Phytochemistry, 1979, Vol. 18, pp. 427–430 Pergamon Press Printed in England.

DISTRIBUTION OF DIFFERENT FORMS OF STEROLS IN THREE CELLULAR SUBFRACTIONS OF *CALENDULA OFFICINALIS* LEAVES

WIRGINIA JANISZOWSKA, EWA SOBOCIŃSKA and ZOFIA KASPRZYK
Institute of Biochemistry, University of Warsaw, Al. Żwirki i Wigury 93, Poland

(*Revised received* 10 *August* 1978)

Key Word Index—*Calendula officinalis*; Compositae; intracellular distribution; sterols; 24-methylene-cholesterol, clerosterol; free and bound steryl forms.

Abstract—From *Calendula officinalis* leaves three cellular subfractions (mitochondrial, Golgi membranes and microsomal) were obtained and enzymatically characterized. The contents of Δ^0, Δ^5, Δ^7, $\Delta^{5,22}$ sterols, as well as those of 24-methylenecholesterol and clerosterol, in the free and bound in the form of esters, glucosides and acylated glucosides were determined in these fractions. The results revealed the predominance of free sterols in the microsomal fraction, of esters in the mitochondrial fraction and of steryl glucosides and acylated glucosides in the Golgi fraction.

INTRODUCTION

The variety of plant sterols differing by the number of carbon atoms and the position of double bonds in the molecule, their presence in at least four different forms: i.e. free (FS), esters (ES), glucosides (GS) and acylated glucosides (AGS), as well as qualitative and quantitative variations in their distribution among different plant organelles, create great difficulties in investigations on the transformation of these compounds in different cell compartments. Several investigations have been done on the subcellular distributions of different free and bound steryl types but the complete characterization of these compounds in all cell organelles of one plant has not been reported. However, this information is very important for further investigations on the fate of sterols synthesized in the microsomal fraction.

We have previously [1] isolated and identified 13 sterols from *C. officinalis* leaves. All these sterols occurred as free, esters, glucosides and acylated glucosides. In subsequent studies [2] on the distribution of different steryl forms in cellular subfractions of *C. officinalis* leaves, they were shown to occur in various proportions in all the investigated subfractions. Most sterols (66%) were detected in the microsomal fraction and in the fraction consisting of a mixture of mitochondria and Golgi membrane fragments (24%); the sterol content was lower in the chloroplasts (5%), plasmolemma (4%) and cytosol (1%) fractions. However, great differences were observed in the proportions of the different steryl forms in the different subcellular fractions. With the exception of the cytosol, in which steryl esters consisted 65% of all sterols, free sterols predominated (60–70%) in the other subfractions. Steryl esters were present in greatest quantities in chloroplast (29%) and in plasmalemma (23%), but in smaller amounts in mitochondria and Golgi membrane fractions (14%), as well as in the microsomal fraction (9%). The glucosidic forms, i.e. GS and AGS, were present in considerable quantity in the mitochondria and Golgi membranes (19% GS, 7% AGS) as well as in the microsomal fraction (16% GS and 12% AGS) and cytosol (11% GS and 4% AGS). In plasmalemma only GS were present (16%) and chloroplasts were practically devoid of GS and AGS.

The present study was designed to prepare and characterize, using marker enzymes, the three cellular subfractions with the highest sterol contents, i.e. the microsomal, mitochondrial and Golgi membrane fractions, and subsequently to quantitatively isolate and determine the various types of sterols occurring in free and bound form.

Table 1. Glucose-6-phosphatase, UDPG:sterol glucosyltransferase and succinic dehydrogenase activities in subcellular fractions from *C. officinalis* leaves

Fraction	Protein (mg)	Glucose-6-phosphatase (μM/hr/mg protein)	UDPG:sterol glucosyltransferase (μM/hr/mg protein)	Succinic dehydrogenase (μM/min/mg protein)
Pellet 14000 *g*	3.0	0.042	84	7.550
Mitochondria	2.4	0.006	2	43.4
Golgi membranes	0.03	0	102	0.026
Microsomes	0.2	58	0.270	0.002

Table 2. Quantitative determination of the free sterols and sterols from the steryl esters, glucosides and acylated glucosides in subcellular fractions of *C. officinalis* leaves

Sterol form*	Subcellular fractions: Microsomes (μg/100 g fr. wt)	Microsomes (μg/mg protein)	Mitochondria (μg/100 g fr. wt)	Mitochondria (μg/mg protein)	Golgi membranes (μg/100 g fr. wt)	Golgi membranes (μg/mg protein)
FS	214	1070	70	29	22	739
ES	67	335	267	111	19	633
GS	51	255	23	10	28	933
AGS	35	175	14	6	22	733
Sum	367	1835	374	156	91	3038

*FS = Free sterols; ES = steryl esters; GS = steryl glucosides; AGS = acylated steryl glucosides.

RESULTS AND DISCUSSION

The mitochondrial and Golgi membrane fractions were obtained by the modified method of Powell and Brew [3], originally developed for preparation of Golgi membranes from onion stem. The 14000 *g* pellet was suspended in buffer and centrifuged as described in the Experimental, yielding both subfractions. In turn the 105000 *g* pellet was, as previously [2], regarded as the microsomal fraction.

The cellular subfractions prepared were characterized by determining the activity of marker enzymes: glucose-6-phosphatase for microsomes [4], UDPG: sterol glucosyltransferase for Golgi membranes [5], and succinic dehydrogenase for mitochondria [6]. Results presented in Table 1 indicate that the three fractions were not contaminated with each other. In the 105000 *g* pellet we obtained the microsomal fraction of high purity, this confirming the earlier electron microscopic results [2]. The microsomal and Golgi membrane fractions were virtually uncontaminated with each other or with microsomes. These purified subfractions were used for further studies; it was found that the total sterol content in the microsomal and mitochondrial fractions obtained from 100 g of fresh leaves was similar, whereas in the Golgi membrane fraction it was almost three times lower (Table 2). Sterol content, calculated per mg protein, was highest in the Golgi membrane fraction, *ca* two times lower in the microsomal fraction and almost twenty times lower in the mitochondrial fraction. The results obtained for the microsomal fraction were similar to the earlier reports, FS was present in greater quantity, ES and GS in smaller and AGS in the smallest. The purified mitochondrial fraction was characterized by predominance of ES accounting for *ca* 70% of all sterols, followed by FS, GS and AGS. In the separated Golgi membrane fractions all forms of sterols occurred in comparable amounts, with slight predominance of GS and a smaller amount of ES. These findings slightly differ from the recent results of Duperon *et al.* [7] concerning the contents of various forms of sterols in the cellular subfractions of a nonphotosynthesizing tissue, cauliflower inflorescence. According to Duperon *et al.* [7], in the microsomal fraction free sterols also predominated, but the content of AGS was relatively high, whereas ES and GS was lower; in the mitochondrial fraction FS predominated. Inconsistencies between the present results and those of Duperon *et al.* [7] could be due to the differences in the kind of tissue studied and in the methods applied for preparation of cellular subfractions.

The contents of various types of sterols occurring in different forms in the cellular subfractions studied are recorded in Tables 3, 4 and 5. In all three subfractions, Δ^5 (mainly sitosterol) and $\Delta^{5,22}$ (mainly stigmasterol) sterols were the main constituents, i.e. 84% in the microsomal, 66% in the Golgi membranes and 75% in the mitochondrial fraction. However, some differences were observed between the proportions of Δ^5 and $\Delta^{5,22}$ sterols. In the microsomes (Table 3) Δ^5 predominated, in mitochondria (Table 4) the contrary was observed, while in Golgi membranes (Table 5) both these steryl types were present in comparable quantities.

Considerable differences were however noticed in the presence of Δ^5 and $\Delta^{5,22}$ sterols in the different forms present in cellular subfractions. In the microsomal fraction, $\Delta^{5,22}$ sterols were present in greater quantities in FS and ES (76%) than Δ^5 sterols, but the contrary was true for glycosidic forms. This result indicates that Δ^5 sterols are better substrates for the glucosylation

Table 3. Quantitative determination of the sterols from the different steryl forms in the microsomal fraction of *C. officinalis* leaves (100 g)

Type of sterol	FS (μg)	FS (%)	ES (μg)	ES (%)	GS (μg)	GS (%)	AGS (μg)	AGS (%)	Sum (μg)	Sum (%)
Δ^0	2.3	1.1	0	0	0.7	1.4	0.8	2.3	3.8	1.0
Δ^7	9.0	4.2	0.7	1.0	1.5	2.9	10.0	28.6	21.2	5.8
Δ^5	53.0	24.8	7.2	10.8	42.6	83.5	16.0	45.7	118.8	32.4
$\Delta^{5,22}$	130.5	61.0	51.0	76.1	2.8	5.5	6.0	17.2	190.3	51.4
Clerosterol	14.4	6.7	3.0	4.5	2.4	4.7	1.2	3.4	21.0	5.7
24-Methylenecholesterol	4.8	2.2	5.1	7.6	1.0	2.0	1.0	2.8	11.9	3.2
Sum	214.0	100	67.0	100	51.0	100	35.0	100	367.0	100
%	58		19		13		10			100

Table 4. Quantitative determination of the sterols from the different steryl forms in the mitochondrial fraction of *C. officinalis* leaves (100 g)

Type of sterol	FS		ES		GS		AGS		Sum	
	(μg)	(%)	(μg)	(%)	(μg)	(%)	(μg)	(%)	(μg)	(%)
Δ^0	2.8	4.0	18.5	6.9	0.3	1.3	3.7	26.4	25.3	6.8
Δ^7	6.1	8.8	26.7	10.0	0.5	2.2	2.1	15.0	35.4	9.4
Δ^5	32.1	45.9	118.6	44.4	0.6	2.6	4.2	30.0	155.5	41.0
$\Delta^{5,22}$	25.4	36.3	54.5	20.4	18.5	80.4	1.5	10.7	99.9	26.5
Clerosterol	1.8	2.5	40.0	15.0	1.5	6.6	1.4	10.0	44.7	12.0
24-Methylenecholesterol	1.8	2.5	8.7	3.3	1.6	6.9	1.1	7.9	13.2	3.5
Sum	70.0	100	267.0	100	23.0	100	14.0	100	374.0	100
%	19		72		6		3			100

and $\Delta^{5,22}$ sterols for the esterification reactions. In earlier work [2] on the labelling of the steryl forms in the microsomal fraction with mevalonate-[2-^{14}C], it was found that FS and ES were labelled in parallel and attained peaks of radioactivity earlier than GS, while the radioactivity in AGS was increasing up to 280 hr at the end of the experiment. The previous and the present results suggest that Δ^5 sterols in the microsomal fraction are primarily transformed to $\Delta^{5,22}$ sterols, the main final products of sterol metabolism in the plant, which then accumulate in this fraction and are simultaneously esterfied. With some delay Δ^5 sterols are subjected to the glucosylation reaction and the synthesized GS are then transformed to AGS. Also notable were the accumulation of clerosterol in FS, absence of stanols in ES and accumulation of Δ^7 sterols in GS. This indicates that not only Δ^5 sterols but earlier products of sterol metabolism are good substrates for glucosylation in this fraction.

The separation of the pure mitochondrial and Golgi membrane fraction permitted the quantitative determination of different steryl types present in different steryl forms in these two fractions. The interpretation of the results is not easy because it is not known in what form sterols of different types synthesized in the microsomal fraction, and at least partially acylated and glucosylated there, are transported to other cell organelles. In the mitochondrial fraction Δ^5 sterols prevailed over $\Delta^{5,22}$ sterols in FS and ES. This may be explained in two ways. First, Δ^5 sterols may be transported from the microsomal fraction to the mitochondria more readily than $\Delta^{5,22}$ sterols in these forms and $\Delta^{5,22}$ sterols in the FS and ES forms therefore accumulate in the microsomal fraction. Second, Δ^5 sterols in FS form, which in the microsomal fraction is the main substrate for glucosylation, may be quickly transported to the mitochondria where they are preferentially esterified. The similar quantitative compositions of all steryl types in the mitochondria seems to point to the second suggestion. By contrast, in the microsomal fraction there were great differences in the steryl components between FS and ES; in the latter form some sterols were present in very small amount and stanols were lacking. The other difference between microsomes and mitochondria was the fact that $\Delta^{5,22}$ sterols were the main components of GS and sterols with two double bonds in the molecule were present in greater amount than Δ^7 sterols and stanols in this fraction. In AGS, however, stanols and sterols with one double bond (Δ^5 and Δ^7) constituted a greater proportion than sterols with two double bonds.

This result may be explained in a similar way to the result obtained for FS and ES in this fraction, glucosides of $\Delta^{5,22}$ sterols may be more readily transported from the microsomal fraction and accumulate in mitochondria or they may be transported preferentially from the Golgi membranes, which are the main site of the glucosylation reaction in accordance with the finding of Wojciechowski [8] that Δ^5 and $\Delta^{5,22}$ sterols are the best substrates for UDPG:sterol glucosyltransferase. The great quantity of stanols and sterols with two double bonds in AGS may be explained also by the preferential acylation of these steryl types in GS or by their transport from Golgi membranes.

In Golgi membranes (Table 5) $\Delta^{5,22}$ sterols prevail over Δ^5 sterols in FS but the contrary is true for the other forms. In addition to these two steryl types (which constitute in the bound forms, 73–85% of all sterols) sterols of other types are present in similar proportions.

Table 5. Quantitative determination of the sterols from the different steryl forms in the Golgi membrane fractions of *C. officinalis* leaves (100 g)

Type of sterol	FS		ES		GS		AGS		Sum	
	(μg)	(%)	(μg)	(%)	(μg)	(%)	(μg)	(%)	(μg)	(%)
Δ^0	1.0	4.6	1.0	5.3	1.0	3.6	0.7	3.2	3.7	4.0
Δ^7	0.9	4.1	1.5	7.9	1.0	3.6	1.0	4.5	4.4	4.8
Δ^5	3.3	15.0	7.8	41.0	12.7	45.3	11.6	52.7	35.7	39.1
$\Delta^{5,22}$	10.4	47.2	6.1	32.1	11.1	39.6	5.5	25.0	33.1	36.3
Clerosterol	5.4	24.5	1.5	7.9	1.2	4.3	1.2	5.5	9.3	10.2
24-Methylenecholesterol	1.0	4.6	1.1	5.8	1.0	3.6	2.0	9.1	5.1	5.6
Sum	22.0	100	19.0	100	28.0	100	22.0	100	91.3	100
%	25		20		30		25			100

However, in FS, in which the sum of Δ^5 and $\Delta^{5,22}$ sterols is lower (62% of all sterols) a large amount of clerosterol (24%) was found. These results are also difficult to explain. It is known from the work of Lercher and Wojciechowski [5] that the Golgi membranes fraction is most probably the main site not only of UDPG:sterol glucosyltransferase but also of complex lipid:sterol glucoside transacylase. The distribution of steryl ester synthetase is not known, but according to the preliminary findings of Zimowski [9] it is bound with the heavy membrane fraction and according to our results probably with the mitochondria.

It seems therefore that the main problems to be elucidated to understand the distribution of different steryl types bound in different steryl forms in the plant cell are first, the problem of the transport of these forms among different organelles and second, the localization of the enzymes transforming the free sterols into the bound forms. We assume that all steryl types are formed in the microsomal fraction as the free compounds before being transformed into the bound forms. We do not know however, if some transformations, such as the introduction of the double bonds and alkyl groups in the sterol side chain, can proceed in the bound steryl forms.

EXPERIMENTAL

Experiments were performed on leaves of 3-month-old *C. officinalis* L. var. Radio plants cultivated in a lumistate [10].

Preparation of cellular subfractions. Cut leaves were ground in a mortar 3 × for 20 sec with an equal amount of Kieselgel (0.2–0.5 mm) in 0.3 M sucrose containing 0.01 M Tris–HCl buffer (5 ml of soln, 3 g of leaves); the suspension was filtered through 4 layers of cheesecloth and centrifuged successively at 4000 *g*, 10 min; 14000 *g*, 20 min; 105000 *g*, 60 min. The 14000 *g* pellet was suspended in the homogenization soln and further fractionated by sucrose density gradient centrifugation at 95000 *g*, exactly as described by Powell and Brew [3]. Two membrane layers localized at the 1.0/1.25 M sucrose and 1.25/1.5 M sucrose interfaces were collected as the Golgi fraction and the pellet was the mitochondrial fraction.

Extraction, preparative chromatography of 4 forms of sterols, their hydrolysis and separation of stanols, Δ^7 sterols, Δ^5 sterols, $\Delta^{5,22}$ sterols, campesterol and 24-methylenecholesterol from each steryl form have been described previously [1]. Quantitative determinations of steryl types were performed by the method with Ac_2O-[1,1-^{14}C] [2]. Protein content of the different subfractions was assayed by the method of Lowry *et al.* [11].

REFERENCES

1. Adler, G. and Kasprzyk, Z. (1975) *Phytochemistry* **14**, 723.
2. Janiszowska, W. and Kasprzyk, Z. (1977) *Phytochemistry* **16**, 473.
3. Powell, J. T. and Brew, K. (1974) *Biochem. J.* **142**, 203.
4. Hübscher, G. and West, G. R. (1965) *Nature* **205**, 799.
5. Lercher, M. and Wojciechowski, Z. A. (1976) *Plant Sci. Letters* **7**, 337.
6. Colowick, S. P. and Kaplan, N. O. (1955) *Methods Enzymol.* **1**, 725.
7. Meance, J., Dupéron, P. and Dupéron, R. (1976) *Physiol. Vég.* **14**, 745.
8. Wojciechowski, Z. A. and Van Uon, N. (1975) *Acta Biochim. Polon.* **22**, 25.
9. Zimowski, J., unpublished data.
10. Kasprzyk, Z., Wojciechowski, Z. and Czerniakowska, K. (1968) *Physiol. Plant.* **21**, 966.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.