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Metabolism of Apigenin and Related Compounds in the Rat

METABOLITE FORMATION *IN VIVO* AND BY THE INTESTINAL MICROFLORA *IN VITRO*

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1. The metabolism of a group of flavonoid compounds related in structure to apigenin (4′,5,7-trihydroxyflavone) and including apigenin, apiin, naringin, phlorrhizin, acacetin, kaempferol, robinin, chrysin, tectochrysin and 4′,7-dihydroxyflavone, was 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. The rat intestinal microflora is capable of effecting degradation of flavonoid compounds to metabolites observed in the urine after oral administration of the specific flavonoid. 3. All compounds 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, which included 4′-hydroxyphenylacyl derivatives. 4. On anaerobic incubation in a thioglycollate medium, intestinal micro-organisms can effect flavonoid-ring fission, cleavage of glycosidic bonds and the reduction of double bonds in the side chains of certain metabolites. 5. Two flavonoids (chrysin and tectochrysin) undergo hydroxylation in the 4′-position *in vivo* but not during incubation with the intestinal microflora *in vitro*. 6. Observations on the metabolism of other compounds substituted in the 4′-position, e.g. epiafzelechin, pelargonin and the isoflavones, genistein, biochanin A, daidzein and formononetin, by the intestinal microflora of the rat are also reported.

Flavonoids possessing a 4′-monohydroxylated B ring are known to be widely distributed in plants of dietary importance (Harborne, 1967). Glycosides of the anthocyanidin pelargonidin have been shown to occur in the fruits of the strawberry *Fragaria vesca*, in various *Rubus* spp. and in the scarlet radish *Raphanus sativus* (Sondheimer & Karash, 1956; Harborne, 1964; Harborne & Hall, 1964), and kaempferol glycosides have been found in the grape *Vitis vinifera* (Ribereau-Gayon, 1964) and in tea-leaf extracts (Roberts, 1962). The flavone glycoside naringin is restricted to citrus fruits, where it contributes to the taste of juice and peel (Horowitz, 1964). The corresponding flavone, apigenin, is of limited dietary importance, its apiosyl glucoside having been found in the leaves of parsley, *Apium petroselinum* L. (Schmidt, 1930). In addition, isoflavones monosubstituted in the 4′-position in the B ring occur widely in forage legumes (Guggolz *et al.*, 1961).

Since previous work on the metabolism in mammals of this group of flavonoids has largely been restricted to naringin and phlorrhizin (Braun *et al.*, 1957; Booth *et al.*, 1958), it was decided to study a number of structurally related flavonoids so that some assessment could be made of the importance of substitution and other structural modifications in determining susceptibility to ring fission. Further, in view of recent reports which have implicated the intestinal microflora not only in the dehydroxylation of flavonoid metabolites, as reported by Booth & Williams (1963*a*,*b*), Nakagawa *et al.* (1965) and Dacre *et al.* (1968), but also in the ring fission of certain flavonoids both *in vivo* (Griffiths, 1964; Das & Griffiths, 1968, 1969; Smith & Griffiths, 1970) and *in vitro* (Scheline, 1968, 1970; Das, 1969; Smith & Griffiths, 1970), studies have been carried out to identify the metabolites of the 4′-hydroxy-substituted flavonoids and also to determine whether the pathways of these compounds *in vivo* are identical with those observed *in vitro* under conditions of anaerobic incubation.

Experimental

Methods

Animals. Male albino Wistar rats, each weighing approx. 350 g, were housed in individual metabolism cages designed to permit the separate collection of urine from faeces. The animals were fed at 24 h intervals, but unrestricted water intake was allowed throughout the experimental period.

Diet. The animals were fed on a standardized powder-type diet described previously (Griffiths, 1964).

Administration of compounds to animals. The compounds were administered orally at the stated doses 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 been previously maintained for a minimum period of 2 weeks on this diet.

Collection and extraction of urine and faeces. The urine and faeces were collected over a period of 4 days. Continuous ether extraction of the acidified urine was carried out as described previously and, after removal of the ether, each residue was dissolved in 1 ml of methanol (Griffiths, 1964). The individual faecal samples (each relating to a 24-h period) were freeze-dried and then extracted with 5 × 10 ml portions of hot methanol, and the suspensions were filtered. The filtrates were evaporated to dryness and methanol (1 ml) was added to each residue. Samples (0.1 ml) of the methanolic solutions of extracts of both urine and faeces were then submitted to paper chromatography or t.l.c. as described below. In each experiment the urine or faeces or both obtained from a similar number of control animals maintained under the same conditions but not receiving the test substance were examined similarly.

Recovery of metabolites from urine and faeces. The efficiency of the standard extraction methods for flavonoid metabolites was determined by adding known amounts of each to samples of control urine and faeces. The compounds were then extracted and quantitatively measured by the methods described below. The recovery of the phenolic acids and kaempferol from urine was 60% or above, but for apigenin, a low recovery from urine (e.g. 20%) was observed. By contrast, the recovery of metabolites from faeces was always greater than 90%.

Stability of test substances under experimental conditions. The stability of all compounds administered, and of metabolites detected under the conditions of ether extraction from acidified urine and from autoclaved samples from acidified broth, was investigated and it was found that only apiin and robinin gave rise to artifacts. Apiin showed limited decomposition to apigenin 7-glucoside (Table 2) and robinin to kaempferol 7-rhamnoside (Table 2). Test compounds were also examined under the conditions of methanolic extraction (as described for the extraction of phenolic compounds from faeces), but they did not give rise to artifacts.

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 3 MM paper was used. R_F values were measured on paper chromatograms after the appropriate solvent had ascended a distance of 28 cm from the origin. For t.l.c., glass plates (20 cm × 20 cm) of silica gel G (E. Merck A.G., Darmstadt, W. Germany), 250 μm thick, were used (Stahl, 1962). The thin layers were dried at room temperature overnight before use. 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, water; G, aq. 15% (v/v) acetic acid; H, hexane–2-methylpropan-2-ol–methanol–water (10:5:5:4, by vol.). The solvent mixtures employed in t.l.c. were: I, benzene–pyridine–formic acid (36:9:5, by vol.); J, benzene–acetic acid–water (2:3:1, by vol.); K, chloroform–acetic acid–water (4:1:1, by vol.); L, benzene–dioxan–acetic acid (90:25:4, by vol.); M, benzene–methanol (19:1, v/v); N, ethyl acetate–cyclohexane (1:1, v/v); O, chloroform–methanol (9:1, v/v).

Extracts containing phenolic acid metabolites were submitted to a preliminary screening procedure based on two-dimensional paper chromatography in solvent A followed by solvent B. Subsequent chromatographic examination utilized other paper and thin-layer systems listed above.

The detecting reagents used were: 1, diazotized *p*-nitroaniline (Swain, 1953); 2, diazotized sulphanilic acid (Smith, 1969); 3, $FeCl_3$–$K_3Fe(CN)_6$ (Barton *et al.*, 1952); 4, ethanolic 5% (w/v) $AlCl_3$ (Gage & Wender, 1950); 5, aq. 5% (w/v) Na_2CO_3 (Harborne, 1959); 6, 1% (w/v) $NaBH_4$ in propan-2-ol (Horowitz, 1957); 7, Folin–Ciocalteu reagent (Lindner, 1967); 8, *p*-dimethylaminobenzaldehyde in acetic anhydride (Altman) reagent (Smith, 1969); 9, benzidine–trichloroacetic acid (Bacon & Edelman, 1951).

Gas–liquid chromatography. A Pye series 104 chromatograph fitted with a dual-head flame ionization detector (W. G. Pye and Co. Ltd., Cambridge, U.K.) was used. Argon was passed through columns (4 mm diam.) packed with acid-washed silanized Supasorb (60–80 mesh) coated with either 5% QFI or 10% SE-30, maintained at 125°C, at a flow rate of 45 ml/min. The reference compounds and microfloral metabolites in methanol were applied directly to the columns.

Acid hydrolysis of urine. Conc. HCl was added to the urine to give a final concentration of 20% (v/v). The mixture was then heated at 100°C for 1 h.

Enzymic hydrolysis of conjugates. After chromatographic purification, the conjugates were submitted to the following procedures.

(*a*) β-Glucuronidase. The conjugate was dissolved in 20 ml of 0.1 M-KH_2PO_4–Na_2HPO_4 buffer (pH 6.8) and divided into two equal portions. To both portions one drop of chloroform was added to inhibit bacterial growth, and to one portion was added 5000 Fishman units (Talalay *et al.*, 1946) of β-glucuronidase (β-D-glucuronide glucuronohydrolase; EC 3.2.1.31; bacterial type 1; Sigma Chemical Co., St. Louis, Mo., U.S.A.). Control and test solutions were incubated for 16 h at 37°C.

(*b*) Arylsulphatase. The conjugate was dissolved in

20ml of 0.2M-sodium acetate buffer (pH5.0) and divided into two equal portions. To one portion was added 50 units of arylsulphatase (arylsulphate sulphohydrolase; EC 3.1.6.1; type III from limpets; Sigma). The two solutions were then incubated for 24h at 37°C.

Spectra. Ultraviolet-absorption spectra were determined with a Unicam SP.800 recording spectrophotometer.

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

Determination of glucuronides. The colorimetric method of Bray *et al.* (1952) was employed.

Determination of ethereal sulphate. Ethereal sulphates in urine were determined by the method of Sperber (1948). Measurements of turbidity were made in a Unicam SP. 600 spectrophotometer at 605 nm.

Determination of phenolic acids. Phenolic acids isolated from urine by continuous ether extraction or from faeces by methanolic extraction were purified by band chromatography. The residue from each eluate was dissolved in water (10ml) and the phenolic acid determined by a modification of the Folin–Ciocalteu method (Griffiths, 1964). Standard curves over the range 0–100μg were prepared for each of the compounds determined. *p*-Hydroxybenzoic acid, which gives a weak colour with the Folin–Ciocalteu reagent, was determined by a method employing diazotized *p*-nitroaniline (Bray & Thorpe, 1954).

Determination of flavonoids. Kaempferol present in extracts of urine and faeces was, after separation and purification by band chromatography, determined as the $AlCl_3$–flavonoid complex by the u.v.-spectrophotometric method of Dowd (1959). Absorption was measured at 428nm, the observed $\lambda_{max.}$ of the kaempferol–$AlCl_3$ complex. The flavone apigenin was determined by a method based on the yellow–green coloration produced when certain flavonoids are treated with NH_3 (Bate-Smith, 1956). After purification by band chromatography, 1ml of aq. NH_3 (sp.gr. 0.88) was added to the flavonoid in 10ml of ethanol. Colour development was measured at the observed $\lambda_{max.}$ (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 0–80μg.

Carbazole reaction. The carbazole reaction (Dische, 1947) was used for the detection of glucuronic acid in conjugated metabolites.

Examination of urine for apiose. The 24h urines of apiin-fed animals and of a control group were freeze-dried and extracted with hot pyridine (5ml). The extracts were filtered and evaporated to dryness. Each extract was then dissolved in 1ml of water and portions (0.1ml) were submitted to chromatography with authentic specimens of apiose on Whatman no. 1 paper in solvent E. The chromatograms were sprayed with benzidine in trichloroacetic acid (reagent 9), when the apiose standards appeared as white fluorescent spots (R_F 0.37). The extraction procedure was shown to be efficient by determining the recovery of added apiose from control urine samples.

Preparation of inocula. The rats were killed by cervical fracture and dissected to expose the alimentary tract. With the use of sterile instruments three incisions were made in the wall of the caecum and two in the lower part of the ileum. A sterile platinum needle was then introduced through the incisions and a small quantity of the caecal and intestinal contents removed. The material withdrawn was suspended in 5ml of sterile 0.9% NaCl. The suspensions were then centrifuged at low speed to remove debris. Portions (0.2ml) of the supernatant were transferred to tubes containing sterile media.

Bacteriological media. Thioglycollate medium was prepared by dissolving granules of Thioglycollate Medium U.S.P. (Oxoid) (29.5g) in 1 litre of 0.1M-KH_2PO_4–Na_2HPO_4 buffer (pH7).

Conditions of incubation. After inoculation or addition of a sterile suspension of the flavonoid under investigation (Table 1) or both, tubes containing

Table 1. *Experiments on microfloral degradation in vitro*

The table shows the distribution of substrate and inocula in tubes of series nos. 1–4. The additions were made to 10ml of thioglycollate medium.

Tube series no.	Substrate* (mg)	Inoculum (ml)	Sterile water (ml)	Sterile 0.9% NaCl (ml)
1	—	0.2	1	—
2	10	0.2	—	—
3	—	—	1	0.2
4	10	—	—	0.2

* Sterile substrate was added as a suspension in 1 ml of water.

thioglycollate medium were placed in B.T.L. anaerobic jars, which were evacuated and filled with N_2. The jars were then incubated at 37°C for 7 days.

Extraction of phenolic metabolites from culture medium. After incubation, the culture medium was made up to 15ml with water, acidified to pH 1–2 and continuously extracted with ether for 12h. Each extract was evaporated to dryness, the residue dissolved in acetone (1ml) and a portion (0.1ml) chromatographed. The recovery was approx. 70% for *p*-hydroxyphenylpropionic acid and other phenolic acid metabolites, but low for flavonoids.

Incubation of flavonoids with microflora. Incubation of specific flavonoids with microfloral inocula was carried out in accordance with the scheme shown in Table 1, which permitted phenolic metabolites arising from the test flavonoid to be distinguished from phenolic constituents of the medium, phenolic metabolites of the basal medium constituents and possible artifacts arising from the flavonoid during heat sterilization.

Materials

4′,7-Dihydroxyflavone was prepared from 7-hydroxy-4′-methoxyflavone by the demethylation procedure of Blakley & Simpson (1964). The product (m.p. 310°C) showed similar spectral properties (Table 5) to those reported for 4′,7-dihydroxyflavone by Mabry *et al.* (1970). Kostanecki & Osius (1899) give m.p. 315°C.

p-Ethylanisole (1-ethyl-4-methoxybenzene) was obtained by the action of dimethyl sulphate on a solution of *p*-ethylphenol in dry acetone in the presence of anhydrous K_2CO_3. The product had b.p. 195°C (see Klages, 1903; Johnson & Hodge, 1913). Apiin, m.p. 227°C, was prepared from fresh parsley leaves by the method of Gupta & Seshadri (1952). Phloretin, m.p. 262°C, and naringenin, m.p. 251°C, were obtained by acid hydrolysis of phlorrhizin and naringin respectively. Kaempferol and (−)-epiafzelechin were kindly given by Dr. J. W. Clark-Lewis, University of Adelaide, Australia, genkwanin by Professor M. Hasegawa Tokyo, Metropolitan University, Tokyo, Japan, equol by Professor W. Klyne, Westfield College, University of London, and genistein, biochanin A, formononetin and daidzein by Dr. E. M. Bickoff, U.S. Dept. of Agriculture, Albany, Calif., U.S.A. Other chemicals used were purchased commercially.

Results

Phenolic constituents of the urine of rats maintained on the standard diet

Chromatographic examination of the ethereal extracts of the urine of six rats maintained on the standard diet over a period of 3 weeks indicated a

Table 2. *R_F values and colour reactions of ring-fission metabolites of flavonoids*

The solvents and sprays are described in the text. All compounds gave a blue colour with $FeCl_3 - K_3Fe(CN)_6$. On exposure to NH_3 vapour, *p*-hydroxycinnamic acid and phloroglucinol gave blue colorations in u.v. light.

	R_F values						Colour on paper chromatograms	
Solvent ...	A	B	C	D	J	K	With diazotized *p*-nitroaniline	With diazotized sulphanilic acid
p-Hydroxybenzoic acid	0.22	0.30	0.41	0.60	0.48	0.49	Red	Yellow
p-Hydroxycinnamic acid	0.33	0.35	0.50	0.35, 0.79	0.48	0.51	Grey–blue	Red
p-Hydroxyphenylacetic acid	0.40	0.26	0.44	0.80	0.39	0.40	Purple	Purple–red
p-Hydroxyphenyl-lactic acid	0.41	0.05	—	—	0.10	0.05	Purple	Red–pink
p-Hydroxyphenylpropionic acid	0.50	0.40	0.61	0.72	0.48	0.50	Purple	Purple–red
Phloroglucinol	0.24	0	0	0.46	—	—	Dark brown	Yellow–brown
Metabolite Ep_2*	0.79	0.05	—	—	—	—	Purple	Red–pink
Metabolite Ep_3	0.79	0.60	—	—	—	—	Purple	Red–pink

* Metabolite detected by two-dimensional chromatography, but absent from chromatograms run in solvent B only.

Table 3. *Ultraviolet-absorption maxima of phenolic acid metabolites*

Experimental details are given in the text.

	Absorption maxima (nm)			
	In 0.1 M-HCl	In 0.1 M-NaOH	In methanol	In methanol+$AlCl_3$
p-Hydroxybenzoic acid	254	281	254	262
p-Hydroxycinnamic acid	310	335	310	321
p-Hydroxyphenylacetic acid	275	294	279	279
p-Hydroxyphenylpropionic acid	276	294	278	278

small phenolic excretion, as reported by Griffiths (1964). Small amounts of *p*-hydroxyphenylacetic acid and trace amounts of *m*-hydroxyhippuric acid, *p*-hydroxybenzoic acid and *p*-hydroxyphenylpropionic acid were detected as reported by Griffiths (1964).

Apigenin (*4',5,7-trihydroxyflavone*). Ethereal extracts of the urine of six rats each given apigenin (200mg) contained large amounts of compounds showing chromatographic and spectral characteristics identical with those of *p*-hydroxyphenylpropionic acid, *p*-hydroxycinnamic acid and *p*-hydroxybenzoic acid (Tables 2 and 3). Three further compounds, Ya, Yb and Ap, which occupied the same area on the two-dimensional chromatograms (R_F in solvent A, 0.12; R_F in solvent B, 0.05) were separated by using other solvent systems (Table 4), and compound Ap had similar spectral and chromatographic properties to apigenin (Tables 4 and 5). Compounds Ya and Yb gave reactions characteristic of flavonoids (Table 4) and had spectra similar to that of apigenin, except that no bathochromic shift in band 2 was obtained with fused sodium acetate (Table 5). Compound Yb gave a strong pink colour with the carbazole reagent and gave apigenin on treatment with β-glucuronidase. Both compounds Ya and Yb gave apigenin on acidic hydrolysis. The excretion of phenolic acid metabolites (Table 6) and of glucuronide and ethereal sulphate (Table 7) before and after oral administration of apigenin was determined.

After incubation of apigenin with intestinal microflora in thioglycollate medium (tube 2, Table 1) together with the appropriate controls (tubes 1, 3 and 4, Table 1), and subsequent extraction of the phenolic metabolites, a metabolite possessing chromatographic and spectral characteristics identical with those of *p*-hydroxyphenylpropionic acid (Tables 2 and 3) was formed in tube 2 extracts only. No flavonoid conjugates were detected, but residual apigenin was detected. Two other phenolic compounds, IF_1 and IF_2, were detected, but since they were present in similar amounts in extracts of the control tubes 1, 3 and 4 it was evident they were not derived from apigenin but were probably from the thioglycollate medium. Similar results were obtained with ten sets of four tubes, with inocula from the intestines of different rats. Formation of *p*-hydroxyphenylpropionic acid was completely suppressed by the addition of aureomycin (2.5mg/tube) before incubation or by heating the inocula at 100°C for 5min.

Apiin (*apigenin 7-apiosylglucoside*). Administration of the glycoside apiin (200mg) orally to each of six rats gave rise to *p*-hydroxyphenylpropionic acid, *p*-hydroxycinnamic acid, *p*-hydroxybenzoic acid, apigenin and the conjugates, compounds Ya and Yb, in the urine. The amount of apigenin and the concentrations of phenolic acids present in urinary extracts were determined (Table 6). The urine samples were also examined for the presence of apiose, the sugar moiety of apiin (see the Experimental section), but this was not detected. After the separate administration of apigenin (100mg) and apiin (100mg), 15.2mg and 3.95mg respectively of apigenin was excreted in the faeces over a period of 3 days. Incubation of apiin (10mg) with intestinal microflora under the standard conditions gave rise to *p*-hydroxyphenylpropionic acid (3.0mg) and apigenin (2.7mg); unchanged apiin was also detected.

Naringin (*4',5,7-trihydroxyflavanone 7-rhamnoglucoside*). Oral administration of naringin (200mg) to each of four rats resulted in the excretion of *p*-hydroxyphenylpropionic acid, *p*-hydroxycinnamic acid, *p*-hydroxybenzoic acid and naringenin in the urine, but incubation of naringin (10mg) with the microflora gave rise to *p*-hydroxyphenylpropionic acid and naringenin only.

Phlorrhizin (*2',4,4',6'-tetrahydroxydihydrochalcone 2'-β-glucoside*). Administration of phlorrhizin (200mg) orally to each of four rats caused the excretion of *p*-hydroxyphenylpropionic acid, phloretin and smaller amounts of *p*-hydroxycinnamic acid and *p*-hydroxybenzoic acid. Incubation of phlorrhizin with the microflora gave rise to *p*-hydroxyphenylpropionic acid, phloretin and a third metabolite chromatographically identical with phloroglucinol (Table 2).

Acacetin (*4'-methylapigenin*). After the administration of acacetin (200mg) to each of four rats, the pooled ether extracts of the urine were divided into

Table 4. *R_F values and colour reactions of apigenin and related flavonoids*

The solvents and sprays are described in the text. All compounds gave a blue colour with spray 3 and dull yellow–orange colorations with sprays 1 and 2, except epiafzelechin, naringenin and phloretin, all of which gave bright-orange colorations with the diazotized sprays. In addition, naringenin gave a red colour with spray 6. st indicates streaking of spot.

	R_F values							Colours on paper chromatograms		
Solvent ...	A	B	E	F	G	I	L	In u.v. light	With spray 4 in u.v. light	With spray 5 in u.v. light
Acacetin	0.62	0.68	0.91	—	—	0.72	0.69	Brown	Yellow–green	Brown
Apigenin	0.25	0.02	0.90	—	—	0.60	0.42	Brown	Yellow–green	Green
Apigenin 7-glucoside	—	—	0.65	0.04	0.25	—	—	Brown	Yellow–green	—
Apiin	0.20	0	0.57	0.05	0.42	0	0	Red–brown	Yellow–green	Yellow–green
Chrysin	0.70	0.73	0.94	—	—	0.78	0.70	Brown	Dull yellow	Brown
Conjugate Ya	0.10	0.05	0.55	0.40	0.19	—	—	Brown	Yellow–green	Yellow
Conjugate Yb	0.15	0.05	0.68	0.60	0.30	—	—	Brown	Yellow–green	Yellow
4′,7-Dihydroxyflavone	0.19	0.02	0.90	—	—	0.36	0.20	Light blue	Bright blue	Light blue
Epiafzelechin	0.45	—	0.71	—	—	0	0.16	—	—	—
Genkwanin	0.59	—	0.92	—	—	0.73	0.67	Brown	Yellow–green	Yellow–green
Kaempferol	0.12st	0.03	0.84	0	0.02	0.62	0.47	Yellow	Bright green	Golden yellow
Kaempferol 7-rhamnoside	—	—	0.75	0.02	0.18	—	—	Yellow	Yellow–green	Bright green
Naringenin	0.50	0.15	0.90	—	—	0.64	0.67	—	—	—
Phloretin	0.58st	0	0.91	—	—	0.36	0.34	—	—	—
Tectochrysin	0.70	0.98	0.95	—	—	0.92	0.90	Brown	Dull yellow	—

Table 5. *Ultraviolet-absorption properties of apigenin and related flavonoids*

Experimental details are given in the text. The u.v.-absorption spectra were determined in ethanol, except for 4′,7-dihydroxyflavone, when methanol was used. –, Not measured.

	$\lambda_{max.}$ (nm) in ethanol		Δλ (nm) in ethanol+sodium acetate	Δλ (nm) in ethanol+$AlCl_3$	Δλ (nm) in ethanol+sodium acetate+H_3BO_3
	Band 1	Band 2	Band 2	Band 1	Band 1
Acacetin	328	273	+2	+14	0
Apigenin	336	269	+4	+47	0
Apiin	335	268	0	–	–
Chrysin	314	270	+3	+15	0
Conjugate Ya	330	269	0	–	–
Conjugate Yb	325	269	0	–	–
4′,7-Dihydroxyflavone	328, 312*	253*	–	–	–
Genkwanin	335	269	0	–	–
Kaempferol	368	267	+7	+60	0
Naringenin	325*	288	–	–	–
Phloretin	—	287	–	–	–
Tectochrysin	310	269	–	–	–

* Shoulder or inflexion.

Table 6. *Excretion of p-hydroxyphenylpropionic acid, p-hydroxycinnamic acid, p-hydroxybenzoic acid and apigenin in urine after the oral administration of apigenin and apiin in the rat*

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

				Excretion (mg/24h per rat)			
Day	Group of rats	Apigenin administered (mg)	Apiin administered (mg)	*p*-Hydroxyphenylpropionic acid	*p*-Hydroxycinnamic acid	*p*-Hydroxybenzoic acid	Apigenin
0	A	—	—	0	0	0.15	0
	B	—	—	0	0	0.15	0
1	A	100	—	0.41	0.31	0.42	0.38
	B	—	100	1.10	0.40	0.70	1.50
2	A	—	—	1.30	0.11	0.61	0.50
	B	—	—	2.10	0.19	0.36	0.53
3	A	—	—	0	0	0.21	0.30
	B	—	—	0.12	0	0.15	0.25

Table 7. *Excretion of glucuronide and ethereal sulphate in urine before and after oral administration of apigenin to rats*

Experimental details are given in the text. The results are expressed as mg of glucuronic acid or sulphate/24h, and are the mean values obtained with a group of six rats. The animals were each given 100mg of apigenin on day 1.

	Excretion (mg/24h per rat)	
Day	Glucuronide	Ethereal sulphate
0	25.7	4.55
1	37.3	12.53
2	38.2	8.45
3	26.1	5.13
4	24.0	4.20

two equal parts and evaporated to dryness. The dried residue from one part (*A*) was then demethylated by the method of Blakley & Simpson (1964), and the residue from the other part (*B*) was dissolved in aq. 10% (v/v) $NaHCO_3$. Each solution was acidified and re-extracted with ether. Chromatographic examination indicated that although trace amounts of *p*-hydroxyphenylpropionic acid were present in fraction *B*, slightly larger amounts were present in fraction *A*, which also contained small amounts of apigenin. Acacetin was detected in fraction *B*.

After incubation of acacetin with intestinal microflora the culture medium was divided into equal fractions and extracted and demethylated as described above for urine. *p*-Hydroxyphenylpropionic acid and apigenin were found only in the demethylated fraction.

Submission of the urine samples from undosed control rats to the above procedure showed only trace amounts of *p*-hydroxyphenylpropionic acid in fractions *A* and *B*.

Kaempferol (3,4′,5,7-*tetrahydroxyflavone*). Ethereal extracts of the urine of four rats, each given kaempferol (100mg), contained small but detectable amounts of *p*-hydroxyphenylacetic acid and kaempferol (Tables 2 and 4). These compounds were also found after incubation of microflora with kaempferol under the standard conditions.

Robinin (*kaempferol 7-rhamnosido-3-galactorhamnoside*). Administration of robinin (100mg each) to four rats resulted in the urinary excretion of kaempferol with smaller amounts of *p*-hydroxyphenylacetic acid. Formation of *p*-hydroxyphenylacetic acid was increased in animals that had previously received a diet containing kaempferol or robinin. The same metabolites were shown to be formed on incubation of robinin with the microflora.

Residual robinin was also detected. A substance showing chromatographic properties identical with kaempferol 7-rhamnoside (Table 4) was also detected in the control tubes of series 4 (Table 1).

Chrysin (5,7-*dihydroxyflavone*). Administration of chrysin (200mg) by stomach tube to each of six rats resulted in the urinary excretion of apigenin and unchanged chrysin (Tables 4 and 5). No phenolic acid metabolites of chrysin nor any increase in the size or intensity of hippuric acid spots in the test urine samples was observed. No detectable metabolite formation occurred after the incubation of chrysin with the microflora under the standard conditions.

Tectochrysin (*5-hydroxy-7-methoxyflavone*). Oral administration of 100mg of tectochrysin to four rats gave rise to metabolites in the urine with spectral and chromatographic properties similar to those of apigenin, genkwanin (apigenin 7-methyl ether) and tectochrysin (Tables 4 and 5). Comparison of the chromatographs of urine extracts obtained on successive days showed that the excretion of apigenin, the main metabolite, was maximal on the first and second days after dosing, whereas only trace amounts of genkwanin and tectochrysin were detected. It was found that metabolite formation did not occur on incubation of tectochrysin with rat intestinal microorganisms under the standard conditions.

4′,7-*Dihydroxyflavone*. On oral administration (100mg) to each of three rats, the unchanged compound was detected in the urine with a metabolite (R_F 0.85 in solvent A and R_F 0.05 in solvent B), which gave a brown colour with diazotized *p*-nitroaniline but was not identified. *p*-Hydroxyphenylpropionic acid, *p*-hydroxycinnamic acid and *p*-hydroxybenzoic acid were not detected on chromatograms of the urine of the dosed animals. The compound was not metabolized by gut microorganisms *in vitro*.

(−)-*Epiafzelechin* (4′,5,7-*trihydroxyflavan*-3-*ol*). Incubation of epiafzelechin with gut micro-organisms gave rise to three phenolic metabolites. Metabolite EP_1 was shown to be *p*-hydroxyphenylpropionic acid (Tables 2 and 3). Metabolites EP_2 and EP_3, which behaved as neutral compounds on partitioning between $NaHCO_3$ and diethyl ether, reacted with diazo reagents (Table 2), but were not identified.

Pelargonin (3,4′,5,7-*tetrahydroxyflavylium* 3,5-*diglucoside*). Incubation of this compound with the gut microflora resulted in the formation of a phenolic metabolite, which was tentatively identified by chromatography as *p*-hydroxyphenyl-lactic acid (Table 2).

Genistein (4′,5,7-*trihydroxyisoflavone*). This isoflavone was metabolized by rat gut microflora to a compound that was identified by paper chromatography and t.l.c. (Table 8) as *p*-ethylphenol, and confirmed by g.l.c. (Table 9). Only traces of the unchanged compound persisted in tubes of series 2.

Table 8. *R_F values and colour reactions of isoflavonoids and simple phenols*

The solvents and sprays are described in the text. All compounds gave deep-blue colours with reagents 3 and 7, except formononetin, which gave faint-blue colorations with these sprays. Daidzein and formononetin appeared as light-blue spots under u.v. light after exposure of chromatograms to NH_3 fumes. st indicates streaking of spot.

	R_F values					Colour with diazotized sulphanilic acid	
	On paper chromatograms		On silica-gel plates				
Solvent ...	B	H	M	N	O	On paper chromatograms	On silica-gel plates
Biochanin A	0.83 st	0.90 st	0.45	0.74	0.83	Dirty yellow	Deep orange
Daidzein	0.04	0.09	0.03	0.39	0.44	Pink	Pale orange
Equol	0.35	0.35	0.15	0.72	0.59	Salmon-pink	Yellow
Formononetin	0.40 st	0.45	0.20	0.56	0.72	Pale yellow	Pale yellow
Genistein	0.08	0.17	0.12	0.60	0.58	Orange–brown	Deep orange
p-Ethylphenol	0.92	0.97	0.62	0.90	0.82	Violet–pink	White–pink
Phenol	0.95	—	0.62	—	—	Yellow	Yellow

Table 9. *Retention times on g.l.c. of simple phenols*

For experimental details see the Experimental section.

	Retention time (min)	
Coating ...	5% QF-1	8% SE-30
p-Cresol	1.30	1.50
p-Ethylanisole	—	1.8
p-Ethylphenol	1.80	2.20
Phenol	0.90	1.01

Biochanin A (*5,7-dihydroxy-4′-methoxyisoflavone*). Biochanin A was found to be largely resistant to microbial catabolism, large amounts of the unchanged compound being observed in tubes of series 2. However, analysis of tube-2 extracts by g.l.c. revealed the presence of a small peak [R_T (min) 1.35], which was absent from tubes of series 1 and 4, but this was not *p*-ethylanisole, a possible fission product of biochanin A.

Daidzein (*4′,7-dihydroxyisoflavone*). Incubation of daidzein resulted in the formation of a metabolite with chromatographic properties similar to equol (4′,7-dihydroxyisoflavan) (Table 8).

Formononetin (*7-hydroxy-4′-methoxyisoflavone*). Incubation of formononetin with the intestinal microflora showed it to be largely resistant to microbial degradation, since large amounts of the unchanged compound and only trace amounts of a single metabolite (showing similar chromatographic properties to equol) were observed (Table 8).

Phenolic acids. Possible interconversions of certain of the phenolic acid metabolites were studied *in vivo* and *in vitro*. Administration of *p*-hydroxycinnamic acid (200mg each) to three rats resulted in the excretion of *p*-hydroxyphenylpropionic acid and *p*-hydroxybenzoic acid together with the unchanged compound, confirming the report by Booth *et al.* (1959). Incubation of *p*-hydroxycinnamic acid with the microflora resulted in the formation of *p*-hydroxyphenylpropionic acid and two trace metabolites, neither of which was *p*-hydroxybenzoic acid.

Administration of *p*-hydroxyphenylpropionic acid (200mg) to rats resulted in the excretion of *p*-hydroxycinnamic acid, *p*-hydroxybenzoic acid and unchanged *p*-hydroxyphenylpropionic acid in the urine, but the compound was not metabolized by the microflora *in vitro*.

Discussion

The results presented here show that many of the apigenin-derived compounds were readily metabolized when administered to rats or when incubated with rat-intestinal microflora *in vitro*. The flavones apigenin and apiin were metabolized to *p*-hydroxyphenylpropionic acid, *p*-hydroxycinnamic acid and *p*-hydroxybenzoic acid. Similar products were formed from the related dihydrochalcone, phlorrhizin, and the flavanone, naringin, *in vivo* (as reported by Booth *et al.*, 1958) and *in vitro*. The 3-flavonols kaempferol and robinin undergo a cleavage reaction analogous to that reported for quercetin and rutin by Booth *et al.* (1956), in that derivatives of phenylacetic acid instead of those of the C_6–C_3 type were detected. The hydroxyflavan epiafzelechin was also readily metabolized by the microflora to *p*-hydroxyphenylpropionic acid and two neutral products which, although unidentified, were shown to possess chemical

and chromatographic behaviour analogous to phenyl-valerolactones, reported previously as metabolites of (+)-catechin (Das & Griffiths, 1969), and it appears probable that metabolite EP_3 is δ-(4-hydroxyphenyl)-γ-valerolactone and that metabolite EP_2 is an artifact derived from it by interaction with the ammonia present in solvent A. The anthocyanidin pelargonin was degraded by the microflora *in vitro* to a compound chromatographically identical with *p*-hydroxyphenyl-lactic acid. The susceptibility of pelargonin to ring fission was unexpected, since another flavylium compound, cyanidin, has been reported by Scheline (1968) not to undergo detectable metabolism, a finding which has been confirmed in this laboratory. The isoflavone genistein was readily metabolized by the rat microflora to *p*-ethylphenol, which has been detected in urine as a metabolite of genistein in sheep (Batterham *et al.*, 1965; Braden *et al.*, 1967).

Certain compounds, although possessing similar carbon skeletons to the readily metabolized flavonoids, were found to be largely resistant to ring fission. Structural modifications of flavonoids that appeared to decrease their susceptibility to ring fission include the following.

(*a*) Absence of a free hydroxyl group in position 5. Thus apigenin (4′,5,7-trihydroxyflavone), but not 4′,7-dihydroxyflavone, undergoes cleavage, and likewise genistein (4′,5,7-trihydroxyisoflavone), but not daidzein (4′,7-dihydroxyisoflavone). DeEds (1968) also noted that 5-methoxyquercetin on administration to the rat withstands ring fission.

(*b*) Absence of a free hydroxyl group in position 4. Administration of acacetin (apigenin 4-methyl ether) to rats resulted in the excretion of very small amounts of ring-cleavage products, including small amounts of a compound (probably *p*-methoxyphenylpropionic acid) giving *p*-hydroxyphenylpropionic acid on demethylation. The flavones chrysin (5,7-dihydroxyflavone) and tectochrysin (5-hydroxy-7-methoxyflavone) were apparently resistant to ring fission *in vivo* and *in vitro*, as no free aromatic acid metabolites were detected. It is also noteworthy that no ring-fission products of the parent compound, flavone, were detected in an earlier investigation (Das & Griffiths, 1966). Although genistein (4′,5,7-trihydroxyisoflavone) was found to be completely metabolized by the microflora, incubation of biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) or of formononetin (7-hydroxy-4′-methoxyisoflavone) under similar conditions did not give rise to ring-fission products.

A specially noteworthy point in this investigation is that, with the exception of *p*-hydroxybenzoic acid and of the flavonoid conjugates, all of the metabolites detected in the urine after oral administration of the flavonoids were also formed *in vitro* by rat intestinal micro-organisms under anaerobic conditions. It is evident therefore that the microflora are able to effect all the reactions leading to the urinary ring-fission products reported here, with the exception of *p*-hydroxybenzoic acid, independently of the enzymes of rat tissues. These reactions include the splitting of the glycosidic bonds in apiin, robinin, naringin and phlorrhizin, the heterocyclic ring fission of the flavonoids possessing free 5- and 4′-hydroxyl groups, and the reduction of the side chain of cinnamic acid intermediates. The formation of *p*-hydroxybenzoic acid from flavonoids *in vivo* but not *in vitro* suggests that this acid is essentially a tissue metabolite probably derived from *p*-hydroxycinnamic acid, which is metabolized to it by a mitochondrial enzyme of rat liver (Ranganathan & Ramasarma, 1971).

The conjugates Ya and Yb (Tables 4 and 5) were found in ether extracts of urine after oral administration of apiin and apigenin. Degradation of conjugate Yb to apigenin by β-glucuronidase indicates that it is probably a glucuronic acid conjugate of apigenin. Although the absence of a bathochromic shift with sodium acetate (Table 5) indicates that the 7-hydroxyl groups are masked, recorded chromatographic data (Harborne, 1965) show that neither of those metabolites is apigenin 7-glucuronide or apigenin 4′,7-diglucuronide. The possibility that other flavonoid conjugates of a more polar character might remain in the aqueous phase after ether extraction was not explored. The detection of flavonoid conjugates in the urine of orally dosed animals is evidence that at least part of the administered flavonoid was absorbed. The detection of flavonoid conjugates in the bile of biliary cannulated rats (Barrow & Griffiths, 1971; Das & Sothy, 1971) suggests that in the intact animal the phenolic acid metabolites in the urine may be derived in part from flavonoid conjugates entering the intestine through the bile. The observation of Marsh *et al.* (1952) that the intestinal contents show a high β-glucuronidase activity suggests a mechanism for the release of the free flavonoid.

It is noteworthy that, although ring-fission products showing similar hydroxylation patterns to the B ring of the flavonoid compounds administered were readily detected *in vivo* and *in vitro*, possible phenolic metabolites of the A ring were detected only with the dihydrochalcone phlorrhizin and the hydroxyflavan epiafzelechin, under conditions *in vitro*. It appears probable that the A ring of the flavanone, flavanol, flavones and flavonols investigated in the current study is degraded to CO_2 as has been shown for (+)-catechin by Das & Griffiths (1969). They showed that (+)-[*ring A*-^{14}C]catechin, on oral administration to both guinea pigs and rats, gave rise to labelled phenyl-γ-valerolactones and $^{14}CO_2$.

Comparison of the metabolism *in vivo* of chrysin, 4′,7-dihydroxyflavone, and tectochrysin indicates that the flavone molecule can be hydroxylated in the 4′-position. 4′-Hydroxylation of flavone on oral administration to guinea pigs has been shown by Das

& Griffiths (1966). Demethylation was shown to occur with tectochrysin (5-hydroxy-7-methoxyflavone) but not with acacetin (5,7-dihydroxy-4'-methoxyflavone). The selective demethylation of a 7-methyl ether of a flavonoid has not been described previously, although the demethylation of other substrates, for example substituted anisoles, by liver preparations has been demonstrated (Bray *et al.*, 1955).

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References

Bacon, J. S. D. & Edelman, J. (1951) *Biochem. J.* **48**, 114
Barrow, A. & Griffiths, L. A. (1971) *Biochem. J.* **125**, 24P
Barton, G. M., Evans, R. S. & Gardner, J. A. F. (1952) *Nature (London)* **170**, 249
Bate-Smith, E. C. (1956) *Sci. Proc. Roy. Dublin Soc.* **27**, 165
Batterham, T. J., Hart, N. K., Lamberton, J. A. & Braden, A. W. H. (1965) *Nature (London)* **206**, 509
Blakley, E. R. & Simpson, F. J. (1964) *Can. J. Microbiol.* **10**, 175
Booth, A. N. & Williams, R. T. (1963*a*) *Biochem. J.* **88**, 66P
Booth, A. N. & Williams, R. T. (1963*b*) *Nature (London)* **198**, 684
Booth, A. N., Murray, C. W., Jones, F. T. & DeEds, F. (1956) *J. Biol. Chem.* **223**, 251
Booth, A. N., Jones, F. T. & DeEds, F. (1958) *J. Biol. Chem.* **233**, 280
Booth, A. N., Masri, M. S., Robbins, D. J., Emerson, O. H., Jones, F. T. & DeEds, F. (1959) *J. Biol. Chem.* **234**, 3014
Braden, A. W. H., Hart, N. K. & Lamberton, J. A. (1967) *Aust. J. Agr. Res.* **18**, 335
Braun, W., Whittaker, V. P. & Lotspeich, W. D. (1957) *Amer. J. Physiol.* **190**, 563
Bray, H. G. & Thorpe, W. V. (1954) *Methods Biochem. Anal.* **1**, 27
Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952) *Biochem. J.* **52**, 416
Bray, H. G., Craddock, V. M. & Thorpe, W. V. (1955) *Biochem. J.* **60**, 225
Dacre, J. C., Scheline, R. R. & Williams, R. T. (1968) *J. Pharm. Pharmacol.* **20**, 619
Das, N. P. (1969) *Biochim. Biophys. Acta* **177**, 668
Das, N. P. & Griffiths, L. A. (1966) *Biochem. J.* **98**, 488
Das, N. P. & Griffiths, L. A. (1968) *Biochem. J.* **110**, 449
Das, N. P. & Griffiths, L. A. (1969) *Biochem. J.* **115**, 831
Das, N. P. & Sothy, S. P. (1971) *Biochem. J.* **125**, 417
DeEds, F. (1968) *Compr. Biochem.* **20**, 127
Dische, Z. (1947) *J. Biol. Chem.* **167**, 189
Dowd, L. E. (1959) *Anal. Chem.* **31**, 1184
Gage, T. B. & Wender, S. H. (1950) *Anal. Chem.* **22**, 708
Griffiths, L. A. (1964) *Biochem. J.* **92**, 173
Guggolz, J., Livingstone, A. L. & Bickoff, E. M. (1961) *J. Agr. Food Chem.* **9**, 330
Gupta, S. R. & Seshadri, T. R. (1952) *Proc. Indian Acad. Sci. Sect. A.* **35**, 242
Harborne, J. B. (1959) *J. Chromatogr.* **2**, 581
Harborne, J. B. (1964) *Phytochemistry* **3**, 151
Harborne, J. B. (1965) *Phytochemistry* **4**, 107
Harborne, J. B. (1967) *Comparative Biochemistry of the Flavonoids*, p. 296, Academic Press, London and New York
Harborne, J. B. & Hall, E. (1964) *Phytochemistry* **3**, 453
Horowitz, R. M. (1957) *J. Org. Chem.* **22**, 1733
Horowitz, R. M. (1964) in *Biochemistry of Phenolic Compounds* (Harborne, J. B., ed.), p. 545, Academic Press, London and New York
Johnson, T. B. & Hodge, W. W. (1913) *J. Amer. Chem. Soc.* **35**, 1018
Jurd, L. (1956) *Arch. Biochem. Biophys.* **63**, 376
Jurd, L. (1969) *Phytochemistry* **8**, 445
Jurd, L. & Horowitz, R. M. (1957) *J. Org. Chem.* **22**, 1618
Klages, A. (1903) *Ber. Deut. Chem. Ges.* **36**, 3593
Kostanecki, St. V. & Osius, F. W. (1899) *Ber. Deut. Chem. Ges.* **32**, 321
Lindner, H. R. (1967) *Aust. J. Agr. Res.* **18**, 305
Mabry, T. J., Markham, K. R. & Thomas, M. B. (1970) *The Systematic Identification of Flavonoids*, p. 70, Springer-Verlag, New York
Marsh, C. A., Alexander, F. & Levvy, G. A. (1952) *Nature (London)* **170**, 163
Nakagawa, Y., Shetlar, M. R. & Wender, S. H. (1964) *Anal. Biochem.* **7**, 374
Nakagawa, Y., Shetlar, M. R. & Wender, S. H. (1965) *Biochim. Biophys. Acta* **97**, 233
Ranganathan, S. & Ramasarma, T. (1971) *Biochem. J.* **122**, 487
Ribereau-Gayon, P. (1964) *Les Composés Phenoliques du Raisin et du Vin*, Institut National de la Recherche Agronomique, Paris
Roberts, E. A. H. (1962) in *The Chemistry of Flavonoid Compounds* (Geissman, T. A., ed.), p. 468, Pergamon Press, Oxford
Scheline, R. R. (1968) *Acta Pharmacol. Toxicol.* **26**, 332
Scheline, R. R. (1970) *Biochim. Biophys. Acta* **222**, 228
Schmidt, O. T. (1930) *Justus Liebigs Ann. Chem.* **483**, 115
Smith, G. E. & Griffiths, L. A. (1970) *Biochem. J.* **118**, 53P
Smith, I. (1969) *Chromatographic and Electrophoretic Techniques*, 3rd. edn., vol. 1, p. 370, W. Heinemann (Medical Books), London
Sondheimer, E. & Karash, C. B. (1956) *Nature (London)* **178**, 648
Sperber, I. (1948) *J. Biol. Chem.* **172**, 441
Stahl, E. (1962) *Dünnschicht Chromatographie*, Springer-Verlag, Berlin
Swain, T. (1953) *Biochem. J.* **53**, 200
Talalay, P., Fishman, W. H. & Huggins, C. (1946) *J. Biol. Chem.* **166**, 757