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Flavanone Glycoside Biosynthesis in *Citrus*

Chalcone Synthase, UDP-Glucose:Flavanone-7-*O*-Glucosyl-Transferase and -Rhamnosyl-Transferase Activities in Cell-Free Extracts

Efraim Lewinsohn[1], Lothar Britsch, Yehuda Mazur[2], and Jonathan Gressel*[3]

Departments of Plant Genetics and Organic Chemistry, The Weizmann Institute of Science, Rehovot, IL-76100, Israel (E.L., Y.M., J.G.), *and Lehrstuhl für Biochemie der Pflanzen, Institut für Biologie II, der Albert-Ludwigs Universität, D-7800 Freiburg i. Br., Federal Republic of Germany* (L.B.)

ABSTRACT

Previous indirect evidence suggested that the biosynthesis of flavonoids in *Citrus* may not proceed via the usual chalcone synthase reaction and that glycosylation occurs during chalcone formation and not afterward, as has been reported in other species. We detected chalcone-synthase and UDP-glucose:flavanone-7-*O*-glucosyl-transferase activities in cell-free extracts of *Citrus*. The glucosylated flavanone was further rhamnosylated when exogenous UDP-glucose and NADPH were added to the extract. Chalcone-synthase activity was detected in cell-free extracts derived from young leaves and fruits. Young fruits (2 millimeter diameter) had the highest chalcone synthase activity. UDP-glucose:flavanone-7-*O*-glucosyl-transferase activity was measured in cell-free extracts derived from young leaves and fruits of *Citrus mitis* and *Citrus maxima*. The highest UDP-glucose:flavanone-7-*O*-glucosyl-transferase activity was found in young *C. maxima* leaves. These data indicate that *Citrus* contains a flavonoid pathway similar to that studied in other species.

Several *Citrus* spp. (Rutaceae) contain bitter tasting flavanone-disaccharides (18). They are used as food additives for their flavor or can be converted by hydrogenation into the extremely sweet dihydrochalcone derivatives (18). Little information is available on the biosynthetic pathways to flavanone-glycosides in *Citrus* (16). Chalcone-synthase is a key enzyme in the flavonoid pathway in several plants (13), but has not previously been reported in *Citrus* tissues. Chalcone-synthase catalyzes the condensation of *p*-coumaryl-CoA with three molecules of malonyl-CoA yielding naringenin-chalcone (20). The substrate specificity of a 'naringin-chalcone-cyclase' activity, from immature grapefruit fruit (25), suggested that the flavonoid pathways in *Citrus*, might be different from the more studied flavonoid pathways of other plants (13, 14) (Fig. 1). They interpreted their results (25) as suggesting that glucosylation and rhamnosylation occurred during chalcone formation in *Citrus*, as the aglycones were not cyclized. Chalcone isomerases (EC 5.5.1.6) isolated from other plants only reversibly cyclized aglycones and were unable to isomerize glycosides (8, 12). The lack of the free aglycones naringenin and hesperetin in *Citrus* tissues was used as support for their theory of a different flavonoid biosynthetic pathway in *Citrus* (25). They suggested that hydroxylation and methylation of naringenin to form hesperetin occurred after glycosylation as the chalcone isomerase did not isomerize neohesperidin chalcone (25). This is in contrast to other plants where hydroxylations and methylations occur before glycosylation (13, 14).

Young grapefruit (*C. paradisi*) tissue metabolize [^{14}C]phenylalanine (9) and [^{14}C]acetate (2) to [^{14}C]-prunin and [^{14}C] naringin indicating that there could be a flavonoid pathway in *Citrus* paralleling that of other plants. Indeed, known enzymes of the flavonoid pathway in plants (13, 14) have been found in *Citrus* tissues, including phenylalanine ammonia-lyase (23, 26, 30), cinnamate 4-hydroxylase (15) and chalcone isomerase (23, 28). The chalcone isomerase from *Citrus* peels catalyzed the reversible conversion of chalcones to their isomeric flavanones (28). This enzyme isomerized the chalcone glycosides of naringin and poncirin, and isosakuranin. Aglycones were not tested (28), but a chalcone isomerase from *Citrus* using the aglycone isoliquiritigenin as a substrate has been described (23). A chalcone-isomerase from *Citrus* catalyzing the isomerization of naringenin chalcone has not been reported.

Our earlier studies showed that the glycosylation of the aglycone in undifferentiated *Citrus* cells is not by the addition of a disaccharide; there was an initial glucosylation (21, 22) and in one case a further rhamnosylation (22) of exogenous presented aglycones. Prunin (naringenin-7-*O*-glucoside) was shown to be a likely intermediate in the biosynthesis of naringin in immature grapefruit fruits (2). There exists the vague possibility that the terminal sugar is added as glucose, which is later metabolized to a rhamnose residue, but such a post-glycosylation modification has never been reported. It is more likely that the monoglucoside was further glycosylated by a rhamnosyl-transferase, using UDP-rhamnose. Enzymatic preparations from plants catalyze the conversion of UDP-glucose to UDP-rhamnose in the presence of NADPH (1). The conversion is due to at least three different enzymatic

[1] Supported by a Minerva foundation travel grant. Present address: Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340.

[2] Rebecca and Israel Sieff Chair of Organic Chemistry.

[3] Gilbert de Botton Chair of Plant Science.

activities requiring NADH and/or NADPH. It was not previously known how this conversion is catalyzed in *Citrus*.

We explore below the possibility that differentiated plant tissues can express the typical enzymatic pathway toward flavanone-glycosides. Flavonoid specific glycosyltransferases are crucial for the biosynthesis of flavonoid glycosides (17). They catalyze the transfer of a glycosyl group from a nucleotide-activated sugar to a specific position on a flavonoid. Enzymes catalyzing the transfer of a glucose to position 3 or 5 of flavonols and anthocyanidins have been described (17). An enzyme activity from irradiated parsley cells glucosylated flavones and naringenin at position 7 (29).

We present here evidence for the presence of chalcone synthase and UDP-glucose:flavanone-7-*O*-glucosyl-transferase activities. Additionally we show that in a coupled assay, UDP-glucose with NADPH formed a rhamnosylated flavanone glucoside indicative of a UDP-rhamnose:flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase activity.

MATERIALS AND METHODS

Chemicals

Bio-Gel P4 was purchased from BioRad. Dowex 1-X2 was from Serva (Heidelberg). Sodium ascorbate, polyvinylpolypyrrolidone, naringenin, hesperetin, and malonyl-CoA were from Sigma. *p*-Coumaryl-CoA was from the Freiburg laboratory collection. UDP-[^{14}C]glucose (11.93 GBq/mmol) was from NEN. 2-[^{14}C]malonyl-CoA (2.18 GBq/mmol) was from Amersham.

Plant Material

Calamondin (*Citrus mitis* Blanco = *Citrofortunella mitis* = *Citrus madurensis* = *Citrus reticulata* var *austera* × *Fortunella* sp.) plants *ca.* 4 years old were purchased from Getzler Nurseries, Moshav Bnei Zion, D.N. Sharon, Israel, and grown in greenhouses in Freiburg and Rehovot on a mixture of ground tuff-rock:compost (1:1, v/v). Young leaves and fruits were collected, immediately extracted and assayed for enzymatic activity.

Pummelo (also called shaddock) (*Citrus maxima* [J. Burman] Merril = *C. Grandis* [L] Osbeck [27]) leaves (0.5–2.5 cm long) and flowers were collected from trees growing in a commercial grove (Schwarz Farms, Moshav Arugot, D.N. Northern Lachish, Israel), frozen and stored for up to 1.5 years at −70°C.

Grapefruit (*Citrus paradisi* Macf.) 20 × 2 mm leaves were collected from trees grown in Rehovot and were immediately extracted.

Most experiments with calamondin were carried out in Freiburg; the rest in Rehovot.

Extraction of Chalcone-Synthase Activity

Chalcone-synthase activity was extracted by the method of Britsch and Grisebach (4). *Citrus* tissues were frozen under liquid N_2 and homogenized for 5 min in a mortar together with 1 weight of sand, 0.5 weight of Dowex 1-X2 (previously washed with extraction buffer) and 20 volumes of extraction buffer (100 mM Tris-HCl (pH 7.5), containing 10% v/v glycerol and saturated with N_2). Solid Na-ascorbate was added to reach a final concentration of 10 mM. The homogenates were centrifuged for 10 min, 4°C (Eppendorf). The cleared supernatants were assayed for chalcone synthase activity.

Chalcone-Synthase Activity Measurements

Chalcone-synthase activity was assayed as described elsewhere (4). Cell-free extracts from *Citrus* spp. (previous section) were incubated with 15.5 μM [^{14}C]malonyl-CoA (0.92 GBq/mmol) and 10 μM *p*-coumaryl-CoA in 100 mM phosphate buffer (pH 6.8) at 37°C for 1 h, in a total volume of 100 μL. Crude extracts from *Petroselinum hortense* were incubated for 10 min. The samples were then extracted with 200 μL ethylacetate. Five mL of a scintillation cocktail containing 5 g/L 2,5-diphenyloxazol in toluene were added to aliquots of the ethylacetate extracts and assayed for radioactivity in a LKB121 liquid scintillation counter. At times, the ethylacetate extracts were directly applied to 20 cm long cellulose TLC plates (Merck) and developed with 15% acetic-acid (4). Radioactivity was then detected with a TLC-Analyzer (Berthold, Wildbach, FRG).

Extraction of Flavanone Glycosylating Activities

Tissues were frozen in liquid N_2 and homogenized for 5 min in a mortar containing two weights of polyvinylpolypyrrolidone (previously washed with methanol and with extraction buffer), one weight of acid-washed sand, solid Na-ascorbate added to make 125 mM, and four volumes of extraction buffer. The homogenates were centrifuged for 10 min at 4°C (Eppendorf). Prior to the glucosyltransferase activity assays, 300 μL aliquots of the supernatant were applied to 0.8 × 1.2 cm columns of Bio-Gel P4 previously equilibrated with 50 mM Hepes-NaOH (pH 7), and eluted with 1 mL buffer to remove flavonoids. For the coupled assay for rhamnosylated products, the supernatants were added to one weight of dry Sephadex G-10 to remove impurities. After swelling, the crude enzyme was collected by filter centrifugation and used for the coupled rhamnosyl-transferase activity assays.

UDP-Glucose: Flavanone-7-O-Glucosyl-Transferase Activity Assay

Aliquots of the Bio-Gel P-4 purified, flavonoid free material containing enzyme (4–12 μg protein), 100 μM naringenin or hesperetin (diluted from a 20 mM stock solution in 50% DMSO) and 25 nCi UDP-[^{14}C]glucose were incubated in 50 mM Hepes-NaOH (pH 7) in a total volume of 100 μL for 1 h at 30°C. The mixtures were then extracted with 500 μL ethylacetate to extract the glucosylated flavanones. Four mL of Insta-Mix 40 (Lumax)-xylene (3:7, v/v) were added to 200 μL aliquots of the ethylacetate and counted in a Tri-Carb 1500 liquid scintillation analyzer. The remaining ethylacetate extract was evaporated under reduced pressure and the residue applied to a 0.2 mm thick × 20 cm long Silica Gel 60 F_{254} TLC plate and developed with ethylacetate:methanol:H_2O (10:2:1, v/v). Radioactive prunin and hesperetin-7-*O*-glucoside were detected by autoradiography against Kodak X-OMAT OR diagnostic x-ray film(1 d exposures).

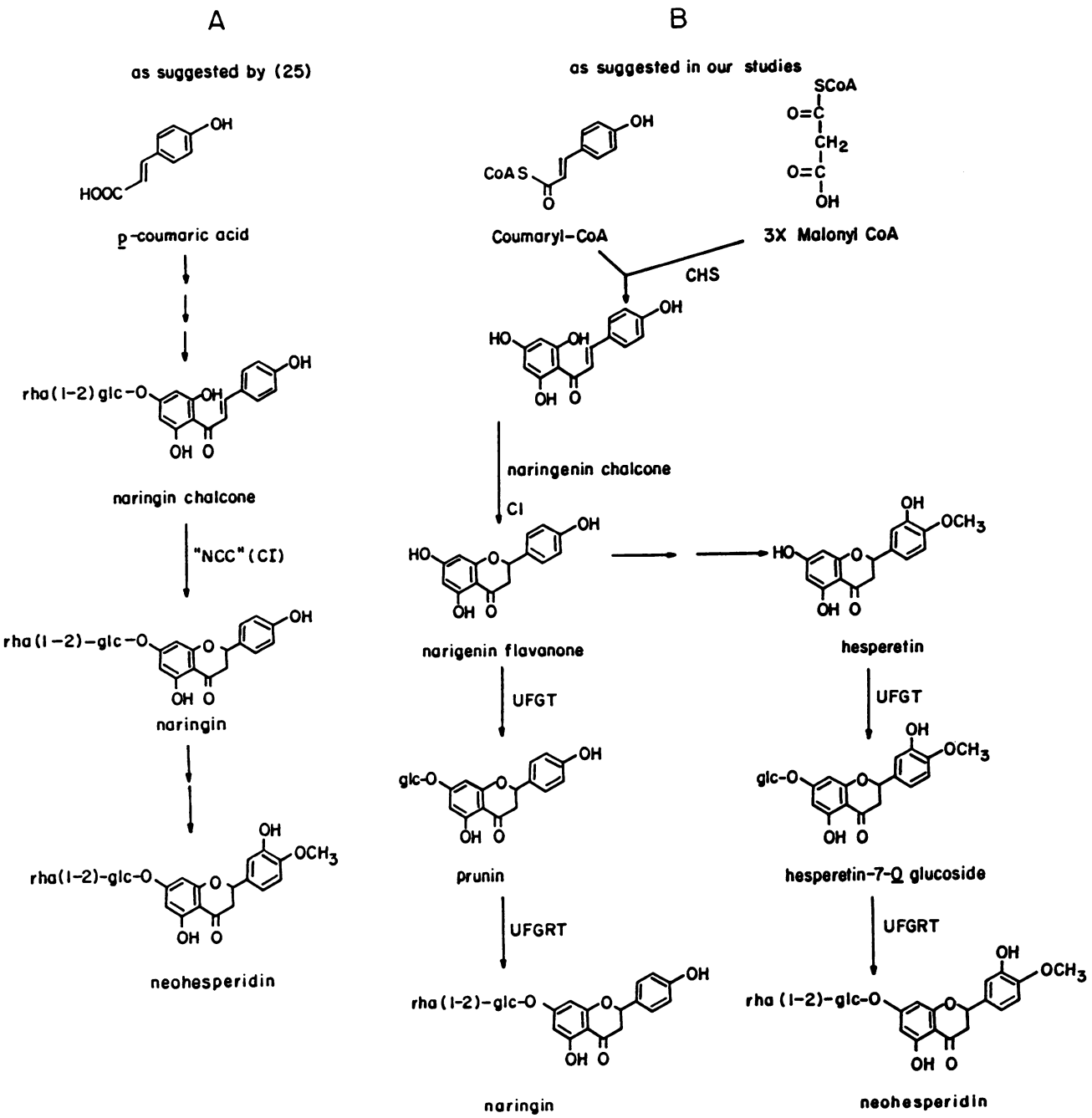

Figure 1. Proposed pathways of naringin and neohesperidin biosyntheses in *Citrus*. A, As proposed by Raymond and Maier (25). B, As proposed in these studies. Enzymes involved: NCC, naringin-chalcone-cyclase (25); CHS, chalcone-synthase reported in this work; CI, chalcone-isomerase reported in Maier and Hasegawa (23) using isoliquiritigenin, and in Shimokoriyama (28) using isosakuranin, naringin, or poncirin chalcones but not naringenin chalcone as a substrate. UFGT, UDP-glucose: flavanone-7-*O*-glucosyl-transferase. Reported in this work. UFGRT, UDP-rhamnose flavanone-glucoside rhamnosyl-transferase. Reported in this work.

Coupled Assay for Conversion of Flavanone Glucosides to Rhamnosyl Glycosides

Due to the unavailability of radiolabeled UDP-rhamnose, we developed an assay whereby the crude extracts both generate UDP-rhamnose and perform the rhamnosylation. This assay is modified from Barber (1). Crude enzyme preparations (120 μg protein) containing both of the abilities to convert UDP-glucose to UDP-rhamnose and rhamnosyl-transferase activity, were incubated with 10 mM NADPH and 1 μCi UDP-glucose in a total volume of 100 μL for 1.5 h at 37°C with or without the addition of 100 μM hesperitin-7-*O*-glucoside (from a 2 mM stock solution in 50% DMSO). The samples were extracted with 400 μL *n*-butanol, evaporated to dryness and the residue applied to a Silica Gel-60 plate (0.2 mm thick) and developed with ethylacetate: methanol:water, 10:2:1 v/v.

The radioactive spots were detected as above and the spots scraped and eluted into 200 μL methanol for elution. The methanol was evaporated and 200 μL of 2 N HCl were added to hydrolyze the glycosides to free-sugars at 100°C for 1 h (9). Upon cooling, the samples were extracted with 200 μL ethylacetate to remove aglycones, and the aqueous phase was concentrated under reduced pressure and applied to 10 cm long cellulose TLC plates. The plates were developed with formic acid: *t*-butanol:methylethylketone:water (15:30:40:5, v/v/v) and autoradiographed. The plates were then sprayed with the aniline phthalate reagent to detect the glucose and rhamnose internal standards.

Protein Determination

Protein was measured by the method of Bradford (3) using the Bio-Rad Coomassie reagent in a total volume of 200 μL. A Titertek-Twinreader (Flow Laboratories) was employed to determine absorbance at 595 nm. BSA (Sigma) was used as a standard.

RESULTS AND DISCUSSION

Chalcone Synthase

Calamondin is an ornamental species that flowers continuously throughout the year (in contrast to other *Citrus* species), enabling the collection of various ages of tissues as necessary. We chose young leaves and fruits because of their high flavanone-glycoside content (19). The cell-free extracts derived from young calamondin fruits contained relatively high levels of chalcone synthase activity (Table I). Younger fruits had a five-fold greater level of activity (Table I), as would be expected from the timing of maximum flavanone-glycoside accumulation (23). Identically prepared cell-free extracts from irradiated parsley cell cultures were tested as controls. The extracts were further analyzed by radio-TLC. Only one radioactive spot corresponding to [^{14}C]naringenin was detected in young calamondin fruit extracts (data not shown), identically to what was found with irradiated parsley cells (4). This permits measuring radioactivity as an index of activity in ethylacetate extracts without further TLC, as previously shown (4).

Table I. *Chalcone-Synthase Activity in Citrus mitis and Petroselinum hortense Cell-Free Extracts*[a]

Source	Tissue Analyzed	Activity
		pmol formed/g fresh wt/min
C. mitis	2 mm diameter fruits	421
C. mitis	15 mm diameter fruits	79
C. mitis	17 × 35 mm young leaves	216
P. hortense	Irradiated cell cultures	1,347

[a] Chalcone-synthase activity was assayed after incubating *C. mitis* cell-free extracts with 15.5 μM [^{14}C]malonyl-CoA (0.92 GBq/mmol) and 10 μM *p*-coumaryl-CoA in 100 mM phosphate buffer (pH 6.8) at 37°C for 1 h and *P. hortense* extracts were incubated for 10 min according to Britsch and Grisebach (4). [^{14}C]Naringenin was the only radioactive product formed as detected by radio-TLC.

These data indicate that chalcone synthase activity is present in cell-free extracts of *Citrus* spp. in greatest amounts at the time of maximum flavanone glycoside accumulation. This demonstrates that chalcones need not be glycosylated prior to their formation, as previously suggested by (25).

UDP-Glucose: Flavanone-7-O-Glucosyl-Transferase

According to the pathway of (25), glucosylation and rhamnosylation of naringenin occur before flavanone formation in *Citrus* tissues (Fig. 1A). Glycosylation occurs after the flavanone is formed and further modified in other species (14). We therefore tested cell-free extracts of *Citrus* for UDP-glucose: flavanone-7-*O*-glucosyl-transferase activity to ascertain whether a flavanone glycosylation pathway is operative (Fig. 1B). We incubated calamondin-derived cell-free enzymatic preparations with the flavanone aglycone naringenin, together with UDP-[^{14}C]glucose. The extracts catalyzed the transfer of a glucose from UDP-glucose to position 7 of naringenin, synthesizing prunin (Table II and Fig. 2, lane N). Extracts obtained from young pummelo leaves had *ca* 100 fold more activity than the other tissues examined (Table II). This demonstrates that the monoglucoside prunin is an intermediate in the biosynthesis of naringin, and is corroborated by the low levels of [^{14}C]prunin found in tracer studies (2). The pummelo extracts also glucosylated H^+ to form hesperetin-7-*O*-glucoside but at half the rate measured for naringenin (Table II and Fig. 2, lane H), even though hesperetin is not usually present in pummelo. This indicates that there is hydroxylation and methylation of naringenin to form hesperetin before glucosylation. The enzymes catalyzing the hydroxylation and methylation of naringenin to form hesperetin have not been described in *Citrus*. The enzymatic hydroxylation of naringenin to eriodictyol is known in *Haplopappus* (10) and *Petroselinum* (11) cell cultures but not in *Citrus*. The *O*-methylation of eriodictyol at position 4′ to form hesperetin has not been reported from any source. Enzymes from parsley (7) and soybean (24) cell cultures *O*-methylated eriodictyol at position 3′. Several flavonoids were *O*-methylated at position 4′ by cell-free extracts of *Citrus mitis* (5) and *Chrysoplenium*

Table II. *UDP-Glucose: Flavanone-7-O-Glucosyl-Transferase Activity in Citrus Cell-Free Extracts*[a]

Source	Tissue Analyzed	Activity Toward	
		Naringenin	Hesperetin
		pmol formed/g fresh wt/min	
C. mitis	2 mm diameter fruits	4	0.5
C. mitis	15 mm diameter fruits	11	2
C. mitis	17 × 35 mm young leaves	21	10
C. maxima	5 × 25 mm young leaves	963	413

[a] UDP-glucose: flavanone-7-*O*-glucosyl-transferase activity was assayed after incubating *Citrus* cell-free extracts with 100 μM flavanone and 925 Bq UDP-[^{14}C]glucose (11.93 GBq/mmol) in 50 mM Hepes-NaOH buffer (pH 7) at 30°C for 1 h. [^{14}C]Prunin (naringenin-7-*O*-glucoside) and [^{14}C]hesperetin-7-*O*-glucoside were formed, respectively, as detected by radio-TLC (Fig. 2). Values were calculated using the specific activity of the UDP-[^{14}C]glucose supplied, ignoring the possible presence of endogenous (cold) UDP-glucose.

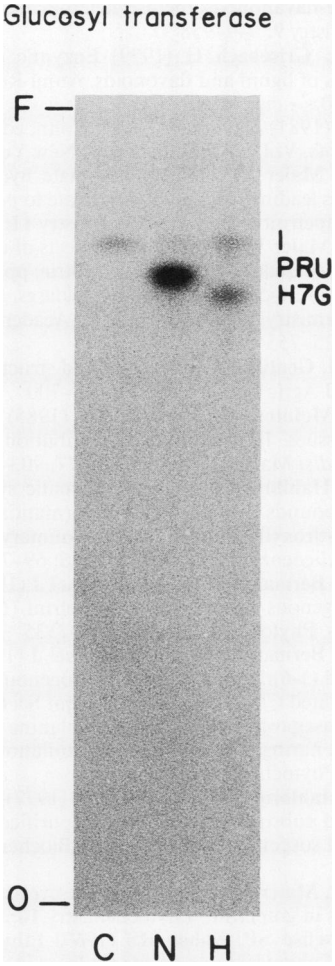

Figure 2. Autoradiogram of a UDP-glucose: flavanone-7-*O*-glucosyltransferase assay in cell-free extracts derived from young *C. maxima* leaves. Conditions as in Table II. Aliquots of the ethylacetate extracts were separated by TLC as described in "Materials and Methods." C, Control, no flavanone addition; N, naringenin (100 μM) added; H, hesperetin (100 μM) added; O, origin; F, solvent front; I, an unidentified impurity; PRU, [^{14}C]prunin; H7G, [^{14}C]hesperetin-7-*O*-glucoside.

(6), but flavanones were not tested. The glucosylation of naringenin and hesperetin at position 7 is catalyzed by cell free extracts of *Citrus* (Fig. 2, Table II), indicating that methylation of flavanones occurred before glycosylation.

Rhamnosylation of Flavanone Glucosides

To ascertain whether the monoglucosides could be further rhamnosylated, hesperetin-7-*O*-glucoside was provided as a substrate to cell-free extracts of grapefruit and pummelo. UDP-[^{14}C]rhamnose was simultaneously provided by the coupled *in situ* synthesis from UDP-[^{14}C]glucose and NADPH. The cell free extracts synthesized a large number of compounds (Fig. 3, lanes 3), including one spot that co-migrated with authentic neohesperidin (hesperetin rhamnoglucoside), the expected product. All spots were eluted and the radioactive sugars removed by acid hydrolysis and analyzed by radio-TLC. The spots comigrating with neohesperidin were the only spots in lanes 3 yielding rhamnose upon hydrolysis (data not

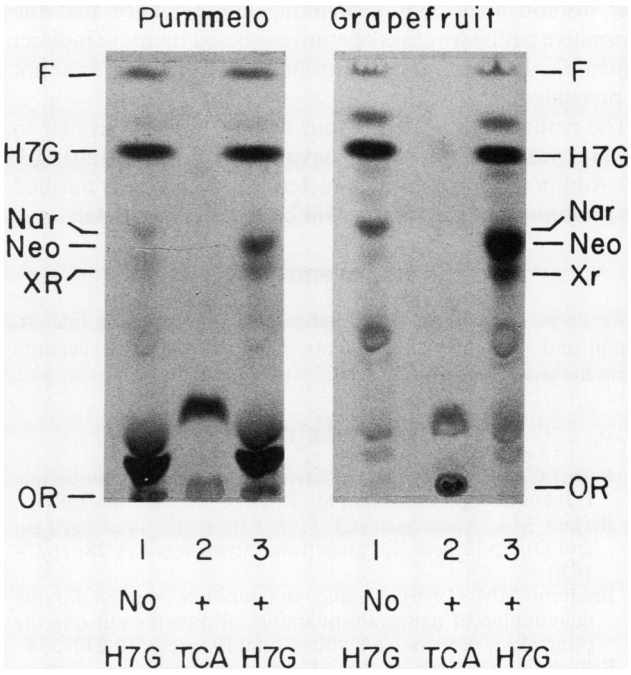

Figure 3. Rhamnosylation of flavanone glucosides by a coupled assay using UDP-[^{14}C]glucose and NADPH. Extracts of pummelo flowers and young grapefruit leaves were prepared as outlined in "Materials and Methods." Lanes 1 contain no exogenous H7G (hesperetin-7-*O*-glucoside), lanes 2 contain the whole reaction mixture with 10% TCA-treated enzyme and lanes 3 contain the complete mixture. The positions of migration of H7G, naringin (NAR) and neohesperidin (NEO) are shown. Acid hydrolysis of all spots from the radio-TLC plates yielded [^{14}C]glucose except the NAR, NEO, and the unidentified XR spots that yielded [^{14}C]rhamnose.

shown), corroborating that these spots were truly neohesperidin. It also indicates that the extracts can convert the glucose in UDP-[^{14}C]glucose to [^{14}C]rhamnose. The spots corresponding to a naringin standard (Fig. 3, lanes 1) also yielded [^{14}C] rhamnose, probably due to the presence of the monoglucoside prunin, in the cell-free extracts. With the exception of an unidentified rhamnoside (XR), all the other compounds in Figure 3, lanes 1 and 3, yielded [^{14}C]glucose upon hydrolysis, as expected from a heterologous system starting with UDP-[^{14}C]glucose. Very young leaves from grapefruit and young open flowers from pummelo gave similar results.

In conclusion, we have demonstrated the enzymatic formation of the aglycone naringenin from *p*-coumaryl-CoA and malonyl-CoA in cell-free extracts derived from young *Citrus* tissues (Table I), indicating that chalcones are biosynthesized before their glucosylation at the developmental times expected. We have also shown that the glucosylation of naringenin and hesperetin at position 7 occurs in *Citrus* cell cultures (21, 22), and is catalyzed by cell-free extracts of *Citrus* (Table II and Fig. 2). The cell-free extracts seemed to be able to synthesize UDP-rhamnose from UDP-glucose as they biotransformed exogenous hesperetin-7-*O*-glucoside into neohesperidin and endogenous prunin into naringin (Fig. 3). These findings clearly indicate that flavanones can be hydroxylated, methylated, glucosylated and then rhamnosylated in a step-

wise fashion after their formation. The evidence that the alternative pathway (25) is operative is based on more indirect evidence and on certain assumptions, but its existence cannot be precluded.

The pathway supported by our data (Fig. 1B) is similar to the biosynthetic pathways of flavonoids in other plants (13, 14). Additionally, we have characterized and partially purified the rhamnosyl-transferase, as will be reported separately.

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