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THE METABOLISM OF [3-^{3}H]OLEANOLIC ACID-3-*O*-MONO-[^{14}C]GLUCOSIDE IN ISOLATED CELLS FROM *CALENDULA OFFICINALIS* LEAVES

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Key Word Index—*Calendula officinalis*; Compositae; isolated cells; oleanolic acid glycosides; labelling dynamics.

Abstract—The radioactive precursor, [3-^{3}H]oleanolic acid-3-*O*-mono-[^{14}C]glucoside was administrated to isolated cells obtained from the leaves of *Calendula officinalis*. The radioactivity of the precursor was incorporated into fractions containing free oleanolic acid, individual glucosides, glucuronide F and other glucuronides. The ratio of ^{3}H:^{14}C radioactivity in these fractions indicated that glucosides were formed in a process involving direct glycosylation of the precursor, whereas the glucuronides were formed from oleanolic acid released by hydrolysis of the precursor. Dynamics curves showed that glucoside II formed by direct glycosylation of the precursor was intensively transformed to other derivatives.

INTRODUCTION

From the previous work [1, 2] on the labelling dynamics of oleanolic acid glycosides belonging to series I and II in *Calendula officinalis* (marigold) flowers after administration of the 3-*O*-monoglucoside (a precursor of series II) and the 3-*O*-monoglucuronide (precursor of series I) of oleanolic acid, it was evident that glycosylation to yield the glycoside of the opposite series must proceed after hydrolysis of supplied precursor to oleanolic acid. However, it was not elucidated to what extent the supplied monoglycoside is transformed into derivatives of its own series and how much is synthesized from free oleanolic acid. The resolution of this problem was the aim of the present investigation using the cells from the leaves of *Calendula officinalis* instead of ligulate flowers. The flowers of *Calendula officinalis* are not the best experimental material due to the fact that some oleanolic acid glycosides appear in the flowers as the result of their transportation from the shoots. Our preliminary investigation [3] showed that after administration of radioactive acetate free oleanolic acid and both series of its glycosides were well labelled in isolated marigold cells. As the precursor in the present study the [3-^{3}H]oleanolic acid-3-*O*-mono-[^{14}C]glucoside was used. It was expected that examination of the ^{3}H:^{14}C ratio in the glycoside derivatives would allow a correlation between glycosylation and hydrolysis of administrated precursor.

RESULTS AND DISCUSSION

As a first step in the present work the quantity of oleanolic acid, both free and bound in monoglucoside I, glucoside VII, other glucosides and glucuronides, was determined. The results are presented in Table 1. They confirmed earlier results [3] showing that the quantity of glucuronides was more than twice the quantity of glucosides.

In some of our previous investigations [4, 5] we found that different triterpene compounds could be partially destroyed during the TLC on silica gel and hydrolysis procedures. In the same manner TLC separation of oleanolic acid glycosides gave some interfering compounds showing similar R_f values. About 9% of tritium and 8% of carbon radioactivity were located in these artefacts as shown in Table 2.

Table 1. The quantities of free oleanolic acid and oleanolic acid bound in glucosides and glucuronides in cells (10 g) isolated from the leaves of *Calendula officinalis*

	Oleanolic acid	
Fraction	μg	%
Free oleanolic acid	14.8	8.4
Glucoside I	7.9	4.5
Glucoside VII	6.8	3.8
Other glucosides	35.7	20.1
Total in glucosides	50.4	28.4
Glucuronide F	15.9	9.0
Other glucuronides	96.2	54.5
Total in glucuronides	112.1	63.2
Total in all fractions	177.3	100.0

The major part of the tritium radioactivity was found at the level of oleanolic acid due to non-enzymatic hydrolysis of the precursor. The radioactivity due to carbon which was located on the level of oleanolic acid and glycosides of both series must arise from degradation of the sugar moiety of the administrated precursor. It corresponds to the quantity of freed oleanolic acid. In further experiments this amount of radioactivity was considered as the control level.

The principle experiments were connected with the labelling dynamics of different glycoside fractions. The amount of radioactivity was determined for all individual

Table 2. Radioactivity in the artefacts formed during TLC of the 3-*O*-[^{14}C]glucoside of [3-^{3}H]oleanolic acid and their location at the R_f values of oleanolic acid and its glycosides.

Band	^{14}C cpm	^{14}C %	^{3}H cpm	^{3}H %
Glucoside I				
Starting material	69504	92.0	130111	91.0
Oleanolic acid	2410	3.1	9490	7.0
Glucosides				
II, III, VI, VII	1102	1.5	1714	1.2
Glucuronides	2584	3.4	1181	0.8
Total	75600	100.0	142496	100.0

Radioactivity applied to TLC plate: ^{14}C—84000 cpm; ^{3}H—157200 cpm.

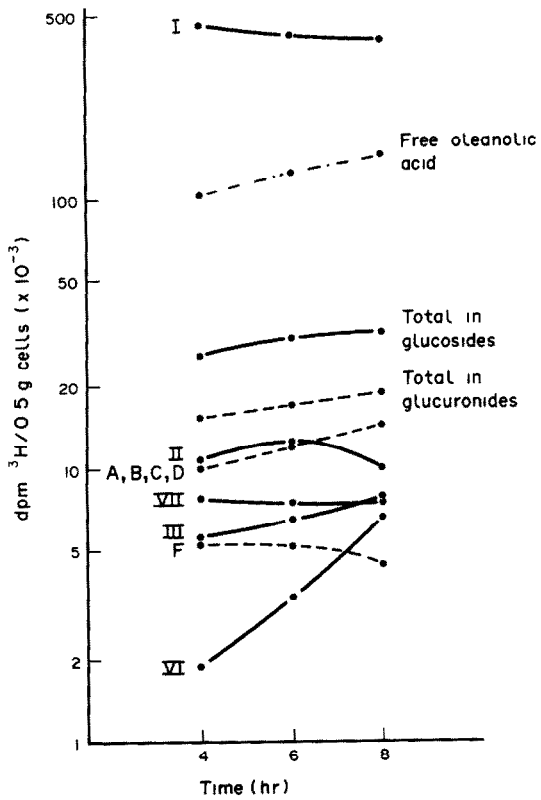

Fig. 1. The dynamics of incorporation of [^{3}H] radioactivity into oleanolic acid and its glycosides after incubation with the 3-*O*-[^{14}C]glucoside of [3-^{3}H] oleanolic acid.

glucosides, glucuronide F and for the total other glucuronides. The results presenting incorporation of tritium radioactivity into both series of glycosides are shown in Fig. 1. Tritium radioactivity of administrated precursor (glucoside I) decreased constantly during the experiment. The process of precursor hydrolysis to [3-^{3}H]oleanolic acid and [^{14}C]glucose is responsible for this decrease. Hence, as was expected, the radioactivity in free oleanolic acid increased accordingly. The total tritium radioactivity in glucosides increased, but at a higher rate from 4 to 6 hr than from 6 to 8 hr. In glucoside II (3-*O*-[gal(1 → 4)glc]-oleanolic acid), the derivative of glucoside I, in radioactivity increased up to 6 hr and then decreased, reaching after 8 hr a lower level than after 4 hr. This showed that glucoside II was actively metabolized to successive derivatives, i.e. glucoside III (3-*O*-[gal-gal(1 → 4)glc]-oleanolic acid) and glucoside VII (3-*O*-[gal(1 → 4)glc-1,glc-glc-glc(1 → 3)glc-1]-oleanolic acid [6]. The radioactivity in glucoside III increased 1.3 times during 8 hr, but a much higher increase was observed in its derivative, glucoside VI (3-*O*-[gal-gal(1 → 4)glc-1,glc-glc(1 → 3)glc-1]-oleanolic acid), into which three-fold more radioactivity was incorporated after 8 hr than after 4 hr. Glucoside VI is the 'transport glucoside' which in the marigold plant is transported from leaves to roots [7]. Synthesis in isolated cells from the leaf is hence very intensive and because isolated cells have no possibility of transportation of their metabolites, glucoside VI is accumulated in them. The amount of tritium radioactivity in all glucuronides (A, B, C, D) except F increased constantly. In glucuronide F radioactivity decreased 1.2 times during the course of the experiment.

The incorporation of carbon radioactivity into both series of glycosides is shown in Fig. 2. The labelling curves for the administered precursor, total glucosides and individual glucosides were very similar to those observed with tritium radioactivity and they confirmed our con-

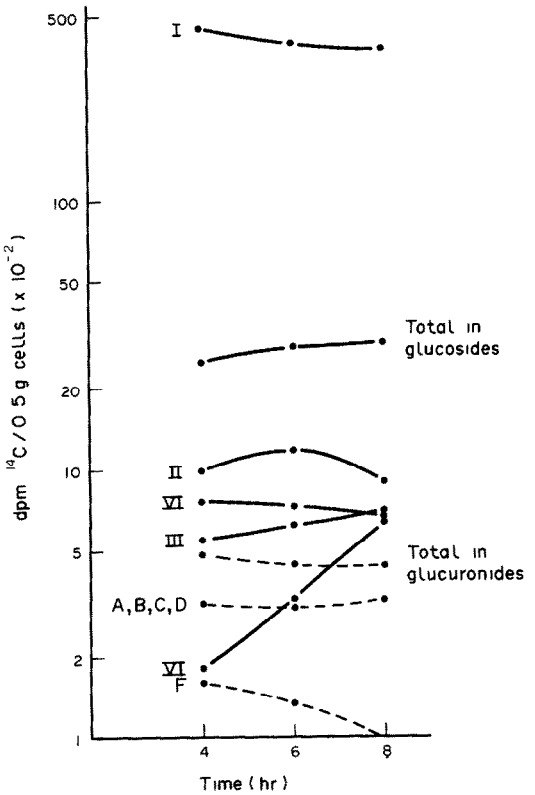

Fig. 2. The dynamics of incorporation of [^{14}C] radioactivity into the glycosides of oleanolic acid after incubation with the 3-*O*-[^{14}C]glucoside of [3-^{3}H] oleanolic acid.

Table 3. Changes in the $^3H:^{14}C$ ratios in the fractions of free oleanolic acid and its glycosides obtained from the isolated cells after incubation with the 3-*O*-[^{14}C]glucoside of [3-^3H]oleanolic acid.

Fraction	$^3H:^{14}C$ ratio		
	4 hr	6 hr	8 hr
Oleanolic acid	—	—	—
Glucoside I	10.87	10.89	10.89
Glucoside II	10.87	10.80	11.23
Glucoside III	10.55	10.60	11.11
Glucoside VI	10.33	10.48	10.88
Glucoside VII	10.37	10.50	10.86
Mean in glucosides	10 60	10.65	11.03
Glucuronide F	33 35	40.07	44.20
Glucuronides A, B, C, D	32.10	38.86	43.72
Average in glucuronides	32.52	39.30	43.83

clusions drawn from the tritium radioactivity results. The total carbon radioactivity of glucuronides was constant, whereas radioactivity in glucuronide F decreased rapidly. The different profiles of the tritium and carbon radioactivity curves for the glucuronides changes the $^3H:^{14}C$ ratios in these compounds. The $^3H:^{14}C$ ratio in different glycoside fractions is shown in Table 3.

The unchanged $^3H:^{14}C$ ratios in the glucosides in comparison to the administered precursor proves that glucosides are formed in isolated cells by the pathway of direct glycosylation of the precursor. The $^3H:^{14}C$ ratio in glucuronides was much higher and increased during incubation. This shows that glucuronides are synthesized by secondary glycosylation of oleanolic acid freed during hydrolysis of the precursor. [^{14}C]Glucuronic acid is formed from [^{14}C]glucose but the radioactive material is considerably diluted by endogenous glucuronic acid.

Our results allow us to draw the following conclusions. [3-^3H]Oleanolic acid-3-*O*-mono-[^{14}C]glucoside is effectively absorbed and metabolized by isolated cells from *Calendula officinalis* leaves. Hydrolysis of the precursor gives [3-^3H]oleanolic acid and [^{14}C]glucose while direct glycosylation of precursor yields glucosides II, III, VI and VII. The free oleanolic acid undergoes subsequent glycosylation to produce glucuronides. [^{14}C]Glucose freed during hydrolysis of the precursor can be used after oxidation to [^{14}C]glucuronic acid or epimerization to [^{14}C]galactose for further glycosylation reactions.

EXPERIMENTAL

Isolation of cells. This procedure and conditions of administration of precursor were described previously [8].

Isolation of oleanolic acid and its glycosides and their quantitative determination in isolated cells. Compounds were separated by TLC as described earlier [6] and their quantity was determined by measurement of oleanolic acid by GLC on an SE-30 column.

Preparation of the radioactive precursor. The 3-*O*-[^{14}C]glucoside of [3-^3H]oleanolic acid was synthesized by reaction of tetraacetylbromide [^{14}C]glucose with [3-^3H]oleanolic acid [9]. The obtained precursor had a specific activity of ^{14}C 0.27 mCi/mmol and ^3H 2.93 mCi/mmol. The ratio of $^3H:^{14}C$ was 10.85.

Administration of the radioactive precursor. The soln of radioactive precursor in 5% EtOH–H_2O was administrated to isolated cells of marigold leaves for 4, 6 and 8 hr.

Extraction and preparative chromatography. The crude fraction of oleanolic acid and its glycosides was obtained by extraction of cells several times with hot MeOH. The crude fraction was purified and separated into individual compounds by TLC in systems described earlier [6].

Radioactivity measurement. The radioactivity of compounds was measured in a scintillation counter and calculated by the use of simultaneous equations as in Gilbert's method adapted to two beta-emitters (^3H and ^{14}C) [10].

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