The Bacterial Degradation of Flavonoids

HYDROXYLATION OF THE A-RING OF TAXIFOLIN BY A SOIL PSEUDOMONAD

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Cell-free extracts prepared from a *Pseudomonas* sp. grown on (+)-catechin oxidized taxifolin in the presence of NAD(P)H and molecular oxygen. The enzyme catalysing the reaction was partially purified and shown to be a flavoprotein. The product was identified as 3',4',5,7,8-pentahydroxyflavanonol (dihydrogossypetin).

An investigation by I. S. Farr & W. C. Evans (unpublished work) showed taxifolin (3',4',5,7-tetrahydroxyflavanonol; II) to be an intermediate in the aerobic metabolism of (+)-catechin (3',4',5,7-tetrahydroxyflavanol; I) by an organism isolated from rat faeces. Taxifolin was shown to be further oxidized to protocatechuic acid, which by isotopic experiments was shown to arise from the B-ring of the flavanonol. The present study has shown the initial stage in the metabolism of taxifolin to be its hydroxylation to dihydrogossypetin (3',4',5,7,8-pentahydroxyflavanonol; III). A preliminary report has been made (Jeffrey *et al.*, 1969).

Materials and Methods

Micro-organism

Maintenance and growth of bacteria. The micro-organism used was *Pseudomonas* sp. (N.C.I.B. 9940)

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isolated under sterile conditions from rat faeces by I. S. Farr and W. C. Evans and was used in the work described above. It was maintained by monthly sub-culture on Oxoid nutrient agar slopes. Media for liquid cultures contained (g/l): KH_2PO_4 , 5.4; $(\text{NH}_4)_2\text{SO}_4$, 1.3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; also disodium succinate hexahydrate, 2.7, or (+)-catechin, 1.0, or protocatechuic acid, 1.15. The pH was adjusted to 7.0 with 2.5M-NaOH. Aromatic substrates were sterilized by filtration and added to the basal medium (sterilized by autoclaving at 121°C) immediately before inoculation. In the early exponential phase 0.2ml of a solution of trace elements (Bauchop & Elsdon, 1960) was added to 10-litre cultures. Large cultures (10 litres) were grown at 25°C in Pyrex glass reagent bottles, from 10% (v/v) inocula of catechin-grown cells in 1-litre and 250ml Erlenmeyer flasks. Cultures in Erlenmeyer flasks were aerated by shaking in a Gallenkamp Orbital shaker; 10-litre cultures were aerated through sintered-glass spargers.

Cells from the 10-litre cultures were harvested in the late exponential phase in a Sharples air-turbine

continuous-flow centrifuge. The cell paste was washed with 10 vol. of 0.1 M-potassium phosphate buffer, pH 7.0, by centrifugation at 10000g for 15 min (20°C) and was used immediately.

Preparation of cell-free extracts. The washed cells were suspended in an equal volume of 0.1 M-potassium phosphate buffer, pH 7.0, and disrupted in a Biox X-press (AB Biox, Nacka, Sweden). After treatment with deoxyribonuclease (about 0.1 mg/ml of extract) the extract was centrifuged for 30 min at 38000g. The supernatant was termed the 'crude cell-free extract'; it contained between 20 and 30 mg of protein/ml. All extracts were stored at -25°C; enzyme activities were stable for several months.

Enzyme assays

Taxifolin hydroxylase. This activity was measured from the decrease in extinction at 325 nm, the $\lambda_{\max}$ of the mono-anionic salt of taxifolin. Cuvettes contained, in a final volume of 2.5 ml: taxifolin, 0.125 μ mol; NADH, 0.15 μ mol; potassium phosphate buffer, pH 7.7, 200 μ mol. The change in E_{325} observed is due to changes in the spectra of both taxifolin and NADH. When measurements of taxifolin disappearance were made, NADH was replaced by an NADH-regenerating system, consisting of: NAD⁺, 0.1 μ mol; ethanol, 200 μ mol; alcohol dehydrogenase, 5 μ l (35 units). Assuming that the sample of (+)-taxifolin used was in the dihydrated form, $\epsilon_{325\text{nm}}$ in potassium phosphate buffer, pH 7.7, was calculated to be 3.03×10^4 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹.

Dihydrogossypetin oxygenase. This was assayed by following the decrease in absorption of dihydrogossypetin at 331 nm. Assays contained, in a final volume of 0.4 ml: dihydrogossypetin, 20 nmol; GSH, 0.4 μ mol; potassium phosphate buffer, pH 7.7, 40 μ mol; enzyme. When taxifolin hydroxylase was used to prepare the dihydrogossypetin *in situ*, the reaction was carried out as for that enzyme.

The ϵ_{331} for dihydrogossypetin was determined by enzyme-catalysed hydroxylation of (+)-taxifolin. The value obtained was 2.4×10^4 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹.

Protocatechuate 3,4-oxygenase. This was assayed by following the change in E_{290} (Stanier & Ingraham, 1954).

Spectrophotometric methods

Measurement in the visible or u.v. light were made with either a Unicam SP. 800B recording spectrophotometer or a Unicam SP. 500 spectrophotometer, with silica cuvettes of 1 cm light-path and of either 0.4 ml or 4.0 ml capacity. All kinetic determinations were made at 25°C. Anaerobic assays were performed in double-side-arm cuvettes (Evans, 1965). Oxygen was removed by first flushing the main volume of liquid with N₂ for 5 min and then evacuating with a rotary pump.

Infrared spectra were determined with a Perkin-Elmer model 137B Infracord spectrophotometer. Fluorimetric measurements were made with a Farrand spectrofluorimeter (Farrand Optical Co. Inc., New York, N.Y., U.S.A.).

Acidimetry

Measurements of acid uptake or release during enzyme reactions were made with an automatic titration assembly (Radiometer, Copenhagen, Denmark). Reactions were done at pH 7.7, this pH being maintained by the addition of 1 mM-NaOH or 1 mM-HCl to a reaction mixture (initial volume 1.0 ml) containing 0.1–0.2 μ mol of substrate: at higher taxifolin concentrations, enzyme inactivation occurred. Extracts were dialysed against 10 mM-potassium phosphate buffer, pH 7.7, before use. Measurements were made at 25°C under a slight positive pressure of CO₂-free air.

Determination of oxygen and protein

Changes in O₂ concentration were measured at 25°C with a Clark-type electrode (Rank Bros, Bottisham, Cambridge).

Protein concentration was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Thin-layer chromatography

Analytical t.l.c. was done on plates spread to a thickness of 0.25 mm, or for preparative work 1.0 mm, with cellulose powder MN 300 (Macherey, Nagel and Co.), silica gel (Kieselgel G; E. Merck A.G.) or polyamide (Polyamide Woelm TLC).

The solvents used were: (a) acetic acid–water (1:50, v/v); (b) butan-1-ol–acetic acid–water (63:10:27, by vol.); (c) the organic phase of butan-1-ol–acetic acid–water (4:1:5, by vol.); (d) butan-1-ol–ethanol–3% (w/v) disodium tetraborate (1:1:1, by vol.) (plates were first sprayed with satd. aq. disodium tetraborate and dried); (e) aq. 95% ethanol; (f) redistilled phenol–acetic acid–water (40:1:9, w/v/v) (chromatographed in the dark); (g) the organic phase of chloroform–acetic acid–water (2:2:1, by vol.); (h) benzene–dioxan–acetic acid (90:25:4, by vol.). Before cellulose plates were run in the second dimension, a line was cut immediately behind the material deposited at the solvent front so that the flow of the second solvent should not be retarded. Plates were dried in a current of air without heating.

Phenols were detected with the following spray reagents. (1) Doty's reagent for catechols (Chafetz *et al.*, 1968). (2) Folin-Ciocalteu reagent and NH₃ (Mitchell & Davies, 1954). Catechols often gave a faint-blue colour before exposure to NH₃, and the

flavonols, fisetin and quercetin, were always slow to react even in the presence of NH_3 ; gossypetin and quercetagenin reacted normally. (3) Vanillin (10%, w/v) in aq. 70% (v/v) H_2SO_4 (Swain, 1956). (4) Zirconium oxychloride (2%, w/v) in methanol (Hörhammer & Müller, 1954).

Special chemicals

Yeast alcohol dehydrogenase was obtained from Boehringer Corp. (London) Ltd., London W.5, U.K.; NAD^+ and NADH were from Sigma (London) Chemical Co. (Kingston-on-Thames, Surrey, U.K.); deoxyribonuclease was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; Folin-Ciocalteu reagent, bismuth oxycarbonate and zirconium oxychloride were from BDH Chemicals Ltd, Poole, Dorset, U.K.

Solvents used in enzyme assays, or for extracting products of enzyme reactions, were redistilled before use.

(+)-Catechin was isolated from block gambier by the method of Perkin & Yoshitake (1902) and (+)-taxifolin from *Pseudotsuga douglassi* (Pew, 1948). ($\pm$)-Taxifolin was prepared from quercetin by the procedure of Geissman & Lischner (1952). It was homogeneous on t.l.c. in four solvents (Table 1).

Reduction of flavanols by sodium amalgam

The method used was based on that of Hurst & Harborne (1967), with the following modifications. The quantities of reagents were decreased by a factor of ten and the sample (about 0.1 mg in ethanolic solution) was added after being adsorbed on to filter paper. The nitrogen (High Purity; Air Products Ltd., Manchester, U.K.) used was first passed through alkaline pyrogallol and then NaOH soln. to remove traces of O_2 . The reaction products were extracted three times with ethyl acetate (2 ml) and separated by two-dimensional t.l.c. on cellulose with solvent (g) followed by solvent (d), a system which gave clear separation of the phenols being investigated. Plates were sprayed either with reagent (1) followed by reagent (2), or with reagent (3).

Bismuth oxycarbonate oxidation of flavanols

The method of Mahesh & Seshadri (1955) was used, but with the following modifications. The flavanone (0.5–2.0 mg) was refluxed with 5 mg of bismuth oxycarbonate, 50 μl of acetic acid and 0.5 ml of AnalaR 'amyl alcohol'. Taxifolin was refluxed for 1.5 h; synthetic dihydrogossypetin and dihydroquercetagenin and enzyme reaction products were refluxed for 4 h. After being cooled, sufficient 1 M-HCl was added to dissolve the precipitate, and the organic phase was collected. This was extracted under

N_2 with 1% (w/v) disodium tetraborate, which was then acidified and extracted with ethyl acetate. After evaporation of the ethyl acetate under N_2 , the residue was analysed by two-dimensional t.l.c. on cellulose plates with solvents (c) and (f). Plates were sprayed with reagent (4) followed by (2), or the spots were eluted immediately for u.v.-spectral analysis.

Results

Growth of micro-organisms on catechols has often proved difficult because of the formation of inhibitory products by autoxidation. When catechin media were inoculated directly from nutrient agar slopes there was a long lag phase before growth started, during which autoxidation of the catechin occurred. The lag period could be shortened by using a 10% (v/v) inoculum grown on succinate medium. It was essential to restrict aeration during the lag phase by leaving flasks undisturbed in the incubator until growth was established. The addition of iron above the concentration used (18 μg of Fe^{2+} /litre) led to the formation of a blue catechin-iron chelate, which was readily autoxidized to products that inhibited growth: at the concentration used the chelate could not be seen and growth was not inhibited. With these precautions, catechin was found to be a good substrate for the organism, yielding about 0.3 g dry wt. of cells/litre of culture. The supernatant fluid of the cultures was pale yellow, and never brown.

Experiments with crude cell-free extracts

Cell-free extracts of catechin-grown cells catalysed changes in the spectrum of taxifolin only in the presence of both O_2 and NAD(P)H. Extracts prepared from organisms grown on protococatechuate did not oxidize taxifolin and the protococatechuate 3,4-oxygenase activity was essentially unchanged (0.49 unit/mg of protein in catechin-grown cells against 0.56 unit/mg of protein in protococatechuate-grown cells).

The maximum observed rate of taxifolin oxidation was at pH 8.0, above which measurements could not be made because of rapid autoxidation; below pH 8.0 activity decreased linearly until at pH 6.0 it was 20% of that at pH 8.0. The pH used in the taxifolin hydroxylase assay (pH 7.7) was a value that gave high activity without autoxidation. The rate of hydroxylation at this pH in 0.1 M-glycylglycine-KOH buffer was the same as in 0.1 M-potassium phosphate buffer; in 0.1 M-tris-HCl buffer the initial rate was 20% lower and did not remain linear with respect to time.

Crude extracts (20 mg of protein/ml) lost 25% of their taxifolin hydroxylase activity when heated for 10 min at 40°C and immediately cooled in ice-water; when heated to 50°C the proportion lost was 95%.

The protocatechuate oxygenase activity was unaffected. Taxifolin hydroxylase activity was not sedimented by centrifugation at 120000g for 1 h at 0°C, the pellet containing only 5% of the total activity. Both (+)- and (±)-taxifolin reacted at essentially the same rates (1.0 and 0.95 respectively). The enzyme did, however, show a specificity for reduced coenzyme, NADH being oxidized at twice the rate of NADPH. Neither phloroglucinol nor phloroglucinol carboxylic acid nor 4-hydroxybenzoic acid caused any decrease in the NADH concentration when added in the presence of crude cell-free extracts. NADH-dependent changes in the u.v. absorption were observed when (–)-fustin (3',4',7-trihydroxyflavanonol) was incubated with extracts, the absorption maximum shifting from 334 nm to 361 nm.

Partial purification of taxifolin hydroxylase

Since I. S. Farr & W. C. Evans (unpublished work) had obtained no evidence of intermediates occurring between taxifolin and protocatechuate, an attempt was made to identify such compounds by using fractionated cell-free extracts. All fractionation procedures were done between 0°C and 4°C in

0.1M-potassium phosphate buffer, pH7.0, except where stated.

Solid ammonium sulphate was slowly added to the stirred extract to 35% saturation. After equilibration for 30 min the precipitate was collected by centrifugation at 15000g for 20 min and redissolved in 0.1M-potassium phosphate buffer, pH7.0. This material was then applied to a column of Sephadex G-200, operating under reverse-flow conditions; the elution pattern is shown in Fig. 1. The combined active fractions (42–54) were dialysed for 3 h against water (10 vol.) before separation on a DEAE-cellulose column. Taxifolin hydroxylase was eluted from this column with 50 mM-potassium phosphate buffer, pH7.0; details of the conditions and results are given in Fig. 2. These treatments separated the enzyme from protocatechuate 3,4-oxygenase and from dihydrogossypetin oxygenase, an enzyme catalysing the oxidation of the product of taxifolin hydroxylase.

Resolution of the prosthetic group

Eluates from the DEAE-cellulose column that contained taxifolin hydroxylase activity were yellow with a fluorescence excitation spectrum having maxima at

Fig. 1. Separation of ammonium sulphate precipitate on Sephadex G-200

The sample (50 mg of protein in 2.5 ml of 0.1 M-potassium phosphate buffer, pH7.0) was applied to a column (90 cm × 1.5 cm diam.), operating under reverse-flow. Flow rate, variable between 0.12 and 0.20 ml/min. Fraction size, 2.0 ml. Taxifolin hydroxylase (.....), dihydrogossypetin oxygenase (—), protocatechuate 3,4-oxygenase (----) and percentage transmission at 254 nm (— — —) are all shown in arbitrary units. The abscissa is linear with respect to time but, owing to variation in the flow rate, not to volume of effluent.

Fig. 2. Separation of eluates from Sephadex G-200 on DEAE-cellulose

The sample (35 ml) was applied to a column (30 cm $\times$ 1.5 cm diam.) and eluted successively with 125 ml portions of 50 mM-potassium phosphate buffer, pH 7.0, and of 0.25 M-KCl in the same buffer. Fraction size, 2.0 ml. Taxifolin hydroxylase (—), dihydrogossypetin oxygenase (— · — · —) and the percentage transmission at 254 nm (—○—) are all shown in arbitrary units. The abscissa is linear with respect to time (see Fig. 1).

288, 373 and 560 nm, when measured at its emission maximum of 520 nm. This was consistent with a flavoprotein being present. Addition of riboflavin, FMN or FAD (all at 50 μ M) did not stimulate the initial rate of reaction. The flavin moiety was partially resolved by the following treatment. A sample (2 ml) of the enzyme preparation (DEAE-cellulose column eluate; 1 mg of protein/ml) was dialysed for 30 min against 100 ml of 0.1 M-potassium phosphate buffer, pH 7.0, containing ammonium sulphate (40% satd.) and taxifolin (50 μ M). The pH was then adjusted to 3.0 with 2 M-HCl and dialysis was continued for a further 2 h with occasional mixing of the precipitate. The dialysis residue was then further dialysed against 0.1 M-potassium phosphate buffer, pH 7.0, for 1 h. After the precipitate had been removed by centrifugation at 10000g for 10 min, the preparation had lost 95% of its original taxifolin hydroxylase activity. The residual activity was lost on keeping the preparation at 0°C for several hours. Restoration of about 60% of the original activity was obtained by the addition of FAD but not of FMN or riboflavin. After acid ammonium sulphate treatment, the rate of enzymic hydroxylation was not linear with respect to time, an effect which was accentuated by the addition of FMN. Linearity could be restored by preincubation of the enzyme for 2 min with 0.4 μ M-FAD. At this concentration, without preincubation, there was a lag of 15–20 s before the maximum velocity was reached, though at 4 μ M there was no detectable lag. As a precaution against loss of activity, 1 μ M-FAD was therefore added to all reaction mixtures when eluates from DEAE-cellulose were used.

Stoichiometry

Measurements of O₂ usage showed consumption of 0.8 mol and 1.0 mol of O₂/mol of NADH or of taxifolin respectively, whichever was limiting. With an NADH-generating system (ethanol and alcohol dehydrogenase) no acid production or consumption was observed during the reaction. However, if substrate quantities of NADH were used then 1.1 mol of acid was consumed/mol of taxifolin added, indicating that the phenolic product was in the same ionic state as taxifolin, which has pK_a 6.5.

Isolation and identification of the product of taxifolin hydroxylase

Although the preparation at this stage of purification could still be resolved into several components by disc-gel electrophoresis on polyacrylamide, repeat-scan analysis of the enzyme assay (Fig. 3a) indicated the formation of intermediates not previously detected. The changes in the life-time of absorbing products during the purification as enzymes catalysing latter steps in the degradation were removed also made it difficult to estimate the specific activity of the hydroxylase at later stages. By using fractionated enzyme preparations, which had been dialysed against 200 vol. of 10 mM-potassium phosphate buffer, pH 7.0, containing 1 μ M-FAD, reagents prepared in deionized water, and sufficient enzyme to bring the reaction to completion within 15 min, a compound was produced absorbing at 331 nm, the spectrum of which appeared stable for at least 30 min (Fig. 3b). The product was, however, sensitive to traces of metal ions, particularly Mn²⁺,

Fig. 3. Spectral changes associated with the hydroxylation of taxifolin catalysed by purified taxifolin hydroxylase

The assay was carried out under standard conditions as described in the text. (a) Reagents prepared with distilled water: curve 1 at 0min, curve 5 at 55min. (b) Reagents prepared in deionized water: curve 1 at 0min, curve 5 at 12min. Intervals between the scans in (a) and (b) are not constant.

in the presence of which the spectrum rapidly changed to one with maxima at 320 and 383 nm (Fig. 3a). This reaction was shown to be non-enzymic but was not further investigated.

The 331 nm-absorbing compound was isolated from scaled-up (200ml) assay mixtures. When the reaction was complete, as determined by following the spectral changes in a sample, the solution was acidified to pH 3.5 with 2M-HCl and evaporated to dryness under reduced pressure at <30°C. The residue was extracted twice with 5 ml of ethanol and the extract was evaporated to dryness under a stream of N₂. When the material was chromatographed on cellulose in solvent (b) it separated as a yellow band with *R_F* 0.64, and was almost pure as determined by u.v. absorption and reaction with spray reagent (2). As soon as the plates were dry the bands were scraped off and the material was eluted from the cellulose with ethyl acetate saturated with 50mM-HCl. The eluate, after evaporation to dryness under N₂, was chromatographically pure in all systems

Table 1. *R_F* values of the compound formed from taxifolin

The compounds were chromatographed and revealed by sprays as described in the text.

Support	Solvent	<i>R_F</i>	
		Enzymically produced compound	Taxifolin
Cellulose	(a)	0.30	0.37
Cellulose	(b)	0.64	0.81
Polyamide	(e)	0.21	0.34
Silica gel	(h)	0.00	0.17

tested (Table 1). If the plates were left after drying, the yellow areas developed a greenish tinge within a few hours.

When plates were sprayed with vanillin reagent, the compound became reddish purple, a reaction similar to that which was given by hydroxyquinol. Taxifolin initially gave a spot that was yellow, rather than red as would be expected from a resorcinol derivative; this is probably caused by hydrogen bonding between the 5-hydroxyl group and the carbonyl group in the 4-position. The u.v. spectrum of the isolated compound exhibits a bathochromic shift of 36nm from 295nm at pH 3.5 to 331 nm at pH 7.7. It gives a purple colour with zinc-HCl reagent (Pew, 1948). Both these properties are typical of flavanols.

T.l.c. analysis of the products of sodium amalgam reduction showed them to be identical with those obtained from taxifolin: phloroglucinol, resorcinol, 3,4-dihydroxyphenyl-carboxylic acid and -propionic acid, together with one catechol that did not co-chromatograph with any of the standard 3,4-dihydroxyphenyl compounds available. This was probably 3,4-dihydroxyphenylpropanol, which has been identified as a product from the reduction of 3',4'-dihydroxyflavonoids by Hurst & Harborne (1967). These results indicated that both aromatic rings were still intact and were consistent with hydroxylation having occurred in the A-ring. No pyrogallol was detected.

To confirm that hydroxylation had occurred in the A ring and to determine the position of the new group, the compound was specifically oxidized at positions 2 and 3 with bismuth oxycarbonate. The quercetin produced in preliminary experiments with taxifolin was contaminated with only small amounts of starting material. The product expected from the oxidation of the enzymically produced material was either gossypetin or quercetagenin. Difficulty was experienced in the chromatographic separation of these two flavonols. They could, however, be distinguished: (i) by the slightly higher *R_F* of gossypetin

in solvent (c) (0.65 compared with 0.60 for quercetagenin); (ii) by the greater tendency of gossypetin to autoxidation on standing, giving a blue-green spot; (iii) by the difference in colour reaction when sprayed with zirconium oxychloride, gossypetin giving a reddish-brown complex whereas that of quercetagenin was yellow. Their u.v. spectra in ethanol showed considerable differences: gossypetin had two peaks ($\lambda_{\max}$, 334 and 392nm), changing to a single peak ($\lambda_{\max}$, 418nm) on addition of zirconium oxychloride; that of quercetagenin had a single peak ($\lambda_{\max}$, 363nm), shifting to 442nm on addition of zirconium oxychloride. Gossypetin and quercetagenin differ from other flavonols such as fisetin and quercetin in that they absorb u.v. radiation rather than give a strong-yellow fluorescence, and react normally with spray reagent (2).

The enzymically produced compound, on specific oxidation of the 2,3-dihydro group with bismuth oxycarbonate, gave a flavonol indistinguishable by all the above criteria from gossypetin.

The i.r. spectrum of isolated enzyme-product (carbonyl frequency, 5.8 μ m in CHCl_3) was consistent with its structure being that of dihydrogossypetin (Briggs & Colebrook, 1962).

The mass spectrum was obtained with an AEI MS902 spectrometer, the direct insertion probe being used at a source temperature of 250°C and a 70eV beam. Ions at m/e 302 and 292, corresponding to losses of 18 and 28 mass units respectively from the molecular ion ($m/e=320$, 23%, $\text{C}_{15}\text{H}_{12}\text{O}_8$) (the calculated and experimental values of formulae of ions agreed within 2.25 p.p.m.), occurred at less than 1% relative intensity, but these transitions were supported by the presence of metastable peaks in the spectrum, as was the loss of CHO from m/e 152 (18%, $\text{C}_8\text{H}_8\text{O}_3$) to m/e 123 (18%, $\text{C}_7\text{H}_7\text{O}_2$). It is proposed that the major fragmentation process is a retro-Diels-Alder rearrangement, with both hydrogen and charge transfer, yielding ions at m/e values 169 (100%, $\text{C}_7\text{H}_5\text{O}_3$), 168 (28%) and 152 (cf. Pelter *et al.*, 1965). This process is compatible with either 6- or 8-hydroxytaxifolin.

Synthesis of dihydrogossypetin

An attempt was made to synthesize dihydrogossypetin by the following procedure. 3,3',4',7-Tetramethylquercetin was prepared by heating quercetin (17g dissolved in 200ml of acetone) with excess of dimethyl sulphate in the presence of anhydrous K_2CO_3 (45g) at 55°C for 2 days. The product was recrystallized from ethanol, in which quercetin is soluble; the unchanged quercetin was remethylated. The final yield was 10g, m.p. 158–159°C (lit. 159–160°C).

The 3,3',4',7-tetramethylquercetin was oxidized to 5,8-dihydroxy-3,3',4',7-tetramethoxyflavone with

potassium persulphate (Rao & Seshadri, 1947). It was recrystallized from 'amyl alcohol' to give 7.0g, m.p. 240–242°C, decomp. (lit. 250–252°C). When the 5,8-dihydroxy-3,3',4',7-tetramethoxyflavone was demethylated with either 48–50% (w/v) hydrobromic acid or freshly distilled hydriodic acid, it gave a heterogeneous product, separating on t.l.c. in solvent (b) into four distinct spots. Rao & Seshadri (1947) do not report any attempt at the demethylation of their product but only a further methylation to hexamethylgossypetin. This was done; the yield of hexamethylgossypetin, twice recrystallized from ethanol was 1.5g, m.p. 169–170°C (lit. 170–172°C). Demethylation of this product by refluxing with freshly distilled hydriodic acid for 35 min gave, after recrystallization from aq. ethanol saturated with SO_2 , almost pure gossypetin, contaminated only by traces of quercetagenin, as judged by t.l.c. and by the u.v. spectrum. Refluxing with hydrobromic acid for 6 h resulted in 95% conversion into quercetagenin, as determined spectrophotometrically.

The products of reduction of gossypetin and quercetagenin with alkaline dithionite (Pew, 1948) were extracted with ethyl acetate and separated as yellow bands by paper chromatography on Whatman 3MM paper with solvent (a). The product of both reductions had R_F 0.45; unchanged starting material remained almost at the origin (R_F 0.05). When the separation was done by t.l.c. in solvent (a) there were always two overlapping spots with R_F values 0.28 and 0.30. After elution with ethyl acetate saturated with 50mm-HCl, and oxidation with bismuth oxycarbonate for 4 h, the products were separated by two-dimensional t.l.c. with solvents (a) and (b). These flavanonols were more resistant to oxidation at the 2,3-dihydro positions than taxifolin and even after 4 h some had not reacted. The flavonols were eluted with 0.5 ml of aq. 95% (w/v) ethanol and their spectra recorded.

Both gossypetin and quercetagenin were recovered from the reaction mixture after this cycle of reduction and back-oxidation, regardless of which one was used as the starting material. The proportion of gossypetin recovered from gossypetin and quercetagenin in this way was calculated from the u.v. spectra, the values being 64% and 50% respectively. On oxidation the natural product gave only traces (<3%) of quercetagenin; the isomerization of the synthetic gossypetin must therefore have occurred during its reduction to dihydrogossypetin rather than during the back-oxidation. The above percentages can therefore be taken to represent the proportions present in the dihydro derivatives. Making the assumption that only the dihydrogossypetin is metabolized and that the $\epsilon_{331\text{nm}}$ of dihydrogossypetin and dihydroquercetagenin are identical, these values are in agreement with the proportions of the synthetic flavanonols degraded enzymically. Infrared spectra of the natural and

synthetic dihydrogossypetin were very similar, despite the known contamination of the latter by dihydroquercetagenin. The u.v.-spectral changes observed with the natural product in the presence of manganese ions occurred also with the synthetic material.

Discussion

In many respects taxifolin hydroxylase is similar to other bacterial hydroxylases that have been investigated: e.g. *p*-hydroxybenzoate hydroxylase (Hosokawa & Stanier, 1966) or salicylate hydroxylase (Katagiri *et al.*, 1965). Both enzymes contain bound FAD and require a reduced nicotinamide nucleotide coenzyme for activity; the former enzyme is specific for NADPH and the latter for NADH. The *p*-hydroxybenzoate hydroxylase is more restricted in its substrate specificity than salicylate hydroxylase. It would hydroxylate none of the substrates at more than 5% of the rate with *p*-hydroxybenzoate whereas salicylate hydroxylase will also hydroxylate several compounds with a hydroxyl group in the *o*-position to a free carboxyl.

Although substrate specificity for taxifolin hydroxylase could not be adequately tested with the limited range of flavonoids available it seems to be restricted to flavanonols: catechin (a flavanol), quercetin (a flavonol) and mollisacacin (a flavan-3,4-diol) were not metabolized by eluates from DEAE-cellulose, whereas (–)-fustin, (3',4',7'-trihydroxyflavanonol) did undergo the spectral changes and gave the O₂ consumption for hydroxylation, although the product was not identified. Mollisacacin was, however, oxidized by crude cell-free extracts in the presence of NAD⁺ to fustin (Jeffrey, 1969).

The differentiation of 6- and 8-substituted flavonoids is often difficult owing to the possibility of isomerization. For example, Chopin (1958) found that during the partial methylation of 5,6,7-trihydroxyflavanone either by treatment with diazomethane at room temperature or by refluxing with dimethyl sulphate and potassium carbonate in acetone the product obtained was 5,8-dihydroxy-7-methoxyflavanone; complete methylation caused the reversion to 5,6,7-trimethoxyflavanone.

The technique of complete methylation followed by reductive fission with sodium amalgam and identification of the products from the A-ring is therefore ambiguous when dealing with 6- and 8-hydroxyflavanones. The observation (Hurst & Harborne, 1967) that, on treatment with sodium amalgam, 5,7,8-trihydroxyflavones gave pyrogallol, and 5,6,7-trihydroxyflavones gave phloroglucinol may, however, still be applicable to other classes of flavonoids in which the heterocyclic ring is stable to heating in alkali. When the compound is able to

undergo isomerization, as is dihydrogossypetin, the A-ring behaves as a substituted 1,2,3,5-tetrahydroxybenzene, yielding phloroglucinol. Tetrahydroxybenzene itself gave phloroglucinol (cf. Posternak, 1936).

None of the compounds discussed in the literature contains a hydroxyl group in the 3-position, which increases the rate of cyclization of chalcones in acid solution, so no assessment can be made regarding the general way in which this might affect the final equilibrium. Lindstedt & Misiorny (1951) were unable to differentiate between 5,7-dihydroxy-6-methylflavanonol and the 8-methyl isomer. Jain & Seshadri (1953) found that flavonols were more resistant to isomerization than the corresponding flavone. Apart from this, the only case of isomerization of flavanonols studied is that reported here of dihydrogossypetin and dihydroquercetagenin, a transposition in which the equilibrium appears to lie between 50% and 65% in favour of dihydrogossypetin.

The method of oxidation by bismuth oxycarbonate has in this particular instance given unequivocal information regarding the structure of the flavanonol, presumably because the rate of oxidation is much greater than isomerization under the conditions used. It would be interesting to know how wide an application this method has in the differentiation of 6- from 8-hydroxyflavanones.

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