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# Metabolism of [3-$^3$H]oleanolic acid in the isolated *Calendula officinalis* leaf cells and transport of the synthesized glycosides to the cell wall and the extracellular space

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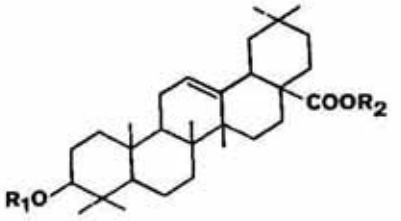
**It has been shown for the first time that [3-$^3$H]oleanolic acid glycosides formed in the cytosol of *C. officinalis* leaf cells are transported to the extracellular space in the form of pentaglucoside VI (44%), whereas glucuronides derived from [3-$^3$H]oleanolic acid 3-*O*-monoglucuronide (29%) as well as a part of glucosides (24%) were transported into the cell walls.**

*Calendula officinalis* leaves contain two series of oleanolic acid glycosides, i.e. derivatives of 3-*O*-monoglucuronide (F) and 3-*O*-monoglucoside (I) (Fig. 1) [1, 2]. As shown previoulsy [3, 4] oleanolic acid glycosides are involved in haemolysis, inhibition of *Trichoderma viride* growth and in allelopatic inhibition of germination and growth of several higher plants. In our earlier studies [5] it has also been demonstrated that, although biosynthesis of the glycosides of both series takes place in the cytoplasm, more than 35% of all cellular glycosides is located in non-cytoplasmic compartments, i.e. vacuoles and cell walls. In general, mechanisms of the transport of various compounds across the tonoplast are well established and the transport of oleanolic acid glycosides into intact vacuoles from *C. officinalis* leaf protoplasts has been reported [6]. In contrast, no data are available concerning the transport of triterpenic compounds into the cell wall. In a preliminary experiment [7] it was found that 3-*O*-monoglucoside (I) of [3-$^3$H]-oleanolic acid effectively penetrated into the cells (80%), where it was partly (10%) hydrolysed to free [3-$^3$H]oleanolic acid. Then both compounds, i.e. the labelled glucoside I and liberated [3-$^3$H]oleanolic acid, were further




OLEANOLIC ACID $R_1$=$R_2$=H

Series I

| $R_1$ | $R_2$ | | |
|---|---|---|---|
| GlcUA | H | glucuronide | F |
| Gal(1→3) GlcUA | H | | D |
| Gal(1→3) GlcUA | Glc | | C |
| Gal(1→3) Glc(1→4) GlcUA | H | | B |
| Gal(1→3) Glc(1→4) GlcUA | Glc | | A |

Series II

| $R_1$ | $R_2$ | | |
|---|---|---|---|
| Glc | H | glucoside | I |
| Gal(1→4) Glc | H | | II |
| Gal–Gal(1→4) Glc | H | | III |
| Glc–Glc(1→3) Gal–Gal(1→4) Glc | H | | VI |
| Glc–Glc–Glc(1→3) Gal(1→4) Glc | H | | VII |

*Fig. 1. Structure of two series of oleanolic acid glycosides present in Calendula officinalis leaves.*

glycosylated. Some of the newly formed [3-$^3$H]oleanolic acid glycosides remained in the cytoplasm (38%), whereas others were transported into the extracellular space (32%) or accumulated in the cell walls (30%). These results [7] did not clearly elucidate which glycosides of either series remained inside the cell and which were transported outside.

The present studies were designed to examine transformations of [3-$^3$H]oleanolic acid in the whole cell and in the fractions of cytoplasm and cell walls including distribution of the formed glycosides between the cytoplasm, the cell wall and the extracellular space.

## MATERIALS AND METHODS

***Isolation of cells.*** Cells were isolated from the leaves of *C. officinalis* by hydrolysis with macerozyme, as previously reported [8].

***Radioactive precursor.*** [3-$^3$H]Oleanolic acid was synthesized as described earlier [9] and had a specific activity of 96.8 mCi/mmol.

***Administration of radioactive precursor.*** A solution of radioactive precursor ($320 \times 10^{-3}$ d.p.m. in 200 µl of 5% EtOH/$H_2O$) was administered to isolated cells (300 mg in 7.5 ml of 0.3 M mannitol) for 8, 24 or 48 h.

***Preparation of the intracellular fraction, cell walls and extracellular space.*** After incubation, the cells were treated by repeated centrifugation ($300 \times g$, 5 min) and resuspension in 0.3 M mannitol to wash off the remaining precursor as well as the compounds transported outside the cell to extracellular space. Then the pellet containing the cells was homogenized in a Potter homogenizer with subsequent centrifugation at $600 \times g$ for 10 min. The pellet contained the crude fraction of cell walls and the supernatant constituted the intracellular fraction (cytoplasm, membranes and the soluble vacuolar compounds). The cell wall fraction was purified by centrifugation ($600 \times g$, 10 min) through a layer of 50% (w/v) sucrose solution according to Greve & Ordin [10]. The supernatant was combined with the intracellular fraction, whereas the pellet represented the purified cell wall fraction. The contents of chlorophyll and marker enzymes [5] indicated that the fraction of cell wall contained about 10% of impurities resulting from contamination with other cell fractions.

***Extraction.*** The fraction containing the compounds of the extracellular space washed off with mannitol and the fraction of intracellular components were separately extracted four times with etyl ether and butanol. The pellet containing the purified cell walls was extracted four times with hot methanol and once with 50% solution of ethyl ether in methanol. Extracts were separated by t.l.c. as earlier described [11], whereupon the radioactivity of the individual compounds was measured.

## RESULTS AND DISCUSSION

The dynamics of incorporation of [3-$^3$H]oleanolic acid supplied to the isolated cells from *C. officinalis* leaves into glucuronides (A, B, C, D, F) and glucosides (I, II, III, VI, VII) in the fractions of the intracellular compounds, cell walls and extracellular space is presented in Fig. 2 (a, b, c). Total changes of the amount of [3-$^3$H]oleanolic acid and its glycosides in the extracellular space, the intracellular fraction and the cell wall are presented in Table 1. The results are mean values from four replicate experiments. The scatter of the results does not exceed 15%.

The supplied [3-$^3$H]oleanolic acid was glycosylated inside the cell to glycosides of both the glucuronide and glucoside series at different rates (Table 1, Fig. 2a). After 48 h incubation glucosides were labelled over five times more rapidly than glucuronides. This is consistent with the earlier findings that biosynthesis of glucosides is several times faster than that of glucuronides [12]. Among glucosides, monoglucoside I was labelled most intensively and at the end of the experiment exceeded the amount of free [3-$^3$H]oleanolic acid. The labelling of the remaining glucosides (II, III, VI and VII) increased throughout the experiment, especially rapidly in the case of glucoside VII. On the contrary, the glucuronides displayed different labelling dynamics. The amount of glucuronides A, B, C and D rose during the first 24 h and then the rate declined slightly, whereas labelling of monoglucuronide F decreased rapidly throughout the experiment, probably as a result of its transformation to other glucuronides.

In the cell wall fraction (Fig. 2b), the general course of labelling of glucosides was similar to that of the intracellular components but in this

fraction glucoside III was the most abundant, whereas the amount of glucoside VII decreased significantly after 24 h of incubation. In turn, the labelling of glucuronides A, B, C and D rose throughout the experiment. Similarly as before, the amount of glucuronide F decreased rapidly.

Radioactivity of the supplied [3-$^3$H]oleanolic acid decreased uniformly in the extracellular space throughout the incubation time (Table 1, Fig. 2c), due to lowering penetration of the acid into the cells and its transformation into glycosides of both series. After 48 h incubation, the most abundant compound in the extracellular space was glucoside VI, the labelling of which increased very rapidly throughout the experiment. In turn, the labelling of glucoside I decreased uniformly in this fraction, whereas the amount of glucosides II and III rose during the first 24 h and then declined slightly. The labelling of glucoside VII was practically stable. Glucuronides A, B, C and D displayed similar labelling dynamics as in both previous experiments, whereas the labelling of glucuronide F increased slowly after 24 h of incubation.

As demonstrated in our earlier studies [13, 14] biosynthesis of oleanolic acid and its two monoglycosides takes place in the microsomal fraction, in which also other glucosides are formed as a result of the addition of the chains containing several sugar residues. On the other hand, the remaining glucuronides originate in the Golgi membranes due to addition of single sugar molecules. Although the possibility of some transformations of oleanolic acid glycosides in the cell wall cannot be ruled out definitively, these findings allow us to assume that all glycosides located in the cell walls and in the

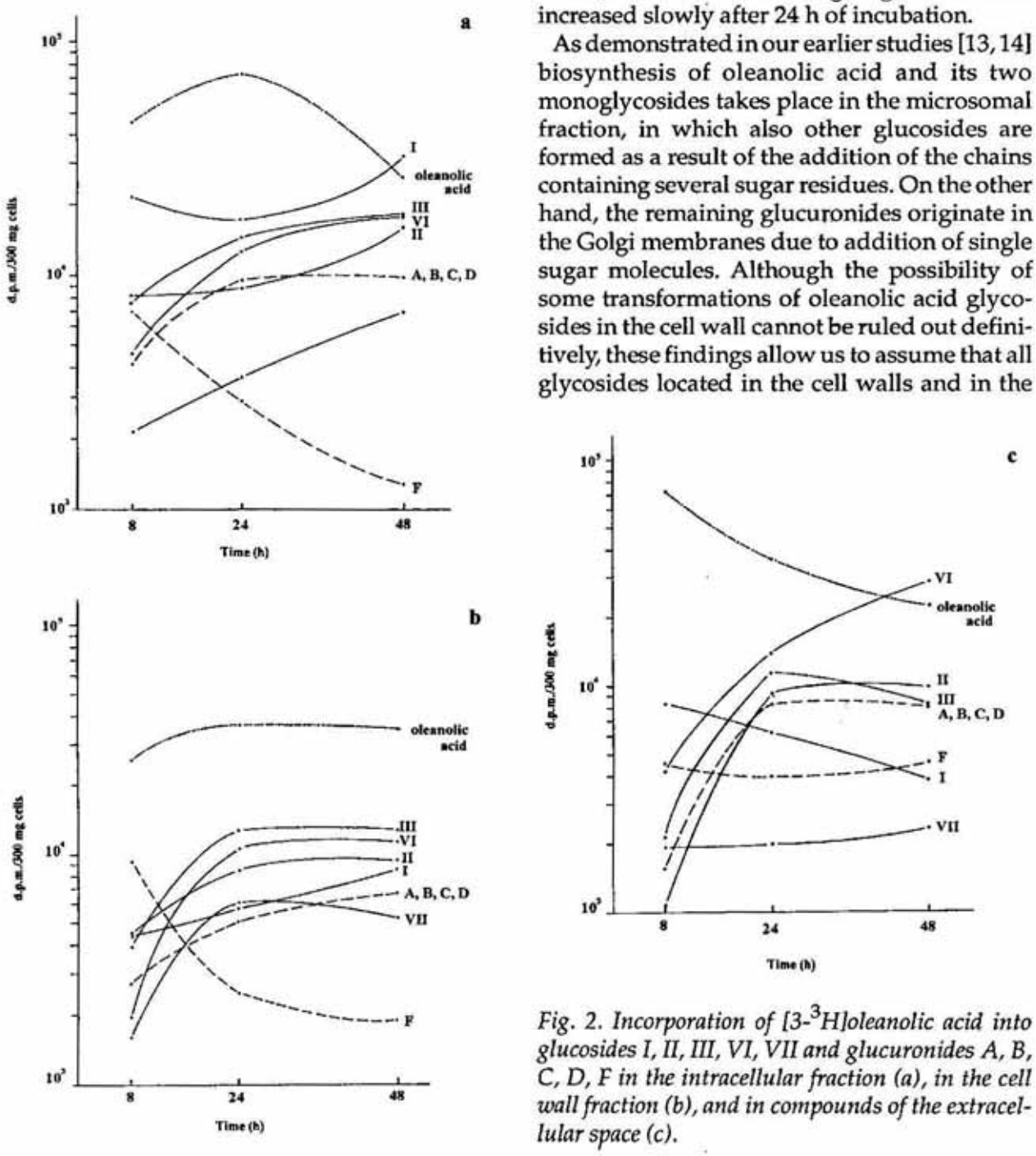



*Fig. 2. Incorporation of [3-$^3$H]oleanolic acid into glucosides I, II, III, VI, VII and glucuronides A, B, C, D, F in the intracellular fraction (a), in the cell wall fraction (b), and in compounds of the extracellular space (c).*

Table 1

*Distribution of [3-$^3$H]oleanolic acid and its glycosides in the extracellular space, the intracellular fraction and the cell wall of Calendula officinalis leaf cells*

| Fraction | | Incubation time (h) | | | | | |
|---|---|---|---|---|---|---|---|
| | | d.p.m. × $10^{-3}$ | % | d.p.m. × $10^{-3}$ | % | d.p.m. × $10^{-3}$ | % |
| Extracellular space | oleanolic acid | 82 | 31 | 37 | 12 | 22 | 7 |
| | glycosides | 33 | 12 | 44 | 15 | 64 | 21 |
| | **sum** | **115** | **43** | **81** | **27** | **86** | **28** |
| Intracellular fraction | oleanolic acid | 45 | 17 | 72 | 25 | 26 | 9 |
| | glycosides | 55 | 20 | 58 | 19 | 98 | 32 |
| | **sum** | **100** | **37** | **130** | **44** | **124** | **41** |
| Cell wall | oleanolic acid | 26 | 9 | 37 | 12 | 36 | 12 |
| | glycosides | 29 | 11 | 52 | 17 | 55 | 19 |
| | **sum** | **55** | **20** | **89** | **29** | **91** | **31** |
| Total | oleanolic acid | 153 | 57 | 146 | 49 | 84 | 28 |
| | glycosides | 117 | 43 | 154 | 51 | 217 | 72 |
| | **sum** | **270** | **100** | **300** | **100** | **301** | **100** |

extracellular space, are transported there across the plasma membrane from the sites of their biosynthesis in cytoplasm, resembling their transport to vacuoles [6]. The present results indicate that, following biosynthesis, 26% of the newly formed glycosides is transported to the cell wall and 29% to the extracellular space. Four glucuronides A, B, C, D (29%) as well as glucosides (24%) containing one, two or three moieties of sugar are accumulated in the cell wall. The main compound transported outside the cell is pentaglucoside VI (44% of all glycosides in the extracellular space), whereas in the cell wall glycosides with shorter sugar chains are found. These results are also consistent with our earlier findings [15] that large amounts of oleanolic acid are transported in *C. officinalis* from shoot to root in the form of pentaglucosides (mainly VI). Moreover, biosynthesis of glucosides is several times faster than that of glucuronides, whereas the final level of glucuronides in the leaves is more than twice as high as that of glucosides [8]. This may be due to the fact that glucuronides being the probable typical secondary metabolites accumulate in the vacuole and the cell wall, whereas glucosides serve as a transport form of oleanolic acid in the plant.

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