hanced agglutinability, whereas a batch of pooled normal rabbit sera (Cappel Laboratories) increased embryo agglutinability over 60%. This was not surprising since rabbit sera are known to contain natural antibodies reactive with mouse tissues, and individual rabbits differ among themselves in the amount of such antibodies[9]. Thus, it is better to use hyperimmune serum in this procedure to ensure the presence of agglutinating antibodies.

Trials were made to determine whether antibody treatment affected the capacity of cleavage stage embryos to form blastocysts. The results indicate that exposure to antibody has no adverse effects on blastocyst formation by DBA/2J morulae. This was the case with regard to the percentage of embryos reaching blastocyst stage as well as the time at which they were formed. 39 of 43 morulae (91%) treated with antibody reached the blastocyst stage, in comparison to 36 of 39 (92%) control embryos. Moreover, we have begun to employ the rabbit antiserum to facilitate aggregation in the routine production of allophenic mice and have already produced 85 chimeric animals with the technique.

This report shows that rabbit antisera can be used to promote aggregation in the production of allophenic mice. It is effective, easy to apply, and results in no adverse effect on the embryo development. This procedure represents an alternative to that reported by Mintz et al.[10], where it was shown that the plant lectin phytohemagglutinin is also effective in mediating blastomere aggregation. Use of antibody represents an easier means of agglutinating the embryos. Application of phytohemagglutinin involves agglutinating the embryos in its presence for several minutes, followed by extensive washes to remove excess lectin from the delicately bound embryo pairs. Complete removal of unbound lectin is likely to be important, considering the well known toxic effect it has on cultured cells (for example, see Stanley et al.[11]). The procedure with antibody involves bathing the embryos in antiserum and washing them prior to aggregation, with no further need for manipulation following the formation of embryo pairs.

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New metabolites of the naturally-occurring mutagen, quercetin, the pro-mutagen, rutin and of taxifolin

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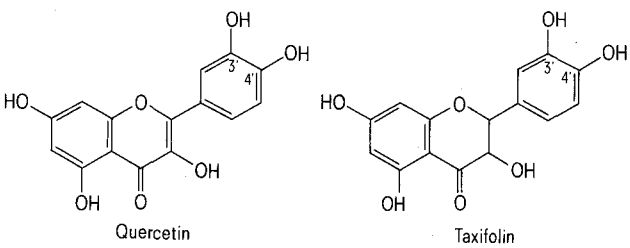
Summary. The major biliary metabolites of the mutagen, quercetin, the pro-mutagen rutin and taxifolin have been identified by EI-mass spectrometry, UV-spectroscopy and chromatographic methods as conjugates of the corresponding 3′-O-methyl ethers. The toxicological significance of these findings is discussed.

The flavonol, quercetin, which has recently been shown not only to be mutagenic in the Salmonella/microsomal assay[3,4] but also to be carcinogenic in the rat[5], is known to be widely distributed in glycosidic forms in the plant kingdom[6] and recently evidence has been presented that the quercetin glycoside, rutin, which is known to be present in edible plants, although non-mutagenic in the Salmonella/microsomal assay, is activated by incubating with a glycosidase preparation ('fecalase'), derived from the human intestine[7].

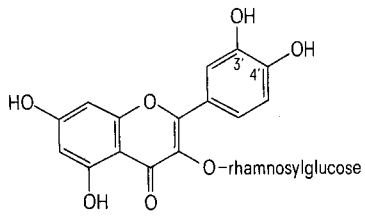
Since evidence has recently been presented that the related 3′,4′-*o*-dihydric flavanols are largely metabolized in the liver by 3′-O-methylation and that conjugates of the 3′-O-methyl ethers are subsequently excreted in large amounts in bile and urine[8–10], it appeared of interest to establish whether the mutagen, quercetin, and the 2 structurally-related compounds, rutin (quercetin-3-rhamnoglucoside) and taxifolin (dihydroquercetin) are metabolized by this route.

Experimental. Animals. Groups of 3 rats (300–350 g) of the Wistar strain were employed under conditions and on a diet previously described[11].



Quercetin

Taxifolin



Rutin

Chromatographic and spectral characteristics of the aglycones of the biliary metabolites of quercetin, taxifolin and rutin

Aglycones	R_f-values Untreated paper (solvent A)	Mo-impregnated paper (solvent A)	Cellulose TLC (solvent B)	Polyamide TLC (solvent C)	UV-spectra In methanol λ max (nm)	+Na OMe $\Delta\lambda$ (nm)	+$AlCl_3$ $\Delta\lambda$ (nm)	+Na OAc $\Delta\lambda$ (nm)	+Na OAc/H_3BO_3 $\Delta\lambda$ (nm)
Q_1	0.74	0.27	0.20	0.02	370	−40	+86	+14	+18
Q_2	0.81	0.57	0.69	0.11	370	+66	+58	+2	2
T_1	0.84	0.52	0.44	0.26	290	ND	ND	ND	ND
T_2	0.84	0.97	0.81	0.52	288	ND	ND	ND	ND
T_3	0.84	0.26	0.31	0.8	370	ND	ND	ND	ND
Rl_a	0.81	0.57	0.67	0.12	370	+68	+58	+2	0
Rl_b	0.74	0.26	0.20	0.02	370	−40	+88	+12	+18
Standards									
Isorhamnetin	0.81	0.57	0.69	0.11	370	+68	+58	+2	0
Quercetin	0.74	0.27	0.20	0.02	370	−40	+88	+12	+18
Taxifolin	0.84	0.52	0.46	0.26	290	ND	ND	ND	ND

ND, not determined.

Compounds. Quercetin and taxifolin were supplied by Koch Light Ltd and rutin by the Aldrich Chemical Co. Ltd.
Chromatography. Paper chromatography was carried out under conditions previously described[12] but employing the solvent system: sec. butanol-acetic acid-water (5:1:2 by vol.). HPLC separations were carried out using a Pye Unicam LC-XPD system. Separation of quercetin and isorhamnetin was carried out on a prepacked μ Bondapak C_{18} column using the solvent system: water-acetic acid-methanol (80:6:65 by vol.) at a flow rate of 1 ml/min; detection was carried out at 370 nm. Separation of flavonoid glycosides and conjugates was carried out on a Spherisorb 10 μm ODS column. The solvent system used was water-acetic acid-methanol (70:6:50 by vol.) at a flow rate of 1 ml/min, whilst detection was at 352 nm.

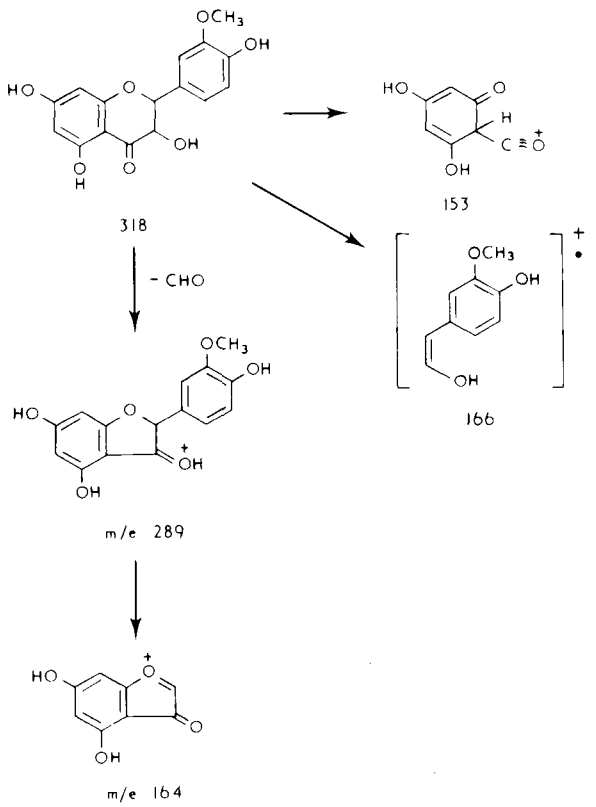


Figure 1. Proposed MS fragmentation pathway of the major biliary metabolite of taxifolin.

Enzyme hydrolysis. Conditions were as earlier described[13].
Mass spectrometry. Mass spectra were determined in an AEI MS 902 or Kratos MS 80 with direct probe insertion. The electron energy was 70 eV.
UV-spectroscopy. The procedures used were those described by Mabry et al.[14].
Biliary cannulation procedures. These were as previously described[15].
Results. Taxifolin. Following the i.p. administration of taxifolin (dihydroquercetin) (10 mg) to 3 bile duct cannulated rats, 13 metabolites were detected in bile, all of which were absent from control bile collected for 3 h prior to dosing. Each of these metabolites were hydrolyzed by a mixed glucuronidase/aryl sulphatase preparation to give 1 of 3 aglycone products T_1, T_2 and T_3. Separation of these aglycones was effected by TLC and the eluted compounds were submitted to mass spectrometry and UV-spectroscopy. The mass spectrum obtained in respect of the aglycone T_2 showed a molecular ion at m/e 318 and other ions at m/e 289, 166, 164 and 153. The fragmentation pattern indicates that methylation has occurred in ring B to give 3′ or 4′-O-methyltaxifolin (fig. 1). Further evidence for 3′ and 4′-O-methyl substitution is provided by the absence of retarda-

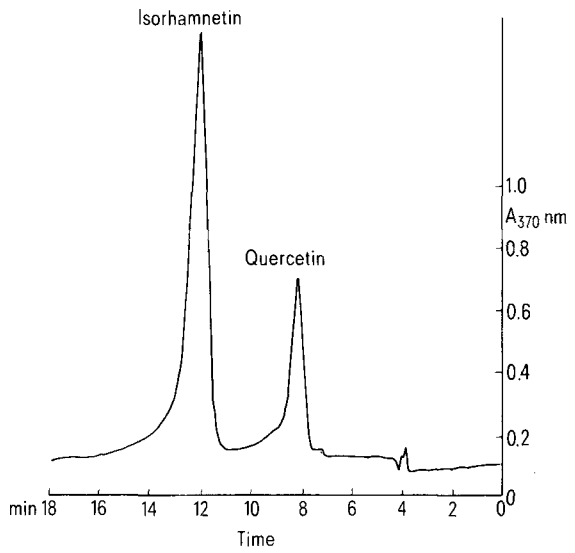


Figure 2. HPLC separation of the aglycones present in the bile of the rutin-dosed rat following acid-hydrolysis. (For conditions see text.)

tion on molybdate-treated paper (table), as flavonoid compounds possessing free 3′,4′-dihydroxylation are known to display lower R_f-values on paper pretreated with sodium molybdate than on untreated paper[9,16]. Due, however, to the absence of authentic standards it was not possible to determine whether T_2 was 3′-methyltaxifolin or the 4′-methylisomer.

The aglycone, T_1, was shown to possess chromatographic properties identical with those of taxifolin (table). Although T_3 was not identified, the compound showed a higher λ max (370 nm) than taxifolin and also greater retardation on molybdate-impregnated paper (table) suggesting that it may be a hydroxylation product of taxifolin. Mass spectra of this compound were not obtained.

Following the oral administration of taxifolin, the same metabolites were detected in bile but in reduced amounts.

Rutin. Following the i.p. administration of rutin to 3 bile duct cannulated rats, 3 flavonol conjugates, R_1, R_2 and R_3 (R_fs 0.57, 0.44 and 0.28 respectively) were detected as biliary metabolites on paper chromatograms employing solvent A. Submission of the eluted conjugate R_1 to HPLC revealed the presence of a major component R_{1a}, the aglycone of which was subsequently identified by spectral and chromatographic methods as isorhamnetin. Whereas the aglycone of the minor component R_{1b} was identified as quercetin (table).

Confirmatory evidence that the aglycone produced by acid hydrolysis of R_{1a} was a methylquercetin was obtained by mass spectrometry when a molecular ion of m/e 316 was observed. Although mass spectrometry did not permit the position of the methyl group to be established due to the absence of significant fragmentation under conditions of EI mass spectrometry, the UV-spectral characteristics of the biliary metabolite and its aglycone are in good agreement with those of isorhamnetin rhamnoglucoside[14] and isorhamnetin respectively (table).

Conjugates R_2 and R_3 were not fully characterized but each was degraded by acid hydrolysis to give a mixture of isorhamnetin and quercetin, which were separated and identified by HPLC (fig. 2). The parent conjugates are believed to be mixed isomeric glucuronides of rutin and isorhamnetin rhamnoglucoside, since they were hydrolysed by β-glucuronidase.

Following oral administration of rutin to bile-duct cannulated rats at the 10 mg dose level, no flavonol aglycones were detected in bile following hydrolysis. At higher dosage levels (50 mg) oral administration resulted in the detection of both isorhamnetin and quercetin in bile following hydrolysis together with an unidentified aglycone.

Quercetin. Following i.p. administration of quercetin (10 mg) to 3 bile duct cannulated rats, flavonol conjugates but not free aglycones were detected in bile. Following hydrolysis with glucuronidase/aryl sulphatase preparation, 2 products, Q_1 and Q_2 were obtained (table). Q_1 was shown by chromatographic and UV-spectral studies to be identical with quercetin, whereas Q_2 was shown by similar methods to be identical with isorhamnetin.

Following oral administration the same metabolites were detected in bile but in much reduced quantities.

Discussion. The detection of the corresponding 3′-O-methyl ethers in bile following the administration of quercetin and compounds of related structure indicates that a metabolic mechanism is available in mammalian tissues for the 3′-O-methylation of *o*-dihydric flavonols. Since it is known that the 3′-O-methyl ether of quercetin, namely isorhamnetin, is considerably less mutagenic in the Ames test than quercetin[3] it appears possible that metabolic 3′-O-methylation may be of importance in protecting the body against the carcinogenic action of certain naturally-occurring flavonoids of dietary origin. Although the quercetin glycoside rutin is reported to be non-mutagenic in the Salmonella/microsomal assay[3] it is known that following pre-treatment with glycosidases of intestinal microfloral origin, considerable mutagenicity is induced probably associated with the release of quercetin[7,17]. 3′-O-Methylation of the rutin molecule prior to its excretion in conjugated form in bile would therefore be expected to markedly reduce mutagenicity resulting from hydrolytic release of the flavonol aglycone in the intestinal lumen. Although the quantities of 3′-O-methyl ethers detected in bile following oral administration are relatively small it is important to note that HPLC, paper and TLC techniques indicated that the amounts of the conjugates of methyl ethers found in bile exceed those of the unchanged flavonols, which would suggest that although absorption of these flavonols from the gastrointestinal tract is small, a major proportion of the absorbed flavonol undergoes methylation in the tissues. Although the dihydroflavonol, taxifolin also gives rise to the corresponding 3′-O-methyl ether in bile this is unlikely to be of toxicological interest as evidence has been presented that the presence of a 2,3-double bond is essential for mutagenic activity in the flavonol series[3].

Chromatographic examination of the urines following parenteral administration of quercetin, taxifolin and rutin indicated that considerably lower levels of flavonol conjugates are present in urine than were observed in bile, confirming earlier observations that flavonoid conjugates generally are selectively excreted via the biliary route[18].

Although methylated flavonols were readily detected in bile, their presence in faeces and urine was not observed. This suggests that following biliary excretion in the intact mammal, the 3′-O-methyl flavonols are degraded in the intestine by the microflora which has been implicated in the catabolism of a wide range of flavonoid compounds[18,19]

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