3. The five compounds and their conjugates in this Table account for nearly all the radioactivity excreted in the urine in both normal and pretreated rats. The main urinary metabolites in the normal rats are protocatechuic acid (36% of dose) and vanillic acid (21%) including their conjugates, but there are also three minor metabolites, namely, 3-hydroxybenzoic acid (2·1%), 4-hydroxybenzoic acid (1·2%) and 3-methoxybenzoic acid (2%). One of the conjugates of protocatechuic acid appeared to be 3,4-dihydroxyhippuric acid, which was identified by its Rf value, colour reactions and its radioactivity, and it amounted to about 7·6% of the dose. No radioactive isovanillic, veratric, anisic or benzoic acids were detected. In the neomycin-pretreated rats, the main metabolites are protocatechuic acid (55%) and vanillic acid (22%) including their conjugates, but the other three metabolites have virtually disappeared. 3,4-Dihydroxyhippuric acid, however, was still present to the extent of about 8% of the dose. It is to be noted that in the normal rats, the radioactive metabolites accounted for 62% of the dose in 24 hr whereas in the treated rats they accounted for a larger amount, namely 77%.

On incubating [^{14}C]protocatechuic acid with rat gut contents, small amounts of radioactive *m*- and *p*-hydroxybenzoic acids were formed and these were detected chromatographically. Radioactive CO_2 was also detected and the amount formed suggested that the main reaction in the incubate was decarboxylation. The amounts of each metabolite found after incubation for 6 and 12 hr were CO_2, 7·9 and 12·4%, *m*-hydroxybenzoic acid, 0·8 and 1·6%, *p*-hydroxybenzoic acid, 0·3 and 0·7%, and unchanged protocatechuic acid, 90 and 83·3%, respectively, of the protocatechuic acid added initially. Scheline (1966) has reported that both protocatechuic acid and *p*-hydroxybenzoic acid, but not *m*-hydroxybenzoic

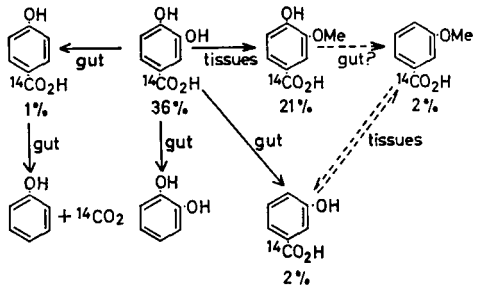
acid, are decarboxylated when incubated with rat caecal contents, the first to catechol and the second to phenol.

[^{14}C]Protocatechuic acid (100 mg/kg; 1·4 μc of ^{14}C/rat) in 1 ml water was injected intraperitoneally into biliary cannulated female rats (200 g each) and the bile collected for 24 hr. The average bile volume was 12·5 ml (range 12·0–13·0 ml in 4 rats) and the average biliary excretion of ^{14}C was 13% (range 11·6–14·4) of the dose. On chromatographing the bile in solvent A (Table 1) a spot (Rf 0·1) was found reacting positively in the naphthoresorcinol test for glucuronic acid, but the bile was non-reducing. Ether extraction of the bile brought to pH 2, gave an extract containing 28·5% of the ^{14}C in the original bile. Chromatography of this extract (solvent A) gave two radioactive spots which were not identified with any of the reference compounds (Table 1). The residual bile was made 5N with respect to HCl and the mixture heated under reflux for 2 hr. The hydrolysed bile was extracted with ether, the ether removed and the residue chromatographed in solvent A. The residue was largely vanillic acid (identified by Rf and colour reactions) with a small amount of protocatechuic acid (probably an artifact produced by demethylation of vanillic acid by HCl). These observations suggest that the main biliary metabolite of protocatechuic acid was a non-reducing glucuronide of vanillic acid, probably 4-carboxy-2-methoxyphenylglucuronide (Sammons & Williams, 1941), which accounted for about 70% of the biliary ^{14}C. *m*-Methoxybenzoic and *m*- and *p*-hydroxybenzoic acids were not detected in the bile.

Discussion

The results show that in the rat protocatechuic acid undergoes methylation of the 3-hydroxy group and dehydroxylation at positions 3- or 4-, although dehydroxylation does not occur simultaneously in both positions since radioactive benzoic acid was not detected as a metabolite. Methylation is a relatively major reaction occurring to the extent of about 20% of the dose (Table 3) whereas dehydroxylation is relatively minor, occurring to the extent of about 5% (sum of 3- and 4-hydroxy- and 3-methoxybenzoic acids in Table 3).

Omitting conjugation reactions with glucuronic acid, sulphate and glycine, the metabolic reactions of protocatechuic acid in the rat appear to be the following, the percentages given under the formulae being the amounts found in the urine 24 hr after dosing:



Both the tissues and gut flora appear to play a role in the metabolism of protocatechuic acid. The methylation of protocatechuic acid to vanillic acid is an example of a well-known metabolic reaction of catecholamines and catechol acids in animal tissues (e.g. Axelrod & Tomchick, 1958). It is a reaction of the tissues and not gut flora, for the extent of methylation is approximately the same in normal and in neomycin-treated rats (Table 3), and the main biliary metabolite of protocatechuic acid is a vanillic acid glucuronide. Isovanillic acid was not detected in the urine and it appears that methylation occurs exclusively at the 3-OH group.

Dehydroxylation appears to be carried out by the gut flora, since the *m*- and *p*-hydroxybenzoic acids and *m*-methoxybenzoic acid were almost absent from the urine of neomycin-treated rats in which the gut flora had been reduced. When the neomycin-treated rats were kept for several weeks on a normal diet, the ability to produce these three compounds from protocatechuic acid was restored. Dehydroxylation also occurred when protocatechuic acid was incubated with rat intestinal contents.

The presence of *m*-methoxybenzoic acid as a minor metabolite of protocatechuic acid raises the problem of whether it is formed by the methylation of *m*-hydroxybenzoic acid or the dehydroxylation of vanillic acid. Its formation is reduced in neomycin-treated rats and it is not formed when protocatechuic acid is incubated with rat intestinal contents. If dehydroxylation is carried out by the gut flora, then its absence in the neomycin-treated rats could be due either to lack of formation of *m*-hydroxybenzoic acid from protocatechuic acid (if *m*-hydroxybenzoic acid were formed in the gut, it could be absorbed and then methylated in the tissues, and, in fact, Tompsett, 1960, has claimed that this acid undergoes some methylation in man) or to the inability to dehydroxylate vanillic acid which is formed in the tissues and then partly excreted into the intestine through the bile.

Table 3 shows that the output of total protocatechuic acid in the urine is much greater in neomycin-treated rats (55%) than in normal rats (36%). This could be due to another reaction carried out by gut bacteria, namely, aromatic decarboxylation. In untreated rats, some 20–30% of the ^{14}C of the [*carboxy*-^{14}C]protocatechuic acid is unaccounted for. At least part of this ^{14}C can probably be accounted for as CO_2 lost by decarboxylation by the gut flora. Incubation of [^{14}C]protocatechuic acid with gut contents results in the formation of $^{14}CO_2$, and Booth & Williams (1963) and Scheline (1966) have shown that this acid is converted extensively to catechol by rat faecal and caecal extracts. Some of the *p*-hydroxybenzoic acid formed from protocatechuic acid may be decarboxylated in the gut to phenol. Scheline (1966) has shown that *p*- but not *m*-hydroxybenzoic acid is decarboxylated by rat caecal contents. This type of observation could account for the fact that the amount of *m*-hydroxybenzoic acid (2%, or 4% if *m*-methoxybenzoic acid is included, see Table 3) is greater than that of *p*-hydroxybenzoic acid (1%), if the assumption is made that the *m*- and *p*-OH groups of protocatechuic acid are equally susceptible to microbial dehydroxylation.

References

Abou-El-Makarem, M. M., Millburn, P., Smith, R. L. & Williams, R. T. (1967). *Biochem. J.*, **105**, 1269–1274.
Axelrod, J. & Tomchick, R. (1963). *J. biol. Chem.*, **233**, 702–705.
Booth, A. N., Murray, C. W., DeEds, F. & Jones, F. T. (1955). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **14**, 321.
Booth, A. N., Murray, C. W., Jones, F. T. & DeEds, F. (1956). *J. biol. Chem.*, **233**, 251–257.
Booth, A. N. & Williams, R. T. (1963). *Nature, Lond.*, **198**, 684–685.
Bray, H. G. & Thorpe, W. V. (1954). *Meth. biochem. Analysis*, **1**, 27–52.
Bruce, H. M. & Parkes, A. S. (1956). *J. Anim. Techns Ass.*, **7**, 54–56.
Dacre, J. C. & Williams R. T. (1962). *Biochem. J.*, **84**, 81P.
DeEds, F., Booth, A. N. & Jones, F. T. (1955). *Fedn Proc. Fedn Am Socs exp. Biol.*, **14**, 332.
DeEds, F., Booth, A. N. & Jones, F. T. (1957). *J. biol. Chem.*, **225**, 615–621.
Dodgson, K. S. & Williams, R. T. (1949). *Biochem. J.*, **45**, 381–386.
Elliott, T. H., Parke, D. V. & Williams, R. T. (1959). *Ibid.*, **72**, 193–200.
Gaffney, G. W., Schreier, K., DiFerrante, N. & Altman, K. I. (1954). *J. biol. Chem.*, **206**, 695–698.
Neish, A. C. (1959a). *Can. J. Biochem. Physiol.*, **37**, 1431–1438.
Neish, A. C. (1959b). *Ibid.*, **37**, 1439–1446.
Perkin, W. H. Jun. & Stoyle, F. W. (1923). *J. chem. Soc.*, **123**, 3171–3176.
Sammons, H. G. & Williams, R. T. (1941). *Biochem. J.*, **35**, 1175–1189.
Scheline, R. R. (1966). *J. Pharm. Pharmac.*, **18**, 664–669.
Scheline, R. R., Williams, R. T. & Witt, J. G. (1960). *Nature, Lond.*, **188**, 849–850.
Shaw, K. N. F., Gutenstein, M. & Jepson, J. B. (1961). *Proc. Intern. Congr. Biochem. 5th, Moscow*, **9**, 427.
Tompsett, S. L. (1959). *J. Pharm. Pharmac.*, **11**, 32–35.
Tompsett, S. L. (1960). *Ibid.*, **12**, 62–64.