Changes in Neodiosmin Levels during the Development of *Citrus aurantium* Leaves and Fruits. Postulation of a Neodiosmin Biosynthetic Pathway

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Neodiosmin (5,7,3′-trihydroxy-4′-methoxyflavone 7β-neohesperidoside) levels have been analyzed during the developmental stages of *Citrus aurantium* leaves and fruits. Neodiosmin reaches its maximum concentration in the organs studied during the first stages of growth, gradually decreasing until the organs reach full development. However, the maximum rate of neodiosmin synthesis and/or accumulation occurs during the lineal phase of leaf and fruit growth. The study of 4′-*O*-methyltransferase substrate specificity, the capacity of glucosyltransferase for flavone glucosylation, and the correlation between the levels of neodiosmin and the flavanone neohesperidin during leaf and fruit growth lead us to propose a biosynthetic pathway for neodiosmin in *C. aurantium*.

INTRODUCTION

The flavonoids are one of the widest and most diverse groups of plant secondary metabolites from both structural and functional points of view (Horowitz and Gentile, 1977). The flavonoids of *Citrus* have been the subject of intense biochemical studies in past years (Ebel and Hahlbrock, 1982).

The genus *Citrus* is characterized by its synthesis and accumulation of flavanones in their free form and/or as glycosides rather than of the more common flavones, flavonols, or anthocyanins (Albach and Redman, 1969). Flavones and their glycosides form an important subgroup within the flavonoids, their basic structure being the 2-phenylbenzo-γ-pyrone skeleton. The introduction of the C2–C3 double bond into flavanone forms flavone, an important "end product", generated in the flavonoid biosynthesis (Wollenweber and Jay, 1988; Harborne and Willians, 1988). We have recently isolated and identified neodiosmin (5,7,3′-trihydroxy-4′-methoxyflavone 7β-neohesperidoside) from *Citrus aurantium* (Del Rio et al., 1991). Bitter or sour orange contains a certain percentage of methoxyflavones (nobiletin, tangeretin, sinensetin), although neodiosmin is the only glycosylmethoxyflavone identified to date in *C. aurantium*.

Neodiosmin has been used to reduce the bitterness of naringin and limonin in fruit juices (Guadani et al., 1976, 1977). This fact is of commercial interest because it could lead to increased acceptability of juices containing relatively high levels of naringin and/or limonin, such as grapefruit or lemon juices.

Little information is available on the individual reactions during the biosynthesis of flavonoids in *Citrus*. Enzymes that take part in naringenin biosynthesis and in its subsequent glycosylation to naringin have been characterized (Berhow and Vandercook, 1989; Gavish et al., 1989; Lewinsohn et al., 1989a,b; Maier and Hasegawa, 1970; Maier and Metzler, 1967; McIntosh and Mansell, 1990; McIntosh et al., 1990). However, the biosynthetic pathways of 4′-methoxylated glycosylflavones and flavanones have not been fully described (Lewinsohn et al., 1989a; Raymond and Maier, 1977).

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We report the changes in the neodiosmin content during the development of leaves and fruits of Seville orange. In addition, the substrate specificity of the enzyme 4′-*O*-methyltransferase (OMT) and the capacity of glucosyltransferase for flavone glucosylation, both isolated from *Citrus aurantium*, are analyzed to help clarify the biosynthetic pathway of this flavone.

MATERIALS AND METHODS

Plant Material. Leaves and immature fruits of *C. aurantium* were obtained from 5-year-old trees, grown in greenhouses of the University of Murcia.

Extraction of Flavonoids. Ten leaves and five fruits were collected and immediately dried at 50 °C (Hosoda and Noguchi, 1988). They were then ground and the flavonoids extracted with dimethyl sulfoxide in the ratio 2 mg/mL. The solutions were filtered through a 0.45-μm nylon membrane. These measurements were repeated for four trees, and the mean values obtained at each age were used to express the distribution of neodiosmin in leaves and fruits.

Chromatographic Analysis. For the quantification of neodiosmin in the leaf and fruit extracts of *C. aurantium*, we used a μBondapak C_{18} (250 × 4 mm i.d.) analytical column with an average particle size of 5 μm, with the isocratic solvent H_2O–MeOH–acetonitrile–HOOAc (15:2:2:1); the flow rate was 1 mL/min at room temperature. The absorbance change was monitored at 280 nm with a UV–vis diode array detector.

Preparation of Cell-Free Extracts. Cell-free extracts were obtained as described previously (Larson, 1989) for *Zea mays*, using the eluate of a Sephadex G-50 column as enzyme source. The protein content was determined according to the Bradford method (Bradford, 1976).

4′-*O*-Methyltransferase (OMT) Assay. OMT activity was determined as described previously (Larson, 1989). Product formation was determined by HPLC of the reaction medium in a μBondapak C_{18} (250 × 4 mm i.d.) with an average particle size of 5 μm, using methanol–water (50:50 v/v) as the eluent with a flow rate of 1 mL/min at room temperature. The column eluent was monitored at 280 nm for flavanones and at 345 nm for flavones. The concentration of the reaction product was determined by comparison of peak area data with those obtained for the relevant standard chromatographed at different concentrations.

UDPG:7-*O*-Glucosyltransferase (GT) Assay. The assay contained Hepes buffer (50 mM, pH 7.5) with 2 mM DTE, 200 μM UDP-glucose, 100 μM diosmetin, and 2.8 mg of enzyme in a total volume of 3 mL. Controls without substrate or enzyme were run with the samples and all incubated at 40 °C for 60 min. The assays were terminated by the addition of 200 μL of 2 N

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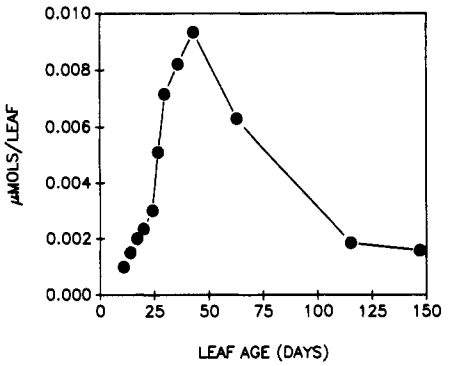


Figure 1. Changes in the mean total content per leaf of neodiosmin according to age in *C. aurantium*.

HCl. Product formation was determined by HPLC of the reaction medium of a μBondapak C_{18} (250 × 4 mm i.d.) with an average particle size of 5 μm, using methanol–water (40:60 v/v) as the eluent with a flow rate of 1 mL/min at room temperature. The column eluent was monitored at 345 nm.

RESULTS AND DISCUSSION

Changes in the Levels of Neodiosmin during the Development of *C. aurantium* Leaves. Neodiosmin levels (micromoles per leaf) increase as the leaf develops (Figure 1), reaching its maximum value when the organ ceases to grow (43 days). Subsequently, not only does flavone accumulation cease but a sharp drop in total content occurs during the maturation phase, probably due to degradative processes (Bartz, 1977; Curir et al., 1990).

During leaf development, neodiosmin concentration is at its highest at the end of the logarithmic phase of leaf growth and decreases during the lineal phase due to a dilution effect caused by water entering the growing cells, as was observed for naringin and neohesperidin in the same species (Castillo et al., 1992).

These levels of neodiosmin in *C. aurantium* seem to suggest that the synthesis or accumulation of this flavone is higher in the lineal phase of leaf growth. This is not the case for the other flavonoids described in this plant, particularly the most abundant flavanones, naringin and neohesperidin, which show maximum accumulation rates during the logarithmic phase of development, coinciding with a period of intense cellular division (Castillo et al., 1992). This last fact is also supported by the strong increase in flavonoids in vitro when certain vegetal tissues are treated with cytokinins (Curir et al., 1990), active inducing agents of cellular division. It also agrees with the data obtained for naringin synthesis in *Citrus paradisi* (Albach et al., 1969).

The fact that neodiosmin shows a higher synthesis or accumulation rate in the stages immediately following cellular division suggests that it is an end product in the biosynthetic pathway of flavonoids in *C. aurantium* and that the specific enzyme responsible for its synthesis (flavone synthase) acts later than the characteristic enzymes of flavonoid biosynthesis (chalcone/flavanone synthase) and after the other activities involved in the different stages of this biosynthesis.

Changes in the Levels of Neodiosmin during the Development of *C. aurantium* Fruits. The fruit of Seville orange presents a sigmoidal growth curve (Castillo et al., 1992), reaching its maximum size (61-mm diameter) at approximately 200 days. From this time, the processes of maturation begin, with no appreciable change in diameter.

The accumulation curve for neodiosmin as a function of the age of the fruit (Figure 2) shows a profile that is

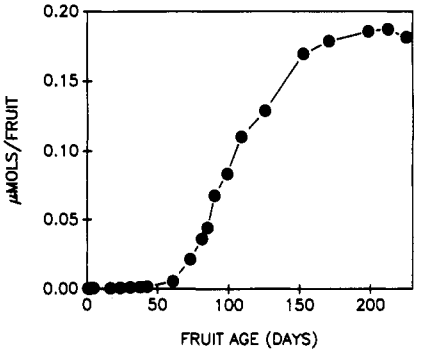


Figure 2. Changes in the mean total content per fruit of neodiosmin according to age in *C. aurantium*.

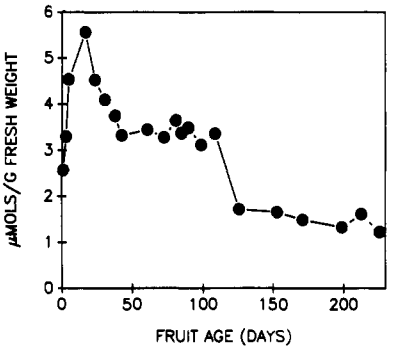


Figure 3. Neodiosmin concentration during the development of *C. aurantium* fruits. Fruit growth is determined according to age.

identical to the growth curve. An initial latency during the logarithmic phase of fruit growth is observed, followed by rapid accumulation during the lineal phase and a final stage of stabilization, where no increases (with time) are produced and which coincides with the beginning of maturation. This profile is similar to that for other flavonoids in the same plant material and in immature fruits of *Citrus limonia* (Vandercook and Tisserat, 1989) and *C. paradisi* (Albach et al., 1969), although neodiosmin shows a longer period of latency.

The fact that no neodiosmin was detected in stems or flower receptacle and stem-end fluids seems to suggest that the neodiosmin found in *C. aurantium* fruits is the result of the in situ synthesis of this flavone.

Neodiosmin concentration (Figure 3) is highest in the first stages of fruit development (17 days). After this, the concentration falls by approximately 40% over a short period of time, primarily due to the dilution effect caused by the growth in cell volume during fruit development. Between 43 and 110 days, which coincides with the lineal phase of growth, the concentration of neodiosmin remains practically constant, due to the fact that its rate of synthesis and/or accumulation during this period is maximal and overlapped the dilution effect. After this, neodiosmin concentration falls again and then stabilizes when the lineal phase of growth finishes.

Possible Neodiosmin Biosynthetic Pathway. Possible biosynthesis pathways of neodiosmin in *C. aurantium* are shown in Figure 4. The flavonoid compounds possibly involved in the pathways can be seen, both those that have been isolated and identified in *Citrus* species and those whose structure might make them intermediaries in the pathways described but whose presence has not been reported in *Citrus*.

Two pathways can essentially be distinguished in this complex scheme: one through the flavanone aglycons and their subsequent glycosylation (Lewinsohn et al., 1989b;

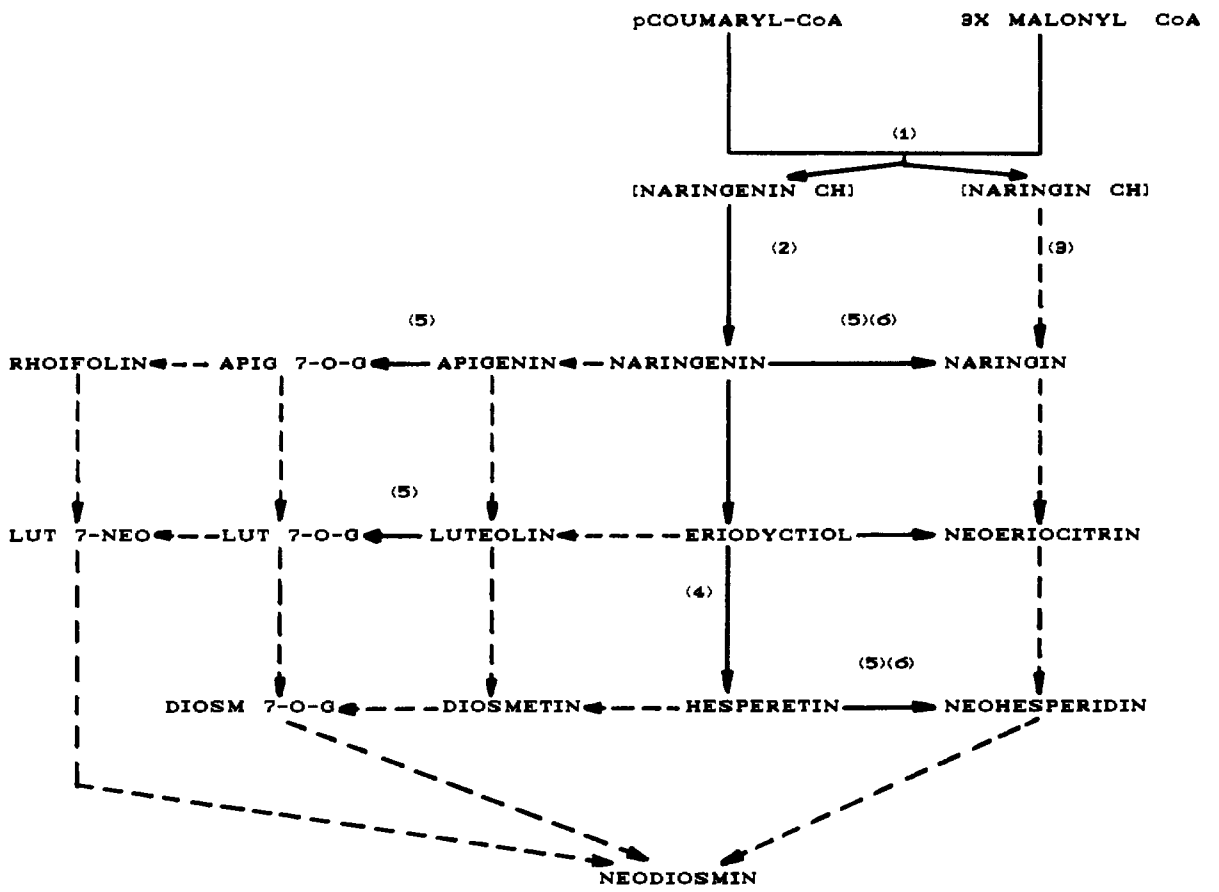


Figure 4. Possible interrelationship of flavonoid compounds based on the proposed pathways for flavanone and flavone biosynthesis in *Citrus*. Enzymes involved: (1) chalcone synthase (Lewinsohn et al., 1989b); (2) chalcone isomerase (Maier and Hasegawa, 1970); (3) naringin chalcone cyclase (Raymond and Maier, 1977); (4) 4′-*O*-methyltransferase (this work); (5) glucosyltransferase (UFGT) (McIntosh et al., 1990; McIntosh and Mansell, 1990; Lewinsohn et al., 1989b); (6) rhamnosyltransferases (UFGRT) (Lewinsohn et al., 1989b; Bar-Peled et al., 1991).

Table I. Substrate Specificity of 4′-OMT in *C. aurantium*

acceptor	relative activity[a]	product
naringenin	0	ND[b]
eriodictyol	100	hesperetin
apigenin	0	ND
luteolin	0	ND
naringin	0	ND
neoeriocitrin	0	ND
luteolin 7β-neohesperidoside	0	ND

[a] 100% corresponding to 992 pmol min^{-1} (mg of protein)$^{-1}$ of hesperetin. [b] None detected.

Table II. Glucosyltransferase Activity of Cell-Free Extracts of *C. aurantium*

substrate	specific activity[a]	product
naringenin	1.980	prunin
hesperetin	1.870	hesperetin 7-glucoside
apigenin	0.000	ND[b]
diosmetin	0.000	ND

[a] pmol of product seg^{-1} (mg of protein)$^{-1}$. [b] None detected.

McIntosh and Mansell, 1990; McIntosh et al., 1990) and another one that develops through the flavanone 7-β-neohesperidosides (Raymond and Maier, 1977). However, in neither pathway have the hydroxylation steps (naringenin/naringin to eriodictyol/neoeriocitrin) and subsequent methylation (eriodictyol/neoeriocitrin to hesperetin/neohesperidin) been completely characterized in *Citrus* species. Only some processes of flavone and flavonol methylation by cell-free extracts of *Citrus mitis* have been described (Brunet and Ibrahim, 1980; Brunet et al., 1978).

Since neodiosmin is a methoxylated flavone in position 4′ of the B ring of the flavonoid structure, we studied the substrate specificity of the 4′-*O*-methyltransferase (OMT) in *C. aurantium* to determine which of the pathways shown in Figure 4 might lead to neodiosmin.

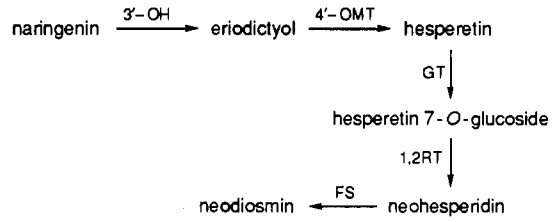
Table I shows the relative activity of the OMT enzyme with different substrates. It can be seen that, of the compounds tested, only eriodictyol could act as a substrate of this enzyme; the possible steps which lead from neoeriocitrin to neohesperidin, from luteolin to diosmetin, and from luteolin 7-β-neohesperidoside to neodiosmin itself could not be demonstrated. This suggests that the neodiosmin may be synthesized via hesperetin through flavone synthase acting on the flavanone glycoside neohesperidin and/or catalyzing the conversion of hesperetin to diosmetin with subsequent conversion to neodiosmin by glycosyltransferase enzymes.

However, the data available on glucosyltransferase activities in *Citrus* (McIntosh et al., 1990; McIntosh and Mansell, 1990) show a very high specificity for flavanones in comparison with other groups of flavonoids (flavonols and flavones). Thus, diosmetin is not glucosylated by glucosyltransferase of *C. paradisi* (McIntosh and Mansell, 1990). Our experiments on flavanone and flavone glucosylation by cell-free extracts of *C. aurantium* (Table II) showed relatively high activity levels with naringenin and hesperetin; however, they did not show any activity with their respective flavones, apigenin and diosmetin.

Additional data from other sources show that flavanones can serve as substrates in enzymatic glycosylation reactions in vitro with the subsequent formation of the corresponding flavone (Britsch, 1990).

On the basis of all of the above information, we suggest

the following scheme for neodiosmin biosynthesis in *C. aurantium*:



This scheme is supported by the other data obtained from a comparison of the accumulation curves of neodiosmin (Figures 1 and 2) and those previously published for neohesperidin (Castillo et al., 1992), where a delay in the start of neodiosmin synthesis with respect to neohesperidin can be observed.

In conclusion, both leaves and fruit of *C. aurantium* synthesize neodiosmin, the latter accumulating greater quantities than the former. In both organs this synthesis is more intense at the beginning of the period of cell growth, following active cellular division, in contrast to that describe for other flavonoids in *Citrus* species. Thus, we propose a scheme for neodiosmin biosynthesis in which neohesperidin is its immediate precursor.

Studies on the isolation and characterization of flavone synthase activity in *C. aurantium* tissues are in progress.

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