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INTRACELLULAR DISTRIBUTION AND ORIGIN OF STEROLS IN *CALENDULA OFFICINALIS* LEAVES

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Key Word Index—*Calendula officinalis*; Compositae; intracellular distribution; labelling dynamics; free sterols and their derivatives.

Abstract—In *Calendula officinalis* leaves 66% of all steryl forms are present in the 'microsomal fraction' (IV), 24% in the mitochondrial and Golgi membranes (III), 5% in the 'chloroplast' (II), 4% in the 'cell wall and membrane' (I) fraction and 1% in the cytosol. Free sterols, their esters, glycosides and acylated glycosides are present in varying proportions in all cellular subfractions. Mevalonate-[$2^{14}C$] labelling of sterols derived from various steryl forms showed that free sterols and all their derivatives, i.e. steryl esters and glucosides, are formed in fraction IV and are then translocated to other organelles. Fraction III is the main site of glycosylation of transported sterols as well as of acylation of steryl glycosides.

INTRODUCTION

Plant sterols occur as the free compounds and in bound form as esters, glycosides and acylated glycosides. It is generally assumed that all forms of sterols occur in various cellular compartments but quantitative differences occur between individual isolated cellular subfractions, with species, age and part of the plant, as well as with the various methods used for preparing subfractions [1–7].

Previous investigations on whole plants indicate that free sterols are formed first, which are converted to esters and glycosides. The glycosides may then be acylated [8–14]. The microsomal fraction is the site of squalene cyclization and of sterol biosynthesis, while there is no information on the site of free sterol esterification. A number of papers deal with the site of biosynthesis of steryl glycosides and acylated glycosides. Some authors consider the microsomal fraction [15–19] and others the mitochondrial fraction [11, 20, 21] to be the site where these processes take place. The latest studies showed that in *Calendula officinalis* seedlings and in onion stem UDPG:sterol glucosyltransferase and acyltransferase are associated with the membranes of the Golgi apparatus [22, 23] which in most procedures sediment in the mitochondrial fraction. In the present paper, data are presented on the distribution, site of biosynthesis and translocation of the various forms of sterols in the cellular subfraction of *C. officinalis* leaves.

RESULTS AND DISCUSSION

Light microscopy showed that the 600 *g* pellet [fraction I] contained, apart from fragments of the cell walls and membranes, also nuclei and a proportion of the chloroplasts. The 3000 *g* pellet (mainly chloroplasts) and 10000 *g* pellet (mainly mitochondria) were assumed, from the work of Wojciechowski, to be contaminated also by some of the Golgi membrane fragments. The

Table 1. Quantiative determinations of steryl forms in subcellular fractions of 10 g *Calendula officinalis* leaves

Fraction	Protein (mg)	Steryl forms: Free µg	%	Esters µg	%	GS µg	%	AGS µg	%	Sum µg	%
I	2.3	0.40	4	0.15	8	0.10	4	0.00	0	0.65	4
%		61		23		16		0		100	
II	0.4	0.52	6	0.22	13	0.01	1	0.01	1	0.75	5
%		69		29		1		1		100	
III	0.3	2.07	22	0.50	27	0.58	22	0.44	38	3.58	24
%		58		14		16		12		100	
IV	1.8	6.29	68	0.90	48	1.87	73	0.69	60	9.75	66
%		65		9		19		7		100	
V	1.6	0.03	1	0.09	4	0.02	1	0.01	1	0.14	1
%		22		63		11		4		100	
Sum	6.4	9.31	100	1.85	100	2.58	100	1.15	100	14.87	100
%		63		12		17		8		100	

GS—steryl glycosides; AGS—acylated steryl glycosides.

chloroplast fraction (II) and the mitochondrial fraction (III) were purified by filtration through 1.6 M sucrose. This procedure removed some of the Golgi fragments from fraction III. The 105000 *g* pellet (IV) was considered to contain the membranes of the endoplasmic reticulum, and the supernatant (V) the cytosol fluid.

In a study of the steryl forms in the subcellular fraction (Table 1), 66% of all steryl forms are present in fraction IV, 24% in III, 5% in II, 4% in I and less than 1% in V. Free sterols constitute 63% of all steryl forms, steryl esters 12%, steryl glycosides 17% and acylated glycosides 8%. Sterols occurring in the four forms were present in all cellular subfractions but in different proportions.

In fractions I, II, III and IV free sterols constitute 58–69% of all steryl forms but in the fraction V only 22%. In fraction V steryl esters are the main form, constituting 63% of all sterols. Steryl esters constitute 23 and 29% in fraction I and II respectively being present in lowest proportion in fractions III and IV (14 and 9%). The steryl glycosides constitute a considerable proportion in fractions I, III, IV and V (from 11 to 19%) and are insignificant in fraction II. Acylated steryl glycosides were found in greatest proportions only in fractions III, IV and V while they are absent in fraction I and present in traces in fraction II.

In the investigations on both the sites of biosynthesis and translocation of free sterols and their derivatives within the cell, isotope experiments were performed to study the dynamics of labelling of squalene and of the different forms of sterols present in the cellular subfractions.

In Tables 2–6 the mean cpm values of steryl forms in cellular fractions obtained from 10 g of fresh leaves of *C. officinalis* are presented. The mean values are calculated from two parallel experiments. The differences between results obtained in these experiments were not higher than 10%. Moreover the specific radioactivity of all these compounds was calculated.

For squalene only total radioactivity could be recorded because the quantity of this compound was too small to permit quantitative determination. As is shown in Table 7 radioactive squalene was detected only in fractions IV and V, over 100 times more radioactivity being localized in the former fraction; this indicated contamination of the soluble fraction with light microsomes. The rapid drop in the radioactivity of squalene pointed to its transformation into triterpenoids, among others into sterols of the microsomal fraction.

Table 2 presents the incorporation of MVA-[2^{14}C] into different steryl forms in the whole cell after 4, 27, 70 and 280 hr. The total radioactivity incorporated into all

Table 2. MVA-[2^{14}C] incorporation into free sterols and sterols isolated from steryl conjugates of *C. officinalis* leaves (per 10 g fr. wt)

Time	4 hr cpm	%	24 hr cpm	%	70 hr cpm	%	280 hr cpm	%
Free sterols	14600	87	136000	89	73300	58	62200	51
Steryl esters	1950	12	9830	6	6200	5	5130	4
GS	300	1	6570	4	38700	30	27300	22
AGS	0	0	1160	1	8410	7	28000	23
Sum	16800		154000		127000		123000	

GS—sterol glycosides; AGS—acylated sterol glycosides.

Table 3. MVA-[2^{14}C] incorporation into free sterols in various cellular subfractions obtained from 10 g of *C. officinalis* leaves

Time / Fraction	4 hr cpm	%	24 hr cpm	%	70 hr cpm	%	280 hr cpm	%
I	0	0	180	<1	6020	8	14100	22
II	0	0	730	<1	25000	34	36700	59
III	1000	6	12300	9	31200	42	9770	16
IV	13500	93	123000	90	11100	15	1590	3
V	100	<1	100	<1	40	<1	0	0
Sum	14600		136000		73300		62200	

Table 4. MVA-[2^{14}C] incorporation into sterols occurring as steryl esters in various cellular subfractions obtained from 10 g of *C. officinalis* leaves

Time / Fraction	4 hr cpm	%	24 hr cpm	%	70 hr cpm	%	280 hr cpm	%
I	0	0	70	1	900	14	1280	25
II	0	0	1180	12	1620	26	2050	40
III	350	18	1390	14	1700	28	1670	32
IV	1580	81	6980	71	1750	28	50	1
V	20	1	210	2	260	4	80	2
Sum	1950		9830		6230		5130	

Table 5. MVA-[2^{14}C] incorporation into sterols occurring as steryl glycosides in various cellular subfractions obtained from 10 g of *C. officinalis* leaves

Time	4 hr		24 hr		70 hr		280 hr	
Fraction	cpm	%	cpm	%	cpm	%	cpm	%
I	0	0	0	0	140	<1	300	1
II	0	0	0	0	70	<1	300	1
III	120	44	3400	52	31100	80	22300	81
IV	150	66	3170	48	7310	19	4400	16
V	0	0	0	0	40	<1	30	<1
Sum	270		6570		38700		27330	

Table 6. MVA-[2^{14}C] incorporation into sterols occurring as acylated steryl glycosides in various cellular subfractions obtained from 10 g of *C. officinalis* leaves

Time	4 hr		24 hr		70 hr		280 hr	
Fraction	cpm	%	cpm	%	cpm	%	cpm	%
I	0	0	0	0	0	0	0	0
II	0	0	0	0	40	1	150	1
III	0	0	930	81	7700	91	25800	92
IV	0	0	230	19	670	8	2070	7
V	0	0	0	0	0	0	0	0
Sum	0		1160		8410		28000	

steryl forms increases from 16700 cpm at 4 hr to 154000 cpm at 24 hr and does not change much during 280 hr (123000 cpm). However distinct changes are observed in the labelling of each steryl form. The radioactivity in the free sterols constituting nearly 90% from 4 to 24 hr decreased to 50% at 280 hr. Steryl esters are the less metabolized fraction, their radioactivity during 280 hr decreases from 12 to 4%. The radioactivity lost from the free sterols appears in the fractions of steryl glycosides and acylated glycosides. The radioactivity of steryl glycosides constituting 1% at 4 hr increases to 30% at 70 hr and then decreases to 22% when the acylated glycosides are labelled only after 24 hr (1%) and their radioactivity increases continually to 23% at 280 hr. These results and the values of labelling obtained for the different steryl forms in various cellular subfractions suggest that the formation of squalene together with its cyclization to free sterols takes place in microsomal fraction (IV). On the basis of specific radioactivity and the time of maximum ^{14}C incorporation into free sterols and their esters, it was found that free sterols are rapidly converted into steryl esters in the same fraction. Total and specific radioactivity of free sterols and steryls esters rises up to 24 hr and then decreases rapidly. This indicates that free sterols are transformed to other steryl forms (i.e. glycosides) which are then metabolized to acylated glycosides in the same fraction and partially transported to other cellular organelles.

In fraction III, radioactive free sterols, their esters and glycosides appeared after 4 hr. Subsequently, the radioactivity of steryl esters increased until 70 hr and then remained at a constant level whilst the radioactivity of free sterols and steryl glycosides dropped after passing the peak at 70 hr. These results suggested that free sterols are transformed into glycosides which are next converted into acylated glycosides which appeared in this fraction after 24 hr and their radioactivity increased rapidly to the end of the experiment.

Both processes, glycosylation and acylation of glycosides, occur in this fraction as in fraction IV but glycosides and acylated glycosides of sterols were much more intensely labelled in fraction III than fraction IV. Their specific radioactivity was about 10 times higher in fraction III than that in fraction IV which indicates their intense biosynthesis in fraction III. These results are in agreement with the localisation of steryl glycosidases and acylases of steryl glycosides in the membranes of the Golgi apparatus which were not separated from the

Table 7. MVA-[2^{14}C] incorporation into squalene in various cellular subfractions obtained from 10 g of *C. officinalis* leaves

Time	4 hr		24 hr		70 hr		280 hr	
Fraction	cpm	%	cpm	%	cpm	%	cpm	%
I	0	0	0	0	0	0	0	0
II	0	0	0	0	0	0	0	0
III	0	0	0	0	0	0	0	0
IV	118200	99	12500	97	1380	100	840	100
V	770	1	310	3	0	0	0	0
Sum	119000		12800		1380		840	

mitochondrial fraction in this procedure.

Beginning from 24 hr, radioactive free sterols and steryl esters appear in fractions I and II. The radioactivity of these compounds increases to 70 hr and then is unchanged to 280 hr. Radioactive steryl glycosides in both fractions and acylated glycosides in fraction II could be detected only after 70 hr, the radioactivity of these compounds increases slightly to 280 hr. These results suggest that after being transported from fraction IV the compounds become incorporated into the structure of the organelles present in fractions I and II.

In fraction V all forms of sterols are present in small amount. Only radioactive free sterols and steryl esters were found after 4 hr and radioactive steryl glycosides not until 70 hr. Their rather high specific radioactivity in this fraction suggests that sterol derivatives are transported from the site of their biosynthesis (fraction IV) to other cellular organelles by means of fraction V. The highest specific radioactivity of steryl esters as well as the biggest proportion of these compounds in this fraction (63% of all steryl forms) indicated that steryl esters are the main transported form of sterols. This suggestion may be supported by the fact that the specific radioactivity of steryl esters in fraction IV is decreasing faster than that of the free sterols and that the specific radioactivity of steryl esters appearing at 4 hr in the fractions I, II and III is higher than that of the free sterols.

In fraction IV, sterols are formed by way of squalene cyclization and then undergo esterification and glycosylation, whereas the resulting steryl glycosides are acylated. The fact of squalene cyclization and the biosynthesis of glycosylated steryl forms in the microsomal fraction is consistent with the finding of other authors [13, 15, 18, 19]. As yet there has been no indication concerning the site of steryl esterification in the plant cell. Compounds formed in fraction IV are transported to other organelles and incorporated into their membranous structures. Steryl esters are probably the main form of transport of sterols in plant cells.

Fraction III is the main site of glycosylation of transported sterols as well as of the acylation of steryl glycosides. It seems that all forms of sterols may perform a structural function in all plant cell membranes; this is suggested by their occurrence and slow accumulation in all the cellular fractions.

EXPERIMENTAL

Experiments were performed on 5-week-old *C. officinalis* L.var. Radio shoots, cultivated in a lumistat as reported in ref. [24].

Administration of MVA-[$2^{14}C$]. Shoots, 2.5 ± 0.1 g, were treated through the cut stem with 25 μCi MVA-[$2^{14}C$] (DBED salt), sp. act. 36 mCi/mmol, in 0.1 ml H_2O; they were exposed to light of 15 000 lx. After the soln was absorbed (*ca* 90 min), the plants were transferred to vessels with H_2O and placed in a lumistat. Plants were processed 4, 24, 70 and 280 hr after precursor uptake.

Preparation of cellular subfractions. Cut leaves were ground in a mortar, ×3 for 15 sec, with 2 g Kieselgel 0.2–0.5 mm in a 0.3 M sucrose soln (10 ml of soln, 1 g of leaves); subsequently, they were filtered through 4 layers of cheesecloth and centrifuged, successively, at 600 *g*, 5 min; 3000 *g*, 5 min; 10000 *g*, 20 min; 105000 *g*, 60 min. Pellets obtained upon centrifugation at 3000 *g* and 10000 *g* were suspended in a 0.3 M sucrose soln and filtered through 1.6 M sucrose at 2200 rpm for 60 min. The resulting fractions were checked by light and electron microscopy using negative staining with a 1.5% soln of sodium tungstate.

Extraction. Every cellular subfraction was successively extracted with boiling MeOH for 30 min and Et_2O for 10 min.

Preparative chromatography. The 4 forms of sterols present in the Et_2OH–MeOH extracts were separated and purified by TLC on Si gel impregnated with rhodamine G6 2 mg/1 g gel, as described in ref. [14].

Hydrolysis. Sterol esters as well as steryl glycosides and acylglycosides were hydrolysed, as reported in ref. [14].

Quantitative determinations. Non-radioactive free sterols obtained from the various forms of sterols were acylated with a mixture of C_5H_5N and Ac_2O-[1,$1^{-14}C$] sp. act. 10.4 mCi/mmol (3:1) at room temp. during 24 hr. The acylated samples were purified by chromatography in petrol–$CHCl_3$–MeOH (20:10:1). The amounts of different compounds were calculated by comparison of their radioactivity with those of appropriate acylated standards.

Radioactivity measurements. The radioactivity of compounds was measured in a scintillation counter with a yield of 80%, as described in ref. [14]. *Protein* content of the different subfractions were determined by the method of ref. [25].

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