

A Cytochrome P450 Mediated Naringenin 3'-Hydroxylase from Sweet Orange Cell Cultures¹

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A microsomal flavonoid 3'-hydroxylase (F3'H) catalyzing the metabolism of naringenin to eriodictyol in *Citrus sinensis* (L.) Osbeck cv. 'Hamlin' cell suspension cultures was shown to be a cytochrome P450 enzyme. This reaction required O₂ and NADPH and was inhibited by CO, with partial reversal of CO-inhibition by light at 450 nm. Cytochrome P450 content ranged from 10–20 pmol (mg microsomal protein)⁻¹. The F3'H reaction was shown to be linear in regard to protein concentration between 2.5 and 25 µg of microsomal protein. The optimum pH for the reaction was 7.4–7.6 and the temperature optimum was between 30 and 37°C. The apparent K_m and V_{max} for naringenin were 24 µM ± 3.2 and 81.4 ± 7.9 pmol eriodictyol min⁻¹ (mg protein)⁻¹, respectively. The microsomal F3'H was also capable of forming dihydroquercetin from dihydrokaempferol (40 pmol min⁻¹ (mg protein)⁻¹) and of quercetin from kaempferol (3.25 pmol min⁻¹ (mg protein)⁻¹). Cytochrome *c* and ketoconazole were the best inhibitors of F3'H activity followed by piperonyl butoxide and *α*-naphthoflavone. Light was shown to be an inducer of the F3'H almost doubling the specific activity and increasing the microsomal cytochrome P450 content by 30% over that of dark grown cells. F3'H activity was also confirmed in microsomal preparations of young (new flush) leaves from 'Hamlin' trees and flavedo of 'Hamlin' oranges, 'Marsh' grapefruit, and 'Lisbon' lemon. No activity was observed in older, hardened leaves and albedo of all the fruit tested. Initiation of embryogenesis in the 'Hamlin' cell suspension cultures by switching from a sucrose medium to a glycerol-based medium resulted in the down-regulation of F3'H.

Key words: *Citrus* — Eriodictyol — Flavonoid — Hydroxylase — Microsomes — Tissue culture.

Flavonoids are common to most plants and have wide ranging physiological and biochemical effects in plants and on other life forms. Plant flavonoids give rise to color (e.g. anthocyanidins) in the fruit and blossoms, ensuring their pollination by insects, and may themselves be involved in sexual reproduction of plants (Harborne 1965, Koes et al. 1994). They can also influence the flavor of plant products, be involved as plant structural components and serve to protect plants from UV damage. The flavonoid comple-

ment in plants is often unique and has been used for taxonomic purposes (William et al. 1993). Flavonoids may serve defensive functions as they have been found to affect larval growth of insects (Elliger et al. 1980) and act as phytoalexins to inhibit infectivity of plant pathogens (Harborne 1965, Koes et al. 1994).

The selective genetic manipulation of citrus flavonoids may be a means for reducing production and postharvest losses in citrus crops which result from pests. This may also increase consumer acceptance of citrus by improving their nutritional and disease prevention properties, since flavonoids are antioxidants (Harborne 1967, 1988, Terao et al. 1994) and have beneficial antiulcer (Martin et al. 1993) and anticancer (Hertog et al. 1992) activities and reduce the risk of heart disease (Hertog et al. 1993) in humans.

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Flavonoids offer many possibilities in accomplishing the goals of reducing production and postharvest losses due to pests and we have, therefore, initiated research in this area. Initial investigations have focused on flavanone metabolism because most classes of flavonoids (e.g., anthocyanins, isoflavones, flavones, flavonols, etc.) are derived from flavanone intermediates (Stafford, 1991). In this report we describe the flavanone 3'-hydroxylase (F3'H) responsible for converting naringenin to eriodictyol (Fig. 1) in *Citrus sinensis* (L.) Osbeck cv. 'Hamlin' cell cultures. Although eriodictyol is not usually found in sweet oranges, the 3' *O*-methylated derivatives of eriodictyol, i.e. hesperetin and its glycoside hesperidin, are (Harborne 1967, Rouseff et al. 1987). In the assay we describe, the conversion of eriodictyol to the 3' *O*-methyl derivatives does not occur because of the lack of the necessary cofactors and, therefore, measurement of eriodictyol is possible. Evidence is presented which indicates that the F3'H is a cytochrome P450 enzyme of microsomal origin.

Materials and Methods

Chemicals—[³H]Naringenin with a specific activity of 98 $\mu\text{Ci } \mu\text{mol}^{-1}$ was prepared by New England Nuclear (Boston, MA) and was 90% pure (determined via high pressure liquid chromatography (HPLC) as described below). NADPH, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) and glacial acetic acid were obtained from Sigma (St. Louis, MO). HPLC solvents were from Baxter (Chicago, IL). Naringenin, eriodictyol, hesperidin, hesperetin, dihydroquercetin, dihydrokaempferol, quercetin, apigenin, and luteolin were purchased from Roth (via Atomergic Chemetals Corp., Farmingdale, NY). The flavonoid analog, 58C80 ((4-*t*-butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone), was obtained from Wellcome Research Laboratories (Beckenham, U.K.). BSTFA (*N,O*-bis(trimethylsilyl)trifluoroacetamide) and all other chemicals were from either Sigma or Aldrich Chemical Co. (Milwaukee, WI). Gases were either from Matheson Gas Products (Morrow, GA) or Florida Airgas, Inc. (Orlando, FL).

Plant materials—An embryogenic callus cell line from *Citrus sinensis* (L.) Osbeck cv. 'Hamlin' (H-89) was maintained as previously described (Niedz 1993). H-89 cell suspension cultures were initiated by inoculating approximately 25 g of callus into each 1 liter Erlenmeyer flask containing 250 ml of liquid medium. The liquid medium was the same as the medium used to culture the callus, but with the agar omitted. The suspension cultures were maintained at 27°C on a gyratory shaker at 150 rpm in low light (3 $\mu\text{E m}^{-2} \text{s}^{-1}$ for 10 h per day). Cells were harvested 10 days after transfer into suspension culture.

Embryogenesis in H-89 was induced by transferring callus into the same liquid medium, but with 2% glycerol

(embryo induction medium) substituted for the 5% sucrose as the main carbon source. The cultures were transferred to fresh 2% glycerol embryo induction medium every 10 days for 8 weeks. In some cases F3'H activities were compared at different stages of growth for cells grown in medium with either sucrose or glycerol.

Samples (10 g) of young leaves (new flush), mature leaves from 3-year old 'Hamlin' scion on 'Carrizo' rootstock, as well as flavedo and albedo from ripe 'Hamlin' oranges, 'Marsh' grapefruit, 'Lisbon' lemons and 'Persian' limes were also used in experiments.

Microsome preparation—Microsomes were prepared by freezing tissues in liquid nitrogen and subsequently disrupting the frozen tissues in an Omni-Mixer (OCI Instruments, Waterbury, CN). Powdered cells were resuspended at 10 g per 100 ml cold homogenization buffer (0.1 M sodium phosphate, pH 7.4, 15 mM mercaptoethanol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 15% glycerol, v/v) and homogenized on ice for 1 min in a Brinkmann Polytron homogenizer (Brinkmann Instruments, Westbury, NY) equipped with a PTG 35/50 generator. The homogenate was passed through two layers of cheesecloth and centrifuged at 15,000 $\times g$ for 20 min at 4°C. The resulting supernatant was passed through two layers of Miracloth® (Calbiochem, La Jolla, CA) and centrifuged at 100,000 $\times g$ at 4°C for 1 h. The microsomal pellet was resuspended in homogenization buffer containing 30% (v/v) glycerol and stored at -80°C.

For the differential centrifugation studies to determine which subcellular fraction(s) contained activity homogenates were prepared as described above. The cheesecloth filtered homogenate was centrifuged at 1,000 $\times g$ for 15 min and the supernatant separated from the pellet at the completion of the run. The pellet was resuspended in a minimum of homogenization buffer and the supernatant was subsequently centrifuged at 10,000 $\times g$ for 15 min. The ensuing pellet was resuspended in homogenization buffer and the supernatant transferred to another centrifuge tube and centrifuged at 100,000 $\times g$ for 1 h. The 100,000 $\times g$ (microsomal) pellet was resuspended in homogenization buffer after the postmicrosomal supernatant had been removed. Each of the fractions were assayed for protein content and F3'H activity as described below.

Flavonoid 3'-hydroxylase reactions—Flavonoid 3'-hydroxylase reactions were generally as follows unless otherwise noted. [³H]Naringenin (0.12 μCi ; 17.8 pmol) in ethanol was added to a 1.5 ml microcentrifuge tube, dried under nitrogen, and resuspended in 50 μl of 100 mM TES buffer, pH 7.4 (concentration and pH used for all reaction components), by vortexing. Microsomes (25 μg protein) were then added and total volume adjusted to 100 μl with TES buffer. The tubes were placed in a gyratory water bath at 37°C and the reaction initiated by the addition of 1.5 μl of 20 mM NADPH in TES buffer. Reactions were ter-

minated by adding 100 μ l of chilled acetonitrile, mixed and then placed on ice for 5 min to aid precipitation of protein. The reaction mixtures were then centrifuged (10,000 \times *g*) for 5 min at 4°C and the supernatant filtered through a 0.2 μ m nylon syringe filter (PGC Scientific, Gaithersburg, MD). The resulting filtrate was used for identification of metabolites. Calculations of the recovery of naringenin and metabolites were accomplished by measuring the radioactivity of aliquots (5 μ l) of the incubation mixtures and filtrates via scintillation counting.

Inhibitors (5 mM stocks in ethanol) were added to the naringenin in the microcentrifuge tubes and dried as described above. Cytochrome *c* (in 100 mM TES) was added to the naringenin after it was resuspended in TES buffer. Microsomes and inhibitors were coincubated on ice for 10 min prior to the addition of the NADPH and incubation at 37°C. Piperonyl butoxide and metyrapone were also preincubated with the microsomes and NADPH at 37°C for 10 min and in this case the reactions were initiated by the addition of naringenin dissolved in dimethylsulfoxide (DMSO) and further incubated for another 10 min at 37°C. Appropriate controls with DMSO were run concurrently.

Experiments utilizing specialty gases were conducted in septum sealed test tubes with syringe needles placed in the septa to allow introduction and exiting of gases. Gases were controlled with calibrated flow meters. Buffer was added to the tubes and the gases bubbled through the buffer for 3 min, microsomes were introduced, followed by gassing over the buffer for an additional 2 min. Reactions were incubated for 20 min in the dark for those experiments using CO. Argon was used for those experiments determining O₂ requirements.

Experiments to determine light reversal of CO inhibition were conducted using an irradiation box containing a Shimadzu densitometer fluorescence attachment xenon lamp (16.5 cm from the sample which was separated by a glass plate) and a blue-violet (#47 Wratten) filter with maximum transmission between 440–450 nm. Samples were irradiated for 1 h and then F³H activities measured. Sample temperature was maintained at 32–35°C using a fan to cool the lamp.

F³H activity and cytochrome measurements reported herein are the means and standard deviations of 3–9 replicates per reactions.

Metabolite analyses—Separation and detection of metabolites was achieved via HPLC. Metabolite separation was accomplished on a C-18 bonded phase column (HP ODS Hypersil, 5 μ m, 200 \times 4.6 mm; HP Analytical, Kennett Square, PA) employing a solvent mix of 80% A (40% acetonitrile in H₂O containing 1% glacial acetic acid) to 20% B (1% glacial acetic acid in H₂O) at a flow rate of 1 ml per min. The HPLC system consisted of a Spectra Physics (San Jose, CA) P2000 pump and AS3500 autosampler, a Flo-One/Beta radiodetector (Packard Instrument Co.,

Meriden, CN) using Packard FloScint A scintillation fluid mixed 3 : 1 (v/v) with eluant and a Waters 996 photodiode array detector (Millipore Corp., Milford, MA). Data analysis was completed using Millipore Millennium 2010 chromatography manager.

Elution times of metabolites were determined by comparison to eriodictyol, dihydrokaempferol, dihydroquercetin, luteolin, apigenin, kaempferol, and naringenin standards that were treated the same as test samples.

Confirmation of eriodictyol production was also achieved via GLC (gas-liquid chromatography). Twenty F³H reactions were conducted simultaneously for 2 h at 37°C using naringenin (100 pmol reaction⁻¹) as the substrate. The reactions were terminated and extracted as described above. The resulting supernatant was mixed with an equal volume of acetonitrile/chloroform (1 : 1, v/v) and the organic phase removed and dried under a stream of N₂. The residue was resuspended in 100 μ l of pyridine to which 50 μ l of BSTFA was added. The mixture was allowed to react for 30 min at room temperature and samples were injected (7–10 μ l) directly into the GC (gas chromatograph). Standards (naringenin and eriodictyol) were silylated as before. The GC was a Hewlett Packard 5890 (Palo Alto, CA) equipped with a flame ionization detector and a DB-5 column (30 m \times 0.25 mm ID, 0.25 μ m thickness; J & W, Alltech, Avondale, PA). Injector and detector temperatures were 300°C. A temperature program was employed (115°C for 0.5 min then 30°C min⁻¹ to 250°C and finally 1°C min⁻¹ to 265°C) with a helium flow rate of 1.2 ml min⁻¹ at 20 psi.

Protein and cytochrome P450 measurements—Protein was determined following the method of Bradford (1976) using bovine serum albumin (fraction V) as the standard. Cytochromes P450 and P420 concentrations were calculated using the reduced CO-binding spectrum (91 mM⁻¹ cm⁻¹ for A_{450–490} and 110 mM⁻¹ cm⁻¹ for A_{420–490}; Omura and Sato 1964) using an SLM Aminco DW2000 UV-visible spectrophotometer (Milton Roy, Urbana, IL).

Results

Induction of embryogenesis—Embryogenesis of H-89 cells was induced by transfer of the cells into a culture medium where the main carbon source was glycerol. Within 6 days of transfer of the cells to the glycerol-based medium, embryo primordia were visible by light microscopy when viewed at 100 \times magnification. The conversion of cells to embryos was complete after 6 weeks, resulting in a suspension culture of globular and heart-shaped embryos. Embryo development did not proceed beyond the heart-shaped stage as long as the embryos were subcultured into the glycerol-based medium. Proliferation of the embryos was by secondary and tertiary embryogenesis. New embryos would form on the surface of an embryo, break off,

Table 1 Flavonoid 3'-hydroxylase activities in 'Hamlin' suspension culture subcellular fractions

Fraction	Volume (ml)	Protein (mg ml ⁻¹)	Cyt. P450 (pmol (mg protein) ⁻¹)	Specific activity (pmol eriodictyol min ⁻¹ (mg protein) ⁻¹)	Total activity (pmol eriodictyol min ⁻¹)	(%)
Homogenate	90	2.2	nd	17	3,366	100
1,000 × g pellet	21	2.9	nd	18	1,096	32.6
10,000 × g pellet	4.4	3.9	nd	64	1,098	32.6
100,000 × g pellet	1.8	7.4	19.2	77	1,025	30.4
100,000 × g supernatant	82	1.04	nd	1.3	111	3.3

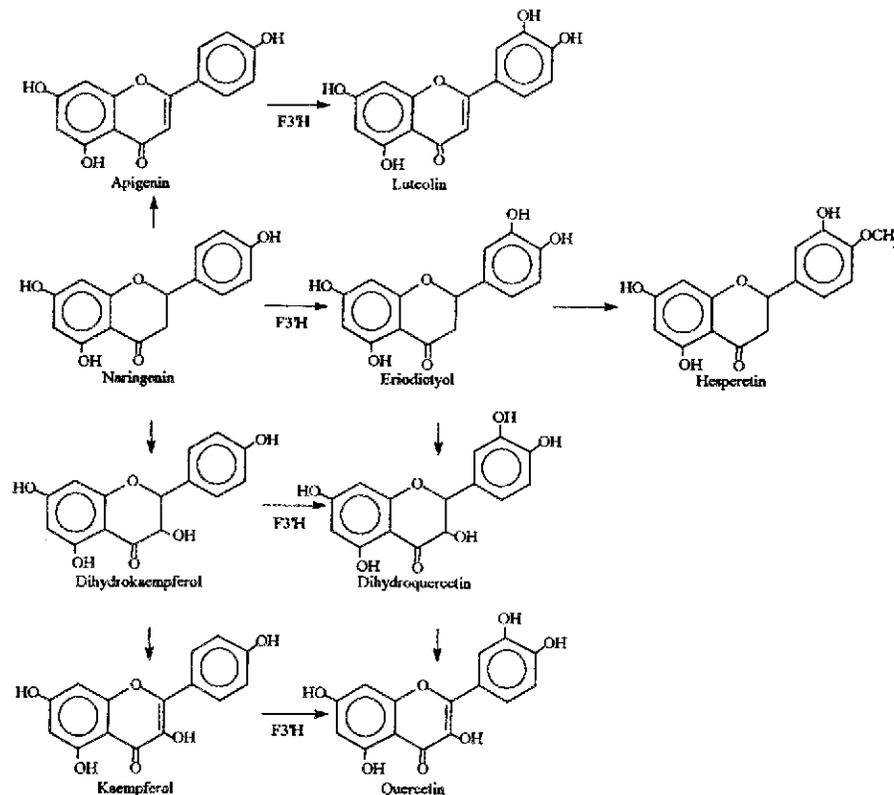
Cells were grown exposed to light (see Methods). Fresh weight of the cells was 15.1 g. nd, not determined.

enlarge, and new embryos would form on these embryos thereby repeating the process. Once the conversion of cells to embryos was complete, the embryos would turn light green indicating the development of chlorophyll.

Activities of subcellular fractions—The hydroxylase involved in the conversion of naringenin to eriodictyol was found to be present in all of the fractions examined (Table 1). The 10,000 × g and 100,000 × g (microsomal fraction) pellets contained almost the same amounts of F3H activity; however, the latter appeared to be a cleaner source of enzyme in that it generated smaller quantities of metabo-

lites other than eriodictyol from the radiolabeled naringenin. Consequently, we used the microsomal fraction as our enzyme source. Also, we determined that the 1,000 × g centrifugation step could be eliminated without affecting the microsomal F3H activity. Microsomal preparations were fully active when frozen at -80°C for at least 6 months.

Characterization of F3H reaction in suspension-grown cells—A typical HPLC tracing of product extracted from a microsomal hydroxylase reaction with naringenin is shown in Figure 2. Product recovery was estimated to be

**Fig. 1** Possible flavonoid 3'-hydroxylase (F3H) reactions.

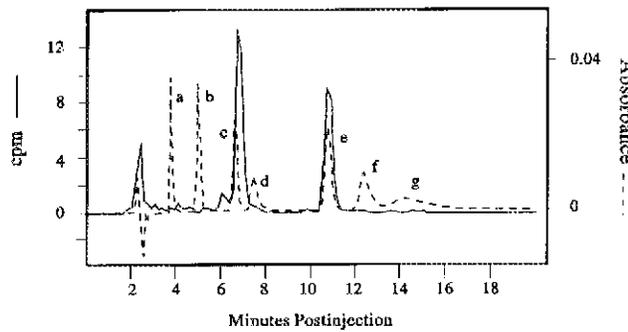


Fig. 2 Chromatograms of HPLC separations of a typical 'Hamlin' microsomal reaction using naringenin as the substrate and radiodetection (—) and flavonoid standards following A_{290} (---) (a) dihydroquercetin, 3.8 min; (b) dihydrokaempferol, 5.1 min; (c) eriodictyol, 6.7 min; (d) luteolin, 6.9 min; (e) naringenin, 10.8 min; (f) apigenin, 12.4 min; and (g) kaempferol, 14.2 min. Separation details are given in Materials and Methods.

90–95%. Figure 2 also shows a typical tracing of the standards detected by monitoring absorbance at 290 nm and indicates that complete separation of naringenin, eriodictyol, and several other possible products is achieved with this method. Elution times of flavonoid standards at these conditions are indicated in Figure 2. Generally, for most of the reactions only naringenin and eriodictyol standards were run to verify elution times; the metabolite peak coincided with the eriodictyol retention time and also exhibited the absorption spectra associated with the standard. The presence of eriodictyol was also confirmed by GLC, as the retention time of the major reaction product coincided with that of the eriodictyol standard (data not shown).

The F3H has an absolute requirement for NADPH and O_2 (Table 2). No activity was observed using NADH as a cofactor. Reactions containing equimolar concentrations

Table 2 Reaction requirements for 3'-hydroxylation of naringenin in 'Hamlin' callous tissue microsomes

Microsomal protein	Reaction components			% Activity
	O_2	NADPH	NADH	
+	+	+	—	100
—	+	+	—	0
+	—	+	—	0
+	+	—	+	0
+	+	+	+	100

Reactions were conducted as described in Methods. Concentrations of cofactors were 0.3 mM in the reactions. 100% Activity is equal to 22 pmol eriodictyol min^{-1} (mg protein^{-1}).

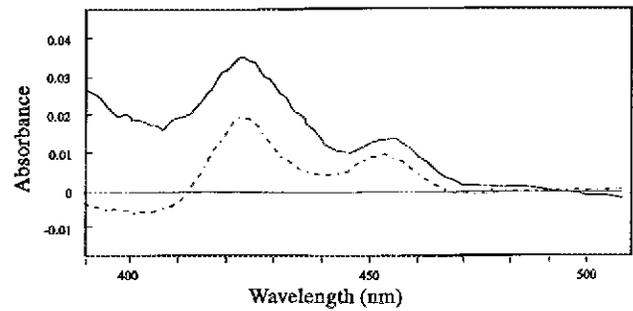


Fig. 3 Reduced cytochrome P450-CO difference spectra of microsomes from (a) 'Hamlin' cells grown in light (—) and (b) cells grown without light (---). Microsomal protein was 7.4 mg ml^{-1} .

of both NADPH and NADH yielded amounts of eriodictyol similar to those with NADPH alone.

The presence of cytochrome P450 was confirmed by the reduced CO-binding difference spectrum of the microsomal suspensions from light and dark grown cells (Fig. 3). Cytochromes P420 and P450 concentrations for microsomes obtained from cell suspensions grown under routine conditions were 43 and 20 pmol (mg protein^{-1}), respectively. Naringenin hydroxylation was reduced $95 \pm 3\%$ when reactions were conducted in a $CO : O_2$ (4/1) atmosphere. CO inhibition was not reversed by irradiation of the reaction mixture with 450 nm light. However, when $CO : O_2$ ratios of 1 : 1 were employed, naringenin hydroxylation was inhibited by 29% and inhibition was reversed to an 8% level after irradiation of the reaction mixture with 450 nm light.

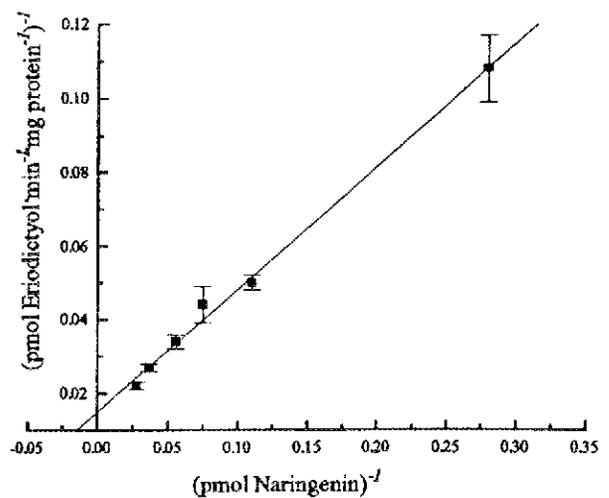


Fig. 4 Typical Lineweaver-Burk plot of naringenin hydroxylation by 'Hamlin' suspension cell culture microsomes. Mean \pm SD; $N=4$ for each data point.

The temperature optimum for the enzymatic reaction was determined to be 30–37°C while hydroxylase activity dropped rapidly above 37°C. The rate of reaction was linear with regard to protein concentration between 2.5 and 25 µg of microsomal protein. At the conditions outlined in Methods the reaction was linear for 15–20 min; consequently, a reaction time of 10 min was routinely used. The optimum pH for the reaction was 7.4–7.6; above or below this range the activity declined.

Graphic determinations of apparent K_m and V_{max} yielded values of $24 \mu\text{M} \pm 3.2$ and 81.4 ± 7.9 pmol eriodictyol min^{-1} (mg microsomal protein) $^{-1}$, respectively (Fig. 4).

Since it is known that some F3'Hs are capable of hydroxylating other flavonoids such as dihydrokaempferol, kaempferol, and apigenin (Hagmann et al. 1993) we tested these as substrates by scaling up the reactions to obtain enough of the products (i.e. dihydroquercetin, quercetin, luteolin) to detect by UV absorbance. We observed formation of dihydroquercetin from dihydrokaempferol (40 ± 6 pmol min^{-1} (mg microsomal protein) $^{-1}$) and of quercetin from kaempferol (3.25 ± 2.5 pmol min^{-1} (mg microsomal protein) $^{-1}$). No conversion of apigenin to luteolin was detected.

Light as an elicitor—Normally the suspension cultures used in our experiments were given 10 h of light (cool white fluorescent lamps) per day. We deprived cell suspension cultures of light for 7 days by wrapping the culture flasks in aluminum foil, harvested the cells as usual, and assayed the microsomal fraction for F3'H activity and cytochrome P450. The wet weight of the cells was 18.7 g and they yielded 8.8 mg microsomal protein. Microsomal F3'H activity

Table 3 Inhibitor effects on the 3'-hydroxylation of naringenin in 'Hamlin' microsomal preparations

Compound	Final concentration (µM)	% Inhibition
Aminobenzotriazole	100	-19.2
α -Naphthoflavone	100	46.8
Cytochrome <i>c</i>	0.5	50
Ketoconazole	50	100
Metyrapone ^a	100	12.5
Metyrapone	100	-7.4
Piperonyl butoxide ^a	100	50
Piperonyl butoxide	100	46.8
Quinine	100	20
Quinidine	100	29.4
Sulphafenazole	100	9.9
58C80	100	7.7

Mean activity of uninhibited reactions was 21.9 pmol eriodictyol min^{-1} (mg protein) $^{-1}$.

^a Preincubated at 37°C prior to initiation of reaction.

was 34 pmol eriodictyol min^{-1} (mg microsomal protein) $^{-1}$ and the cytochromes P450 and P420 contents were 14 and 24 pmol (mg microsomal protein) $^{-1}$, respectively (Fig. 3). Control (i.e. cell cultures grown in light and prepared simultaneously with the cells grown in the dark) values were 77 pmol eriodictyol min^{-1} (mg microsomal protein) $^{-1}$ and the cytochromes P450 and P420 contents were 30% and 44% respectively, greater than those of dark grown cells (Table 1). There were also some apparent qualitative differences in the wavelengths of the absorption maxima for the reduced-CO difference spectra as the cytochrome P450 absorption maximum from dark grown cells was 452 nm vs. 454 nm for light grown cells (Fig. 3).

Inhibitors—Ten inhibitors of cytochrome P450 were used to further characterize the naringenin hydroxylation reaction (Table 3). The reaction was totally inhibited by ketoconazole (50 µM) and approximately 50% inhibited by cytochrome *c* (0.5 µM), piperonyl butoxide (100 µM) and α -naphthoflavone (100 µM), respectively. Each of the other inhibitors (100 µM), however, caused less than 30% inhibition, with aminobenzotriazole stimulating the reaction by 19%. Since metyrapone and piperonyl butoxide can be metabolized to products which form inhibitory complexes with cytochrome P450 (Hodgson and Philpot 1974) these compounds were preincubated for 10 min with 'Hamlin' suspension culture-derived microsomes and NADPH at 37°C. Preincubation increased the inhibition due to metyrapone, although this remained weak (12.5%), but there was no effect on the inhibition due to piperonyl butoxide.

Flavonoid 3'-hydroxylase in other citrus tissues—F3'H activities were determined using naringenin as the substrate and microsomes prepared from other sources. Young and fully expanded leaves were obtained from 'Hamlin' trees. Microsomes prepared from young leaves metabolized nar-

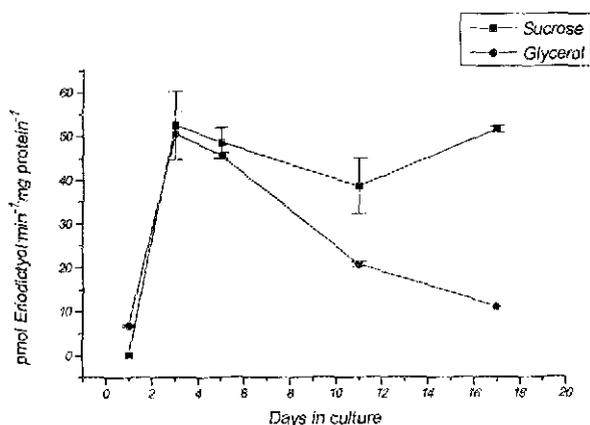


Fig. 5 Flavonoid 3'-hydroxylase activity in 'Hamlin' cell suspension cultures grown in either sucrose- or glycerol-based medium. Substitution of sucrose with glycerol initiates embryogenesis.

ingenin to eriodictyol (19 ± 5 pmol eriodictyol min^{-1} (mg microsomal protein) $^{-1}$); mature, hardened leaves did not.

Microsomal preparations were also made from the flavedo and albedo of 'Hamlin' oranges, 'Lisbon' lemons, 'Marsh' grapefruit, and 'Persian' limes. 'Hamlin' flavedo had an activity of 0.8 ± 0.3 pmol eriodictyol min^{-1} (mg microsomal protein) $^{-1}$. Small amounts of F3'H activity were also observed in 'Lisbon' lemon flavedo, but there was no detectable activity in flavedo of 'Persian' limes. No F3'H activity was seen in microsomal preparations of albedo from any of the varieties examined.

We also used the embryogenic 'Hamlin' cell suspension culture to determine if there was a difference in the expression of F3'H after initiation of embryogenesis using the glycerol-based culture medium. Substitution of glycerol for sucrose in the suspension cell medium induces cell differentiation leading to the formation of embryos. This developmental transition has a profound effect in F3'H activity. Within 5 to 10 days of glycerol treatment the level of F3'H activity starts to decline (Fig. 5) and continues to decline until eventually no F3'H activity can be detected (at ca. 6 weeks when chlorophyll begins to appear in the newly formed globular and heart-shaped embryos).

Discussion

Research on the biosynthesis of flavonoids in plants has made tremendous advances over the last several years due to improved technologies and methods available. Although flavonoids can reach significant levels in *Citrus* (e.g. gram quantities of naringin accumulate in a single grapefruit; Maier 1969, Rouseff et al. 1987), this plant species has not been used to elucidate metabolic pathways for flavonoids. The exact reasons for this are obscure even though bioproduction of flavonoids has been documented in citrus plants, fruit (Vandercook and Tisserat 1989, Berhow and Vandercook 1991, Castillo et al. 1992) and tissue cultures (del Rio et al. 1992).

Flavonoids have long been known to influence the taste of the fruit and juices and many flavonoids have also been suggested to have a defensive function against certain pests (Elliger et al. 1980). Many of these flavonoids occur in significant quantities in *Citrus* and have been found to directly either inhibit or induce specific enzymes in insects. For example, exposure of cabbage looper larvae (*Trichoplusia ni*) to quercetin induced superoxide dismutase levels more than 2-fold (Ahmad and Pardini 1990) while chrysin, apigenin, kaempferol, morin, quercetin, myricetin and phloretin inhibited in a dose dependent manner ecdysone 20-monooxygenase activity in adult female *Aedes aegypti* and larvae of *Drosophila melanogaster* and *Manduca sexta* (Mitchell et al. 1993).

The consumption of flavonoids by insect herbivores may not lead directly to death, but the overall fitness of the

insect could be adversely affected, leading indirectly either to death or physiological dysfunctions. Of particular interest is the report that eriodictyol inhibits the growth of *Helicoverpa zea* and naringenin does not (Elliger et al. 1980). The effects of eriodictyol on insect growth may explain why some limes and lemons resist attack by the Caribbean fruitfly (*Anastrepha suspensa*, Loew) as high levels of eriodictyol in these fruit correlate with resistance (T. Waiss, personal communication; Greany and Shapiro 1993).

We have confirmed the presence of a flavonoid 3'-hydroxylase in citrus tissue cultures and plants. The F3'H isolated from embryogenic 'Hamlin' cells appears to be similar to other cytochrome P450 mediated F3'Hs reported for other plant tissues (Durst 1991). The specific content of cytochrome P450 in 'Hamlin' microsomes is 10-20 pmol (mg protein) $^{-1}$ which is not an unusual level for plant tissues (Durst 1991). Supporting evidence that the 'Hamlin' cell's enzyme is a cytochrome P450 mediated enzyme comes from the fact that it has an absolute requirement for O₂ and NADPH and is inhibited by CO. Photoreversibility of CO inhibition has been reported for the maize F3'H (Larson and Bussard 1986). Reversal of CO inhibition of the 'Hamlin' cytochrome P450 by irradiation with 450 nm light was dependent on the CO/O₂ ratio. We were not able to reverse the 95% inhibition observed at CO/O₂ ratios of 4 : 1, but some reversal (from 29% to 8% inhibition) was achieved at a 1 : 1 ratio.

Light is apparently an elicitor of citrus F3'H as the specific activity of the F3'H in light grown cells is ca. 2-fold greater while cytochromes P450 and P420 contents were 30% and 44%, respectively, larger than those of dark grown cells. The cytochrome contents may be subject to artifacts such as increased light scattering due to turbidity and the possibility that there may be increased absorbance due to other proteins such as peroxidases. Although these factors may affect the cytochrome calculations they would not affect those for specific activity of F3'H which is elevated in light grown microsomal preparations. Consequently, we believe that light is acting as an elicitor.

The apparent K_m (24 μM) for naringenin for the F3'H from citrus cell cultures is higher than those reported for parsley (Haggman et al. 1983) and maize (Larson and Bussard 1986). The apparent V_{max} obtained for the citrus cell culture F3'H (81.4 pmol eriodictyol min^{-1} (mg protein) $^{-1}$) is lower than that for parsley (Haggman et al. 1983). The 'Hamlin' cell's F3'H is similarly capable of hydroxylating dihydrokaempferol to dihydroquercetin and kaempferol to quercetin. These conversions occurred at lower rates than those observed for the naringenin hydroxylation. We did not observe conversion of apigenin to luteolin.

The hydroxylation of naringenin to eriodictyol appears to be specifically carried out by a single cytochrome

P450 which is distinct from other P450 enzymes involved in the flavonoid pathway. 1-Aminobenzotriazole, a suicide inhibitor of the cytochrome P450 enzyme cinnamate 4-hydroxylase (Reichhart et al. 1982), causes an increase in naringenin hydroxylation in 'Hamlin' cell microsomes rather than inhibiting the reaction (Table 3). Cytochrome *c* was the most potent inhibitor of the F3H reaction; similar effects have been reported for other plant cytochrome P450 reactions (Larson and Bussard 1986) and probably results from cytochrome *c* acting as an alternative electron acceptor for the NADPH cytochrome P450 reductase (NADPH cytochrome *c* reductase). The general cytochrome P450 inhibitor, ketoconazole (Maurice et al. 1992), was a potent inhibitor of the F3H reaction in citrus and corroborated the report of Clemens et al. (1993) for ketoconazole inhibition of the formononetin 3'-hydroxylase in chickpea. Piperonyl butoxide, α -naphthoflavone and metyrapone were less effective, a finding similar to that of Pommer and Böhm (1989) for cinnamic acid hydroxylase from maize. It was somewhat surprising that the flavonoid analog 58C80 inhibited the F3H reaction only slightly as this antimalarial compound is extensively hydroxylated by at least one form of cytochrome P450, human CYP2C9 (Weaver et al. 1994). However, since both 58C80 (R.J. Weaver and M.D. Burke, unpublished) and sulphaphenazole (Birkett et al. 1994) selectively inhibit the CYP2C subfamily of cytochrome P450, the failure of both compounds to significantly inhibit naringenin hydroxylation suggests that the F3H responsible is a member of a different cytochrome P450 subfamily.

Flavonoids in citrus plants and fruit are under developmental control and can be influenced by climatic and cultural conditions (Maier 1969). Our findings that F3H activity was present in young leaves and not mature leaves corroborates the findings of others reporting on bioproduction of flavonoids in *Citrus* (Hasegawa and Maier 1972, Berhow and Vandercook 1991, Castillo et al. 1992, del Rio et al. 1992). Of particular interest are the studies on embryogenic cell cultures which indicated that F3H activity is lost when the cells are transferred to a medium that contains glycerol as the carbon source instead of sucrose. Not only is F3H activity diminished, but so are extracellular peroxidase, β -1,3-glucanase, and chitinase activities (unreported data). The process of switching cell metabolism to accommodate another carbon source offers an opportunity to investigate regulation of flavonoid metabolism in vitro and extend our understanding of this important pathway in plants. The 'Hamlin' cell line may prove to be a suitable model for the study of developmental expression of secondary enzymes in *Citrus*.

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