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Naringin Levels in Citrus Tissues[1]

II. QUANTITATIVE DISTRIBUTION OF NARINGIN IN *CITRUS PARADISI* MACFAD.

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ABSTRACT

The quantitative distribution of the flavanone-7-neohesperidoside, naringin, in seeds, seedlings, young plants, branches, flowers, and fruit of *Citrus paradisi* Macfad., cv 'Duncan' was analyzed by radioimmunoassay. High levels of naringin were associated with very young tissue and lower levels were found in older tissues. Seed coats of ungerminated seeds and young shoots had high naringin concentrations whereas cotyledons and roots had very low concentrations. Light-grown seedlings contained nearly twice as much naringin as etiolated seedlings and, in young plants and branches, the naringin content was highest in developing leaves and stem tissue. In flowers, the ovary had the highest levels of naringin, accounting for nearly 11% of the fresh weight. There was a net increase in the total naringin content of fruits during growth. However, due to the large increase in fruit size, there was a concomitant decrease in the naringin concentration as the fruit matured.

The flavonoid pigments of plants have been intensively investigated over the past three decades and it is clear that this group of secondary metabolites is one of the largest and most diverse from both structural and functional standpoints (9). Although the taxonomic distribution of these compounds has been fairly well clarified, many questions concerning their physiological roles remain unanswered. Unique subcellular and tissue distribution patterns have been demonstrated for some of these compounds (25) and metabolic studies have revealed that, in some plant tissues, these compounds are highly dynamic and undergo significant turnover (4). More extensive investigations on the metabolism, transport, and accumulation of flavonoids have been limited, however, by the laborious and relatively insensitive methods used for flavonoid quantification.

Naringin, the 7-β-neohesperidoside of naringenin (4′,5,7-trihydroxyflavanone) is the most abundant flavonoid in grapefruit and can account for 40 to 70% of the dry weight of small green fruit (16). Little information is available on the individual reactions involved in the biosynthesis of this compound (10, 11, 17, 23); however, kinetic studies have suggested that this flavonoid is rapidly synthesized in tissues during the cell division phase and that synthesis slows in the cell enlargement phase. Highest levels are reached at organ maturity and these remain relatively constant (1, 17, 18).

Due to the bitterness which naringin imparts to grapefruit products, the quantitative determination of this compound in fruit tissue on a seasonal basis has received a great deal of attention (1–3, 8, 17; McIntosh *et al.*, in preparation). However, the distribution of naringin in vegetative and floral tissues has not been critically analyzed and little is known about the changes in naringin levels during plant growth and floral development. As an initial part of our studies on the biosynthesis and metabolism of this compound, we have utilized a sensitive and specific RIA[3] (15) to quantitate the naringin levels in *Citrus paradisi* tissues and organs.

MATERIALS AND METHODS

Plant Material. Fruits were obtained from grapefruit trees (var Duncan) grown at the Agricultural Experimental Station, Lake Alfred, FL. Seeds were removed from mature (November) fruit, placed on 1% agar, and were germinated under continuous illumination or in darkness. After 3 weeks, some seedlings were transferred to soil and were grown in a phytotron (25°C, 50% RH) under a 16-h light/8-h dark cycle. The 1-year-old plant used in this study was grown under the same conditions. Buds and flowers at various stages of development were collected from a single mature tree. Stages were defined according to the fresh weight and degree of development of the flowers.

Extraction of Plant Tissues. For seeds, the outer seed coat, inner seed coat, and the cotyledons/embryo were separated before extraction. All tissues were cut into small pieces, placed in 5 ml methanol and boiled for 2 h. Tissues weighing more than 500 mg fresh weight were extracted in 10 ml methanol. After extraction, samples were filtered, brought to the original volume, diluted 50% with water, and stored at −20°C until assay. Fruits and fruit parts were extracted by maceration in a volume of 0.1 M Tris-HCl (pH 8.0) sufficient to prevent gelation of endogenous pectins and the extracts stored at −20°C.

Radioimmunoassay Procedure. The procedures for the naringin RIA have been described elsewhere (15; and part I of this series). Samples were diluted with water and assayed by the [^{3}H] naringinol/oxime-hapten antiserum RIA described in part I.

TLC and HPLC. Chromatographic analysis of the extracts were done using 5× concentrated samples after published procedures (6, 7).

RESULTS AND DISCUSSION

The genus *Citrus* is characterized by the accumulation of flavanone glycosides rather than the more common flavone, flavonol, or anthocyanin glycosides (11) and therefore presents a unique opportunity to study both the biosynthesis and metabolism of this group of compounds. In addition, recent enzymatic studies have suggested that a flavonoid glycoside biosynthetic pathway may exist in grapefruit (20) which is different from that characteristic of other plants (*e.g.* mung bean, parsley, peas, etc.

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[3] Abbreviation: RIA, radioimmunoassay.

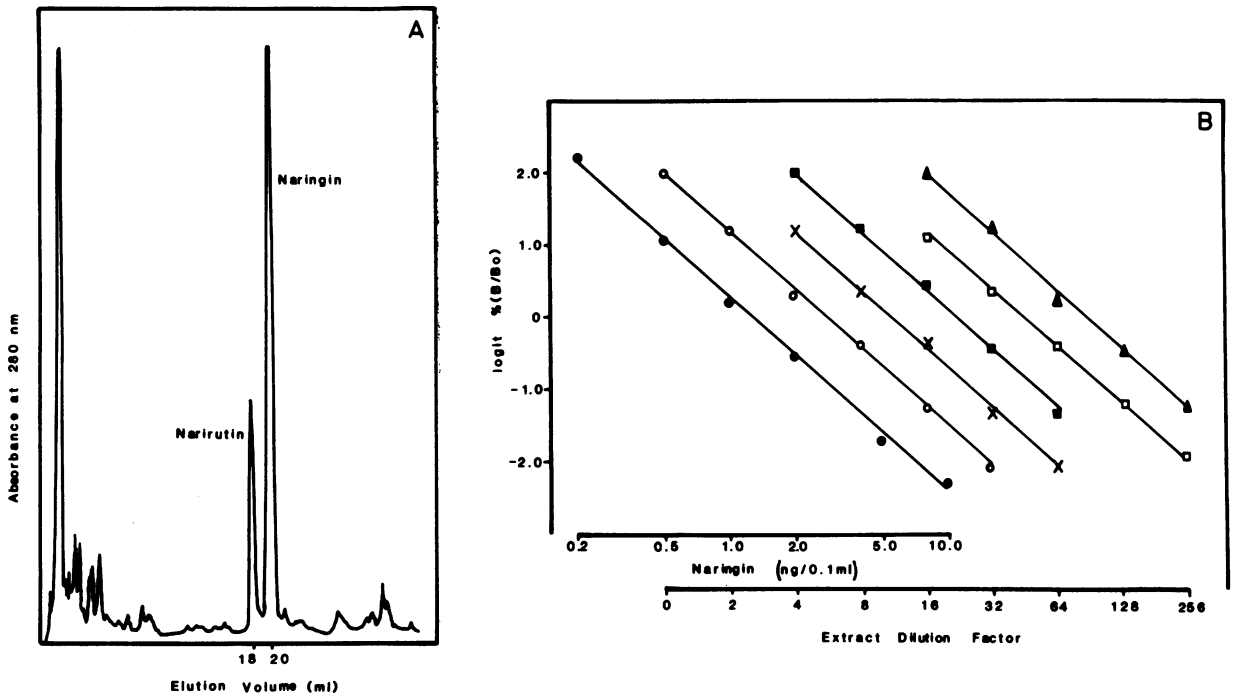


FIG. 1. Identification of naringin as the major flavanone-7-neohesperidoside and principal immunoreactive compound in Duncan grapefruit extracts. A, HPLC pattern of a representative sample (juice vesicles), essentially identical to the pattern of all other tissue extracts examined. B, Comparison of the immunoreactivity of serial dilutions from a standard naringin solution (●) and from various tissue extracts: (○), seed coats; (×), etiolated seedlings; (■), albedo; (□), flushing leaves; (▲), ovary. All serial dilution curves from each of the different tissues and organs extracted were parallel to the dilution curve of the naringin standard. Logit % (B/Bo) = ln [(%B/Bo)/100 − (%B/Bo)] (see 11).

Table I. *Naringin Content of Duncan Grapefruit Seeds*

Seed Part	Naringin Content			
	Total		Concn.	
	nmol[a]		*nmol/g fresh wt*	
Fertilized seeds (n = 16)				
Outer testa	42.5[b]	18.7–86.7[c]	996[b]	309–2477[c]
Inner testa	5.5	3.1–11.6	786	330–1148
Cotyledons/embryo	5.3	1.5–10.5	25	9–43
Whole seed	58.8	37–85		
Unfertilized seeds (n = 7)				
Outer testa	24.8	15.8–30.8	1394	1098–1919
Inner testa/remnants	5.1	2.0–7.7	958	245–1875

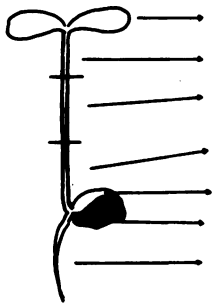
[a] 1 nmol = 0.58 μg. [b] Mean. [c] Range.

[7]). The present study was conducted to identify the grapefruit tissues and stages of development associated with high levels of naringin which could serve as starting material for *in vivo* and *in vitro* biosynthetic and metabolic studies. Logistical problems associated with the analysis of the number of samples necessary to accomplish this have been alleviated through the use of the RIA.

The RIA used in this study is able to detect other flavanone-7-neohesperidosides (part I); however, this broad assay specificity did not affect the present analyses since it was determined that naringin was the most abundant flavanone neohesperidoside in these grapefruit tissues (Fig. 1A). In addition, each tissue extract was analyzed by HPLC and TLC and these analyses showed that the Duncan variety studied here had the same flavonoid composition in flowers, leaves, and fruit as those previously reported (14, 22). Thus, the predominance of naringin in Duncan grapefruit coupled with the extensive extract dilution required for assays (up to 1:50,000) has allowed the measurement of this compound with confidence. Further evidence that naringin was the only immunoreactive compound in the extracts is provided in Figure 1B where the lines representing assays of serial dilutions of crude extracts are parallel to the naringin standard curve. Since previous studies on naringin distribution used unspecific methods for the quantification of this compound (*e.g.* colorimetric test [2, 16]), the analyses of naringin content using the RIA represent an improvement over previous quantitative studies.

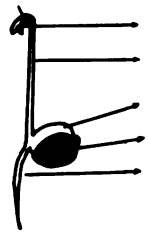
The survey of naringin distribution in grapefruit was initiated by analyzing seeds. The average naringin content per seed was 58.8 nmol and the range was 37 to 85 nmol/seed (Table I). Of the total naringin present, 90% was found in the testae which is maternal in origin (24). Seventy-five % was in the outer testa and 15% was in the inner testa. The naringin concentration, however, was similar in both the outer testa (996 nmol/g fresh weight) and the inner testa (786 nmol/g fresh weight) and only 3% of the total naringin in the cotyledons/embryo was found in the embryo itself. Fertilization and development of embryos (zygotic or nucellar) and cotyledons apparently does not result in naringin accumulation since unfertilized seeds, consisting mainly of a testa, also contained high levels of naringin (Table I). An analysis of seeds washed in warm methanol for 2 min to remove the vesicle mucilage showed that the naringin was intrinsic to the testa. The distribution pattern of naringin in seeds contrasts with that of the other major bitter principle of grapefruit, limonin (a triterpene dilactone), which can account for up to 1% of the fresh weight of the cotyledons (13).

Naringin production in seeds is initiated during germination and the emerging shoot is the major site of either production or accumulation. The average total naringin content in 3-week-old (approximately 3 to 4 cm) light-grown seedlings was 111 nmol (Fig. 2), twice the average of ungerminated seeds (58 nmol) and the shoot contained 88% of the total naringin. The high level of naringin in the shoots (97 nmol average) could not be solely due to transport from the testae and cotyledons (58 nmol average)



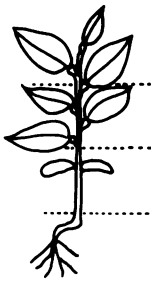
Seedling Part	Total (nmol) Mean	Total (nmol) Range	Concentration (nmol/g fwt) Mean	Concentration (nmol/g fwt) Range
Primary Leaves	74.8	18.7-120.7	12764	3596-27991
Upper One-third Stem	15.3	4.8-23.0	1105	454-1952
Middle One-third Stem	5.3	1.7-14.1	267	87-648
Lower One-third Stem	1.9	0.7-3.4	66	32-104
Cotyledons	1.5	1.4-3.6	58	15-155
Testa	10.5	8.8-12.4	216	61-338
Root	2.4	1.5-3.7	56	24-162
Whole seedling	110.9			

FIG. 2. Distribution of naringin in 3-week-old light-grown seedlings. Twenty seedlings were separated into the various parts shown and the naringin content was determined independently in each.



Seedling Part	Total (nmol) Mean	Total (nmol) Range	Concentration (nmol/g fwt) Mean	Concentration (nmol/g fwt) Range
Plumule	28.6	11.1-46.8	3196	1700-4675
Epicotyl	6.1	3.1-9.4	41	24-78
Cotyledon	3.2	2.2-4.1	17	10-31
Testa	4.9	2.4-12.2	44	31-75
Root	0.7	0.3-1.4	12	3-20
Whole seedling	43.7			

FIG. 3. Distribution and content of naringin in the major organs of 3-week-old etiolated seedlings.



Seedling Part	n	Total (nmol) Mean	Total (nmol) Range	Concentration (nmol/g fwt) Mean	Concentration (nmol/g fwt) Range
Leaves	26	692	63-1198	4932	1510-22326
Stem	8	134	14-333	1165	180-2715
Leaves	24	114	5-343	1185	167-3259
Stem	8	17	2-39	194	97-502
Primary leaves	12	48	7-117	699	141-1434
Stem	8	4	1-10	54	15-117
Root	8	4	1-17	14	2-63

FIG. 4. Distribution of naringin in leaves, stem, and root of 5-month-old plants.

even if a relocation mechanism was active; therefore, it is apparent that either some *de novo* synthesis or conversion from a nonimmunoreactive form(s) occurred. Within the light-grown shoots, the highest levels and concentrations of naringin were found in the primary leaves followed by the epicotyl; the concentration along the length of the stem increased toward the apical region (Fig. 2). Further investigation showed that the apical bud had the highest concentration with the top 2 mm of the epicotyl contributing greatly to the apparent naringin gradient (data not shown).

The naringin content of each pair of primary leaves of a seedling was nearly identical. However, in comparing the naringin content of sets of primary leaves in multiple seedlings from the same seed (polyembryony), no consistent pattern was noted, *i.e.* all seedlings from the same seed did not contain the same amount of naringin. The pattern of distribution of naringin within the etiolated seedlings paralleled that of light-grown seedlings (Fig. 3); however, the average total amount of naringin in etiolated seedlings (43.7 nmol) was lower than the content of ungerminated seeds (58.8 nmol). Thus, it appears that light does not affect the distribution of naringin in seedlings; rather it directly or indirectly affects the absolute amounts of the compound.

Five-month-old seedlings consisted of 6 to 10 foliage leaves in addition to the original pair of primary leaves. The seedlings were divided into three sections (upper, middle, and lower one-third) and all tissues in a section were analyzed. The whole seedlings contained an average of 1013 nmol of naringin of which 84% was found in the leaves (Fig. 4). The highest levels and concentration were found in the upper section where the leaves were actively growing. In the middle section, which contained fully expanded and dark-green leaves, the average naringin concentration was approximately one-fourth that of the younger leaves. The primary leaves contained substantially lower levels (48 nmol) and concentrations (699 nmol/g fresh weight) of naringin than the younger leaves on the same plant (692 nmoles and 4932 nmol/g fresh weight, respectively). Primary leaves of 5-month-old plants were also much lower in naringin content than the primary leaves of 3-week-old seedlings (*cf.* Fig. 2). The pattern of naringin content in the stems also increased acropetally.

In a 1-year-old plant, the naringin concentrations were highest in the youngest leaves (Fig. 5) and mature, dark-green leaves had the lowest concentration. The greatest total amount of naringin per leaf was found in fully expanded, light-green leaves. The naringin concentration of the stem increased acropetally and the concentration in the root was very low.

During the March flush, two branches were collected from a tree in a local grove and the naringin content of the flushing and mature leaves (from the previous flush) was determined. Flushing

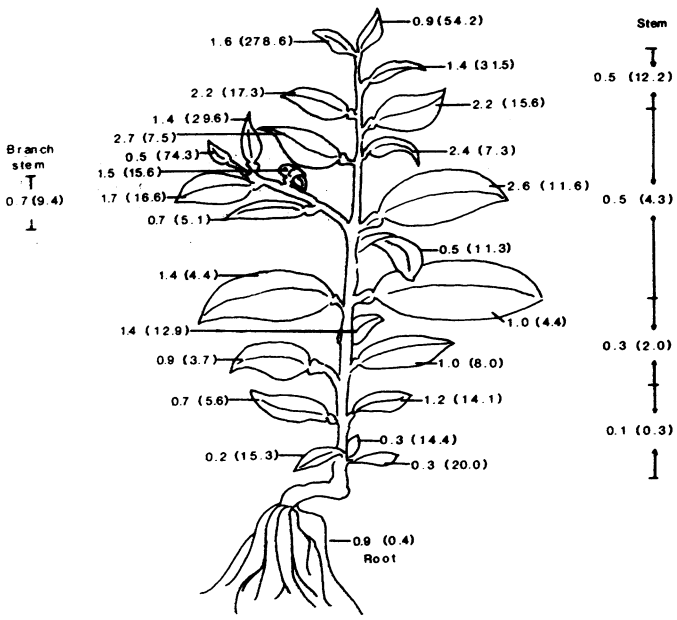


FIG. 5. Distribution of naringin in leaves, stem, and root of a 1-year-old plant. The first number represents the total amount of naringin (μmol) in each leaf, stem section, or root. The numbers in parentheses represent the naringin concentration (μmol/g fresh weight).

leaves contained an average of 12.7 μmol naringin/leaf and the mature leaves had 7.6 μmol/leaf (Fig. 6). The total amount of naringin was high in all flushing leaves; however, the concentrations were highest in leaves nearest the apical bud.

In all cases, very young leaves had the highest concentrations of naringin (up to 10% of the fresh weight). As the leaves expanded and matured, the amount of naringin per leaf increased slightly; however, the concentration dropped due to the increased weight of the leaves. Fully mature leaves contained both lower amounts (40% decrease) and lower concentrations (80% decrease) of naringin. Thus, the net production of naringin appears to occur during development of the leaf. Evidence for the biosynthetic capacity of grapefruit leaves for naringin production had been provided by ^{14}C uptake experiments (5) where it was shown that young leaves readily incorporated phenylalanine into naringin, whereas older leaves did not. Similar situations from other plants have been described for flavonoid biosynthetic enzymes where the highest specific activities were found in the very youngest leaves (25, and references cited therein). In our previous studies (21) on the distribution of limonin in grapefruit leaves, we have observed basically the same pattern as found here for naringin.

Naringin production in flowers appears to parallel that in leaves, that is, the process of development is accompanied by an increase in the total naringin per flower (from 0.5 to 9.9 mg) as the bud develops (Fig. 7); however, the concentration decreases steadily until the stamens and petals drop from the fertilized ovary (stage 9). It is significant that the total amount of naringin in stage 9 flowers was as high as the total amount in the mature flowers which possessed all flower parts (stages 7 and 8). The sharp decrease in the fresh weight of stage 9 flowers, due to loss of stamens and petals, results in a sharp increase in the apparent naringin concentration.

An analysis of the distribution of naringin in flowers (Table

BRANCH I

	Old Leaves			Flush F			Flush F'		
	Fresh Weight (mg)	Total (μmol)	Conc. (μmol/g)	Fresh Weight (mg)	Total (μmol)	Conc. (μmol/g)	Fresh Weight (mg)	Total (μmol)	Conc. (μmol/g)
1	1221	8.7	7.0	a 376	11.9	31.6	a' 387	19.6	50.5
2	1001	10.4	10.3	b 453	13.1	28.9	b' 211	15.0	70.9
3	1616	9.0	5.5	c 521	14.1	27.1	c' 404	17.4	43.8
4	958	7.8	8.1	d 432	12.9	29.9	d' 269	10.7	39.8
5	1175	8.2	6.9	e 188	5.6	29.8	S 307	6.3	20.5
6	1026	8.5	8.2	f 255	6.3	24.7			
7	683	5.6	8.2	S** 299	3.4	11.1			
8	295	2.9	9.8						
S_1*	584	0.5	1.0						
S_2	631	0.5	0.8						
S_3	789	0.5	0.6						

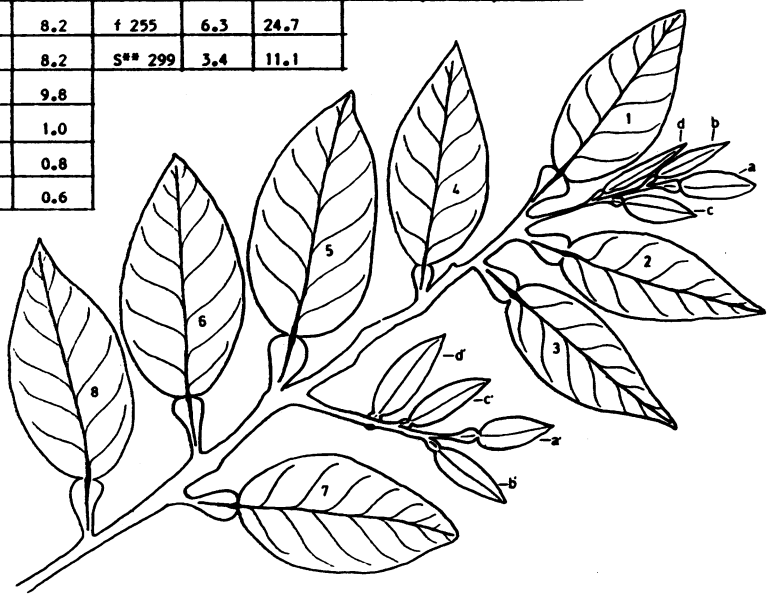


FIG. 6. Naringin content of March flush and adjoining branch. The data represents one of the two branches analyzed; each branch had similar distribution patterns. S_1*, S_2, S_3 = Upper, middle, and lower stem sections, respectively; S** = entire stem of flush.

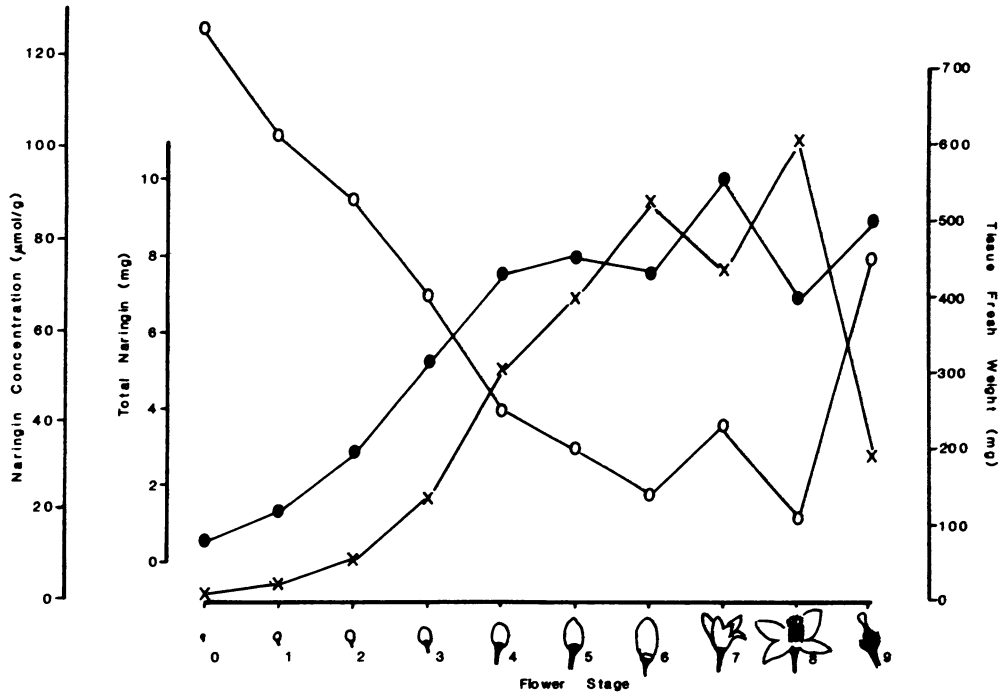


FIG. 7. Changes in naringin content of developing grapefruit flowers. Different stages were selected from a single branch and were established according to fresh weight and degree of development. Two samples were analyzed at each stage and the data represent the average of both. (O), Naringin concentration; (●), total naringin; (×), fresh weight of flowers.

Table II. *Naringin Levels in Stage 8 Flowers*

Flower Part	Naringin Levels			
	Total		Concn.	
	μmol		*μmol/g fresh wt*	
Petals	3.4[a]	2.2–4.3[b]	16.8[a]	8.4–24.6[b]
Stamens	2.1	1.5–3.1	21.1	17.6–26.2
Pistil	9.2	6.9–10.2	133.1	102.8–159.7
Calyx/receptacle	3.4	2.0–5.4	38.1	19.2–67.8

[a] Mean. [b] Range.

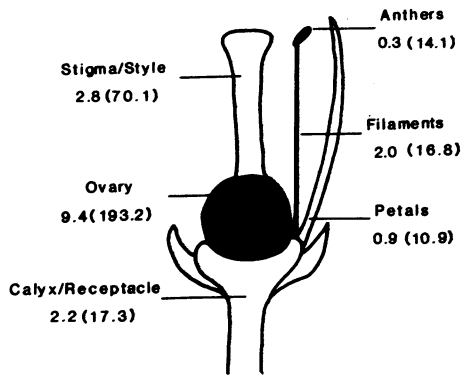


FIG. 8. Naringin content of the different tissues in grapefruit flowers. The first number represents the total amount of naringin (μmol) in each organ; the numbers in parentheses represent the concentration of naringin (μmol/g fresh weight).

II) showed that both the concentration and total content were highest in the pistil followed by the calyx/receptacle, petals, and stamens. The concentrations in the anthers were similar to that in the filaments (Fig. 8) and naringin concentrations in individual petals were similar to each other.

In the pistil, the ovary had the highest naringin content (11% of the fresh weight) and concentration (Fig. 8) and the concentration remained high through the development of this organ and young fruit (Fig. 9). Although the average total naringin per fruit increased from 0.07 mmol in 1-month-old fruit to an average of 5.56 mmol in fully mature fruit, there was a 26-fold decrease in relative concentration (mature fruit having 6.3 μmol/g). This decrease is likely due to the 2000-fold increase in fruit fresh weight relative to the 80-fold increase in the total amount of naringin.

These results confirm previous findings on 'flavanone glycoside' accumulation in grapefruit which demonstrated that synthesis was most active in young fruits (1), and that there is a decrease in naringin concentration in fruit tissues during maturation (2). It is, however, still not clear whether the rapid increase in content during the early stages of fruit development is due to synthesis within the fruit itself or to translocation from the surrounding young leaf tissue which is also capable of synthesis (5). This type of transport mechanism in *Citrus* has been demonstrated for limonin which has been shown to be transported from the leaves into fruit and into seeds (12). The pattern of distribution of naringin within mature fruit was very distinct (Fig. 9). The juice vesicles and the seeds had the lowest concentrations while the segment membranes, albedo, and pith had the highest.

In summary, the results of this study show that the production of naringin is primarily associated with the process of growth and development of leaves, flowers, and fruit. Naringin production or accumulation is very minor in roots whereas in stems it parallels that of leaves, albeit at a significantly lower level. Although the studies described here were not strictly kinetic, the numerous samples that could be analyzed by RIA permitted the analysis of tissues at many different stages of growth, thus allowing inferences about the possible changes accompanying development.

Our finding that young seedlings produce high amounts of naringin provide a good model system for identifying the biosynthetic and metabolic pathway of this flavanone-neohesperidoside. Elucidation of naringin production in seedlings would complement comparative studies of naringin production between different grapefruit organs and between different flavanone compounds (*e.g.* hesperidin) found in other *Citrus* species. In addition, the high levels of naringin and limonin in grapefruit and their unique distribution provide an excellent opportunity

Month	Fruit Weight (g)	Total in Fruit (mmol)	Conc. in Fruit μmol/g	Concentration in fruit tissues (μmol/g) Flavedo	Albedo	Vesicles	Membranes	Pith	Seeds
Apr.	0.45 ±0.18	0.07 ±0.01	162.70 ±24.65	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Sept.	620 ±101	4.66 ±0.68	7.73 ±2.00	13.3 ±4.5	23.6 ±6.6	1.0 ±0.2	16.5 ±4.0	23.6 ±2.7	0.8 ±0.2
Dec.	880 ±90	5.56 ±1.90	6.31 ±1.90	8.3 ±3.2	19.8 ±5.6	0.8 ±0.2	12.6 ±4.0	16.4 ±2.6	0.7 ±0.2

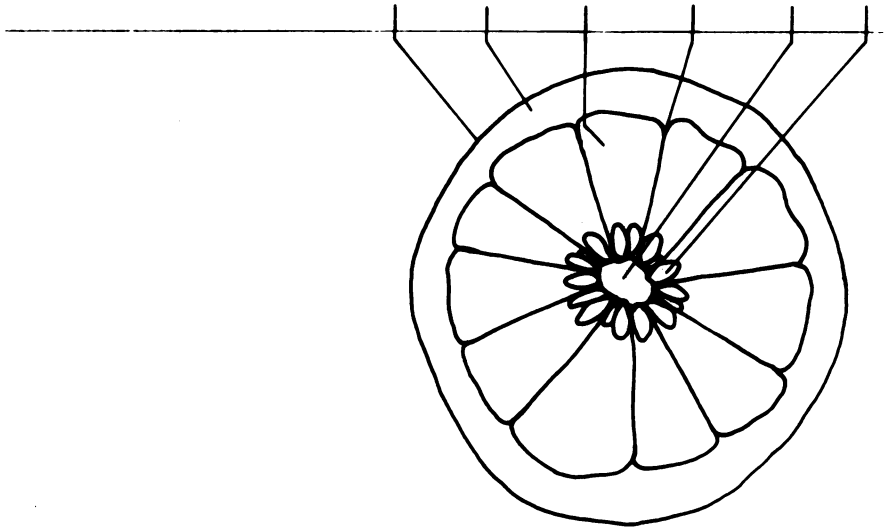


FIG. 9. Naringin content and distribution in fruit. The immature (April) fruit was too small to dissect into the various tissues.

for comparative physiological studies on two chemically unrelated secondary products that share the property of being bitter.

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