Phytochemistry, 1975, Vol. 14, pp. 1749–1753. Pergamon Press. Printed in England.

BIOSYNTHESIS OF OLEANOLIC ACID GLYCOSIDES BY SUBCELLULAR FRACTIONS OF *CALENDULA OFFICINALIS* SEEDLINGS

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(*Received* 5 *December* 1974)

Key Word Index—*Calendula officinalis*; Compositae; biosynthesis; oleanolic acid glycosides.

Abstract—The synthesis of oleanolic acid 3β-D-glucuronoside from oleanolic acid and UDPGlcA has been demonstrated in cell-free preparations from *C. officinalis* seedlings. Moreover, the formation of more complex glycosides by successive additions of galactose and glucose to oleanolic acid glucuronoside was observed when cell-free preparations were incubated with UDPGal or UDPGlc. The consecutive steps of oleanolic acid glycosylation are localized in three different cellular compartments. The biosynthesis of the 3-glucuronoside takes place in the microsomes, the elongation of the sugar chain at C-3 of the aglycone proceeds in heavy membrane structures which are probably fragments of the Golgi complex while a cytosol enzyme(s) is involved in glucosylation of the C-17 carboxyl group of oleanolic acid.

INTRODUCTION

Only sparse data on the biosynthesis of pentacyclic triterpene glycosides are available. These compounds are characterized by a relatively complex structure containing in some cases more than 10 sugar residues, often in the form of branched chains [1, 2].

We have previously proved [3, 4] that in flower and seedlings of *C. officinalis* there are six structurally related oleanolic acid glycosides (A–F; for abbreviated formulae see Scheme 1). Three of them, containing sugars exclusively at the 3β-hydroxyl of oleanolic acid, are the 3β-glucuronoside (F), the 3′ (or 2′)-galactosyl-glucuronoside (D) and the 4′-glucosyl-(3′-galactosyl)-glucuronoside (B), respectively. The remaining three glycosides (D_2, C and A) are glucoside esters of glycosides F, D and B, respectively, containing an additional glucose residue bound to the C-17 carboxyl of the aglycone. Preliminary *in vivo* studies [4, 5] of the dynamics of labelling of the individual glycosides, with acetate-[1-^{14}C] and mevalonate-[2-^{14}C], have suggested that their synthesis probably proceeds by way of stepwise elongation of the sugar chain. These studies, however, permitted no unequivocal conclusions about the order of sugar residue addition. The present study was designed to elucidate this order of addition and to localize the processes of glycoside biosynthesis within the cell.

RESULTS AND DISCUSSION

The material used was 7-day-old *C. officinalis* seedlings, since it has previously been found [4] that during this period triterpenic glycosides rapidly accumulate in the plant.

Biosynthesis of oleanolic acid 3β-D-glucuronoside

Incubation of crude homogenates from *C. officinalis* seedlings with UDPGlcA-[^{14}C] and emulsified oleanolic acid, with the use of various homogenization techniques, buffers of different pH and ionic strength, various commonly used cofactors etc. gave only very low ^{14}C-incorporation (0·01–0·05%) into a compound with the chromatographic properties of oleanolic acid glucuronoside (glycoside F). The subsequent tests

were performed with fractions obtained by partial separation of the homogenate by centrifugation. A much higher ^{14}C-incorporation (0·3–0·5%) was obtained with the 105000 *g* fraction (crude microsomes). The 15000 *g* fraction was six times less active per mg of protein. The identity of the resulting radioactive product was unequivocally confirmed by cocrystalization with non-radioactive glycoside F without any decrease in the specific radioactivity (Table 1). The remaining subcellular fractions failed to synthesize glycoside F in any measurable amounts.

In a search for conditions permitting enhancement of the activity of the microsome preparation, the effect of a number of potential activators was investigated. Highest activity was observed at pH 7·2 with 0·1 M Tris–HCl buffer or pH 7·0 with 0·1 M phosphate buffer, the activity was about 2·5 times higher in Tris–HCl. Divalent metals (Mg^{2+}, Ca^{2+}, Mn^{2+}) or chelating agents (EDTA, dipyridyl) at concentrations of 10^{-4}–10^{-2} M exerted no activating effect. Pronounced stimulation was found, however, in the presence of 2-mercaptoethanol and dithiothreitol (45 and 30% respectively at 10^{-3} M). At 10^{-3} M the oleanolic acid glucuronoside synthesis was slightly stimulated by UTP and ATP (15 and 28% respectively), whereas it was strongly inhibited by UDP (82%). It was found that preincubation (10 min at 10°) of microsomes in the presence of 0·4% Triton X-100 enhanced the glucuronoside synthesis about 2 times. Such preincubation caused partial solubilization of the glucuronosyltranferase activity since about 45% of the activity failed to precipitate during a 1 hr centrifugation at 105000 *g*. However, the solubilized preparation was unstable and completely lost activity within a few hours. Table 2 summarizes some results on enzyme specificity. The observed ^{14}C-incorporation remained relatively low even with a microsome preparation pretreated with Triton X-100 and incubated in the presence of 2-mercaptoethanol. However it is noteworthy that replacement of UDPGlcA-[^{14}C] by GlcA-[^{14}C] caused an about 30-fold reduction of glycoside F synthesis. The pronounced inhibition of the enzyme by UDP (see above) provides additional evidence for the utilization of UDPGlcA as the glucuronic acid donor. It seems that the glucuronosyltransferase is highly specific for oleanolic acid since replacement of this compound by related triterpenes belonging to the Δ^{12}-oleanane group did not result in the stimulation of ^{14}C-incorporation into butanol soluble material. It is stressed that β-amyrin and erythrodiol, considered to be the biogenetic precursors of oleanolic acid, were not utilized as glucuronic acid acceptors. This permits the conclusion that the formation of triterpenic glycosides takes place only after the oxidation of the C-17 methyl group of β-amyrin to give the C-17 carboxyl group of oleanolic acid.

To ascertain the localization of the glucuronosyl transferase the crude microsomes were subjected to sucrose density gradient centrifugation (Fig. 1). The coincidence of the glucuronosyltransferase activity with the microsomal marker, glucose-6-phosphatase [6, 7], confirms the occurrence of glucuronosyltransferase in the microsomes. This is in agreement with the observation [8] that

Table 1. Crystallization of the radioactive product formed during the incubation of a crude 105000 *g* fraction from *C. officinalis* with UDPGlcA-[^{14}C] and oleanolic acid

	Mp	Specific radioactivity (dpm/mg)
Intital		332
1st crystallization	200–206°	359
2nd crystallization	202–206°	386
3rd crystallization	203–208°	390
4th crystallization	204–208°	376

The radioactive product isolated by TLC (8200 dpm) was diluted with non-radioactive oleanolic acid 3β-D-glucuronoside (22 mg). The mixture was methylated with diazomethane and crystallized from aq.EtOH.

Table 2. Incorporation of ^{14}C from UDPGlcA-[^{14}C] or glucuronate-[^{14}C] into the glycoside fraction or glycoside F in the presence of Triton X-100 treated crude microsomes from *C. officinalis* seedlings. For details see Experimental

Sugar-[^{14}C] added	Triterpene added	Radioactivity (dpm) Butanol extract (glycoside fraction)	Glycoside F
UDPGlcA	oleanolic acid	2430	1846
	oleanolic acid*	48	29
	none†	825	324
	methyl oleanolate	924	
	β-amyrin	909	
	erythrodiol	812	
GlcA	oleanolic acid	192	66

* Boiled enzyme control. † Endogenous substrates only.

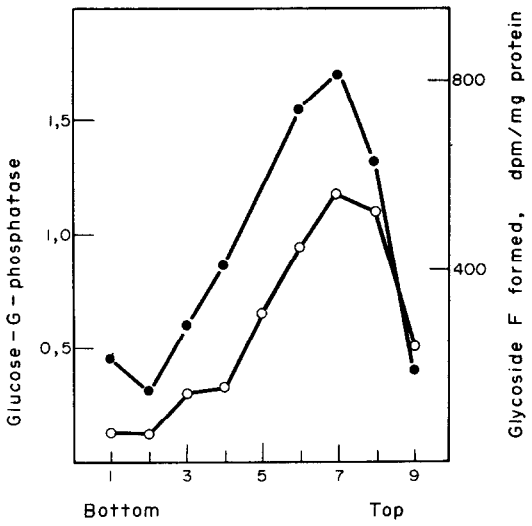


Fig. 1. Sucrose density gradient profile of crude microsomes (105000 *g* pellet) from *C. officinalis* seedlings. —○— relative specific activity of glucose-6-phosphatase (sp. act. in crude microsomes = 1·00); —●— formation of radioactive oleanolic acid 3β-D-glucuronoside (F) in the presence of oleanolic acid and UDPGlcA-[^{14}C].

glycoside F is present mainly in the microsomes in contrast to other glycosides.

Formation of the C-28 glucosidic bond of the oleanolic acid glycosides

Three of the glycosides occurring in *C. officinalis* (D_2, C and A) contain a glucose residue bound to the C-17 carboxyl of the aglycone. To elucidate at which stage addition of this residue occurs various subcellular fractions were incubated with UDPGlc-[^{14}C] and oleanolic acid or glycosides F, D and B. In this case the synthesis of the respective C-28 glucosides occurred mainly in the 105000 *g* supernatant while the activity of the particulate fraction was 4–8 times lower per mg of protein. When oleanolic acid was used as the acceptor no product was detected with chromatographic properties expected for oleanolic acid 28-glucoside. With glycosides F, D and B utilization was definitely highest for glycoside F (Table 3). In this case a radioactive product was formed that was chromatographically identical with glycoside D_2. After alkaline hydrolysis, which selectively releases a sugar bound to a carboxyl group, all the radioactivity was recovered in glucose. The results indicate that although the addition of glucose to the C-28 carboxyl takes place mainly at the level of glycoside F to produce D_2 it may occur to a smaller extent also at the level of glycosides D and B to yield C and A respectively. Replacement of UDPGlc-[^{14}C] by glucose-[^{14}C] or glucose-1-phosphate-[^{14}C] resulted in a several-fold reduction of the synthesis of glycoside D_2 from F.

Elongation of the sugar chain at C-3 of oleanolic acid

The consecutive stages of sugar chain elongation at C-3 of the aglycone were followed by incubation of the subcellular fractions with UDPGlc-[^{14}C] or UDPGal-[^{14}C] and glycosides F, D_2, D, C or B. The best results were always obtained with the particulate fraction sedimenting at 15000 *g*. The activities of the other fractions were 5–15 times lower. The relatively high ^{14}C-incorporation into glycosides even in the absence of any exogenous acceptor by utilization of endogenous compounds created some difficulties (Table 4). The results obtained permit the conclusions that addition of either glycoside F or D_2 to the incubation mixture, in the presence of UDPGal-[^{14}C], enhanced the formation of ^{14}C-3′-galactosyl derivatives (glycosides D or C respectively). On the other hand, no enhancement of the synthesis of the corresponding 3′-galactosyl derivative, B, was observed upon addition of glycoside E* (oleanolic acid 4′-glucosyl-glucuronoside) to the incubation mixture. This leads to the

Table 3. Formation of C-28 glucoside esters of oleanolic acid 3-glycosides by the cytosol fraction from *C. officinalis* seedlings

Sugar-[^{14}C] added	Glycoside added	Glycoside isolated	Radioactivity (dpm) in glycoside isolated
UDPGlc	none	D_2	220
	F	D_2	965
	F*	D_2	22
Glc	F	D_2	49
G-1-Phosphate	F	D_2	66
UDPGlc	none	C	85
	D	C	232
	none	A	62
	B	A	194

* Boiled enzyme control.

* Glycoside E has not been found in *C. officinalis*; however, it can be readily prepared by mild hydrolysis of glycoside B or A [3].

Table 4. Elongation of the sugar chain at C-3 of oleanolic acid in the presence of a 15000 *g* fraction from *C. officinalis* seedlings

Sugar-[^{14}C] added	Glycoside added	Glycoside isolated	Radioactivity (dpm) in glycoside isolated
UDPGal	none	D	825
	F	D	1425
	none	C	620
	D_2	C	1149
	none	B	43
	E	B	59
UDPGlc	none	E	18
	F	E	26
	none	B	740
	D	B	1312
	none	A	130
	C	A	642

conclusion that galactose addition at position 3′ of glucuronic acid precedes the addition of glucose at position 4′. This is confirmed by the lack of synthesis of a compound with the chromatographic mobility of glycoside E in the presence of UDPGlc-[^{14}C] and glycoside F. In this case the use of glycosides D or C as acceptors brought about a marked enhancement of the formation of glycosides B and A respectively.

Microscopic examination of the 15000 *g* fraction showed that it contained mainly mitochondria [8]. To determine if the observed processes were in fact localized in mitochondria the crude

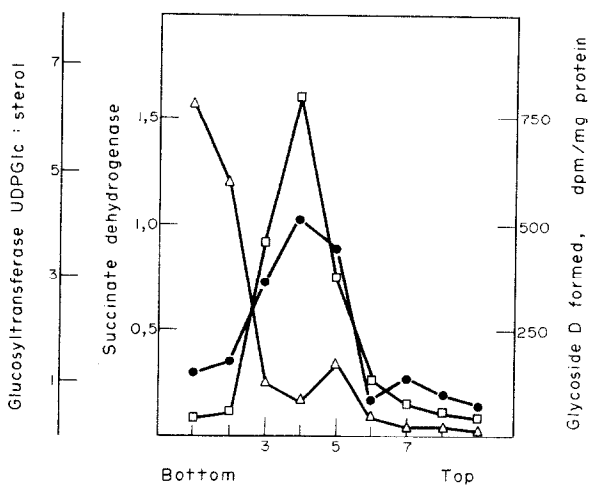
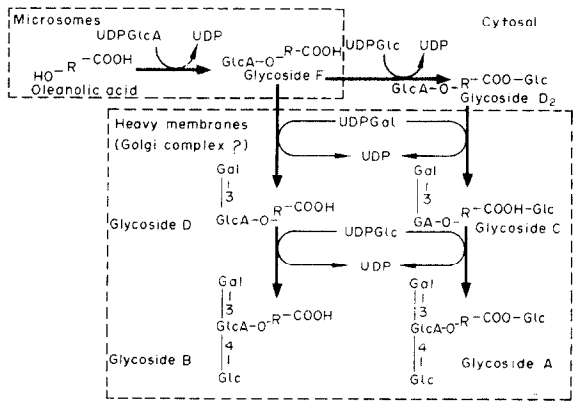


Fig. 2. Sucrose density gradient profile of crude mitochondria (15000 *g* pellet) from *C. officinalis* seedlings. ···△··· relative sp. act. of succinate dehydrogenase; —□— relative sp. act. of UDPGlc:sterol glucosyltransferase; —●— formation of radioactive glycoside D in the presence of UDPGal-[^{14}C] and glycoside F.



Scheme 1. Biosynthesis of sugar chains of *C. officinalis* glycosides.

15000 *g* fraction was centrifuged in a sucrose density gradient (Fig. 2). The following enzyme activities were assayed in the resulting subfractions: (i) succinate dehydrogenase as a mitochondrial marker [7, 9]; (ii) UDPGlc:sterol glucosyltransferase; (iii) galactosyltransferase catalyzing the synthesis of glycoside D in the presence of UDPGal and glycoside F. It is evident that the distribution of the galactosyltransferase activity failed to coincide with that of the succinate dehydrogenase whereas it coincided with the distribution of the UDPGlc:sterol glucosyltransferase, an enzyme recently suggested to be localized within the Golgi complex [10, 11].

On the basis of the present results, especially the data on the localization of the different steps within the cell, a scheme for the sequence of reactions during the biosynthesis of the sugar chains of *C. officinalis* glycosides is suggested in Scheme 1.

EXPERIMENTAL

Subcellular fractions. Whole 7-day-old *C. officinalis* cv Radio seedlings were ground in a mortar (20 sec at 0°) with Si gel [8] (30–70 mesh, Merck) in 0·1 M Tris-HCl buffer, pH 7·2. For 1 g of plants 0·5 g Si gel and 3 ml of buffer were used. The homogenate was squeezed through cheese-cloth and successively centrifuged at 600 *g* (5 min), 3000 *g* (10 min), 15000 *g* (20 min) and 105000 *g* (1 hr). All operations were performed at 0–4°.

Preincubation of microsomes with Triton X-100. The crude 105000 *g* fraction was resuspended using a Potter-Elvehjem homogeniser in Tris-HCl buffer containing 0·4% Triton X-100 (1 ml per 4 mg protein) and incubated with shaking for 10 min at 10°. The suspension was then diluted with buffer (4×) and immediately used for assays.

Glycoside formation assays. The standard incubation mixtures contained: 1 ml of the particle fraction or 105000 *g*

supernatant (1–1·2 mg protein) in 0·1 M Tris-HCl buffer, pH 7·2, containing 1 mM 2-mercaptoethanol; 0·025 ml (0·1 μCi) of labelled sugar or sugar derivative and 0·1 ml (50 μg) of emulsified oleanolic acid or glycoside (see below). Incubations were carried out for 20 min at 30° and stopped by addition of MeOH (1 ml) and boiling for 5 min. Subsequently *n*-BuOH (15 ml) was added and precipitated protein removed by low-speed centrifugation. The BuOH extract was washed with H_2O (5 × 5 ml) and 1 ml of a mixture of oleanolic acid and glycosides A–F (25 μg of each) in MeOH was added to facilitate the chromatographic separation of radioactive products. Individual glycosides were isolated by double TLC on Si gel with PrOH–conc NH_3–H_2O (8:1:1) and $CHCl_3$–MeOH–H_2O (61:32:7) [3, 4, 5]. Radioactivity was measured as described previously [11].

Substrates for glycoside biosynthesis. All ^{14}C labelled sugars were obtained from The Radiochemical Centre (Amersham, U.K.) and had sp. acts. of 90–230 mCi/mM. Oleanolic acid and glycosides A–F were obtained from *C. officinalis* as previously reported [3, 4]. The emulsion of oleanolic acid was prepared as follows: 5 mg oleanolic acid, 40 mg lecithin (commercial preparation from marrow) and 200 mg Triton X-100 were dissolved in 1 ml $CHCl_3$–MeOH (2:1). The solvent was removed at 60° and H_2O (10 ml) was added dropwise with cautious stirring. Glycoside solns were prepared in 2% aq. Triton X-100.

Centrifugation in sucrose density gradients. Gradients were prepared from sucrose solns in an appropriate buffer (0·1 M Tris–HCl, pH 7·2, for determination of the synthesis of oleanolic acid glycosides; 0·1 M phosphate, pH 7·4, for determination of succinic dehydrogenase or 0·1 maleate, pH 6·0, for determination of glucose-6-phosphatase). Four 0·9 ml portions of the sucrose solns (1·8, 1·5, 1·2, 0·9 M for the 105000 *g* fraction or 1·5, 1·2, 0·9, 0·6 M for the 15000 *g* fraction) were layered in 5 ml polythene tubes. Immediately before centrifugation the particulate fraction in 0·6 M sucrose (0·9 ml, about 15 mg protein) was layered onto the gradient. Gradients were spun for 1 hr at 34000 rpm in the SW-50·1 rotor of a Beckman L3-50 ultracentrifuge and nine fractions (0·5 ml each) were collected from the bottom of the tube.

Other methods. The procedure for total and partial glycoside hydrolysis has previously been described [3]. Protein was determined according to Lowry *et al.* [12], chlorophyll according to Arnon [13], succinate dehydrogenase according to Pennington [9] as modified by Porteous and Clark [14] and glucose-6-phosphatase according to Hübscher and West [6]. UDPGlc:sterol glucosyltransferase was determined as previously described [15].

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