Metabolism of Flavonoid Compounds in Germ-Free Rats

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Evidence for the involvement of the intestinal microflora in the metabolism of flavonoid compounds *in vivo* has been presented by Griffiths (1964), Nakagawa *et al.* (1965) and Das & Griffiths (1968), who reported that the formation of ring-fission products from orally administered flavonoids was decreased by the co-administration of oral antibiotics. It was subsequently shown that anaerobic incubation of certain flavonoid compounds with the rat intestinal microflora *in vitro* resulted in the formation of ring-fission products identical with those formed *in vivo* (Das, 1969; Griffiths & Smith, 1972*a,b*). Although the evidence thus presented indicated that the intestinal microflora was capable of effecting flavonoid-ring fission *in vitro* and that it participated in the catabolism of orally ingested flavonoids, it did not permit any conclusion to be drawn as to whether the ring-fission products detected in the urine of orally dosed animals were derived in part from degradative processes occurring in the tissues of the animal or were of wholly microfloral origin. The use of germ-free animals has permitted this problem to be resolved.

Rats of the Wistar strain, which had been bred under germ-free conditions and from which the normal intestinal flora had been shown by bacteriological testing to be absent, were maintained in a Trexler isolator (Trexler & Barry, 1958) on a diet described by Griffiths (1964), modified by the addition of cellulose powder [1 part of cellulose:3 parts of diet (w/w)] and sterilized by irradiation. The following flavonoid compounds, also sterilized by irradiation, were administered orally to two groups of these rats (100 mg/rat): (+)-catechin, apigenin, myricetin, hesperidin, naringin, rutin, 3',4',7-tri-*O*- β -hydroxyethylrutoside and 3',4',5,7-tetra-*O*- β -hydroxyethylrutoside [the last two compounds are constituents of the vascular therapeutic agent Paroven (Venoruton); from Zyma S.A., Nyon, Switzerland]. The urines and faeces were collected separately on the preceding day and for 5 days after flavonoid administration; the faeces were submitted to freeze-drying, powdered and extracted with hot methanol; the urines were divided into two equal fractions, A and B. Fraction A was submitted to our standard ether-extraction procedure for phenolic acid metabolites (Griffiths, 1964) and fraction B to freeze-drying followed by extraction of the residues with cold methanol for flavonoids and flavonoid conjugates.

Examination of the ether extracts by two-dimensional chromatography in the solvents propan-2-ol–aq. NH₃ (sp.gr. 0.88)–water (8:1:1, by vol.) followed by benzene–acetic acid–water (6:7:3, by vol.), or chloroform–acetic acid–water (2:1:1, by vol.) followed by aq. 20% (w/v) KCl, indicated that none of the phenolic acids reported in urine as metabolites of rutin, hesperidin, naringin, (+)-catechin, apigenin and myricetin (Booth *et al.*, 1956, 1958*a,b*; Griffiths, 1964; Griffiths & Smith, 1972*a,b*) were detectable in the extracts obtained from these germ-free rats other than trace amounts of *p*-hydroxyphenylacetic acid and *p*-hydroxybenzoic acid, which are known to be derived from constituents of the standard diet (Griffiths, 1964) and which were detected at the same concentration in rat urine before flavonoid administration. Phenolic acid metabolites were also absent from the urines of the germ-free animals that had received the hydroxyethylrutosides.

Examination by the analytical procedure described above of the urines of a group of rats, of the same Wistar strain but possessing a conventional intestinal microflora, which had received rutin, hesperidin, naringin, (+)-catechin, apigenin and myricetin (after submission to the same irradiation procedure) at the same dosage, confirmed the presence of the ring-fission products reported previously (Booth *et al.*, 1956, 1958*a,b*; Griffiths, 1964; Griffiths & Smith, 1972*a,b*).

Chromatographic examination in butan-1-ol–acetic acid–water (4:1:5, by vol.) of the methanolic extracts of freeze-dried urines of germ-free rats after the administration of (+)-catechin and naringin resulted in the detection of conjugates of the administered compounds, which have previously been detected in the urine (Barrow & Griffiths, 1971) and in the bile (Das & Sothy, 1971) of normal rats.

Hydrolysis of flavonoid glycosides, which is known to occur after oral ingestion by the mammal, was shown to be effected by the intestinal microflora *in vitro* (Griffiths & Smith, 1972*a,b*). The possibility that secretions or enzymes derived from the mammal might also exert a hydrolytic effect on compounds of this type was explored in germ-free animals by examination of methanolic extracts of freeze-dried urines and faeces after glycoside administration, but no aglycones were detected although large amounts of the unchanged glycosides, i.e. naringin, hesperidin, rutin and the hydroxyethylrutosides, were obtained

from the freeze-dried faeces. The detection of large amounts of unchanged rutin in the faeces of germ-free animals is noteworthy, as Clark & McKay (1950) have reported low recoveries of this compound in faeces in the presence of a normal microflora. Recovery of unchanged tri- and tetra-hydroxyethylrutosides from the faeces of germ-free animals indicates that these vascular therapeutic agents are stable to secretions of the alimentary tract and will be split to their aglycones [previously reported as metabolites in faeces by Barrow & Griffiths (1971)] only in the presence of the gut microflora, which is known to be restricted to the lower intestine in man (Hawkesworth *et al.*, 1971).

Extraction of the faeces of (+)-catechin-fed germ-free animals resulted in low recoveries of the administered compound. This may be attributable to more rapid uptake of (+)-catechin from the gut lumen than occurs with other flavonoids, as the urine was found to contain large amounts of two hydroxyflavan conjugates, one of which possessed similar chromatographic properties to a compound identified by Das & Sothy (1971) as (+)-catechin glucuronide. Two conjugates were also detected in the faeces of germ-free rats after (+)-catechin administration, one of which was found to possess similar chromatographic properties to the (+)-catechin glucuronide reported by Das & Sothy (1971). The compound was not detected, however, in the faeces of normal (+)-catechin-fed rats. Since this conjugate is known to be present in large amounts in the bile of normal rats (Das & Sothy, 1971), its presence in the faeces of germ-free rats but absence from faeces of normal rats indicates that it undergoes degradation in the intestine of rats possessing a conventional microflora.

Although conjugates of (+)-catechin, naringin and phlorrhizin and the glycosides 3',4',7-tri-*O*- β -hydroxyethylrutoside and 3',4',5,7-tetra-*O*- β -hydroxyethylrutoside have, after oral administration, been detected in the bile of normal animals (Barrow &

Griffiths, 1971), the presence of these compounds in the bile of germ-free animals was not explored owing to the technical difficulty of implanting cannulae into the bile ducts of animals maintained under germ-free conditions.

These results demonstrate that orally ingested flavonoids do not in the rat give rise to ring-fission products in the absence of the normal intestinal microflora and support the view that the phenolic acids detected in the urine of normal animals after oral ingestion of flavonoid compounds are of wholly microfloral origin.

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