Biochem. J. (1972) **130**, 141–151
Printed in Great Britain

Metabolism of Myricetin and Related Compounds in the Rat Metabolite Formation *in vivo* and by the Intestinal Microflora *in vitro*

By L. A. GRIFFITHS and G. E. SMITH
Department of Biochemistry, University of Birmingham, Birmingham B15 2TT, U.K.

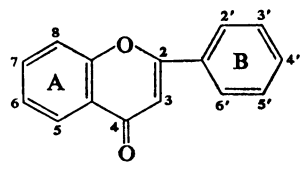
(*Received* 18 *May* 1972)

1. The metabolism of a group of polyphenols related in structure to myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone), including myricetin, myricitrin, 3,4,5-trihydroxyphenylacetic acid, delphinidin, robinetin, tricetin, tricin, malvin and 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone, has been studied both *in vivo* after oral administration to the rat and *in vitro* in cultures of micro-organisms derived from the intestine of the rat. 2. It was shown that the rat intestinal microflora are able to degrade compounds of this group to the ring-fission products observed in urine after oral administration of the specific flavonoid. 3. All flavones and flavonols possessing free 5- and 7-hydroxyl groups in the A ring and a free 4′-hydroxyl group in the B ring gave rise to ring-fission products that included 3′,5′-dihydroxyphenylacyl derivatives. 4. The metabolites 3,5-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,5-dihydroxyphenylpropionic acid and 3-hydroxyphenylpropionic acid were isolated and identified by chromatographic and spectral methods. 5. On anaerobic incubation in a thioglycollate medium it was shown that intestinal micro-organisms can effect cleavage of glycosidic bonds, ring fission of certain flavonoid molecules showing 3′,4′,5′-trihydroxyphenyl substitution and dehydroxylation of certain flavonoid metabolites. 6. The urinary excretion of the metabolites 3,5-dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid was completely abolished when neomycin-treated rats were used.

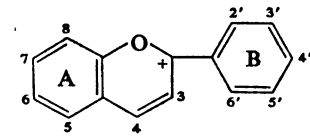
Although polyphenolic compounds showing 3,4,5-trihydroxylation occur less frequently in Nature than the 4-monohydroxyl or 3,4-dihydroxyl derivatives, compounds showing this type of substitution are present in a number of plant materials ingested by mammals. Gallotannins and ellagitannins derived from gallic acid are widely distributed in plants (Roux, 1968). Sinapic acid (4-hydroxy-3,5-dimethoxycinnamic acid) is also of very widespread occurrence in green plants (Bate-Smith, 1954). 3,4,5-Trimethoxycinnamic acid has also been reported as a constituent of certain plants (Corner *et al.*, 1962), and mescaline (3,4,5-trimethoxyphenethylamine) occurs in the fungus *Anhalonium lewinii* (Hefter, 1896).

Flavonoids possessing a 3′,4′,5′-trihydroxylated B ring are known to occur in plants of dietary importance (Harborne, 1967). Glycosides of the flavonol myricetin, for example, have been reported to occur in the grape *Vitis vinifera* (Ribereau-Gayon, 1964) and in tea, *Camellia sinensis* (Roberts, 1962). The flavone tricin occurs in some forage materials, being present in glycosidic form in the leaves of several *Triticum* spp. (Anderson, 1934; Harborne & Hall, 1964*a*) and in the leaves of lucerne, *Medicago sativa* (Bickoff *et al.*, 1964), where it has been reported to be the causative agent of cattle 'bloat' (Ferguson *et al.*, 1950). In addition, the anthocyanidins delphinidin and malvidin, which correspond in B-ring structure to myricetin and tricin respectively, are widely distributed as their glycosides in fruits and vegetables (Harborne, 1967). Thus, delphinidin 3-glucoside occurs in the juice of the 'blood' orange, *Citrus sinensis* (Chandler, 1958), and in the blackcurrant, *Ribes nigrum* (Chandler & Harper, 1962; Harborne & Hall, 1964*b*), whereas both delphinidin and malvidin glycosides occur in the grape (Ribereau-Gayon, 1959, 1964; Singleton & Esau, 1969).

Plant constituents showing 3,4,5-trihydroxylation are thus most commonly of the flavonoid type, but despite intensive investigation of the metabolism of flavonoids possessing a 4′-monohydroxylated or 3′,4′-dihydroxylated B ring the fate of flavonoids showing 3′,4′,5′-trihydroxylation appeared to be largely unknown before our preliminary communication (Smith & Griffiths, 1970), although an investigation by Watanabe & Oshima (1965) had indicated that the trihydroxyphenyl radical of gallocatechin underwent rapid decomposition after ingestion by the rabbit. As it was also known that extensive metabolism of the simpler naturally occurring 3,4,5-trihydroxylated polyphenols, including gallic acid (Booth *et al.*, 1959; Scheline, 1966*a*,*b*; Watanabe & Oshima, 1965) and sinapic acid (Griffiths, 1969, 1970), occurred in



(I)



(II)

the mammal, it was decided to determine whether flavonoids showing similar substitution in the B ring underwent similar metabolic transformations.

The skeletons and numbering systems for flavones and flavonols (I) and flavylium cations (II) are shown above.

Experimental

Animals

Male albino Wistar rats, each weighing approx. 350g, were used. The animals were housed in separate metabolism cages designed for the collection of urine separately from faeces. The animals were fed at 24h intervals but with free access to water.

Diet

The animals were fed on a standard powdered diet (see Griffiths, 1964).

Methods

Administration of compounds to animals. The compounds were administered orally either in admixture with the diet (in the qualitative experiments) or in solution or suspension by stomach tube (in the quantitative studies) to animals that had already been maintained for not less than 2 weeks on this diet.

Collection and extraction of urine and faeces. The urine and faeces were collected for 4 days. Continuous ether extraction of the acidified urine was carried out as described by Griffiths (1964), and after removal of the ether each residue was dissolved in methanol or acetone (1ml). The individual faecal samples (each relating to a 24h period) were freeze-dried and then extracted with five 10ml portions of hot methanol, and the suspensions were filtered. The filtrates were evaporated to dryness and methanol (1ml) was added to each residue. Portions (0.1ml) of the methanolic solutions of the faecal and urine extracts were then submitted to paper chromatography or t.l.c. as described below. In each experiment the urine and/or faeces obtained from a similar number of control animals maintained under the same conditions but not receiving the test substance were submitted to similar examination.

Recovery of metabolites from urine and faeces. The efficiency of the extraction methods used for myricetin and its metabolites, for tricin and for 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone was determined by adding known amounts of each to samples of urine and faeces from control animals. The compounds were then extracted and quantitatively determined by the methods described below. The recovery of phenolic acid metabolites from both urine and faeces was generally greater than 90%. However, for myricetin recoveries were only 40% from urine and 70% from faeces, whereas that of the methoxy-substituted flavonoids from urine was less than 10%.

Stability of test compounds under experimental conditions. The stability of all compounds dosed and of their metabolites during ether extraction from acidified urine or from autoclaved samples of acidified broth was investigated and it was found that myricitrin showed very limited decomposition to myricetin. In addition, 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone gave rise to an artifact with R_F 0.63 in solvent A and R_F 0.80 in solvent B which gave a pink coloration with diazotized sulphanilic acid.

Test compounds added to faeces did not give rise to any artifacts when examined under the conditions of methanolic extraction.

Paper chromatography and t.l.c. Whatman no. 1 paper was used for qualitative paper chromatography, but for the separation of larger amounts of metabolites by band chromatography and in preparative chromatography Whatman 3MM paper was used. R_F values were measured on paper chromatograms after the appropriate solvent had ascended a distance of 28cm from the origin. For t.l.c., plates (20cm×20cm) of silica gel G (E. Merck A.G., Darmstadt, Germany), 250μm thick, were used.

The solvent mixtures employed in paper chromatography were: A, propan-2-ol–aq. NH_3 (sp.gr. 0.88)–water (8:1:1, by vol.); B, benzene–acetic acid–water (6:7:3, by vol.); C, chloroform–acetic acid–water (2:1:1, by vol.); D, aq. 20% (w/v) KCl; E, butan-1-ol–acetic acid–water (4:1:5, by vol.); F, acetic acid–conc. HCl–water (30:3:10, by vol.). The solvent mixtures employed in t.l.c. were: G, toluene–

ethyl formate–formic acid (5:4:1, by vol.); H, benzene–dioxan–acetic acid (90:25:4, by vol.); I, benzene–methanol–acetic acid (45:8:4, by vol.); J, chloroform–ethyl acetate–formic acid (5:4:1, by vol.); K, benzene–pyridine–formic acid (36:9:5, by vol.).

Extracts containing phenolic acid metabolites were submitted to a preliminary screening procedure based on two-dimensional paper chromatography with (1) solvent A followed by solvent B and (2) solvent C followed by solvent D. Subsequent chromatographic examination utilized other paper and t.l.c. systems listed above.

The detection reagents used were: 1, diazotized *p*-nitroaniline (Swain, 1953); 2, diazotized sulphanilic acid (Smith, 1960); 3, $FeCl_3$–$K_3Fe(CN)_6$ (Barton *et al.*, 1952); 4, 5% (w/v) $AlCl_3$ in ethanol (Gage & Wender, 1950); 5, saturated aq. ammonium molybdate (Das & Griffiths, 1966); 6, 3% (w/v) $FeCl_3$ in ethanol; 7, Mäule's reagent (Campbell *et al.*, 1938).

Acid hydrolysis of urine. Conc. HCl (0.25vol.) was added to the urine and the mixture heated at 100°C for 1h.

Spectra. The u.v. spectra of phenolic acids were determined in 0.1M-HCl, in 0.1M-NaOH and in methanol in the presence and in the absence of $AlCl_3$ (Nakagawa *et al.*, 1964) by using a Unicam SP.800 recording spectrophotometer. The spectra of flavonoid compounds were determined in ethanol in the presence and in the absence of $AlCl_3$ (Jurd, 1969), in ethanol in the presence and in the absence of fused sodium acetate (Jurd & Horowitz, 1957) or in ethanol in the presence and in the absence of sodium acetate–boric acid (Jurd, 1956).

Determination of phenolic acids. The phenolic metabolites 3,5-dihydroxyphenylacetic acid and 3,5-dihydroxyphenylpropionic acid were first purified by band chromatography on Whatman 3MM paper in solvent A or by t.l.c. in solvent H, and then determined colorimetrically by using diazotized *p*-nitroaniline (Bray & Thorpe, 1954). 3,4,5-Trihydroxyphenylacetic acid was first purified by band t.l.c. in solvent G and then determined by the Folin–Ciocalteu method (Bray & Thorpe, 1954).

Determination of flavonoids. Myricetin was assayed by a modification of Dowd's (1959) method. The extinction of the $AlCl_3$–myricetin complex was measured at 438nm in a Unicam SP.600 spectrophotometer and the calibration curve for myricetin was linear over the range 0–30μg.

Tricin was determined by means of the yellow-green colour produced when certain flavonoids are treated with aq. NH_3 (Bate-Smith, 1956). After purification by band chromatography, the flavonoid was taken up in ethanol (10ml), and aq. NH_3 (sp.gr. 0.88) (1ml) was added. The colour was measured at 400nm after 5min in a sealed cell in a Unicam SP.600 spectrophotometer. The colour was stable for over 1h, and obeyed the Beer–Lambert Law over the range 0–60μg.

5,7-Dihydroxy-3′,4′,5′-trimethoxyflavone was isolated by t.l.c. in solvent G, and was eluted and determined by Folin's method.

Experiments in vitro. The ability of intestinal micro-organisms to degrade phenolic compounds *in vitro* was studied by incubating test compounds in a thioglycollate broth with mixed inocula of micro-organisms obtained by sterile section of the rat caecum, as described by Griffiths & Smith (1972). Incubations were carried out anaerobically at 37°C, and the metabolites were recovered by continuous ether extraction of the acidified broth. The recovery of test compounds from inoculated thioglycollate broth was approx. 80–90% for 3,5-dihydroxyphenylacetic acid and for 3,4,5-trihydroxyphenylacetic acid and 25% for myricetin.

Materials

All melting points are uncorrected. Elemental analyses were carried out in the Microanalytical Laboratory of the Chemistry Department, University of Birmingham.

3,5-Dimethoxyphenylpropionic acid was prepared by treatment of 3,5-dimethoxycinnamic acid (0.5g) in water (50ml) with 10% sodium amalgam (50g). The sodium amalgam was added slowly to the cooled solution and the reaction was left to proceed for 12h. The aqueous solution was then decanted, acidified with HCl to pH1.0 and then exhaustively extracted with diethyl ether. The extract was evaporated to dryness and the residue recrystallized from benzene–light petroleum (b.p. 40–60°C) as needles, m.p. 61°C, in agreement with Salway (1910).

3,5-Dihydroxycinnamic acid was prepared from 3,5-dimethoxycinnamic acid by demethylation with pyridinium hydrochloride as described by Griffiths (1969).

3,5-Dihydroxyphenylpropionic acid was prepared from 3,5-dimethoxyphenylpropionic acid by the demethylation method of Blakley & Simpson (1964). The product, which was chromatographically identical with the sample prepared by Griffiths (1970), had m.p. 140°C. (Found: C, 59.3; H, 5.6; $C_9H_{10}O_4$ requires C, 59.3; H, 5.5%).

3,4,5-Trihydroxyphenylacetic acid was prepared from 3,4,5-trimethoxyphenylacetic acid (K. & K. Laboratories Inc., Hollywood, Calif., U.S.A.) by the demethylation method of Blakley & Simpson (1964). Repeated recrystallization from ethyl acetate–light petroleum (b.p. 40–60°C) yielded a cream-coloured crystalline solid, m.p. 158°C. (Found: C, 52.0; H, 4.4; $C_8H_8O_5$ requires C, 52.2; H, 4.4%). According to Birkinshaw *et al.* (1951) 3,4,5-trihydroxyphenylacetic acid has m.p. 161°C.

Tricetin was obtained by a modification of the

method of Blakley & Simpson (1964). 5,7-Dihydroxy-3′,4′,5′-trimethoxyflavone (0.5g) was heated gently under reflux with pyridinium chloride (5.0g) for 1h and the residue extracted with aq. 10% (w/v) $NaHCO_3$. After two washings with diethyl ether, the aqueous solution was adjusted to pH2.0 and filtered. The residue obtained was washed with a small amount of water, and was recrystallized with difficulty from aq. ethanol to give a yellow crystalline solid (Found: C, 60.0; H, 3.7; $C_{15}H_{10}O_7$ requires C, 59.6; H, 3.3%) with m.p. 330°C (decomp.), in agreement with that reported by Anderson (1932) and Anderson & Perkin (1932). It showed the same chromatographic and spectral properties as the synthetic tricetin described by Harborne (1967).

3,5-Dihydroxyphenylacetic acid was kindly given by Dr. J. C. Roberts, Department of Chemistry, University of Nottingham, Nottingham, U.K., and by Dr. T. Brunn, University of Trondheim, Trondheim, Norway, myricetin by Dr. H. L. Hergert, Rayonier Inc., Shelton, Wash., U.S.A., and tricin by Dr. E. M. Bickoff, Western Utilization Research and Development Division, U.S. Department of Agriculture, Albany, Calif., U.S.A. Myricitrin and 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone were purchased from Fluka A.G., Buchs, Switzerland, with funds kindly given by Zyma S.A., Nyon, Switzerland.

Other chemicals used were purchased from commercial sources.

Results

Phenolic constituents of urine and faeces

Use of the standard diet resulted in a marked decrease in the urinary excretion of phenolic acids, as reported by Griffiths (1964). No phenolic acids were detected in methanolic extracts of faeces from control animals.

Myricetin (*3,5,7,3′,4′,5′-hexahydroxyflavone*). Ethereal extracts of the urine of six rats that had each received myricetin (200mg) contained two major metabolites, showing chromatographic and spectral characteristics identical with those of 3,5-dihydroxyphenylacetic acid and myricetin respectively (Tables 1–4). In addition, trace amounts of another metabolite, which was identified by its colour reaction with the diazotized sprays and by its chromatographic and spectral properties as 3-hydroxyphenylacetic acid (Tables 1 and 2), were detected. None of these metabolites was detected in urine extracts from a control group of four rats maintained under the same conditions and fed on the standard diet only. On acid hydrolysis of the urine of rats given myricetin no increase in 3,5-dihydroxyphenylacetic acid and myricetin was found, indicating that these compounds are not present as acid-labile conjugates. The excretion of phenolic metabolites in urine and faeces before and after the administration of myricetin was also determined quantitatively (Tables 5 and 6).

To determine whether the metabolism of myricetin is dependent on the activity of the intestinal micro-organisms *in vivo*, the effect of neomycin on myricetin metabolism was studied. Each of two groups of rats, A and B, received a single dose of myricetin (100mg). Animals of group B received neomycin (100mg) orally on each of 4 days preceding and on each day during the period of metabolite collection, whereas group A rats received myricetin only on day 1. Examination of the ethereal extracts of urine by two-dimensional paper chromatography in solvents A and B and by t.l.c. in solvent G revealed that the formation of 3,5-dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid was completely suppressed in the antibiotic-treated animals.

The metabolism of myricetin (10mg) *in vitro* by rat intestinal micro-organisms was investigated, and the metabolites detected on ether extraction of incubation mixtures, subsequently determined colorimetrically, were 3,5-dihydroxyphenylacetic acid (1.58mg), 3-hydroxyphenylacetic acid (trace), 3,4,5-trihydroxyphenylacetic acid (0.62mg) and unchanged myricetin. These results are mean values obtained with five tubes and are uncorrected for percentage recovery.

Myricitrin (*myricetin 3-rhamnoside*). Oral administration of myricitrin to five rats (100mg/rat) resulted in the urinary excretion of 3,5-dihydroxyphenylacetic acid, together with smaller amounts of 3-hydroxyphenylacetic acid and myricetin. The amounts present in urinary and faecal extracts were also determined (Tables 5 and 6).

The formation of 3,5-dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid was completely suppressed by neomycin treatment as before.

Incubation of the glycoside myricitrin (10mg) in a thioglycollate broth with rat intestinal micro-organisms resulted in the formation of 3,5-dihydroxyphenylacetic acid (1.8mg), 3-hydroxyphenylacetic acid (trace), the aglycone myricetin (0.66mg) and 3,4,5-trihydroxyphenylacetic acid (0.56mg). These results are uncorrected mean values obtained with four tubes.

3,4,5-*Trihydroxyphenylacetic acid.* After the oral administration of 3,4,5-trihydroxyphenylacetic acid (200mg) to each of six rats, the presence in urine of metabolites chromatographically and spectrally identical with 3,5-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid and the administered compound was shown (Tables 1 and 2). Another metabolite, S, which appeared as a brown–purple spot on silica gel after spraying with diazotized sulphanilic acid and had R_F 0.51 in solvent G, was also present. This metabolite gave positive reactions with sprays 5 and 6, and after isolation and purification gave 3,4,5-trihydroxyphenylacetic acid as the sole product

Table 1. *R_F values and colour reactions of phenolic acids formed on metabolism of myricetin and related flavonoids*

The solvents and sprays are described in the text. All compounds gave a blue colour with spray 3, whereas only 3,4,5-trihydroxyphenylacetic acid gave orange and grey–black colorations with sprays 5 and 6 respectively.

	R_F values								Colours on paper chromatograms	
Solvent ...	A	B	C	D	G	H	I	J	With diazotized *p*-nitroaniline	With diazotized sulphanilic acid
3,5-Dihydroxyphenylacetic acid	0.23	0.04	0.05	0.72	0.49	0.21	0.24	0.68	Orange–brown	Yellow–orange
3,5-Dihydroxyphenylpropionic acid	0.28	0.06	0.10	0.60	0.55	0.25	0.27	0.72	Orange–brown	Yellow–orange
3-Hydroxyphenylacetic acid	0.39	0.47	0.53	0.77	0.61	0.52	0.62	0.79	Maroon	Yellow
3-Hydroxyphenylpropionic acid	0.44	0.64	0.66	0.75	0.61	0.59	0.66	0.80	Maroon	Yellow
3,4,5-Trihydroxyphenylacetic acid	Decomp.*	0.00	0.00	0.72	0.37	0.13	0.15	0.56	Grey–yellow	Grey–green→ grey–yellow
Metabolite Da	—	—	—	—	0.48	0.25	0.38	0.77	—	Yellow–orange†
Metabolite Db	—	—	—	—	0.42	0.18	0.27	0.25		Yellow–brown†
Metabolite M2	0.40	0.00	—	—	—	—	—	—	Yellow–brown	—
Metabolite M3	0.62	0.00	—	—	—	—	—	—	Yellow–brown	—
Metabolite M4	0.85	0.15	—	—	0.31	—	—	—	Pink–red	—
Syringic acid	0.13	0.76	0.90	0.43st‡	0.61	0.62	0.82	0.85	Blue–yellow	Red

* Decomposition of compound. † Colour reaction on silica-gel plates. ‡ Streaking of spot.

Table 2. *U.v.-absorption maxima of phenolic acids formed on the metabolism of myricetin and related flavonoids*

Experimental details are given in the text.

	$\lambda_{max.}$ (nm)					
Compound	In 0.1 M-HCl	In 0.1 M-NaOH	In methanol	In methanol + $AlCl_3$	In ethanol	In ethanol + H_3BO_3 + sodium acetate
3,5-Dihydroxyphenylacetic acid	275, 280	295.5	277.5, 283	277.5, 283	277.5, 283	277.5, 283
3,5-Dihydroxyphenylpropionic acid	274, 279	294	276, 282	276, 282	276, 282	276, 282
3-Hydroxyphenylacetic acid	272, 278*	239, 291	275.5, 281.5*	275.5, 281.5*	276, 282*	276, 282*
3-Hydroxyphenylpropionic acid	272, 277*	289	274.5, 280*	274.5, 280	275, 280*	275, 280
3,4,5-Trihydroxyphenylacetic acid	222, 270	221, 381†	220, 271.5	235, 271.5	215, 271.5	215, 276

* Shoulder or inflexion. † Probably represents spectral characteristic of decomposition product.

Table 3. *R_F values and colour reactions of myricetin and related flavonoids*

The solvents and sprays are described in the text. Tricin exhibits a bright yellow fluorescence in u.v. light on paper treated with aq. NH_3, and delphinidin and malvin appeared as purple and red–purple spots respectively on paper chromatograms examined in sunlight.

	R_F values						Colours on paper chromatograms			
Solvent ...	E	F	G	I	J	K	Under u.v. light	With spray 4 under u.v. light	With spray 5	With spray 6
Compound										
Delphinidin	0.43	0.33	—	—	—	—	Deep red	—	—	—
5,7-Dihydroxy-3′,4′,5′-trimethoxyflavone	0.85st*	0.86	0.64	0.82	0.91	0.63	Brown	Dull yellow–green	—	—
Malvin	0.28	0.74	—	—	—	—	Cherry red	—	—	—
Myricetin	0.52	0.27	0.57	0.28	0.78	0.14	Golden yellow	Yellow–green	Orange–brown	Grey–black
Myricitrin	0.64	0.72	0.14	0.10	0.13	0.00	Yellow–brown	Deep yellow	Yellow	Grey–black
Robinetin	0.50	0.43	0.35st*	—	—	0.12	Bright yellow	Yellow–green	Orange–brown	Grey
Tricetin	0.65	0.40st*	0.55	0.38	0.74	0.13	Brown	Yellow–green	Yellow–orange	Grey–black
Tricin	0.73	0.73st*	0.62	0.76st*	0.83	0.51	Brown	Yellow–green	—	—

* Streaking of spot.

Table 4. *U.v.-absorption properties of myricetin and related flavonoids*

Experimental details are given in the text.

Compound	$\lambda_{max.}$ in ethanol (nm)		$\Delta\lambda_{max.}$ (nm)		
	Band 1	Band 2	+Sodium acetate Band 2	+$AlCl_3$ Band 1	+H_3BO_3+ sodium acetate Band 1
Myricetin	378	255	15	60	20
Myricitrin	356	257	9	62	24
Robinetin	368, 319	250	—	—	—
Tricetin	358	269, 250	18	38	26
Tricin	355	270.5, 242	5	37	0
5,7-Dihydroxy-3′,4′,5′-trimethoxyflavone	330	272	5	13	0

on demethylation with pyridinium hydrochloride (Blakley & Simpson, 1964). Quantitative determinations of phenolic acid metabolites in urine and faeces before and after the administration of 3,4,5-trihydroxyphenylacetic acid were also made (Tables 5 and 6).

The effect of neomycin on the metabolism of 3,4,5-trihydroxyphenylacetic acid was studied as described for myricetin. The urinary excretion of 3,5-dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid was entirely suppressed by antibiotic treatment. However, metabolite S and unchanged 3,4,5-trihydroxyphenylacetic acid were found in similar amounts in urine extracts from both groups of rats.

Incubation of 3,4,5-trihydroxyphenylacetic acid (10mg) with rat intestinal micro-organisms under anaerobic conditions gave 3,5-dihydroxyphenylacetic acid (4.0mg) and 3-hydroxyphenylacetic acid (trace).

Delphinidin (3,5,7,3′,4′,5′-*hexahydroxyflavylium chloride*). Administration of delphinidin (100mg) to one rat by stomach tube resulted in the urinary excretion of metabolite Da (Table 1), which behaved as a neutral compound on being partitioned between aq. $NaHCO_3$ and diethyl ether, gave negative reactions with sprays 5 and 6 and had $\lambda_{max.}$ at 277 and 283nm in ethanol. Since the delphinidin sample contained small amounts of gallic acid as an impurity, the possibility that metabolite Da was a neutral metabolite of gallic acid, was considered. However, metabolite Da was shown to have different chromatographic properties from resorcinol, a known metabolite of gallic acid (Scheline, 1966*a*). Incubation of delphinidin with intestinal micro-organisms under the standard conditions resulted in the formation of two metabolites, Da and Db (Table 1). Metabolite Db, which was not identified, behaved as an acid on being partitioned between aq. $NaHCO_3$ and diethyl ether.

Robinetin (3,7,3′,4′,5′-*pentahydroxyflavone*). Examination of both urine extracts from robinetin-fed rats (200mg/rat) and ether extracts of robinetin incubation mixtures (10mg/tube) revealed the presence of large amounts of unchanged robinetin. None of the phenolic acids reported as metabolites of myricetin or tricetin was found.

Tricetin (5,7,3′,4′,5′-*pentahydroxyflavone*). Oral administration of tricetin (100mg) to each of four rats resulted in the urinary excretion of unchanged tricetin, accompanied by small amounts of 3,5-dihydroxyphenylpropionic acid. Tricetin on incubation with rat intestinal micro-organisms gave two metabolites, chromatographically and spectrally identical with 3,5-dihydroxyphenylpropionic acid and 3-hydroxyphenylpropionic acid (Tables 1 and 2).

3,5-*Dihydroxyphenylpropionic acid.* On incubation of 3,5-dihydroxyphenylpropionic acid with the microflora the formation of 3-hydroxyphenylpropionic acid was demonstrated (see Tables 1 and 2).

Tricin (5,7,4′-*trihydroxy*-3′,5′-*dimethoxyflavone*). Oral administration of tricin to six rats (100mg/rat) resulted in the urinary excretion of 3,5-dihydroxyphenylpropionic acid (3.1% of dose) and tricin (1.6% of dose). Sinapic acid and dihydrosinapic acid, two compounds that might be expected to arise as a result of heterocyclic ring fission of tricin, were not, however, detected. Further, acid hydrolysis of the urine resulted in no increase in either 3,5-dihydroxyphenylpropionic acid or tricin. Large amounts of tricin, however, were detected in methanolic extracts of faeces on the first and second days after administration of the compound. Incubation of tricin with intestinal micro-organisms under the standard conditions resulted in the formation of small amounts of 3,5-dihydroxyphenylpropionic acid.

Malvin (3,5,7,4′-*tetrahydroxy*-3′,5′-*dimethoxyflavylium chloride* 3,5-*diglucoside*). Administration of malvin (100mg) to one rat by stomach tube resulted

Table 5. *Excretion of metabolites in urine after the oral administration of myricetin, myricitrin and* 3,4,5-*trihydroxyphenylacetic acid in the rat*

Experimental details are given in the text. The results are mean values obtained with five rats, and are not corrected for percentage recovery of metabolites from urine.

Day	Rats	Myricetin administered (mg)	Myricitrin administered (mg)	3,4,5-Trihydroxy-phenylacetic acid administered (mg)	Excretion (mg/24h per rat)		
					3,5-Dihydroxy-phenylacetic acid	Myricetin	3,4,5-Trihydroxy-phenylacetic acid
0	Group A	—	—	—	0	0	0
	Group B	—	—	—	0	0	0
	Group C	—	—	—	0	0	0
1	Group A	100	—	—	0.48	1.39	0
	Group B	—	100	—	0.37	1.46	0
	Group C	—	—	100	0.69	0	0.34
2	Group A	—	—	—	2.22	0.17	0
	Group B	—	—	—	1.42	0.47	0
	Group C	—	—	—	2.75	0	0.13
3	Group A	—	—	—	1.89	0	0
	Group B	—	—	—	1.07	0	0
	Group C	—	—	—	1.32	0	0
4	Group A	—	—	—	1.0	0	0
	Group B	—	—	—	1.65	0	0
	Group C	—	—	—	0.42	0	0

Table 6. *Excretion of metabolites in faeces after the oral administration of myricetin, myricitrin and* 3,4,5-*trihydroxyphenylacetic acid in the rat*

Experimental details are given in the text. The results are mean values obtained with five rats, and are not corrected for percentage recovery of metabolites from faeces. 3,4,5-Trihydroxyphenylacetic acid was not detected as a faecal metabolite.

Day	Rats	Myricetin administered (mg)	Myricitrin administered (mg)	3,4,5-Trihydroxyphenylacetic acid administered (mg)	Excretion (mg/24h per rat)	
					3,5-Dihydroxyphenylacetic acid	Myricetin
0	Group A	—	—	—	0	0
	Group B	—	—	—	0	0
	Group C	—	—	—	0	0
1	Group A	100	—	—	0	0.93
	Group B	—	100	—	0	0
	Group C	—	—	100	0	0
2	Group A	—	—	—	1.05	4.54
	Group B	—	—	—	1.02	0
	Group C	—	—	—	5.0	0
3	Group A	—	—	—	0.13	0
	Group B	—	—	—	0.25	0
	Group C	—	—	—	0.1	0
4	Group A	—	—	—	0	0
	Group B	—	—	—	0	0
	Group C	—	—	—	0	0

in the urinary excretion of several metabolites, one of which (M1) was identical chromatographically with syringic acid (Table 1), which was, however, detected as an impurity in the malvin samples used. The other metabolites M2, M3 and M4, which possessed different chromatographic properties from the known metabolites of syringic acid, behaved as neutral compounds on being partitioned between aq. $NaHCO_3$ and diethyl ether, and reacted with the diazotized sprays (Table 1). On incubation of malvin with gut micro-organisms none of the metabolites found on oral administration of the test compound to the rat was detected, although traces of syringic acid were again found.

5,7 - *Dihydroxy* - 3′,4′,5′ - *trimethoxyflavone*. Four rats were dosed with the compound (100mg/rat) by stomach tube. Examination of the urine collected over 3 days revealed the presence of only two metabolites, namely the administered compound together with traces of 3,5-dihydroxyphenylpropionic acid. However, large amounts of the compound administered were detected in methanolic extracts of faeces in the 2 days after dosing. By the colorimetric method it was shown that 1.67% of the dose was excreted as phenolic compounds in the urine. Chromatographic examination of both untreated ether extracts of the incubation mixture and of their dried residues after application of a demethylation procedure described by Griffiths & Smith (1972) revealed that 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone was not metabolized by the intestinal microflora.

Discussion

The results show that many flavonoid compounds exhibiting 3′,4′,5′-trihydroxylation of the B ring are susceptible to ring fission in the mammal. That similar products are also formed on anaerobic incubation with the intestinal microflora *in vitro* suggests that catabolism *in vivo* could be effected wholly by intestinal micro-organisms, and evidence was obtained that formation of the ring-fission products 3,5-dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid was suppressed by the oral administration of the antibiotic neomycin. By using similar methods it has been shown that the degradation in the mammal of (+)-catechin (Griffiths, 1964; Das & Griffiths, 1968) and of quercetin (Nakagawa *et al.*, 1965) is dependent on the presence of an active intestinal microflora. Although the organisms responsible for these degradative reactions have not yet been isolated from rat intestinal contents, Cheng *et al.* (1969) have reported the isolation from the bovine rumen and identification of bacteria that are capable of degrading rutin to unidentified water-soluble products.

The observations that the C_6–C_2 metabolite 3,5-dihydroxyphenylacetic acid is derived from the flavonols myricetin and myricitrin whereas the C_6–C_3 metabolite 3,5-dihydroxyphenylpropionic acid is derived from the flavones tricetin and tricin are in agreement with the ring-fission mechanisms proposed by Booth *et al.* (1956, 1958*a*) for compounds of the flavonol and flavone type.

Although it is known that monohydroxyphenylpropionic acid metabolites derived from flavonoids possessing a 4′-mono- or 3′,4′-di-hydroxylated B ring (Booth *et al.*, 1956, 1958*b*; Das & Griffiths, 1968; Griffiths & Smith, 1972) are metabolized *in vivo* to the corresponding benzoic acid derivatives, no evidence was found for the formation of either gallic acid or 3,5-dihydroxybenzoic acid from the corresponding phenylpropionic acid compounds, although the former would have been readily detectable by the methods used.

Identification of 3,5-dihydroxyphenylacetic acid and 3,5-dihydroxyphenylpropionic acid as major metabolites indicates that 4-dehydroxylation is of major importance in the metabolism of 3,4,5-trihydroxyphenyl carboxylic acids of the C_6–C_2 and C_6–C_3 types. This contrasts with the reports by Booth *et al.* (1959) and Watanabe & Oshima (1965), who observed that the C_6–C_1 compound gallic acid underwent 4-methylation and decarboxylation but not dehydroxylation. A similar relationship between susceptibility to dehydroxylation and length of the carbon side chain has been noted in the *o*-dihydroxyphenyl carboxylic acid series (DeEds *et al.*, 1957). The detection of 3-hydroxyphenylacetic acid after administration of 3,4,5-trihydroxyphenylacetic acid indicates that dehydroxylation of this acid occurs also in a *meta* position. The *m*-dehydroxylation of 3,5-dihydroxyphenylpropionic acid to 3-hydroxyphenylpropionic acid during anaerobic incubation with the microflora was also noted. The occurrence of *m*-dehydroxylation in the metabolism of homoprotocatechuic acid has been reported by Dacre *et al.* (1968).

Parallel studies on a group of flavonoids showing 4′-monohydroxylation of the B ring (Griffiths & Smith, 1972) showed that the presence of free 5-, 7- and 4′-hydroxyl groups in a flavonoid molecule were essential structural requirements for ring fission to occur *in vivo* or *in vitro*. It is noteworthy that the two compounds in the present study that were resistant to bacterial catabolism were robinetin (3,7,3′,4′,5′-pentahydroxyflavone) and 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone.

Metabolite S, which was shown to arise from 3,4,5-trihydroxyphenylacetic acid after oral administration to the rat, is thought to be 3,4-dihydroxy-5-methoxyphenylacetic acid, as it gave a positive reaction with spray 5, which is known to be specific for compounds containing *o*-dihydroxy groups (Das & Griffiths, 1966), and on dealkylation with pyridinium chloride gave 3,4,5-trihydroxyphenylacetic acid.

The *o*-dihydroxyphenyl carboxylic acids homoprotocatechuic acid and caffeic acid are known to undergo *m*-methylation in the rat (Dacre *et al.*, 1968; DeEds *et al.*, 1957).

This work was supported by a grant from the Agricultural Research Council.

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