Biochem. J. (1966) **98**, 488

Studies on Flavonoid Metabolism

METABOLISM OF FLAVONE IN THE GUINEA PIG

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(*Received* 5 *August* 1965)

1. The metabolism of flavone has been studied in the guinea pig; after administration of the compound either orally or intraperitoneally, 4′-hydroxyflavone is excreted in the urine. 2. Small amounts of 3′,4′-dihydroxyflavone were also detected in the urine after oral administration of flavone. 3. Hydroxyflavone formation is not suppressed by administering high doses of the antibacterial compounds aureomycin and phthaloylsulphathiazole. 4. Although salicylic acid appeared in trace quantities in the urine after administration of flavone, no other aromatic acid metabolites of flavone were detected.

Previous studies on the degradation of flavonoid compounds in the mammal (Booth, Murray, Jones & De Eds, 1956; Booth, Jones & De Eds, 1958*a*,*b*; Griffiths, 1962, 1964) have largely been limited to flavonoid compounds showing a high degree of hydroxylation. It was therefore decided to determine whether the non-hydroxylated parent compound, flavone, is absorbed and metabolized similarly in the mammal.

Administration of naturally occurring flavonoids showing hydroxylation of both ring A and ring B results in the formation of a number of phenolic acid metabolites, which are excreted in the urine. Although certain of these flavonoid metabolites may be products of mammalian metabolism (Booth *et al.* 1956), others have been shown to be formed largely by the microflora of the mammalian intestine (Griffiths, 1964). If flavone undergoes a similar type of ring-fission to that reported for naturally occurring flavonols (Booth *et al.* 1956) the formation of substantial amounts of non-phenolic aromatic acid metabolites from the cinnamoyl portion of the flavone molecule would be expected.

EXPERIMENTAL

Animals. Male guinea pigs each weighing approx. 300g. were used. The animals were housed in separate metabolism cages, designed to permit the separate collection of urine and faeces. The animals were fed at 24hr. intervals before the period of urine collection and unrestricted water intake was allowed throughout the experimental period.

Diet. The guinea pigs were fed on a standardized powder diet of the following composition: skimmed milk powder (fortified with vitamins A and D), 300g.; glucose, 170g.; potato dextrin, 150g.; potato starch, 234g.; arachis oil, 50ml.; vitamin fortification mixture (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), 23g.; cellulose powder, 100g.; salt mixture no. 4 (Hegsted, Mills, Elvehjem & Hart, 1941), 25g. Ascorbic acid (6mg.) was given to each guinea pig daily in aqueous solution.

Although adoption of this diet permits the elimination of plant materials containing flavonoid compounds from the diet it was not adequate for long-term feeding of the animals and was used only over experimental periods of short duration.

Test substance. Flavone, m.p. 99°, was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks. The compound was chromatographically pure. For oral administration flavone was given in admixture with the diet at the doses stated. For intraperitoneal administration, flavone was dissolved in 1ml. of acetone.

Preparation of urinary extracts for chromatography. Collection of the urine followed by ether extraction and paper chromatography of the ethereal extracts was carried out by our standard procedure (Griffiths, 1964).

Spray reagents. The reagent of Barton, Evans & Gardner (1952), diazotized *p*-nitroaniline and diazotized sulphanilic acid (Smith, 1960) were used for the detection of phenolic compounds.

A *p*-dimethylaminobenzaldehyde reagent (Smith, 1960) was used for the detection of aroyl-glycines.

Organic acids were detected by the use of an aqueous solution of 8-hydroxyquinoline sulphate (0·7%) and $ZnSO_4,7H_2O$ (0·5%). After spraying the spots were viewed in ultraviolet light (Pesez & Ferrero, 1957). Organic acids were also detected by the use of ethanolic 0·1% bromocresol green (Smith, 1960).

Flavone was detected by the use of 0·5% dinitrophenylhydrazine in 2N-HCl.

A saturated solution of ammonium molybdate was used for the detection of *o*-dihydroxyphenols.

Solvent systems. Two two-dimensional solvent systems

were used: *A*, propan-2-ol–aq. NH_3 (sp.gr. 0·88)–water (8:1:1, by vol.) followed by benzene–acetic acid–water (6:7:3, by vol.); *B*, chloroform–acetic acid–water (2:1:1, by vol.) followed by 20% (w/v) KCl.

Ultraviolet spectra. Ethanolic solutions of flavones and phenolic acids were examined in a Spectronic 505 recording spectrophotometer.

Infrared spectra. Hydroxyflavones (10mg.) in KBr (200mg.) disks were examined at 40% transmission in a Jasco infrared spectrophotometer [model (IR)–S; Japan Spectroscopic Co. Ltd.].

Alkaline hydrolysis of 4′-hydroxyflavone. Approx. 1mg. of the hydroxyflavone in 10ml. of aq. 50% (w/v) KOH was heated under reflux for 3hr. The degradation products were obtained by acidification of the hydrolysate with 0·1N-HCl to pH1 followed by ether extraction.

Determination of 4′-hydroxyflavone. A method based on the absorption of the compound at 327mμ was employed. The ethereal extract (3ml.) from 100ml. of urine of flavone-fed guinea pigs was applied to washed Whatman no. 3MM paper as a band. Markers of 4′-hydroxyflavone were applied to each side of the main band. The paper was developed (40cm.) with the benzene–acetic acid–water solvent and the chromatogram, after being dried, was examined in ultraviolet light. The fluorescent band corresponding to the fluorescent markers was cut into small fragments and eluted with acetone–ethanol (1:1, v/v) (3×10ml.). The extract was concentrated to 2ml. and applied to a second chromatogram of washed Whatman no. 3MM paper, which was developed with benzene–acetic acid–water solvent. The appropriate band was eluted with 3×7ml. of redistilled A.R. methanol. The extract after filtration was evaporated to dryness *in vacuo* and redissolved in 10ml. of redistilled A.R. ethanol, and this solution after suitable dilution was used for the spectrophotometric determination. The extinction at 327mμ was measured in a Hilger spectrophotometer. Standard curves were prepared by using a pure specimen of 4′-hydroxyflavone.

RESULTS

Aromatic acid constituents of urine of guinea pigs fed on the standard diet. Six guinea pigs were given the standard diet for 3 weeks and during the third week the urine was collected at 24hr. intervals. Examination of the ethereal extracts by two-dimensional paper chromatography indicated the presence of several phenolic compounds giving positive reactions with Barton's reagent and showing colour reactions with the diazotized *p*-nitroaniline reagent. These compounds were provisionally identified by co-chromatography as *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid, *p*-hydroxyphenyl-lactic acid and *m*-hydroxyhippuric acid (Fig. 1). The overall pattern closely resembles the phenolic acid excretion pattern of the rat fed on a diet based on white flour and dried milk (Griffiths, 1964). On spraying duplicate chromatograms with the *p*-dimethylaminobenzaldehyde reagent, an orange–yellow spot appeared that was shown by co-chromatography to be hippuric acid. Examination of the extracts by ascending chromatography with propan-2-ol–aq. ammonia–water revealed the presence of small amounts of benzoic acid (R_F 0·52). The latter compound was detected with the bromocresol green reagent and the 8-hydroxyquinoline sulphate reagent.

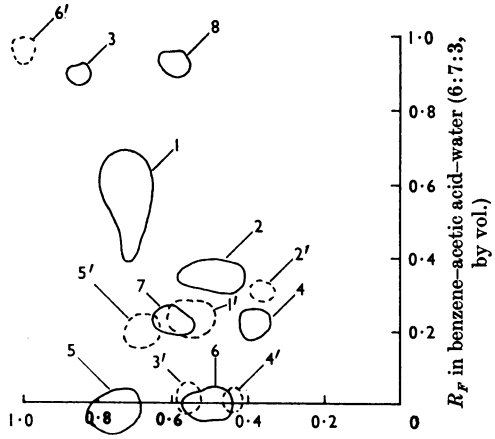

Fig. 1. Composite diagram showing the positions of metabolites of flavone and other aromatic constituents of guinea-pig urine in solvent system *A*. Experimental details are given in the text. Spots shown diagrammatically with a complete outline were detected by their fluorescence in ultraviolet radiation; spots shown with a broken outline were detected by the use of the appropriate spray reagent (see the text). Spot 1 (4′-hydroxyflavone): u.v. fluorescence, blue. Spot 2 (3′,4′-dihydroxyflavone): u.v. fluorescence, blue. Spot 3 (salicylic acid): u.v. fluorescence, blue. Spots 4–8 (unidentified): blue–dark-blue fluorescence. Spot 1′ (*p*-hydroxyphenylacetic acid): purple with diazotized *p*-nitroaniline. Spot 2′ (*p*-hydroxybenzoic acid): red with diazotized *p*-nitroaniline. Spot 3′ (*p*-hydroxyphenyl-lactic acid): purple with diazotized *p*-nitroaniline. Spot 4′ (*m*-hydroxyhippuric acid): red with diazotized *p*-nitroaniline. Spot 5′ (hippuric acid): orange–yellow with *p*-dimethylaminobenzaldehyde. Spot 6′ (flavone): orange with 2,4-dinitrophenylhydrazine, turning red after respraying with aq. 5% Na_2CO_3.

Metabolites of flavone. Twelve guinea pigs were given the standard diet for 2 weeks. At the end of this period the animals were divided into two groups. Each animal of group A received a single dose of 50mg. of flavone in addition to the standard diet, while all the group B animals received the standard diet only. After the first day both groups received the unsupplemented diet only. The 24hr. urine samples collected over 3 days were extracted by the standard procedure and the extracts examined by two-dimensional chromatography with solvent system *A*. Examination of chromatograms from the two groups under ultraviolet light revealed the presence on the chromatograms of group A of

strongly fluorescent compounds that were absent from those of group B (Fig. 1).

Unchanged flavone (spot 6′) was detected by spraying with 2,4-dinitrophenylhydrazine. The colour observed was dark orange on a yellow background. No colour was observed with the other fluorescent spots. The only example of a normally formed carbonyl derivative of a flavone appears to be flavone 2,4-dinitrophenylhydrazone (Baker, Harborne & Ollis, 1952). On spraying with a second spray of 5% sodium carbonate the flavone spot turned a reddish colour. The spot of unchanged flavone showed high R_F values in propan-2-ol–aq. ammonia–water and benzene–acetic acid–water (Fig. 1). Unchanged flavone was observed in the urine extracts of flavone-fed as well as flavone-injected guinea pigs.

Spot 1 was found to give reactions characteristic of a phenolic compound, namely pale blue with the ferric chloride–potassium ferrocyanide spray reagent, yellow with diazotized *p*-nitroaniline and orange–yellow with diazotized sulphanilic acid. It showed blue fluorescence in ultraviolet light becoming green in the presence of ammonia fumes.

Spot 2, which also showed blue fluorescence in ultraviolet light turning to yellow–green in the presence of ammonia, gave a very pale-blue colour with the ferric chloride–potassium ferrocyanide reagent, and on spraying with saturated aq. ammonium molybdate an immediate orange–brown colour was obtained. This has been held by Pridham (1959) to be characteristic of *o*-dihydroxyphenols. The compound was tentatively identified as 3′,4′-dihydroxyflavone by co-chromatography with an authentic specimen of this compound. Comparison of the ultraviolet spectra of the eluted compound and the standard preparation showed that these also were in agreement, showing absorption maxima at 343 and 244mμ. However, since the compound was present in trace quantities only on these chromatograms, it was not possible to obtain the dihydroxyflavone in the crystalline state.

Spot 3 gave a very pale-blue colour with the ferric chloride–potassium ferrocyanide spray reagent, a purple colour with aq. 3% ferric chloride and red–brown and orange–yellow colours with diazotized *p*-nitroaniline and diazotized sulphanilic acid respectively. By co-chromatography with an authentic specimen of salicylic acid in solvent systems *A* and *B*, it was tentatively identified as that compound. However, since it was present on the chromatograms in trace quantities only, it was not possible to characterize it further. No other phenolic metabolites of flavone were detected, nor was there any increase in the amounts of the phenolic acids normally found on the control chromatograms. The other fluorescent spots (4–8) gave no colours with these reagents.

Identification and isolation of 4′-hydroxyflavone. Co-chromatography in solvent systems *A* and *B* showed that the chromatographic behaviour of the compound corresponding to spot 1 (compound 1) was identical with that of 4′-hydroxyflavone. Both showed similar intense blue fluorescence in ultraviolet light, turning to green on exposure to ammonia, identical colour reactions with the diazotized reagents and positive reactions with the ferric chloride–potassium ferrocyanide reagent.

Six guinea pigs were taken and each was given 50mg. of flavone orally for 3 days. Their urines were pooled, hydrolysed with concentrated hydrochloric acid under reflux and extracted with ether in the usual way. Urine collection was continued for 4 consecutive days. The ether extracts were pooled, concentrated and submitted to band chromatography on Whatman no. 17 paper. The eluates of 4′-hydroxyflavone obtained were concentrated and refractionated several times on pre-washed sheets of Whatman no. 17 paper. The final eluate of the 4′-hydroxyflavone in acetone–ethanol (1:1, v/v) was concentrated to 10ml. and to it was added about 30ml. of water. Compound 1 was precipitated as white crystals. The solid was recrystallized several times and the pure crystals were dried for 6hr. under reduced pressure at 120° and over phosphorus pentoxide. The dried solid, which was in the form of white flakes, melted at 269–270°, which is the m.p. of 4′-hydroxyflavone.

The infrared spectra of both compound 1 and 4′-hydroxyflavone over the range 2·5–16μ were also identical.

Determination of the ultraviolet spectra of a pure specimen of 4′-hydroxyflavone and of the compound corresponding to spot 1 showed that both compounds possessed identical absorption maxima at 327 and 254mμ.

After alkaline degradation of compound 1 with aqueous potassium hydroxide, an ether extract of the hydrolysis products was submitted to two-dimensional chromatography in solvent system *A*. With the ferric chloride–potassium ferrocyanide reagent and the diazotized *p*-nitroaniline reagent, the presence of at least four phenolic degradation products was shown (Fig. 2). Similarly it was shown that alkaline degradation of an authentic specimen of 4′-hydroxyflavone gave the same pattern of spots on the chromatogram, and by co-chromatography the identity of the two series was established. By co-chromatography with pure standard substances the chromatographic behaviour of spots 1″ and 2″ was shown to be identical with that of salicylic acid and *p*-hydroxybenzoic acid respectively. Spot 4″, which gave a strong reaction with the ferric chloride–potassium ferrocyanide reagent, was not identified.

It was shown by co-chromatography that spot

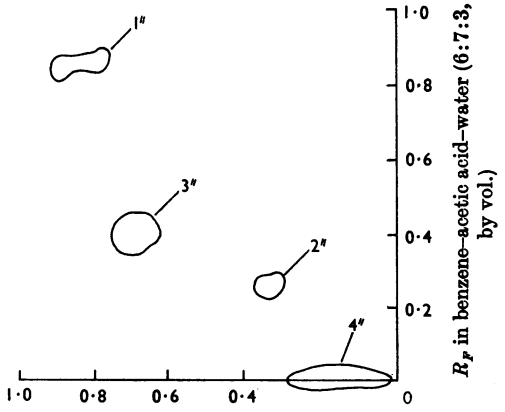

Fig. 2. Two-dimensional chromatogram of degradation products of compound 1 after treatment with 50% KOH, as described in the Experimental section. Colours indicated below were those obtained by spraying with diazotized *p*-nitroaniline. Spot 1″ (salicylic acid): red–brown. Spot 2″ (*p*-hydroxybenzoic acid): red. Spot 3″ (*p*-hydroxyacetophenone): red–brown. Spot 4″ (unidentified).

3″ possessed similar chromatographic properties to *p*-hydroxyacetophenone. The presence of both *ortho* and *para* isomers in the hydrolysate was shown by determination of their absorption spectra. The *o*-hydroxyacetophenone, which formed oily droplets that floated in the alkaline hydrolysate, was removed by physical means and washed with a small volume of water. The absorption spectra, which showed absorption maxima at 252 and 326 mμ in aqueous 0·1 N-hydrochloric acid and 0·1 N-sodium hydroxide respectively, were in agreement with those given by an authentic specimen of *o*-hydroxyacetophenone. Chromatographic separation of the other constituents of the hydrolysate followed by spectrophotometric examination of the eluates showed that the absorption maxima of the compounds believed to be *p*-hydroxyphenylacetic acid, salicylic acid and *p*-hydroxyacetophenone possessed absorption maxima at 256, 305 and 275 mμ respectively in aqueous 0·1 N-hydrochloric acid and at 282, 297 and 325 mμ respectively in aqueous 0·1 N-sodium hydroxide. These values are in agreement with those obtained with solutions of the authentic compounds.

Since it has been shown (Muller, 1915) that flavone under similar conditions of hydrolysis gives rise to salicylic acid, *o*- and *p*-hydroxyacetophenone and benzoic acid, the products reported here are those that would be expected to originate from fission of the 4′-hydroxyflavone molecule. Although spot 4″ was not identified, it was shown that the spot was present on chromatograms of the hydrolysis products of the pure 4′-hydroxyflavone as well as on those of the metabolite. On theoretical grounds, it is thought probable that the compound is the intermediate, 2,4′-dihydroxydibenzoylmethane.

The amount of 4′-hydroxyflavone excreted by flavone-fed guinea pigs was determined by the spectrophotometric method. Six animals maintained on the standard diet for a week before the experiment were each given 30 mg. of flavone. The urines, collected over 24 hr. periods, were pooled and the mean values determined over 4 days. It was shown that 622 μg., 309 μg., 15 μg. and 0 μg./guinea pig/24 hr. were excreted on the first, second, third and fourth day of the experiment respectively. These results show that excretion is maximal during the first 24 hr. after administration. The amount of 4′-hydroxyflavone formed is small in relation to the amount of flavone given.

Formation of 4′-hydroxyflavone. Since previous studies had shown that the degradation of a related substance, (+)-catechin, in the mammal was dependent on the activities of the microflora in the gut (Griffiths, 1964), consideration was given to a possible role of the intestinal bacteria in the degradation of flavone. Flavone (30 mg.) was given to each of two groups (A and B) of guinea pigs daily for 7 days. In group B only, an additional supplement of phthaloylsulphathiazole (70 mg.) and aureomycin (50 mg.) was given to each animal from the third to the seventh day. Although the antibacterial substances at these doses almost completely suppress the degradation of catechin in the rat (Griffiths, 1964), chromatograms showed no decrease of flavone metabolites in the antibiotic-treated group. Further evidence that the intestinal microflora is not of importance in the metabolism of flavone in the guinea pig was obtained by administering 30 mg. of flavone in sterile acetone intraperitoneally to each of a group of six animals while a second group of six each received 30 mg. of flavone orally. Under these conditions chromatograms prepared from the urine of the two groups were identical and it was concluded that formation of the metabolites was not dependent on the action of the intestinal microflora.

In view of the report of Winicki, Chopin, Cier & Nofre (1960) that flavone is hydroxylated by a non-enzymic system containing ascorbic acid, oxygen and ferrous chelate, consideration was also given to the possibility that hydroxylation of ingested flavone in the guinea pig might occur not in the tissues of the animal but in the digestive tract in the presence of dietary ascorbic acid, which is given in large amounts in the standard diet to compensate for the elimination of green leaf material from the diet. However, since flavone administered parenterally gives rise to the excretion of similar amounts

of the metabolites to those found after oral administration, it appears probable that hydroxylation of the flavone molecule normally occurs after absorption from the gut.

Examination of the urine of flavone-fed animals for aromatic acid metabolites. A group of six animals that had been maintained on the standard diet for 3 weeks was divided into two groups. The urine from animals of the first group, which had received a single dose of 50mg. each, was collected over three 24hr. periods and extracted with ether. Urine of the second group, which had received no flavone over this period, was treated similarly. On chromatography of the ethereal extracts in the propan-2-ol–aq. ammonia–water solvent system, followed by the spraying of duplicate chromatograms with ethanolic bromocresol green and the 8-hydroxyquinoline sulphate reagent respectively, it was shown that no additional aromatic acids were present on the chromatograms of the flavone-fed animals other than a trace amount of salicylic acid (R_F 0·90 in propan-2-ol–aq. ammonia–water). Duplicate chromatograms were also dipped in the *p*-dimethylaminobenzaldehyde reagent. No additional aroyl-glycine spots were found on the chromatograms of the urine from flavone-fed animals, but a single spot of hippuric acid, of equal size and intensity, was present on chromatograms of the experimental and control groups.

DISCUSSION

Although hydroxylation of flavone, yielding 4′-hydroxyflavone with small amounts of 3′,4′-dihydroxyflavone, has been shown to occur in the guinea pig, formation of aromatic acids from flavone occurs, if at all, to a very limited extent, as salicylic acid in trace amounts was the only compound of this type detected. In view of previous observations (Booth *et al.* 1956, 1958*a*,*b*; Griffiths, 1964) of the formation of a number of phenyl-acyl acids from ring B of a wide range of naturally occurring flavonoid molecules, it was expected that the corresponding aromatic acids, phenylpropionic acid or phenylacetic acid or their aroyl-glycine derivatives, would be formed in appreciable amounts if ring-fission of flavone occurred. The absence of these compounds suggests that flavone is more resistant to ring-fission than the naturally occurring flavonoids investigated previously (Booth *et al.* 1956, 1958*a*,*b*; Griffiths, 1964). If, however, flavonoid molecules are, as suggested by Masri, Booth & De Eds (1959), degraded from the A ring end of the molecule it would be expected that the absence of hydroxylation in this ring would render the molecule less susceptible to oxidative attack. The possibility that other hydroxyflavones may also be formed in smaller amounts cannot be ruled out, since other fluorescent spots were observed on the chromatograms but were not identified owing to the small amounts present and the absence of suitable chromatographic standards.

We are grateful to Professor T. A. Geissman, Professor T. R. Seshadri and Dr L. Jurd for their gifts of several hydroxyflavones. This investigation forms part of a Thesis to be submitted by N. P. D. to the University of Singapore in partial fulfilment of the requirements for the degree of Ph.D.

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