New Oxygenases in the Degradation of Flavones and Flavanones by *Pseudomonas putida*†

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ABSTRACT: A bacterium, identified as *Pseudomonas putida*, was isolated by elective culture on quercetin (3,3′,4′,5,7-pentahydroxyflavone) as sole carbon and energy source. Protocatechuic acid was the sole product that could be isolated from culture filtrates of quercetin grown bacteria. Quercetin consumed approximately 4 mol of oxygen/mol of substrate when cell-free extracts were supplemented with reduced nicotinamide adenine dinucleotide (NADH). The enzymes involved in the oxidative degradation of quercetin showed a considerable lack of specificity since a number of other flavones and flavanones with a hydroxyl at C_7 (Figure 1) consumed either 2 or 3 mol of oxygen/mol of substrate. The third mole of oxygen was consumed when, in addition to the hydroxyl at C_7, the flavone had a hydroxyl at C_3 and met certain other structural criteria. The consumption of a fourth mole of oxygen in the case of quercetin was shown to be due to the oxidative ring cleavage of protocatechuic acid by a 3,4-dioxygenase. The oxygenases which catalyzed the uptake of the first 2 mol of oxygen/mol of quercetin were separated by ultracentrifugation from those which catalyzed the third and fourth moles of oxygen consumption. Furthermore, the first two oxidative enzymes in the degradation of quercetin were separated from each other by agarose chromatography. The first enzyme in the sequence was shown to be a mixed function oxygenase which introduced a hydroxyl at C_8 of the flavone ring. NADH was demonstrated to be an obligate cofactor for this reaction. The second enzymatic step in quercetin degradation was shown to involve a dioxygenase which cleaved the flavone ring adjacent to the hydroxyl substituent at C_8 (extradiol cleavage) to give a product which rearranged to a 2-pyrone. Studies with flavonoid analogs have facilitated an understanding of flavone binding to these new oxygenases, and a general metabolic pathway for the oxidation of the flavone ring by *Pseudomonas putida* has been elucidated.

Recent work on the degradation of aromatic compounds by soil bacteria has focused primarily on simple one-ring systems. The compounds studied have been either synthetic compounds such as benzene, toluene, and cresol (Gibson *et al.*, 1970a,b; Bayley *et al.*, 1966), or small naturally occurring aromatic compounds such as catechol, protocatechuic acid, and gentisic acid (Dagley, 1971). Studies with the former group have demonstrated the amazing versatility of soil pseudomonads in degrading a wide array of compounds. Studies on the latter group have established a multitude of pathways by which such compounds are metabolized. Compounds such as protocatechuic acid and gentisic acid, however, exist in their natural states for the most part as components of larger more complex natural products, tannins, lignans, and flavones. It is important therefore to study the degradation of these larger natural products in order to gain a fuller picture of the events which comprise the complete assimilation of organic matter in the soil.

We have already investigated the question of lignan degradation (Toms and Wood, 1970a,b). We have now turned our attention to the problem of flavonoid degradation. Quercetin, 3,3′,4′,5,7-pentahydroxyflavone (Figure 1), was chosen as a growth substrate because it is the most common flavone, and flavones are the most widespread of all flavonoids (Harborne, 1967).

Jeffrey *et al.* (1972a,b) studied the degradation of the flavanone taxifolin (dihydroquercetin) by a soil pseudomonad grown with (+)-catechin (3′,4′,5,7-tetrahydroxyflavanonol) as sole carbon source. Oxygenases, which required NAD(P)H for the hydroxylation of taxifolin at C_8 to give dihydrogossypetin, and which cleaved dihydrogossypetin by an extradiol mechanism, were found to be present in cell extracts of this *Pseudomonas spp.* Krishnamurty and Simpson (1970) found that the mold *Aspergillus flavus* induced a dioxygenase which displaced C_3 of the flavone ring as carbon monoxide to give a depside, which then underwent hydrolysis to protocatechuic acid and 2,4,6-trihydroxybenzoic acid. Barz (1969) isolated a bacterium from the rhizosphere of *Cicer certietinum* which metabolized isoflavones, chalcones, flavones, and flavone glycosides, but which did not appear to metabolize flavanones.

In this paper we report a general pathway for the degradation of flavones and flavanones, similar in some respects to that reported by Jeffrey *et al.* (1972a,b), but having a far greater spectrum of activity for the most common flavonoids. A preliminary report of this research has already been presented (Schultz and Wood, 1972).

Materials and Methods

Biological Materials. A gram-negative, motile, asporogenous rod which was classified as *Pseudomonas putida* was isolated from soil by elective culture on quercetin as sole carbon and energy source. This organism was classified as *Pseudomonas putida* on the basis of the following tests (Stanier *et al.*, 1966): King A and King B agar slopes were cultured at 30°; they showed that the organism is a nonfluorescent *Pseudomonas;* ortho cleavage of catechols occurred for both catechol and protocatechuic acid; gelatin was not liquified during 5 days of culture, and the organism did not grow anaerobically with glucose as carbon source. Cells were grown either in 16-l. carboys with forced aeration or in a 120-l.

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fermenter at 30°, serial inoculations having been made from 1-l. starter cultures. Growth media had the following composition: quercetin (0.2 g/l.), Casamino acids (0.04 g/l.), L-threonine (0.04 g/l.), potassium hydrogen phosphate (2 g/l.), ammonium sulfate (1 g/l.), magnesium sulfate (0.2 g/l.), and ferrous sulfate (3.0 mg/l.), adjusted to pH 7.30 with concentrated hydrochloric acid. Casamino acids and L-threonine were added solely to enhance bacterial growth. Quercetin was added as a half-saturated solution in dimethylformamide. Additional quercetin was added during the course of growth. Cells were harvested in the late exponential growth phase (12–24 hr). Whole cells were stored at −20° without significant loss of activity for periods of several months.

Cell extracts were obtained by two methods: (a) cell paste was suspended in 0.05 M phosphate buffer (pH 7.30) (1 g of wet cells/2.0 ml of buffer) and sonically disrupted at the maximum frequency of a Branson sonic probe at 0°, with 20-sec pulses, until breakage was complete. After treatment of this sonicated extract with DNase I and RNase A for 1 hr, particulate matter was removed by centrifugation at 37,500*g* for 1 hr. (b) Frozen cells (20–25 g) were disrupted in a Hughes press. Broken cells were removed from the Hughes press and suspended in 80–100 ml of 0.05 M phosphate buffer (pH 7.30), followed by digestion with RNase and DNase and centrifugation at 37,500*g*.

Enzyme Assays. 7-Hydroxyflavone 8-hydroxylase was assayed by measuring oxygen consumption with a YSI Clark oxygen electrode assembly on a Gilson Model K oxygraph. The reaction mixture, in a final volume of 1.50 ml (pH 7.30), contained 75 μmol of phosphate buffer, 0.33 μmol of NADH, 0.05 μmol of flavone substrate, and enzyme unless otherwise stated. Reactions were initiated by injecting substrate (5 μl dissolved in dimethylformamide) through the capillary port of the vessel. The Gilson oxygraph was polarized at −0.8 V; the vessel was jacketed and the temperature was maintained at 28° during the course of the reaction. One unit of enzyme activity was defined as that amount which consumes 1 μmol of oxygen/min at 28°. Specific activity was units/mg of protein. Protein concentrations were determined by the method of Lowry *et al.* (1951).

7,8-Dihydroxyflavone dioxygenase was also assayed by measuring oxygen uptake under the same conditions as described above for 7-hydroxyflavone 8-hydroxylase except that NADH was not included. Final reaction mixtures (in 1.50 ml), at pH 7.30, contained 75 μmol of phosphate buffer, 0.05 μmol of flavone substrate, and enzyme. Reactions were started by injection of substrate (5 μl dissolved in ethyl alcohol).

Protocatechuate dioxygenase was assayed by following oxygen uptake in a reaction mixture at pH 7.30 containing 75 μmol of phosphate buffer, 0.10 μmol of protocatechuic acid, and enzyme.

Synthesis and Purification of Flavonoids. Norwogonin (5,7,8-trihydroxyflavone) was synthesized from chrysin by the method of Rao *et al.* (1947). 7,8-Dihydroxyflavone was synthesized by fusion of 2′,3′,4′-trihydroxyacetophenone, benzoic acid, and benzoic anhydride according to the method of Venkataraman (1929). 8-Hydroxyacacetin (5,7,8-trihydroxy-4′-methoxyflavone) was synthesized from acacetin by the method of Rao *et al.* (1949). Apigenin (4′,5,7-trihydroxyflavone), chrysin (5,7-dihydroxyflavone), morin (2′,3,4′,5,7-pentahydroxyflavone), techtochrysin (5-hydroxy-7-methoxyflavone), acacetin (5,7-dihydroxy-4′-methoxyflavone), and Biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) were obtained from Aldrich Chemical Co. Galangin (3,5,7-trihydroxyflavone), kaempferol (3,4′,5,7-tetrahydroxyflavone), hesperetin (4′-methoxy-3′,5,7-trihydroxyflavanone), 3-hydroxyflavone, 7-hydroxyflavone, and taxifolin (3,3′,4′,5,7-pentahydroxyflavanone) were obtained from Pfaltz and Bauer. Naringenin (4′,5,7-trihydroxyflavanone), quercetin (3,3′,4′,5,7-pentahydroxyflavone), and rutin (3′,4′,5,7-tetrahydroxy-3-*O*-rhamnoglucosylflavone) were obtained from Sigma. Fisetin (3,3′,4′,7-tetrahydroxyflavone) was purchased from Baker. 8-Hydroxyflavone, 7-methylflavone, and 7-hydroxy-4-methoxy-5-methylflavone were purchased from Alfred Bader Chemicals. 2′,4,4′-Trihydroxychalcone (listed as 2,4,4′) was obtained from K & K Laboratories.

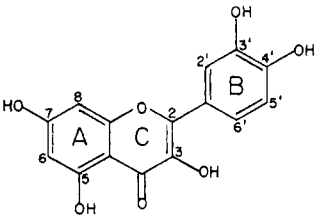


FIGURE 1: Quercetin and the numbering of the flavone ring system and ring designation. Flavanones lack the double bond between C-2 and C-3; isoflavones have the B ring attached at C-3 instead of C-2.

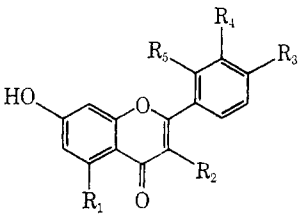
All flavones were purified by recrystallization to at least 99% purity before being used in enzyme assays. Synthetic materials were purified by polyamide chromatography prior to crystallization from appropriate solvents. Commercially available fisetin required extensive purification by polyamide chromatography before material of satisfactory purity could be obtained. Physical and chemical properties of all purified flavones were checked against properties documented by Mabry *et al.* (1970) and from other literature sources to ascertain that the materials had the structure stated by the manufacturer. This was found to be necessary, because several compounds which were available commercially did not have the structure listed.

Other Chemicals and Materials. NADH, NADPH, DNase I, and RNase A were purchased from Sigma. Protocatechuic acid was purchased from K & K Laboratories and was recrystallized from toluene. Polyamide for column chromatography (Polyclar AT) was obtained from GAF. Agarose, Bio-Gel A 1.5m, 100–200 mesh, for protein purification, was purchased from Bio-Rad. All other chemicals were of the highest purity commercially available.

Analytical Methods and Instrumentation. Thin-layer chromatographic (tlc) analyses were done on polyamide and silica plates. MN-Polygram polyamide-6-UV_{254} tlc plates from Brinkmann were developed with the following solvent systems: acetone–water–95% ethanol (2:2:1) and chloroform–methanol–butan-2-one (12:2:1). Spots were resolved under a uv lamp and in addition sprayed with methanolic ferric chloride as a test for phenols. Eastman Silica Chromagram Sheets Type 6060 with fluorescent indicator were used with benzene–dioxane–acetic acid (90:25:5) and chloroform–methanol–water (65:25:4) as developing solvents. Spots were resolved in the same manner as for the polyamide plates.

Mass spectra were obtained on a Varian-MAT CH-5 spectrometer. Infrared spectra were recorded on a Perkin-Elmer infracord with samples prepared as Nujol mulls. Nuclear magnetic resonance spectra were taken routinely on a Varian A60 spectrometer; high-resolution spectra were done on a Varian 220-MHz spectrometer. Ultraviolet-visible spectra were recorded on a Perkin-Elmer/Coleman 124 double

TABLE I: Substrates of Flavone Oxygenases.



Substrate	Moles of O_2 Consumed/Mole of Substrate: Sonified Crude Extract[a]	120,000g Supernatant[b]
R_1–R_5 = H (7-hydroxyflavone)	2.08	
R_1 = OH; R_2–R_5 = H (chrysin)	2.18	1.72
R_1 = R_3 = OH; R_2 = R_4 = R_5 = H (apigenin)	2.10	1.74
R_1 = OH; R_3 = OCH_3; R_2 = R_4 = R_5 = H (acacetin)	2.05	1.71
R_1 = CH_3; R_3 = OCH_3; R_2 = R_4 = R_5 = H (7-hydroxy-5-methyl-4′-methoxyflavone)	1.93	
R_1 = R_2 = OH; R_3–R_5 = H (galangin)	2.69	1.58
R_1–R_3 = OH; R_4 = R_5 = H (kaempferol)	3.09	2.16
R_1 = R_5 = H; R_2–R_4 = OH (fisetin)	2.10	1.83
R_1–R_3 = R_5 = OH; R_4 = H (morin)	2.17	
R_1–R_4 = OH; R_5 = H (quercetin)	3.93	2.12

[a] Values obtained are average of 3–5 different enzyme preparations. A total volume of 1.50 ml contained 75 μmol of phosphate buffer (pH 7.30), 0.33–0.66 μmol of NADH, 0.05–0.10 μmol of flavone substrate, and enzymes. [b] Values obtained are average of two different preparations with the same experimental conditions as above.

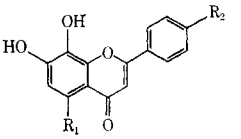
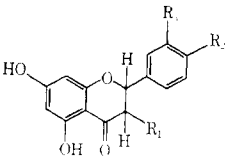
beam spectrometer. Melting points (uncorrected) were determined with a home built hot-stage-microscope apparatus.

Protein solutions were concentrated by pressure dialysis using Amicon Model 52, 401S, and 402 ultrafiltration cells fitted with PM-30 membranes (also from Amicon).

Results

Isolation of Protocatechuic Acid from Culture Filtrates. Culture filtrates of quercetin grown bacteria (120 l.) were acidified to pH 2.50 with concentrated sulfuric acid and extracted with 40 l. of ethyl acetate. The ethyl acetate extract was dried by passage through anhydrous sodium sulfate, and concentrated to 100 ml. Analytical thin-layer chromatography on silica in both solvent systems (Methods) showed four spots other than quercetin; 20 ml of the above material was applied to a polyamide column (21.5 × 2.5 cm) generated in acetone. The column was eluted with an increasing concentration of methanol; 15-ml fractions were collected. Material adjudged to be protocatechuic acid by tlc analyses emerged between tubes 70 and 82 at acetone–methanol 30:70. The contents of tubes were pooled and evaporated to dryness. The resultant white powder was recrystallized from ether–methylene dichloride; upon drying, 25 mg of material was obtained (mp 197–199°, lit. 199°).

TABLE II: Substrates of Flavone Oxygenases.

Substrate	Moles of O_2 Consumed/Mole of Substrate: Sonified Crude Extract[a]	120,000g Supernatant[a]
		
R_1 = R_2 = H (7,8-dihydroxyflavone)	1.08	
R_1 = OH; R_2 = OCH_3 (8-hydroxyacacetin)	1.08	
R_1 = OH; R_2 = H (norwogonin)	1.16	1.08
		
R_1–R_3 = OH (taxifolin)	2.02	
R_1 = R_3 = H; R_2 = OH (naringenin)	1.89	
R_1 = H; R_2 = OCH_3; R_3 = OH (hesperetin)	1.98	

[a] See Table I.

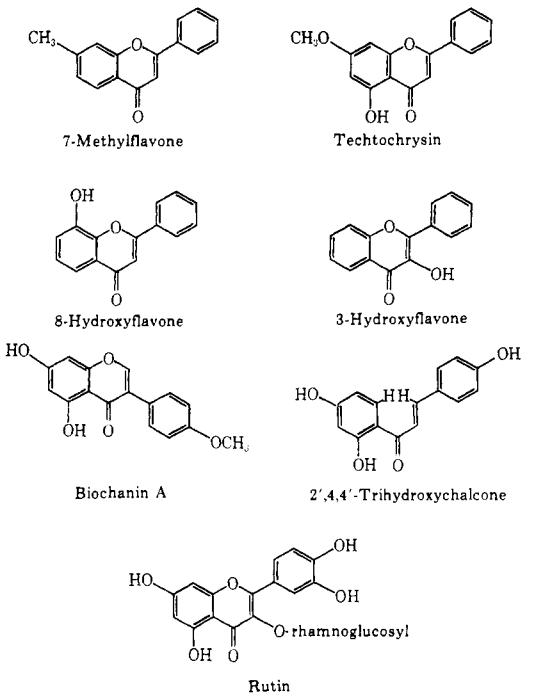
Anal. Calcd: C, 48.84; H, 4.65. C, 48.80; H, 4.47. Nujol mulls on 2 mg of isolated and authentic protocatechuic acid showed identical peaks upon ir analysis. Mass spectral analysis further confirmed the structure as being protocatechuic acid.

Experiments with Cell Extracts. Crude extract, obtained by sonic disruption, showed no oxygen consumption with quercetin unless NADH was added to reaction mixtures; NADPH was totally ineffective in place of NADH. There was no oxygen consumption with NADH in the absence of substrate, therefore no correction for NADH oxidase activity was required. Also crude extracts oxidized protocatechuic acid consuming 1 mol of oxygen/mol of substrate with no external cofactor requirement.

Stoichiometry of oxygen consumption was determined for several flavonoid analogs of the growth substrate quercetin. The compounds in Table I, and also taxifolin, naringenin, and hesperetin in Table II, possessed a hydroxyl at C_7 and were oxidized only when NADH was added. The uptake of oxygen was about 2 mol/mol for each substrate except for kaempferol and galangin (about 3 mol) and quercetin (4 mol). However, 7,8-dihydroxyflavone, 8-hydroxyacacetin, and norwogonin (Table II), which have a hydroxyl at C_8 in addition to a hydroxyl at C_7, each consumed about 1 mol of oxygen/mol of substrate. For the latter substrates, NADH was not required in order to observe oxygen consumption. The following flavones were nonsubstrates: 7-methylflavone, techtochrysin, 8-hydroxyflavone, 3-hydroxyflavone, and rutin. In addition the isoflavone, Biochanin A, and 2′,4,4′-trihydroxychalcone were nonsubstrates (Table III).

The supernatant from the centrifugation of crude extract at 120,000g for 2 hr showed a decrease in the oxygen consumption for quercetin, kaempferol, and galangin to ap-

TABLE III: Nonsubstrates of Flavone Oxygenases.



proximately 2 mol of oxygen/mol of substrate. Chrysin, apigenin, acacetin, and fisetin remained at 2 mol of oxygen consumed/mol of substrate. With the supernatant from ultracentrifuged cell extracts, the 7,8-dihydroxyflavones consumed 1 mol of O_2/mol of substrate (Tables I and II). When the pellet from the ultracentrifuged extracts was resuspended and added back to the products accumulated from the oxidation of quercetin, galangin, and kaempferol, respectively, after catalysis by supernatant, additional O_2 uptake to stoichiometry for these three substrates was observed. When this experiment was repeated with chrysin, apigenin, acacetin, fisetin, or norwogonin as substrate, no additional O_2 consumption was observed (Figure 2).

Assays for the hydroxylase activity using an NADH dependent substrate, and for the dioxygenase using a 7,8-dihydroxylated substrate, indicated that greater than 95% of each activity was recovered in the supernatant upon ultracentrifugation. Assay for the protocatechuic acid dioxygenase activity indicated that no more than 5% was retained in the supernatant. Therefore, for the degradation of quercetin, the first two oxidative enzymes can be separated from the third and fourth oxidative enzymes by ultracentrifugation.

The position of ring cleavage for protocatechuic acid was determined to be between C_3 and C_4 by using the Rothera test (Rothera, 1908).

Separation and Partial Purification of Enzymes. Pressure dialysis of the 120,000*g* supernatant and chromatography on agarose led to the almost complete separation of the enzyme mediating the NADH dependent flavone oxidation (7-hydroxyflavone 8-hydroxylase) from that catalyzing the NADH independent oxidation (7,8-dihydroxyflavone dioxygenase) (Figure 3). The preparation of 7,8-dihydroxyflavone dioxygenase was stable at 4° for several weeks without significant loss of activity. However, 7-hydroxyflavone 8-hydroxylase rapidly lost activity unless ethanol (10%) was added to stabilize the protein. 7,8-Dihydroxyflavone dioxygenase has

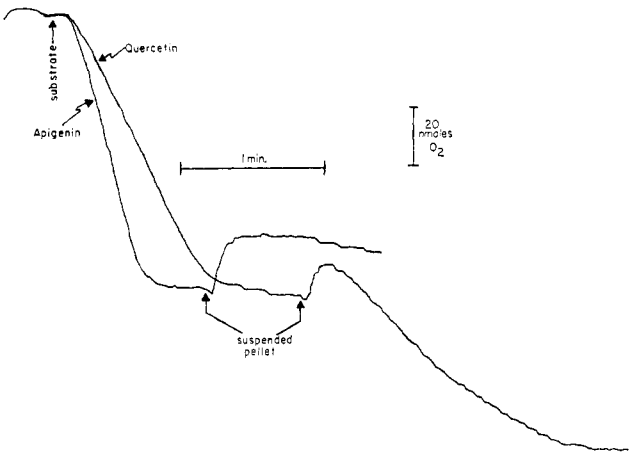


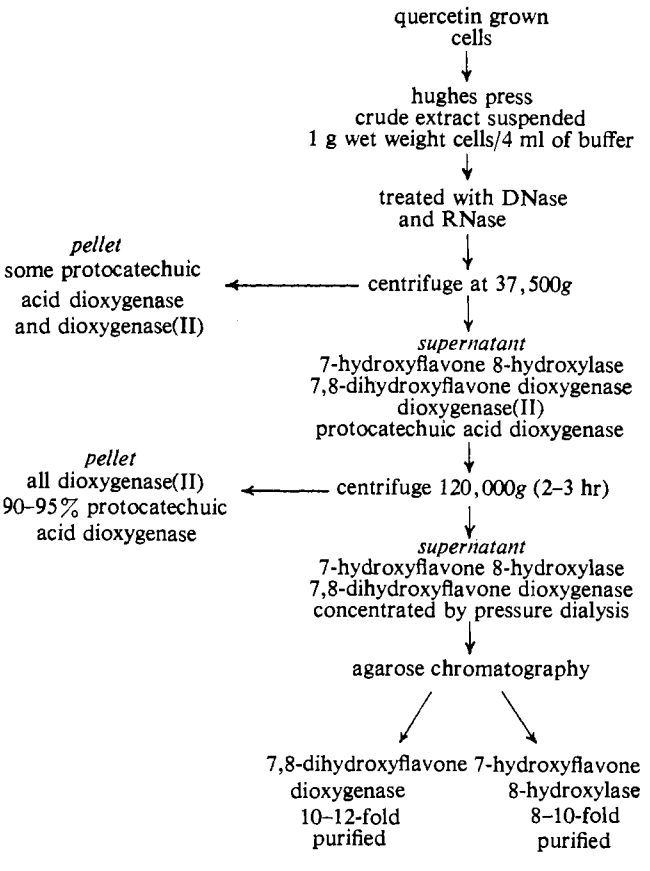
FIGURE 2: Oxygen consumption catalyzed by flavone oxygenases. Oxygen electrode traces of O_2 consumption with 75 μmol of phosphate buffer, 0.33 μmol of NADH, 0.05 μmol of flavone substrate, and 0.10 ml of 120,000*g* supernatant enzyme in a total volume of 1.50 ml (pH 7.30); reaction started by injection of flavone substrate dissolved in dimethylformamide; 0.10 ml of suspended 120,000*g* pellet in 0.05 M phosphate buffer injected after completion of initial oxygen consumption.

now been purified to homogeneity and its properties have been extensively studied (E. Schultz and J. M. Wood, unpublished results).

Scheme I outlines the general methodology involved in the separation and partial purification of the enzymes involved in quercetin degradation.

Hydroxylation of the Flavone Ring. Preparations of 7-hydroxyflavone 8-hydroxylase free of 7,8-dihydroxyflavone dioxygenase were concentrated by pressure dialysis and used

SCHEME I



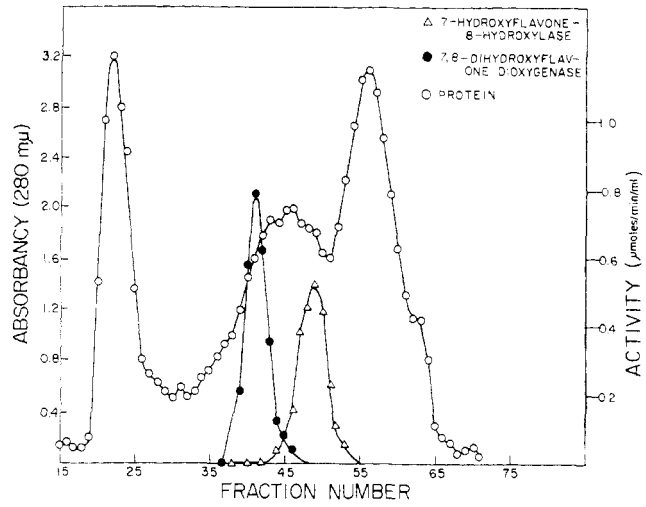


FIGURE 3: Agarose chromatography of concentrated 120,000*g* supernatant; 5 ml of concentrated 120,000*g* supernatant was applied to a column (2.2 × 95 cm) that had been equilibrated in 0.05 M phosphate buffer (pH 7.30), and the protein was eluted with the same buffer. Fractions of 6.5 ml were collected at a flow rate of 39 ml/hr. 7,8-Dihydroxyflavone dioxygenase was assayed with norwogonin as substrate (1.50 ml final reaction volume, pH 7.30, contained 75 μmol of phosphate buffer, 0.05 μmol of norwogonin, and 0.30 ml of column fraction eluate). 7-Hydroxyflavone 8-hydroxylase was assayed with chrysin (1.50 ml final reaction volume, pH 7.30, contained 75 μmol of phosphate buffer, 0.33 μmol of NADH, 0.05 μmol of chrysin, and 0.30 ml of column fraction eluate). Absorbancy at 280 mμ was that of undiluted tubes.

to accumulate the products of the enzymatic reaction with chrysin and 7-hydroxyflavone. Chrysin and 7-hydroxyflavone were used instead of quercetin because of autooxidation problems associated with highly hydroxylated flavones such as gossypetin (*i.e.*, the product expected from quercetin); additionally such products are far more difficult to purify and crystallize. Norwogonin was isolated as the product of the hydroxylation of chrysin from a reaction mixture which contained 75 mmol of phosphate buffer (pH 7.3), 350 mg of NADH, 54 mg of chrysin, and enzyme preparation. The chrysin dissolved in dimethylformamide was added dropwise over a period of 5 min. The total reaction volume was 1500 ml and the temperature 25°. The reaction products were extracted with 3 l. of ethyl acetate followed by filtration of the ethyl acetate extract through anhydrous sodium sulfate and evaporation to dryness. The residue was recrystallized from ethanol and water, and the resulting orange colored needles were dried over P_2O_5 (yield, 40 mg; mp 258–260°).

Thin-layer chromatography in two solvent systems on polyamide plates (Methods) demonstrated that the product comigrates with authentic norwogonin. The product gave an identical ir spectrum to norwogonin. Mass spectral analysis showed identical fragmentation patterns for both the isolated product and norwogonin.

In a similar experiment 7,8-dihydroxyflavone was isolated as the product of enzymatic hydroxylation of 7-hydroxyflavone. Ethyl acetate extracted reaction products were concentrated to 2.0 ml and applied to a polyamide column (2 × 12 cm) in order to remove any nonaromatic impurity. The column which had been equilibrated with acetone–ethanol–water (2:1:2) was eluted with the same solvent, and 2.0-ml fractions were collected. The contents of fractions 51–76 were pooled and evaporated to dryness. The pale brown deposit was recrystallized from ethyl acetate–petroleum ether (yield; 15 mg; mp 227–233° with sublimation). The 220-MHz

TABLE IV: Computer Printout of Mass Spectral Peaks.[a]

	Rel Intensity			
	10 eV		70 eV	
m/e	H	D	H	D
69	0.94	1.76	39.73	24.49
70	0.29	4.36	1.70	40.31
71		0.68	0.28	3.49
77	0.39	0.84	33.08	46.90
78			3.32	8.73
105	100.00	100.00	100.00	100.00
106	8.05	8.10	9.12	9.93
147	5.77	1.93	10.82	1.48
148	0.45	3.79	0.96	4.30
149	0.38	4.24	0.22	6.36
150		0.68		0.62
160	3.06	0.74	2.23	0.45
161	0.27	2.97	0.27	1.38
162		4.85		2.46
163		3.76		1.93
164	0.24	0.38		0.27
187	3.04	0.58	2.72	0.47
188	4.33	1.64	1.51	1.44
189	0.41	5.07	0.29	2.30
190		6.59		1.94
191		5.01		1.55
192		1.01		0.55
197	3.81		3.76	
198	0.97		0.35	0.20
199	0.38	1.26	0.28	0.54
200		2.99	0.12	1.20
201		3.05	0.11	1.61
202	1.34	0.54	0.98	0.31
203	0.28	0.32		0.37
215	43.81	5.51	39.80	4.28
216	4.84	26.44	5.72	16.58
217	0.51	33.31	0.72	20.84
218		5.44		2.64
230	18.74	1.19	6.28	0.46
231	2.45	2.30	0.92	0.89
232	0.74	5.17	0.10	1.45
233	0.27	8.53		2.73
234		7.91		2.68
235		4.00		1.14
236		1.32		0.33
257	48.60	0.76	20.54	0.38
258	7.43	7.13	3.28	1.49
259	1.08	23.80	0.39	5.37
260		32.79		7.59
261		16.24		3.06
262		2.20		0.45
302	66.11	0.25	25.68	
303	8.74	2.59	4.46	0.27
304	2.00	12.37	0.74	1.14
305		36.17		2.91
306		47.25		3.35
307		10.33		0.94
308		2.22		0.16

[a] Peaks above *m/e* 60 with greater than 3% relative intensity and their isotopes.

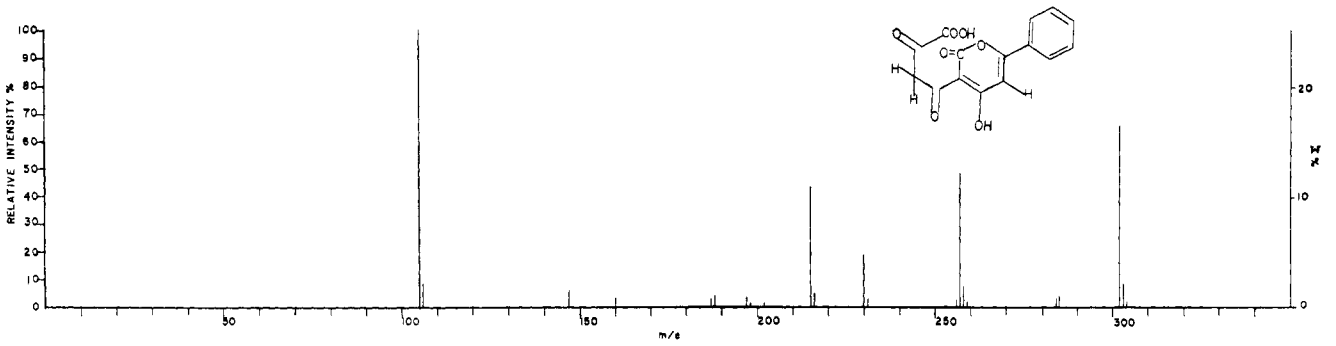


FIGURE 4: The mass spectrum at 10 eV of the isolated product of enzymatic reaction with norwogonin.

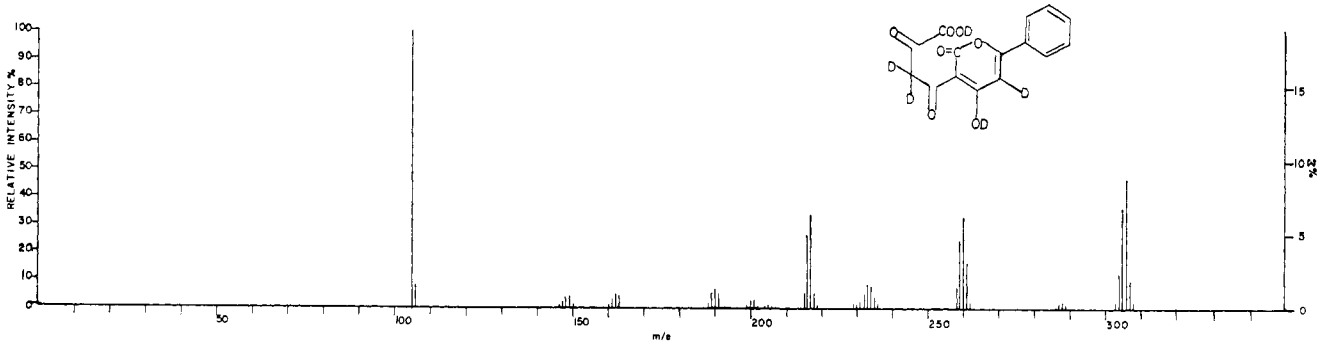


FIGURE 5: Mass spectrum at 10 eV of the deuterium exchanged product of enzymatic reaction with norwogonin.

nmr spectrum taken on 4 mg of this reaction product established the structure of the product as 7,8-dihydroxyflavone. The hydrogen at C_2 appeared as a singlet at δ 6.90. The hydrogen at C_6 was an ortho split doublet at δ 6.98 with J_{56} = 8.5 H_z; similarly the 5 H appeared as a symmetric ortho split doublet at δ 7.43; the B ring showed a complex signal centered at δ 7.60 (3′,4′,5′) and 8.16 (2′,6′) which integrates to five protons.

Cleavage of the Flavone Ring. The ring cleavage product formed by the enzymatic oxidation of norwogonin was isolated and characterized. The final reaction mixture of 300 ml contained 15 mmol of phosphate buffer and enzyme preparation (7,8-dihydroxyflavone dioxygenase obtained by agarose chromatography). A 0.02 M solution of norwogonin in ethanol was added in 0.5-ml aliquots until 13.0 ml of norwogonin (70 mg) had been added to the reaction mixture. The reaction was carried out at 25° over the course of 15 min. Extraction with 900 ml of ethyl acetate at pH 7.30 removed any unoxidized norwogonin. The resulting aqueous solution was then adjusted to pH 2.50 with H_2SO_4 and extracted with a further 900 ml of ethyl acetate. The ethyl acetate extract was dried over anhydrous sodium sulfate and evaporated to dryness. The amorphous product was dissolved in 10 ml of acetone and recrystallized from acetone and water (yield, 10 mg).

The isolated product melted at 191–193° with decomposition. Mass spectral analysis indicated the incorporation of 32 mass units (O_2) into norwogonin giving a molecular ion of *m/e* 302. The 10-eV mass spectrum indicated a base peak at *m/e* 105 with significant peaks at *m/e* 257, 230, 215, 197, 188, 187, 160, and 147 (Figure 4). The 70-eV spectrum showed large decreases in the relative intensity of the molecular ion as well as peaks at *m/e* 257, 230, and 188. New large peaks appeared at *m/e* 77 and 69, but the base peak remained *m/e* 105 (Table IV, under H).

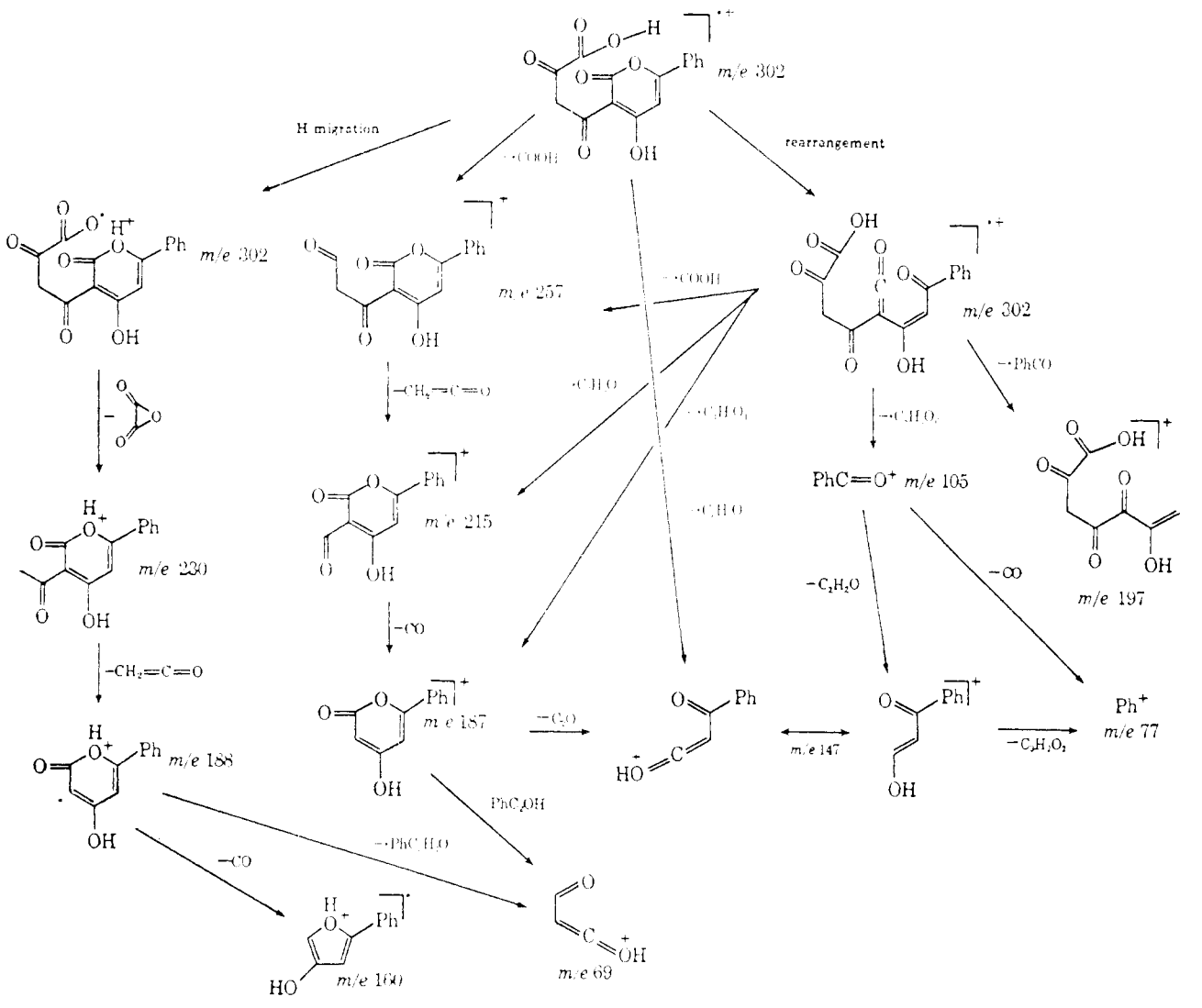
The method of deuterium exchange was employed in order to confirm the tentative assignments of mass spectral fragments; 2 mg of crystallized product was dissolved in several drops of acetone-d_6 and D_2O was added until the solution was slightly turbid. The solution was then heated gently to 50° and allowed to cool at room temperature. Yellow crystals were obtained after 30 min (yield, less than 1 mg). The 10-eV mass spectrum of this product (Figure 5) indicated a significant incorporation of up to five atoms of deuterium. The molecular ion now appeared as a cluster at *m/e* 304–307, *m/e* 307 having a relative intensity greater than that attributable to a natural isotope effect (especially evident in the 70-eV spectrum; see Table IV, under D). In addition, the deuterated compound showed the following clusters of peaks in the 10-eV spectrum: *m/e* 258–261 corresponding to *m/e* 257 for the undeuterated sample; *m/e* 231–235 corresponding to *m/e* 230; *m/e* 216, 217 to *m/e* 215; *m/e* 199–202 to *m/e* 197; *m/e* 189–191 to *m/e* 188; *m/e* 188, 189 to *m/e* 187; *m/e* 161–163 to *m/e* 160; *m/e* 148, 149 to *m/e* 147. The base peak remained at *m/e* 105, and there was no deuterium incorporation into this species as indicated by the equivalence of the *m/e* 106 peak for both the original and deuterium exchanged sample (Table IV).

The 70-eV spectrum showed the same qualitative changes in relative intensities as those observed upon going from 10 to 70 eV for the undeuterated sample. The new peaks corresponding to *m/e* 77 and 69 of the undeuterated sample are *m/e* 77 and the *m/e* 69, 70 cluster, respectively. Calculations of deuterium effects indicated that the peaks *m/e* 230, 188, 160, and 69 are related.

On the basis of these results, the structure of the ring-cleavage product was adjudged to be 3-(1,3-diketo-4-carboxybutyl)-4-hydroxy-6-phenyl-2-pyrone (or a keto–enol tautomer of this compound). Scheme II depicts the fragmentation pattern which can be deduced for this compound.

Fragmentation occurs preferentially *via* intermediates having an even number of electrons (McLafferty, 1966).

SCHEME II

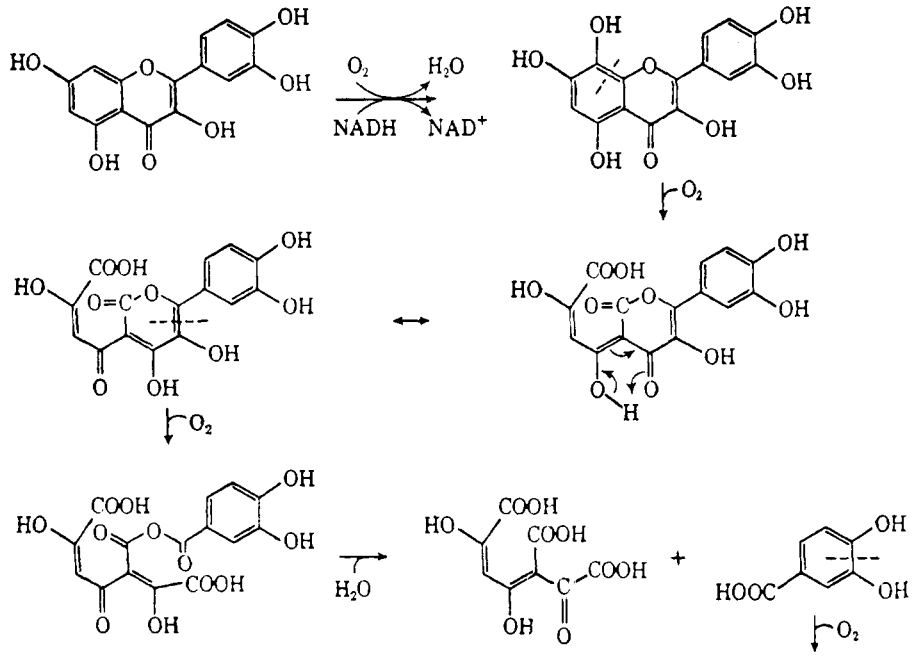


Loss of the carboxyl radical yields *m/e* 257 which subsequently loses ketene and carbon monoxide as neutral particles giving *m/e* 215 and 187, the latter fragment generating *m/e* 147 and 69 (at high electron voltage) by different retro-Diels–Alder reactions. (The *m/e* 147 peak can also arise from a retro-Diels–Alder reaction of the molecular ion.) Opening of the pyrone ring (Scheme II, rearrangement) and cleavage yield the very stable phenyl acylium ion (*m/e* 105) and to a lesser extent *m/e* 197 as the charged fragment. At 70 eV the base peak, *m/e* 105, gives rise to the phenyl ion (*m/e* 77) by loss of CO. Another mode of fragmentation involves the migration of a hydrogen radical from a position sufficiently removed from the center of initial ionization, making the charged center even electroned (Budzikiewicz *et al.*, 1967). Such a process readily explains the appearance of the peaks at *m/e* 230, 188, and 160; the transfer of hydrogen from the carboxyl is not unexpected considering the number of keto–enol resonance isomers that would place the carboxyl hydrogen in close proximity to the ring oxygen. In addition, such a process would be less likely at higher ionizing voltages (Table IV). This fragmentation pathway involves facile losses of small neutral molecules, which can give *m/e* 69 from *m/e* 188 by a retro-Diels–Alder reaction. The deuterium exchange data confirm the above assignments since there are a possible five exchangeable hydrogens at acidic and keto–enol tautomer protons.

Discussion

From the data presented, a general pathway for the degradation of 7-hydroxyflavones and 7-hydroxyflavanones can be proposed (Scheme III). The initial reaction involves hydroxylation at C_8 by a mixed function oxygenase that requires NADH. This enzyme has now been partially purified and has the properties of a typical flavoprotein (E. Schultz and J. M. Wood, unpublished results). The substrate must be a 7-hydroxyflavone or 7-hydroxyflavanone; if the 7-hydroxyl position is occupied by another functional group (7-methylflavone and techtochrysin), or if it is absent altogether (3-hydroxyflavone), there is no enzymatic reaction; 8-hydroxyflavone cannot serve as a substrate causing "inverse" hydroxylation at the 7 position, nor is this compound a substrate for the ring cleavage enzyme. 2′,4,4′-Trihydroxychalcone and the isoflavone, Biochanin A, both of which are isomeric to known flavone and flavanone substrates, are not oxidized, indicating that the substrate specificity does not extend to the chalcone or isoflavone series of compounds. It is interesting to note that the 3-glycoside of quercetin, rutin, is not a sub-

SCHEME III



strate, suggesting that a bulky group at C_3 either prevents access to the active site or causes a disorientation of the flavone ring with respect to the catalytic units on the protein.

The second step in the pathway is an oxidative fission of the aromatic ring. Cleavage is of the extradiol type, occurring between C_8 and the flavonoid C ring. If a 5-hydroxyl is present, rearrangement occurs to the corresponding 2-pyrone as is observed for the product isolated from ring fission to norwogonin. Other possible modes of ring cleavage can easily be ruled out. Intradiol cleavage between C_7 and C_8 of the flavone ring could yield only a 4-pyrone as product. The mass spectral data are not consistent with the formation of a 4-pyrone. The mass spectrum of the 4-pyrone from norwogonin should have a large peak at *m/e* 258 due to loss of CO_2 from either carboxylate group of the diacid which would be the product of intradiol cleavage; a peak at *m/e* 243 $(M - CH_2CO_2H)^+$ would also be expected. The peaks actually observed at *m/e* 188, 187, and 160 are difficult to reconcile with the 4-pyrone that would result from intradiol cleavage. Furthermore, the semialdehyde product which would be formed by extradiol cleavage of norwogonin between C_6 and C_7 would show the incorporation of only four atoms of deuterium instead of the five actually observed; additionally such a product would likely cyclize with loss of water to give a molecular ion of *m/e* 284.

This ring-cleavage reaction is catalyzed by a dioxygenase which has now been purified to homogeneity (E. Schultz and and J. M. Wood, unpublished results). We have synthesized three different 7,8-dihydroxyflavones and each one functions as a substrate for this dioxygenase. It should be emphasized that all substrates that are hydroxylated at C_8 by the mixed function oxygenase to give their respective 7,8-dihydroxyflavones provide a product which is cleaved by the dioxygenase.

The product formed by ring cleavage of the flavonoid A ring can be a substrate for a second dioxygenase if certain structural criteria are met. There must be a hydroxyl at C_3, and this hydroxyl must be at an unsaturated site (3-hydroxyflavanones, such as taxifolin, are nonsubstrates). Additionally, the formation of a diol in the C ring at C_3 and C_4 is dependent upon the isomerization of the product of ring cleavage to a 2-pyrone. This process can only occur if there is a hydroxyl at C_5 (Scheme III). Fisetin which has a C_3 hydroxyl but lacks a C_5 is thus not a substrate for the second dioxygenase. The ring fission of the 2-pyrone-4,5-diol by a typical dioxygenase can occur between the two hydroxyls or to either side of the two hydroxyls. Cleavage between C_5 and C_6 of the 2-pyronediol is favored for several reasons. The fact that morin can isomerize to the diol, but cannot consume a third mole of oxygen, suggests that the presence of a 2′-hydroxyl does not allow morin to align itself correctly at the active site of the enzyme. This effect would certainly be most pronounced if cleavage is between C_5 and C_6. Secondly, this cleavage would yield an anhydride that would easily hydrolyze to give an unsaturated aliphatic chain and a substituted benzoic acid. For quercetin this substituted benzoic acid would be protocatechuic acid, and protocatechuic acid is actually isolated when bacteria are grown on quercetin. Similarly for kaempferol, one would expect *p*-hydroxybenzoic acid. Thin-layer chromatographic analyses showed *p*-hydroxybenzoic acid to be the sole aromatic product when sonified crude extracts supplemented with NADH oxidize kaempferol. The unsaturated aliphatic compound produced by a cleavage of the 2-pyrone-4,5-diol can be hydrolyzed to give two molecules of oxaloacetic acid. Since oxaloacetic acid provides a readily assimilable carbon source, it is to be expected that the substituted benzoic acid should accumulate to some extent in growing cultures.

Jeffrey *et al.* (1972a,b) have shown that a similar pathway is operative in the degradation of the flavanone taxifolin. With taxifolin, hydroxylation occurred at C_8 with an NAD(P)H dependent mixed function oxygenase, and extradiol cleavage gave oxaloacetic acid and 5-(3,4-dihydroxyphenyl)-4-hydroxy-3-oxovalero-δ-lactone after hydrolysis of the ring fission product. In this system the ring-cleavage product was not isolated, and the enzymes involved in this pathway have limited specificity for flavanones, since flavones such as quercetin are not metabolized. One explanation for the different specificity for flavones and flavanones in *Pseudomonas putida* and the pseudomonad isolated by Jeffrey *et al.* could be the coordinate induction of different sets of enzymes depending on the nature of the inducer (*i.e.*, the growth substrate). Growth

on (+)-catechin induced a system which was only capable of oxidizing flavanones, whereas growth on quercetin induces enzymes which have a far broader spectrum of activity.

Acknowledgment

We thank Mr. Joe Wrona for his excellent work in running our mass spectral samples. We are indebted to Messrs. T. Nicksic, D. Walter Jackson, and C. L. Turnbough for helpful and lively discussions, and to Connie Ogden for her excellent help in the preparation of the manuscript.

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