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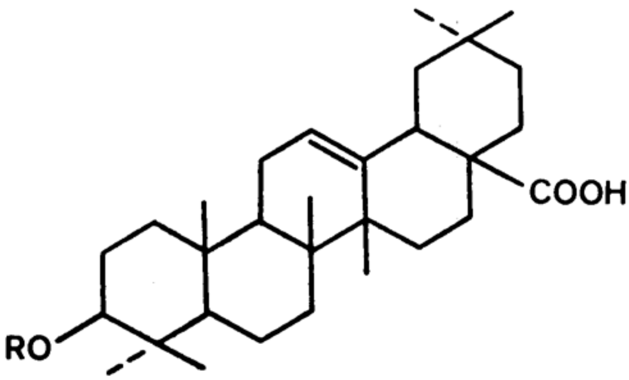
ANNA SZAKIEL and WIRGINIA JANISZOWSKA

THE EFFECT OF pH AND ATP ON THE TRANSPORT OF OLEANOLIC ACID MONOGLYCOSIDES INTO ISOLATED VACUOLES OF *CALENDULA OFFICIALIS* LEAVES

Institute of Biochemistry, Warsaw University, Żwirki i Wigury 93; 02-089 Warszawa, Poland

The transport of oleanolic acid and its glycosides (3-*O*-monoglucuronide F and 3-*O*-monoglucoside I) into the vacuoles of *C. officinalis* leaves was studied. It was found that these monoglycosides are transported into the vacuolar space whereas free oleanolic acid only binds with the tonoplast. The transport of monoglycosides depends on pH of the medium, the optimum pH for monoglucoside I and monoglucuronide F being 6.0 and 7.0, respectively. Moreover, it was demonstrated that the transport of monoglucoside I, in contrast to that of monoglucuronide F, depends on ATP at 0.5 - 2.0 mM concentrations. The presented results indicate that different mechanisms underlie the transport of these two compounds into vacuoles.

Calendula officinalis leaves contain two series of oleanolic acid glycosides: derivatives of 3-*O*-monoglucuronide and 3-*O*-monoglucoside [1, 2] (Fig. 1). Previously [3, 4] we have demonstrated the occurrence of all these compounds



Oleanolic acid R=H

3-O-monoglucuronide F R=GlcUA

3-O-monoglucoside I R=Glc

Fig. 1. The structure of oleanolic acid and its monoglycosides F and I

in vacuoles (23.3% of total cellular oleanolic acid) and we evaluated the dynamics of penetration of [3-^{3}H]-oleanolic acid, its 3-*O*-monoglucuronide (F) and 3-*O*-monoglucoside (I) into isolated vacuoles in only trace amounts. Monoglucuronide F was transported into the vacuoles slowly but uniformly throughout the experiment, whereas monoglucoside I was transported intensively during the first 60 min whereupon the rate of its translocation distinctly dropped. These results testified to differences in the character of transport of the two monoglycosides into the vacuoles.

The aim of the present study was to elucidate whether [3-^{3}H]-oleanolic acid and its monoglycosides only bind with the tonoplast or penetrate into the vacuolar space, as well as to verify whether this process depends on pH of the medium and on the presence of high-energy compounds.

MATERIALS AND METHODS

Isolation of protoplasts and vacuoles. Protoplasts were isolated from leaves of *C. officinalis* as described earlier [5], vacuoles were liberated from protoplasts with DEAE-dextran in an isotonic solution of mannitol and were purified as described previously [3].

Radioactive precursors. [3-^{3}H]-Oleanolic acid, its 3-*O*-monoglucuronide F and 3-*O*-monoglucoside I were synthesized as described earlier [6]. Precursors had a specific activity of 3.82 mCi/mmol.

Administration of radioactive precursors. The solution of radioactive precursor (200000 c.p.m. in 200 µl) in 5% EtOH-H_2O was administered to intact isolated vacuoles (4×10^5 in 1 ml of incubation medium [3]) during 180 min (or during 120 min if the effects of both pH and ATP were determined).

Preparation of the vacuolar fraction. After incubation, the precursor not taken up was washed off by centrifugation of vacuoles in a mannitol/sucrose/Ficoll gradient [3]. To disrupt vacuoles osmotic shock was applied: four volumes of 15 mM phosphate buffer, pH 8.0 were added followed by centrifugation at $105000 \times g$ for 1 h; the pellet contained fragments of tonoplast membranes and the supernatant — the soluble interior of vacuoles.

Extraction. Both fractions like the intact vacuoles, were extracted with ethyl ether and butanol. Extracts were separated by t.l.c., as earlier described [7], whereupon the radioactivity of the different compounds was measured [5].

RESULTS AND DISCUSSION

The transport of [3-^{3}H]-oleanolic acid and its 3-*O*-monoglycosides into isolated vacuoles expressed as the radioactivity incorporated into those particles and their structural components is presented in Table 1. It was found that vacuoles take up monoglucuronide F and monoglucoside I several times more effectively than free [3-^{3}H]-oleanolic acid; namely, the uptake of F and

Table 1

Transport of 3-[^{3}H]-oleanolic acid (OL) and its monoglycosides (I and F) into isolated vacuoles

Compound		Radioactivity incorporated into vacuoles c.p.m.		
added	isolated	intact vacuoles	tonoplast	vacuolar space
I	I	12430	520	11472
	OL	1653	1589	165
F	F	10930	650	10290
	OL	1811	1792	195
OL		1775	1635	103

I accounts for 6 and 7% of the supplied radioactivity, respectively, whereas the uptake of oleanolic acid accounts for only 0.8%. Both monoglycosides are located virtually completely in the vacuole (F — 94%, I — 96%), and free oleanolic acid — in the tonoplast (94%). Thus, results obtained indicate that both monoglycosides are effectively transported into the vacuolar space, whereas free oleanolic acid only binds with the tonoplast. At the same time it was found that upon treating the vacuoles with either labelled monoglycoside free oleanolic acid is obtained. The latter compound is formed as a result of partial hydrolysis of monoglycosides (about 12%) and binds with the tonoplast. It is known that the vacuole contains hydrolases of various types [8]. They could be responsible for partial hydrolysis of the supplied oleanolic acid monoglycosides. On the other hand, the possibility of hydrolysis of these compounds during the transport across the tonoplast cannot be ruled out. The fact that the radioactive monoglycoside located within the vacuole undergoes no further hydrolysis seems to testify to the latter possibility. It is also of interest that the amount of oleanolic acid bound with the tonoplast is identical, irrespective of its level in the medium (high when the incubation is carried out with an excess of [3-^{3}H]-oleanolic acid and low in the case of free oleanolic acid formed as a result of hydrolysis of monoglycosides). This seems to point to a limited number of sites binding free oleanolic acid in the tonoplast. It is not clear whether these sites are located within, or on the surface of, the vacuolar membrane.

The results concerning the dependence of the transport of oleanolic acid monoglycosides into isolated vacuoles on pH and on the presence of ATP are recorded in Figs. 2 and 3. It was found that both monoglycosides differ in optimum pH for transport into the vacuoles; for I and F, optimum pH is 6.0 and 7.0, respectively. Likewise, there were differences between the two monoglycosides in the effect of exogenous ATP on their transport into isolated vacuoles. It was shown that ATP at 0.5 - 2.0 mM concentrations exerts no effect

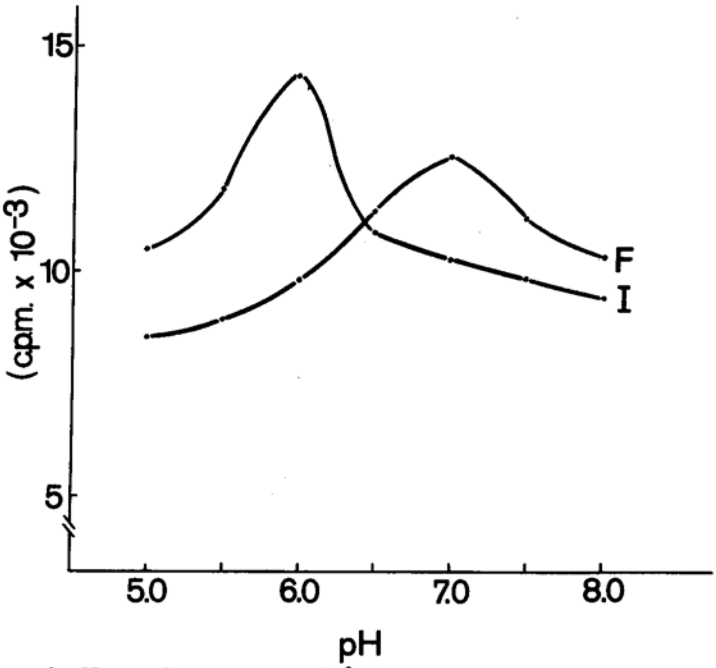


Fig. 2. Effect of pH on the transport of ^{3}H-labelled 3-*O*-monoglucoside I and 3-*O*--monoglucuronide F into isolated vacuoles

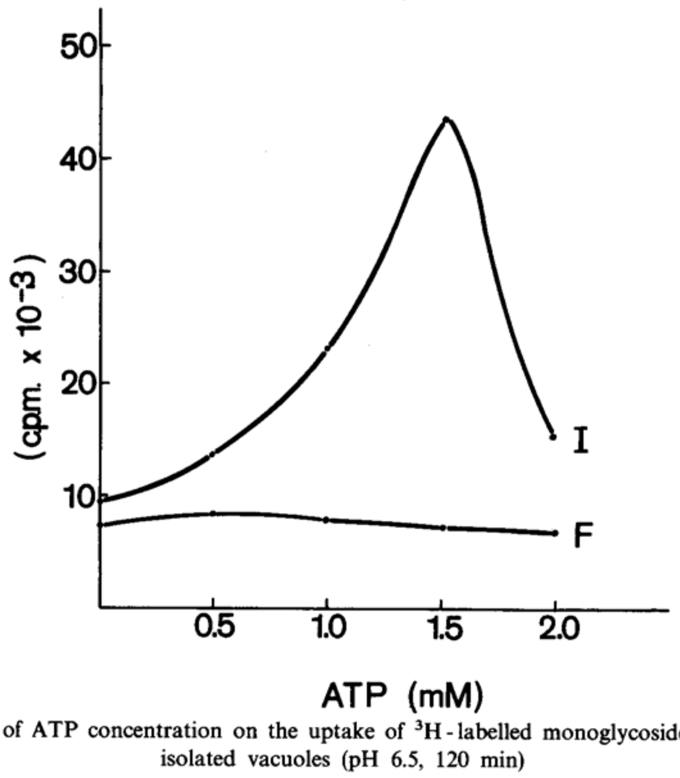


Fig. 3. Effect of ATP concentration on the uptake of ^{3}H-labelled monoglycosides I and F by isolated vacuoles (pH 6.5, 120 min)

on the transport of monoglucuronide F, whereas it stimulates the transport of I. This effect was the strongest at a 1.5 mM ATP concentration when the transport of I was stimulated more than four-fold.

The difference between the two monoglycosides in the dependence of their transport on pH of the medium and the presence of ATP, demonstrated in the present studies, combined with the earlier reported [4] differences between the two monoglycosides in the dynamics of their uptake by isolated vacuoles, suggest the occurrence of different mechanisms of their transport. This is of great interest, since both compounds differ only in the degree of oxidation of the sugar moiety. Perhaps the presence of an additional carboxyl group in monoglucuronide F allows for its transport across the tonoplast without energy expenditure.

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