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The role of the tissues and gut flora in the metabolism of [^{14}C]homoprotocatechuic acid in the rat and rabbit

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The fate of orally administered [*carboxy*-^{14}C]3,4-dihydroxyphenylacetic (homoprotocatechuic) acid has been studied in normal and neomycin-treated rats and rabbits. In normal rats about 93% of the dose (0·1 g/kg) is excreted in 13 days after dosing, although 80% is excreted in the urine within 2 days. In neomycin-treated rats 100% of the ^{14}C is excreted in 13 days, 95% being in the urine within 2 days. The urinary metabolites in 44 hr in normal rats were unchanged homoprotocatechuic acid (55% of the dose), homovanillic acid (19%), *m*-hydroxyphenylacetic acid (6·5%) and *p*-hydroxyphenylacetic acid (1·4%). In neomycin-treated rats, homoprotocatechuic acid (70%) and homovanillic acid (22%) were found but the other two metabolites were virtually absent. In normal and neomycin-treated rabbits, the ^{14}C was excreted in the urine almost quantitatively in 9 days. The same metabolites as in rats were excreted, but there was more *m*-hydroxybenzoic acid (14%) and less homovanillic acid (6%). In neomycin-treated rabbits the excretion of *m*- and *p*-hydroxyphenylacetic acids was suppressed. In the rat, the metabolism of homoprotocatechuic acid, as far as methylation, dehydroxylation and decarboxylation (Scheline, 1967) is concerned, is qualitatively similar to that of protocatechuic acid (Dacre & Williams, 1968). In the rabbit, however, there is little, if any, decarboxylation. Whilst methylation is a reaction of the tissues, dehydroxylation and decarboxylation appear to be carried out by gut micro-organisms.

THE compound 3,4-dihydroxybenzoic (protocatechuic) acid has been shown by Dacre & Williams (1968) to be methylated, dehydroxylated and decarboxylated in the rat. Furthermore, these authors found that, whereas methylation was a reaction of the tissues of the animal, dehydroxylation and decarboxylation was probably carried out by the gut flora. The homologous 3,4-dihydroxyphenylacetic acid has now been examined similarly in rats and it also appears to be methylated, dehydroxylated and decarboxylated. [^{14}C]Homoprotocatechuic acid has also been studied in rabbits but in this species, although methylation and dehydroxylation occurs, there did not appear to be appreciable decarboxylation.

Experimental

MATERIALS

3,4-Dihydroxyphenylacetic (homoprotocatechuic) acid, m.p. 128–129°, 4-hydroxyphenylacetic acid, m.p. 148–150°, 3-methoxyphenylacetic acid, m.p. 67°, and 4-methoxyphenylacetic acid, m.p. 85–87°, were purchased (Koch-Light Laboratories Ltd., Colnbrook) and purified. 3,4-Dimethoxyphenylacetic (homoveratric) acid, m.p. 93–94° (see Blatt, 1943), 4-hydroxy-3-methoxyphenylacetic (homovanillic) acid, m.p. 141–142·5° (Fisher & Hibbert, 1947), 3-hydroxy-4-methoxyphenylacetic (homoisovanillic) acid, m.p. 127–128° (Grundon & Perry, 1954) and 3-hydroxyphenylacetic acid,

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m.p. 128–129° (Kornfield, 1948) were prepared and purified according to the literature. Some of the above acids were converted into *benzylamine salts* according to Wild (1958) and recrystallized from ethyl acetate: *benzylamine 3-hydroxyphenylacetate*, m.p. 147–149° (Found: C, 69·5; H, 6·5; N, 5·4%) and *4-hydroxyphenylacetate*, m.p. 157–159° (Found: C, 69·9; H, 6·5; N, 5·4%. $C_{15}H_{17}O_3N$ requires C, 69·5; H, 6·6; N, 5·4%); *benzylamine* 3,4-*dihydroxyphenylacetate*, m.p. 164–165° (Found: C, 65·0; H, 6·1; N, 5·2%. $C_{15}H_{17}O_4N$ requires C, 65·4; H, 6·2; N, 5·1%); *benzylamine* 3-*hydroxy*-4-*methoxyphenylacetate*, m.p. 166–168° (Found: C, 66·6; H, 6·5; N, 4·3%) and 4-*hydroxy*-3-*methoxyphenylacetate*, m.p. 138–140° (Found: C, 66·1; H, 6·5; N, 4·8%. $C_{16}H_{19}O_4N$ requires C, 66·4; H, 6·6; N, 4·8%).

[carboxy-^{14}C]*Homoprotocatechuic acid.* 3,4-Dihydroxytoluene (8 g) and *N*-bromosuccinimide (7·8 g) were refluxed for 3 hr in CCl_4 (40 ml) containing benzoyl peroxide (1 mg). After cooling, the solvent was removed *in vacuo* and the residue extracted with peroxide-free ether (3 × 50 ml). The extract was taken to dryness and the crystals (8 g) repeatedly recrystallized from benzene. The 3,4-*dihydroxybenzyl bromide* formed long colourless needles, m.p. 92° (Found: C, 40·9; H, 3·4; Br, 38·9%. $C_7H_7O_2Br$ requires C, 41·4; H, 3·5; Br, 39·4%).

To a solution of the above bromide (8·6 g) in dry acetone (100 ml), dry K_2CO_3 (30 g) and benzoyl chloride (11 ml) were added. The mixture was boiled for 8 hr under reflux, cooled, and then water (150 ml) added. The acetone present was removed *in vacuo*. The residue was extracted with ether (3 × 100 ml) and the extract shaken with N NaOH (3 × 50 ml). The ether extract was dried (anhydr. Na_2SO_4), and the ether removed *in vacuo* leaving colourless crystals (9 g). Repeated recrystallization from absolute ethanol gave needles of 3,4-*dibenzyloxybenzyl bromide*, m.p. 107° (Found: C, 65·6; H, 5·15; Br, 20·9%. $C_{21}H_{19}O_2Br$ requires C, 65·8; H, 5·0; Br, 20·85%).

The above compound was converted into 3,4-dibenzyloxy[*carboxy*-^{14}C]-phenylacetic acid using $^{14}CO_2$ from $Ba^{14}CO_3$ (0·4 g) according to Neish (1959 b). The acid was obtained as a reddish-brown gum which resisted all attempts at crystallization. The removal of the benzyl groups from the above acid (2·5 g) was by catalytic hydrogenolysis as described by Neish (1959 a). [*carboxy*-^{14}C]Homoprotocatechuic acid (0·8 g) was repeatedly recrystallized from dry benzene and had m.p. 127–128° and specific activity 1 mc/g.

Some [^{14}C]homoprotocatechuic acid was made from [^{14}C]homoveratric acid starting with [*carboxy*-^{14}C]glycine using the method quoted by Blatt (1944). In this method, however, the radiochemical yield was only 7%, the main losses occurring at the stage of demethylation of homoveratric acid.

ANIMALS, DIET AND DOSAGE

Wistar albino rats, Chinchilla and New Zealand White rabbits were used. Diet, dosing, collection of excreta etc. were essentially as described by Dacre & Williams (1968).

ANALYTICAL METHODS

Glucuronic acid in urine was determined according to Mead, Smith & Williams (1958). Rf values and colour reactions of the relevant compounds are given in Table 1.

MEASUREMENT OF RADIOACTIVITY

Radiochromatogram strips and liquid and solid samples of excreta were examined according to Dacre & Williams (1968).

REVERSE ISOTOPE DILUTION PROCEDURES

The urine, including cage washings, was divided into four equal portions (20–75 ml) and to the appropriate portion was added 250 mg of homoprotocatechuic acid, *m*- or *p*-hydroxyphenylacetic acid or homovanillic acid. An equal volume of 10N HCl was added to each and the whole boiled under reflux for 3 hr. After cooling, the mixture was continuously extracted with ether, the extract dried over anhydrous Na_2SO_4, filtered and evaporated to a small volume.

Homoprotocatechuic acid. The concentrated extract was applied as a band to Whatman 3MM paper which was irrigated with the filtered lower layer of solvent A (Table 1). The paper was allowed to dry overnight, and the homoprotocatechuic acid located with Brentamine fast blue B salt (Table 1). The appropriate area was cut out and extracted with ether in a Soxhlet. The extract was evaporated, the residue counted and twice rechromatographed. The homoprotocatechuic acid (m.p. 128–130°) was then recrystallized repeatedly from benzene to constant activity and converted to the benzylamine salt (m.p. 164–165°) which was recrystallized from ethyl acetate to constant activity.

m-Hydroxyphenylacetic acid. This was determined similarly. The acid finally counted had m.p. 128–129° and its benzylamine salt, m.p. 146–147°.

Homovanillic acid. This was determined as for homoprotocatechuic acid, the acid finally counted having m.p. 141–142° and its benzylamine salt, m.p. 136·5–137·5°. A separate experiment showed that under the above conditions of acid hydrolysis, homovanillic acid was demethylated to the extent of 2·4%.

p-*Hydroxyphenylacetic acid.* The area containing *m*- and *p*-hydroxyphenylacetic acids was eluted with ether. The eluate was taken to a small volume and rechromatographed on Whatman No. 3MM paper with solvent system B (Table 1) allowing the chromatogram to run for 43 hr. In this way the *m*-acid appeared as a sharp band just ahead of a large *p*-acid band. The latter was cut out, eluted with ether and a concentrate of the eluate again chromatographed with solvent B for 46 hr. This time the *p*-band separated sharply 0·5–1″ behind the *m*-band. The *p*-band was eluted as before, suitably diluted with carrier, and the *p*-hydroxyphenylacetic acid (m.p. 149–150°) was repeatedly recrystallized from benzene and finally converted into its benzylamine salt, m.p. 157·5–158·5°.

TABLE 1. Rf VALUES AND COLOUR REACTIONS OF HOMOPROTOCATECHUIC ACID AND ITS POSSIBLE METABOLITES

Descending chromatography on Whatman No. 1 paper. Solvents: A, chloroform–acetic acid–water (2 : 1 : 1, by vol.); B, n-butanol–ammonia (s.g. 0·9)–water, (8 : 1 : 1, by vol.). Sprays: 2% aqueous Brentamine fast blue B salt (I.C.I. Ltd.) followed by 2% aqueous Na_2CO_3; Gibbs reagent, 0·1% 2,6-dichloroquinonechloroimide in ethanol followed by saturated aqueous $NaHCO_3$; 2% aqueous $KMnO_4$.

Compound*	Rf × 100 in solvent		Colour reactions	
	A	B	Brentamine fast blue B salt	Gibbs reagent
3,4-Dihydroxyphenylacetic (homoprotocatechuic) acid	22	2	pale violet (5 μg) violet (>10 μg)	green brown
4-Hydroxy-3-methoxyphenylacetic (homovanillic) acid	69	13	weak brown (5 μg) brown (>10 μg)	light grey
3-Hydroxy-4-methoxyphenylacetic (homoisovanillic) acid	69	13	intense purple (5 μg)	bright blue
3-Hydroxyphenylacetic acid	47	18	purple brown (5–10 μg) red (25–50 μg)	bright blue
4-Hydroxyphenylacetic acid	47	15	brown	none
3-Methoxyphenylacetic acid	—	39	none	none
4-Methoxyphenylacetic acid	—	37	none	none
3,4-Dimethoxyphenylacetic (homoveratric) acid	—	26	none	none

* All the compounds gave pale green spots, which fade, on a violet background, with 2% $KMnO_4$ solution.

Isotope dilutions were not made for homoisovanillic, *m*- and *p*-methoxyphenylacetic, and homoveratric acids since other methods failed to detect them in the urine (see below).

The mono- and di-methoxyphenylacetic acids. In solvent B (see Table 1) these acids travel on chromatograms ahead of the hydroxy acids. Strip counting of chromatograms on Whatman No. 1 paper with solvent B (Table 1) of extracts of urine of rabbits which had received [^{14}C]homoprotocatechuic acid showed that there was no radioactivity in the areas of the strip corresponding in Rf to the methoxy acids (see Table 1). Of the radioactivity chromatographed on the strips, 81% was associated with homoprotocatechuic acid, 8% with homovanillic acid and 11% with *m*-hydroxyphenylacetic acid, these figures being the average for three strips.

Homoisovanillic acid. Homovanillic and homoisovanillic acids are not separated by solvents A or B (see Table 1), nor are they distinguishable spectroscopically or fluorimetrically. However, they can be distinguished by a colour reaction with Brentamine Fast Blue B salt (Table 1). Homoisovanillic acid in amounts of 5 μg on paper gives an intense purple colour with this reagent; 1 μg gives a distinct purple spot and 0·1 μg a faint spot. Homovanillic acid at 5 μg gives a faint brown spot with the reagent and 10 μg is needed for the spot to be easily recognized. When both acids occur together, the purple colour of homoisovanillic acid always predominates. In numerous paper chromatograms of rabbit urine after dosing with homoprotocatechuic acid, the homovanillic acid spot with the above reagent was never purple. If homoisovanillic acid is a metabolite of homoprotocatechuic acid in rabbit urine, it was calculated that it could not occur in amounts >0·5% of the dose (0·1 g/kg) and escape detection.

Results and discussion

The excretion of ^{14}C by rats and rabbits receiving [*carboxy*-^{14}C]homoprotocatechuic acid is shown in Table 2. In the normal rat some 80% of the ^{14}C dose is excreted in the urine in 44 hr and 93% is accounted for in the urine and faeces 13 days after dosing. After neomycin treatment, the ^{14}C appears to be more rapidly excreted, for 95% appears in the urine in 44 hr and all the ^{14}C can be accounted for in the urine and faeces in 13 days. There is a difference of 7% in the recovery of administered ^{14}C between normal and neomycin-treated rats and this may suggest some loss of ^{14}C as CO_2 due to decarboxylation of homoprotocatechuic acid by gut micro-organisms. That decarboxylation occurs in the rat is supported by the finding of Scheline (1967) that homoprotocatechuic acid gives rise to 4-methylcatechol when incubated with rat faecal extracts. Furthermore, 4-methylcatechol has been detected in the urine of rats given homoprotocatechuic acid orally but not when given intraperitoneally (Scheline, 1967). In the rabbit, however, the recovery of ^{14}C in the urine is almost the same in normal and neomycin-treated animals and possibly little decarboxylation occurs in this species.

TABLE 2. THE EXCRETION OF ^{14}C BY RATS AND RABBITS RECEIVING [^{14}C]HOMOPROTOCATECHUIC ACID ORALLY BEFORE AND AFTER TREATMENT WITH NEOMYCIN
Wistar albino rats (about 210 g) and Chinchilla and New Zealand White rabbits (about 3 kg) were used. The dose of homoprotocatechuic acid was 100 mg/kg and of ^{14}C, 1·4 μc/kg. The neomycin-treated rats and rabbits had received orally 100 mg/kg of neomycin sulphate daily for 3 days before dosing with homoprotocatechuic acid. Average values are quoted with ranges in parentheses

	% of dose excreted in the urine			
	Rats		Rabbits†	
Days after dosing	Normal (3 animals/group)	Neomycin treated (3 animals/group)	Normal (5 animals/group)	Neomycin treated (3 animals/group)
1	76 (73–81)	—	—	—
1·83	80 (77–86)	95 (92–98)	89 (80–100)	95 (93–98)
7	88 (83–92)*	—	—	—
9	—	—	99 (96–101)	100 (99–101)
10	92 (88–95)*	99 (95–101)*	—	—
13	93 (89–96)*	100 (96–102)*	—	—

* Including faeces.
† Only traces of ^{14}C were found in the faeces.

In Table 3, the quantitative aspects of the urinary excretory products of homoprotocatechuic acid 44 hr after dosing, are shown. The main excretory product in both species appears to be unchanged homoprotocatechuic acid, which was not conjugated with glucuronic acid, although conjugation with glycine, which is unlikely, cannot be ruled out. Three other radioactive metabolites were found, namely, the methylated product, homovanillic acid, and two dehydroxylation products, *m*- and *p*-hydroxyphenylacetic acids. In the rat, the amount of homovanillic acid excreted is about the same (about 20% of the dose, or 23–24% of the total ^{14}C in the urine) in normal and neomycin-treated rats. This suggests that methylation is carried out by the tissues and not by the gut flora. About

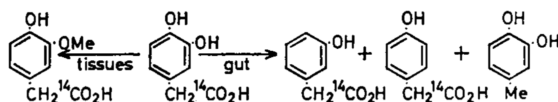
TABLE 3. METABOLITES OF [^{14}C]HOMOPROTOCATECHUIC ACID IN THE URINE OF RATS AND RABBITS BEFORE AND AFTER TREATMENT WITH NEOMYCIN
The dose of [^{14}C] homoprotocatechuic acid as in Table 2. Average values for 3 animals are quoted with ranges in parentheses. Metabolites determined by reverse isotope dilution

	% of dose excreted in the urine in 44 hr (1·83 days)			
	Rat		Rabbit	
Metabolite*	Normal	Neomycin treated	Normal	Neomycin treated
3,4-Dihydroxyphenylacetic acid ..	55 (47–59)	70 (67–73)	63 (58–72)	85 (81–89)
4-Hydroxy-3-methoxyphenylacetic acid	19 (16–24)	22 (20–24)	5·7 (4·6–7·2)	10 (8·1–11·6)
3-Hydroxyphenylacetic acid	6·5 (5·3–8·7)	0·5 (0·0–0·8)	14 (12–18)	0·2 (0·0–0·3)
4-Hydroxyphenylacetic acid	1·4 (1·2–1·6)	0·2 (0·0–0·3)	1·3 (0·0–1·4)	0 (0·0–0·1)
Sum of above metabolites	82 (69–89)	93 (90–98)	84 (79–92)	95 (92–100)
Total ^{14}C in the urine	80 (77–87)	95 (93–98)	85 (80–93)	95 (92–100)

* Determinations of glucuronic acid in the urine showed that there was little or no conjugation of homoprotocatechuic acid or its metabolites with glucuronic acid.

8% of the dose of homoprotocatechuic acid is dehydroxylated in the rat, 6·5% being *p*-dehydroxylation to give *m*-hydroxyphenylacetic acid and 1·4% *m*-dehydroxylation to give *p*-hydroxyphenylacetic acid. Dehydroxylation is suppressed in the neomycin-treated rats indicating that the intestinal flora may be largely responsible as in the case of protocatechuic acid (Dacre & Williams, 1968). In the rabbit, dehydroxylation occurs to the extent of about 15% and this is largely *p*-dehydroxylation (14%). Again dehydroxylation is suppressed by neomycin treatment suggesting that the gut flora are involved. Methylation of homoprotocatechuic acid in the rabbit (5·7%) is much lower than in the rat (19%) and on neomycin treatment there appears to be an increase in methylation (10%) in the rabbit, but whether this increase is significant and related to the suppression of dehydroxylation in this species cannot be assessed without further investigation.

The urinary metabolites of homoprotocatechuic acid in the rat and rabbit may be formed according to the following scheme (approximate % of dose excreted in 44 hr is given under each formula):



rat	19	55	7	1	+
rabbit	6	63	14	1	−?

The metabolism of homoprotocatechuic acid in the rat is similar to that of protocatechuic acid (Dacre & Williams, 1968) except that dehydroxylation is slightly greater, i.e. 8% compared with 3–4%, and decarboxylation probably occurs to a somewhat lesser extent. Methylation of the *m*-hydroxyl group, a reaction of the tissues, occurs to about the same extent (20%) in the rat with both compounds. It thus appears that the gut flora of the rat are able to decarboxylate and dehydroxylate protocatechuic and homoprotocatechuic acid.

In the rabbit, the dehydroxylation of homoprotocatechuic acid occurs to a greater extent than in the rat, i.e. 15% compared with 8%. The difference between the rat and rabbit, however, is in the extent of *p*-dehydroxylation, 14% in the rabbit and 7% in the rat. *m*-Dehydroxylation is about the same (1%) in both species. This species difference might suggest that the two dehydroxylations are carried out by different enzymes, the *p*-dehydroxylating enzyme being more active in rabbit than in rat gut flora. However, methylation is higher in the rat (19%) than in the rabbit (6%) and this might be related to the dehydroxylations. Decarboxylation of homoprotocatechuic acid does not appear to occur in the rabbit to any significant extent.

The nature of the organisms involved in these dehydroxylations is at present unknown, but Perez-Silva, Rodriguez & Perez-Silva (1966) have reported briefly that they have isolated from rat faeces a strain of *Pseudomonas sp.* which converted caffeic acid (3,4-dihydroxycinnamic acid) to *m*-coumaric acid (*m*-hydroxycinnamic acid) and *m*-hydroxyphenylpropionic acid.

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