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Intracellular transport and vacuolar accumulation of *o*-coumaric acid glucoside in *Melitolus alba* mesophyll cell protoplasts

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Sweet clover leaves accumulate *o*-coumaric acid glucoside in the vacuole of the mesophyll cells. Protoplasts isolated from these cells are able to synthesize *o*-coumaric acid glucoside from exogeneous [^{14}C]phenylalanine. Using this simplified system, kinetic analyses have shown that the glucoside is synthesized in the cytoplasm and thereafter transferred into the vacuole. No further degradation of the glucoside occurs in the isolated protoplasts after this transfer. Under our conditions, the vacuolar pH is reduced by light/dark transition and by inhibitors of photosynthesis. These treatments also depressed synthesis and transport of *o*-coumaric acid glucoside. The data support a relationship between the functioning of photosynthesis and pH cytoplasm/vacuole-dependent transport of *o*-coumaric acid glucoside into the vacuole. However as *o*-[^{14}C]coumaric acid glucoside is taken up by isolated vacuoles without any energy requirement, no final conclusions can be drawn as to the coupling of *o*-coumaric acid glucoside transport with the acidification process. In intact plant or isolated protoplasts *o*-coumaric acid glucoside is synthesized as the *trans* isomer but accumulated in the vacuole in the *cis* form. When loaded with *trans o*-[^{14}C]coumaric acid glucoside, reconstituted tonoplastic vesicles released this compound during molecular sieving on a Sepharose 4B column. In contrast, the labelled *cis* form was completely retained inside the vesicular lumen in a parallel experiment. These results suggest a selective permeability of the tonoplast to the two isomers which could be involved, in vivo, in the trapping of the glucoside inside the vacuole and its irreversible accumulation.

Introduction

Higher plants frequently accumulate large amounts of natural substances such as alkaloids, terpenes and phenolics, some of which are of economic importance. Many of these products occur as glycoside [1–4] and most are stored in the vacuoles, as emphasized by cytochemical studies [5–7] and chemical analysis of isolated vacuoles [1,8]. However, although recent studies have dealt with vacuolar transport of substances of the primary metabolism [9–13], the transport processes which result in vacuolar accumulation of secondary compounds remain, as yet, largely unknown. In addition these compounds are retained inside the vacuole against a high cytoplasm/vacuole gradient concentration and do not diffuse from the isolated vacuoles into the isolation medium. Matile's group working on the lutoids of *Chelidonium majus* was the first to give evidence for the occurrence, in these organelles, of non-diffusible alkaloids complexed with tannins [14]. Since

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then, similar trapping mechanisms have been proposed to explain the intravacuolar retention of several other substances [15–18].

Melilotus alba (sweet clover) leaves contain large amounts of *o*-coumaric acid glucoside, The biosynthetic pathway leading to this compound was elucidated in the past decades by Conn's group [19–22]. Moreover, Haskins et al. [20] have demonstrated that, while *o*-coumaric acid glucoside is synthesized under the *trans* isomeric form, it accumulates mainly as the *cis* isomer. As we have recently shown that the *o*-coumaric acid glucoside is located exclusively in the vacuole [23], sweet clover seems to be a good model to study both the transport of the glucoside through the tonoplast and its retention in the vacuole.

Using isolated protoplasts, isolated vacuoles and reconstituted tonoplastic vesicles, we tried to identify the mechanisms involved in the accumulation process of *o*-coumaric acid glucoside.

Preliminary results of this work were presented at the International Workshop 'Plasmalemma and tonoplast in the plant cells' held in Strasbourg (France), September 8–11, 1981.

Materials and Methods

Plant material. Sweet clover plants were obtained as previously described [24]. All experiments were performed on leaves sampled during the first hour of the light phase.

Products. The following compounds were of commercial origin: ^3H_2O (10 mCi/ml) was from CEA (France); [^{14}C]carboxyl dextran (0.5 mCi/mg) was from New England Nuclear (USA); [^{14}C]phenylalanine (495 mCi/mmol), [7-^{14}C]benzylamine hydrochloride (58.7 mCi/mmol), [*pyrrolidine*-2-^{14}C]nicotine (51.7 mCi/mmol) and [^{3}H]inulin (1.78 Ci/mmol) were from Amersham International, U.K.

The chemical used for the preparation of protoplasts and vacuoles have been listed previously [24].

o-[^{14}C]Coumaric acid glucoside was chemically synthesized following a modification of the procedure described by Helferich and Lutzmann [25] 400 μCi [^{14}C]malonate (about 1 mg) were incubated for 10 h at 60°C with 100 μl pyridine, a drop of aniline and 2.3 mg helicin (Ega-Chemie, F.R.G.). Then 100 μl pyridine were added, and the incubation was continued for 15 h. The solution was mixed with 500 μl distilled water and extracted with 3×1 ml diethyl ether. The diethyl ether phase was discarded. Residual diethyl ether was eliminated under reduced presure and one drop of 1 M HCl was added. The synthesised glucoside was then extracted in 5×1 ml ethyl acetate, the solvent was evaporated to dryness and the *o*-[^{14}C]coumaric acid glucoside was purified by successive monodimensional thin-layer chromatography on a cellulose plate (Merck, F.R.G., 200 μm, prewashed with methanol) developed firstly with butyl acetate/acetic acid/water (4 : 1 : 5 (v/v), upper phase) and secondly with chloroform/acetic acid/water (2 : 1 : 1 (v/v), lower phase). The glucoside was detected under ultraviolet light and eluted from the plate with methanol. Generally the yield of *o*-[^{14}C]coumaric acid glucoside was about 25–30% from the starting malonate and the product was composed of 90–92% *trans* *o*-coumaric acid glucoside and 10–8% of the *cis* form.

The *cis* form of the *o*-[^{14}C]coumaric acid glucoside was obtained by treating the *trans* glucoside for 2 h under ultraviolet light (254 nm). It was further separated from the remaining *trans* form by thin-layer chromatography on cellulose plates (Merck) in 2% acetic acid (R_F of the *trans* form 0.70 and of the *cis* form, 0.93).

Preparation of protoplast, vacuole and tonoplast fractions. Protoplasts and vacuoles were prepared as described by Boudet et al. [24]

Vacuoles ($20 \cdot 10^6$) in 25 mM Tris-Mes buffer (pH 6.5)/0.7 M mannitol (medium B [24) were lysed by addition of 25 mM Tris-Mes buffer (pH 6.5)/β-mercaptoethanol (final concentrations of mannitol 0.3 M and of β-mercaptoethanol 5 mM). The suspension was then centrifuged successively for 1 min at $2900 \times g$ (Beckman TJ6R) (and the pellet discarded) and 1 h at $100\,000 \times g$ (Beckman L5-50). The $100\,000 \times g$ pellet (corresponding to the tonoplast fraction) was resuspended in cold deionized water, lyophylised and stored at −20°C until use.

Incorporation of radioactive precursors in protoplasts. A protoplast suspension in medium B was shaken (30 strokes/min) at 20°C with labelled phenylalanine. Experimental details are given in

the appropriate tables and figures. After a given time of incubation, an aliquot of the suspension was centrifuged for 1 min at $75 \times g$ (Beckman TJ6R centrifuge). The protoplasts were washed twice by resuspension in medium B and centrifugation before being used for analysis, or for the preparation of vacuoles.

Isolation of o-coumaric acid glucoside. Sample (protoplasts or vacuoles) were sonicated in ice (MSE sonicator) and adjusted to pH 2 with 1 M HCl. The suspensions were extracted five times with 1.5 vol. of diethyl ether and the diethyl ether extracts were discarded. The residue was extracted five times with 1.5 vol. ethyl acetate, and the organic phases were combined and evaporated to dryness. The residue was then taken up in a minimal volume of methanol and chromatographed on Whatman No. 1 paper in butanol/acetic acid/water (6 : 1 : 2 (v/v)) for 16 h. The chromatogram was dried and the *o*-coumaric acid glucoside was located either under ultraviolet light (254 and 366 nm) and/or with a radioscanner (2723 Berthold scanner I).

The zone corresponding to *o*-coumaric acid glucoside, was cut out and directly counted for radioactivity. In preliminary experiments, the purity of the radioactive *o*-coumaric acid glucoside, isolated after chromatography, was checked by further paper chromatography in the solvents ethanol/ammonia/water (80 : 5 : 15 (v/v)) and solvent B of Reio [26].

In some cases, the extracts were first submitted to descending paper chromatography on Whatman No. 1 paper using 2% acetic acid to separate the *trans*-glucoside from its *cis* isomer. Each isomer eluted from the paper was further purified by paper chromatography in butanol/acetic acid/water as above. All separations were performed in the dark or under green light to avoid light isomerisation of the glucoside [27]. Control experiments using synthetic *o*-[^{14}C]coumaric acid glucoside had shown that, in these conditions, no isomerisation occurred.

Uptake of o-coumaric acid glucoside by isolated vacuoles. All experiments were conducted at 25°C in 0.7 M mannitol/25 mM Tris-Hepes (pH 8) (vacuole isolation medium). 3 μCi of the glucoside (final concentration 7.6 mM) were added to 1 ml of the vacuolar suspension containing $(1.1–2) \cdot 10^6$ vacuoles. Uptake was stopped by gently adding 3 ml of cold (0°C) isolation medium. The suspension was layered onto a Ficoll gradient formed by 3 ml 10% Ficoll and 2 ml 20% Ficoll in the same medium. After 1 min centrifugation at $1900 \times g$ the vacuoles banded at the 20/10% Ficoll interface and were taken up, counted (usually 20–30% of the initial vacuoles were recovered) and the radioactivity determined. Controls for which the uptake was stopped immediately after the addition of the substrate were performed in order to take into account possible adsorption of the phenolic glucoside onto the membranes.

Preparation and loading of tonoplast vesicles with radioactive molecules. The radioactive molecules were dissolved at the appropriate concentration in 25 mM Tris-Mes buffer (pH 6.5)/0.3 M mannitol/5 mM β-mercaptoethanol (vesiculisation medium). 1 ml of this solution was added to the lyophylised membranes and the suspension was transferred to a Potter homogeniser fitted with a Teflon piston (A.H.T., U.S.A.). The membranes were homogenised by 20 strokes of the piston to obtain loaded vesicles.

Separation of the tonoplast vesicles from the radioactive molecules. 500 μl of the loaded tonoplast vesicles suspension were either used as such or diluted 2-fold with the vesiculisation medium. After 15 min, each suspension was put on a Sepharose 4B column (300 × 20 mm, Pharmacia) pre-equilibrated in 25 mM Tris-Mes buffer (pH 6.5)/0.3 M mannitol/5 mM β-mercaptoethanol. Elution of the vesicles was performed with the same medium and fractions of 1 ml were recovered. The radioactivity was determined for each fraction on 100 μl aliquots of the eluate.

Vacuolar pH determination. Vacuolar pH was estimated using radiochemical probes, essentially as described by Kurkdjian and Guern [28] and Kurkdjian [29]: $2 \cdot 10^6$ protoplasts were supplemented with 10 μl of the appropriate probe: [^{14}C]benzylamine or [^{14}C]nicotine. After an incubation time of 30 min, the suspension was layered onto a gradient of 2 ml 20% Ficoll and 2 ml 10% Ficoll in medium B. The gradients were centrifuged for 1 min at $2900 \times g$ (Beckman TJ6R) and the protoplasts which banded at the 10/20% interface were immediately taken up using a pasteur pipette. The number of protoplasts was

counted and their radioactivity was determined.

The vacuolar pH (pH_i) was estimated using the formula:

$$pH_i = pK_a - \log\left(C_i/C_e(1+10^{pK_a-pH_e})-1\right)$$

were pK_a is 9.3 for benzylamine and 8.02 for nicotine; pH_e is the external pH; C_i is the external concentration of the probe at equilibrium; C_i is the vacuolar concentration of the probe. The volume corresponding to $1 \cdot 10^6$ protoplasts was estimated to be 3.9 µl by measurement of the space accessible to ^3H_2O but inaccessible to [^{14}C]dextran [30]. Assuming the vacuole to occupy 80% of the protoplast volume, the volume of $1 \cdot 10^6$ vacuoles was 3.15 µl.

Enzyme measurements. (i) UDPG / o-coumaric acid glucoside transferase activity. This activity was determined as described by Poulton et al. [31].

In some experiments the contents of the vacuoles and protoplasts were compared on the basis of α-mannosidase, a vacuolar marker [32,33]. The α-mannosidase activity was determined using a modification of the method described by Boller and Kende [32]. The assay contained, in a total volume of 800 µl: 1.80 µmol succinate (adjusted to pH 5 with 10 M NaOH); 2.4 µmol β-mercaptoethanol; 0.2% bovine serum albumin and 0.8 µmol *p*-nitrophenyl-α-mannopyranoside. Aliquots (100 µl) were withdrawn at intervals (30–300 min) and added to 400 µl of 1 M Na_2CO_3 and 100 µl 0.1 M succinate buffer (pH 5). The mixture was centrifuged for 5 min (Gelman Microfuge) and the absorbance of the supernatant estimated at 405 nm against a blank containing all the reagents but no substrate.

(ii)trans / cis o-Coumaric acid glucoside isomerase activity.

Enzyme extraction. $100 \cdot 10^6$ protoplasts were sonicated in 10 ml 0.02 M Tris-Mes buffer (pH 7), 0.1% β-mercaptoethanol and 50 mg Polyclar and centrifuged successively for 15 min at $2700 \times g$ and 1 h at $100\,000 \times g$. The $100\,00 \times g$ pellet (P) was resuspended in 700 µl 0.02 M Tris-Mes buffer (pH 7), while the supernatant was treated with solid ammonium sulphate (80% saturation) and centrifuged for 20 min at $20\,000 \times g$. The protein precipitate was desalted on a G-25 Sephadex column using a 0.02 M Tris-Mes buffer (pH 7.5) as eluate. The final volume of the soluble fraction protein (S) was 2.5 ml.

Enzyme assay. The isomerase assay consisted of 100 µl either P or S fractions, 200 µl *o*-[^{14}C]coumaric acid glucoside (150 000 dpm, 97% in the *trans* form), 200 µl of 0.2 M Tris-Mes buffer at pH values between 5 and 8. The assays were incubated for different times from 0 to 6 h in the dark. After acidification to pH 2 by 3 M HCl, the glucoside was extracted with ethyl acetate and chromatographed in Whatman No. 1 paper in 2% acetic acid as solvent. The *trans* and the *cis* forms of *o*-coumaric acid glucoside were detected by radioscanning. The corresponding spots were cut out and counted for radioactivity. Controls, in which the enzyme extracts were boiled for 30 min, were run together with the assays.

Radioactivity measurements. Radioactivity determinations were performed in Ready Solve MP (Beckman) using a 460 C Packard spectrometer.

When the radioactivity was estimated on cut chromatograms, the pieces of paper were maintained in contact with the scintillation cocktail for 12 h before counting.

Results

Synthesis of o-coumaric acid glucoside from [^{14}C]phenylalanine in isolated protoplasts

Protoplasts were easily isolated from leaves of range two to six (counted from the apex) of *Melilotus alba*. These protoplasts were able to convert exogeneously supplied [^{14}C]phenylalanine to *o*-[^{14}C]coumaric acid glucoside and to sustain *o*-coumaric acid glucoside synthesis for several hours. However because of the possible heterogeneity of the protoplast population, we tested the ability of different isolated protoplast subpopulations to form *o*-coumaric acid glucoside. We showed that: (a) protoplasts isolated from leaves of different age synthesized *o*-coumaric acid glucoside with the same efficiency (results not shown); (b) although all the protoplasts, separated on their density basis, did not yield vacuoles, all of them synthesized similar amounts of *o*-coumaric acid glucoside (Table I). Therefore, the subpopulations of protoplasts which yield vacuoles are representative of the total population with regard to *o*-coumaric acid glucoside synthesis.

TABLE I

SYNTHESIS OF *o*-COUMARIC ACID GLUCOSIDE BY DIFFERENT PROTOPLAST SUBPOPULATIONS

Interfaces of the gradient [a] (% Ficoll)	Protoplast distribution [b] (%)	Vacuole yield [c] (%)	Glucoside synthesised (dpm/10^6 protoplasts) [d]
8–16	24	30	10820
16–20	27	17	10090
20–30	40	0	12250

[a] Protoplasts were first separated on their density basis by centrifugation on Ficoll gradient.

[b] Subpopulations corresponding to each Ficoll interface were collected and protoplasts were counted.

[c] Vacuoles were prepared from each protoplast subpopulation and the yield of vacuoles was estimated.

[d] 30 μCi [^{14}C]phenylalanine were fed, in medium B, to $60 \cdot 10^6$ protoplasts collected from each interface for the estimation of the *o*-coumaric acid glucoside synthesis capacity. Light conditions: 6 W/m^2 for 90 min.

Finally, when protoplasts were fed with [^{14}C]phenylalanine and then transferred to a medium without radioactive precursor or containing 0.3 M unlabelled phenylalanine (Fig. 1), the level of the radiaoctive glucoside remained constant indicating no significant *o*-coumaric acid glucoside degradation, for at least 5 h.

Kinetics of o-coumaric acid glucoside accumulation in vacuoles in situ

Vacuoles were obtained from protoplasts by a one-step centrifugation procedure on a Ficoll gradient as described by Boudet et al. [24]. During this centrifugation, the vacuoles are rapidly extruded from the protoplasts (within less than 1 min) and an additional 29 min centrifugation are required for the purification of the vacuolar fraction. Owing to this rapid separation of the vacuole from the extravacuolar part of the protoplast (which immediately stops all metabolic processes), the retained method appears to be highly suitable for kinetic studies.

After feeding radioactive phenylalanine to protoplasts, the appearance with time of *o*-[^{14}C]coumaric acid glucoside in protoplasts and in the corresponding vacuoles was studied. Distinct experiments were performed and Fig. 2 describes three of them. While absolute values were different from one experiment to another (depending on the biosynthetic capacities of the different batch of plants), the same general pattern of variation was

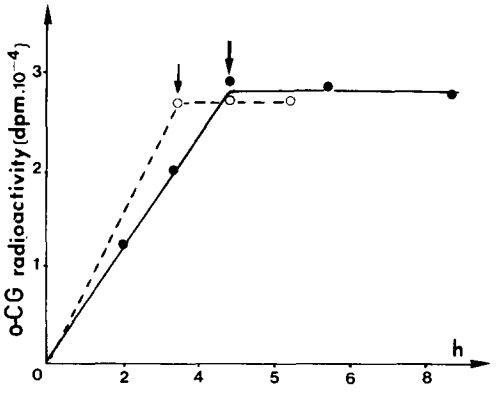

Fig. 1. Absence of *o*-coumaric acid glucoside (*o*-CG) turn over in isolated protoplasts. Experimental conditions: 100 μCi [^{14}C]phenylalanine; $1 \cdot 10^8$ protoplasts in 13.5 ml medium B (results are expressed for $1 \cdot 10^6$ protoplasts). O- - - -O, Protoplasts were transferred to a medium devoid of radioactivity at the time marked by the arrow. ●———●, Protoplasts were transferred to a medium without radioactive phenylalanine but containing 0.3 M unlabelled phenylalanine at the time marked by the bold arrow.

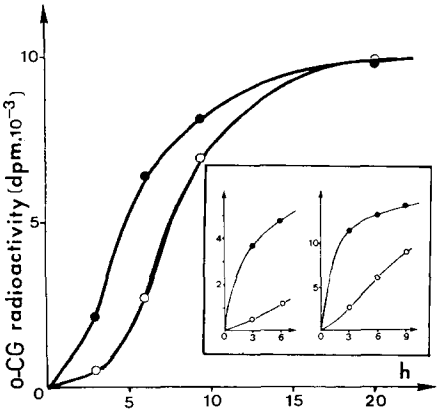

Fig. 2. *o*-Coumaric acid glucoside (*o*-CG) labelling from [^{14}C]phenylalanine in protoplasts and corresponding vacuoles against time. Experimental conditions: 30 μCi [^{14}C]phenylalanine; $64 \cdot 10^6$ protoplasts in 6 ml medium B; light intensity 6 W/m^2. Glucoside radioactivity per 10^6 protoplasts (●———●, 1) or per 10^6 vacuoles (O——— O, 2) (Results were expressed on an α-manosidase basis [28]. We have shown [11] that in *Melilotus* one protoplast contains only one vacuole.). Insets correspond to separate and independent experiments.

found in all cases: after short incubation times most of the newly synthesized radioactive glucoside was absent from the vacuoles, indicating an initial cytoplasmic location. The amount of radioactive *o*-coumaric acid glucoside in the vacuoles increased linearly over the time of the experiment, suggesting a slow but steady transfer from the cytoplasm to the vacuole. After 20 h, synthesis of *o*-coumaric acid glucoside had stooped and all the radioactive *o*-coumaric acid glucoside was found in the vacuole.

In addition, we determined the percentages of *trans* and *cis* radioactive *o*-coumaric acid glucoside. After 2 h of incubation, when *o*-coumaric acid glucoside was mostly extravacuolar, 80% of the compound was under the *trans* form; after 20 h incubation, when the *o*-coumaric acid glucoside was mostly vacuolar, only 25% of the compound was still in the *trans* form, the remainder being under the *cis* isomeric form (75%). It was concluded that isomerisation occurs during accumulation of *o*-coumaric acid glucoside in the vacuolar compartment.

Uptake of o-[^{14}C]coumaric acid glucoside by isolated vacuoles.

When [^{14}C]glucoside was added to a vacuole suspension at a final concentration of 7.6 mM, a slow (1.1 nmol $\cdot$ h^{-1} $\cdot$ 10^{-6} vacuoles) but significant uptake of the phenolic compound was noticed (results not shown), and the rate of incorporation remained constant for at least 3 h. Since the vacuolar concentration of glucoside was about 73 mM (230 nmol in an approximate volume of 3.15 μl), we can assume that the uptake occurred against a concentration gradient. When added to the medium, MgATP did not significantly change the rate of glucoside uptake.

Vacuolar / extravacuolar location of UDPG / o-coumaric acid glucosyl transferase

The results in Table II show that *o*-coumaric acid glucosyl transferase activity is almost entirely absent from the vacuolar fraction. The very slight activity in the vacuoles can be ascribed to the contamination of the preparation by residual protoplasts [24].

TABLE II

VACUOLAR/EXTRAVACUOLAR DISTRIBUTION OF UDPG/*o*-COUMARIC ACID GLUCOSYL TRANSFERASE

Expt. No.	*o*-Coumaric acid glucoside synthesised (nmol $\cdot$ min^{-1})	
	per 10^6 protoplasts	per 10^6 vacuoles [a]
1	2.4	0.02
2	2.45	0.06

[a] Expressed on an α-mannosidase basis.

Effects of light and ΔpH on the synthesis and the transport of o-coumaric acid glucoside into vacuoles, in isolated protoplasts

A protoplast suspension in medium B was incubated in the presence of [^{14}C]phenylalanine in the light. After 3 h, part of the suspension was transferred to the dark (all other conditions remaining the same) while two other portions were kept in the light with or without 5 μM DCMU. After an additional incubation time of 5 h, the protoplasts were harvested and vacuoles prepared. Fig. 3 shows the radioactivity of *o*-coumaric acid glucoside extracted from protoplasts and from the

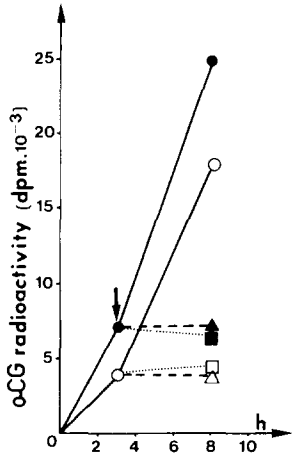

Fig. 3. Influence of light on the synthesis and the transport through the tonoplast of *o*-coumaric acid glucoside (*o*-CG). Experimental conditions: 60 μCi [^{14}C]phenylalanine; 1 $\cdot$ 10^8 protoplasts in 9 ml medium B; light intensity: 22 W/m^2. Radioactivity in *o*-coumaric acid glucoside per 10^6 protoplasts (closed symbols) and per 10^6 vacuoles (on an α-mannosidase basis) (open symbols). ●, ○, under light; ▲, △, in dark; ■, □, under light + 5 μM DCMU.

corresponding vacuoles with time.

Under light conditions, *o*-coumaric acid glucoside was actively synthesized from exogenous [^{14}C]phenylalanine, part of the newly-synthesized compound was transferred into the vacuolar compartment as described above. However, dark conditions or DCMU treatment inhibited both the synthesis of *o*-coumaric acid glucoside and its transport into the vacuole (similar results were obtained in a parallel experiment, although absolute values were different). These results indicate that synthesis and transfer of *o*-coumaric acid glucoside into the vacuole could be related to photosynthesis.

Using the probe [^{14}C]benzylamine, the vacuolar pH changes were determined for protoplasts successively maintained in the dark, transferred to light and then returned to the dark (Fig. 4).

Light caused a marked decrease of the vacuolar pH by about 0.4 pH units after 2 h illumination. This decrease was completely reversed under dark conditions, and the initial pH value was reached after 1 h of darkness. In addition, the light-induced accumulation of protons in the vacuoles was completely prevented by DCMU. Thus, light-induced acidification of vacuoles seems to be related to photosynthesis.

Kurkdjian and Guern [28] have shown that addition of benzylamine to cell suspension cultures induces a rise in the vacuolar pH. A similar experiment was designed, using sweet clover protoplasts, in order to test the influence of the vacuolar pH on *o*-coumaric acid glucoside transport into the vacuole: protoplasts were incubated in medium B containing [^{14}C]phenylalanine for 3.5 h and then transferred to the same medium (but without substrate to stop *o*-[^{14}C]coumaric acid glucoside synthesis), in the presence or in the absence of 5 mM benzylamine (Fig. 5). Such a treatment both increased the vacuolar pH (+0.4 pH unit) and reduced the transport of *o*-[^{14}C]coumaric acid glucoside into the vacuole.

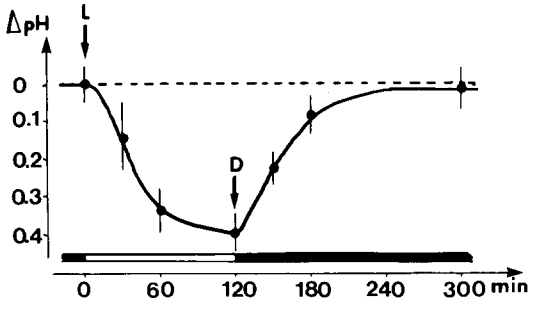

Fig. 4. Vacuolar pH of protoplasts in different experimental conditions. ●———●, in successive dark (D), light (L), dark sequence. - - - - -, in continuous light but in the presence of 5 μM DCMU. Light intensity was 22 W/m^2. Vacuolar pH at time zero 5.47 ± 0.06. Experiments were done in duplicate.

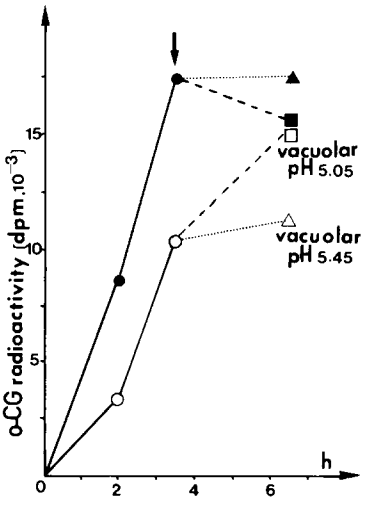

Fig. 5. Influence of artificial alkalinisation of the vacuolar pH on the transfer of *o*-coumaric acid glucoside (*o*-CG) into the organelle. Experimental conditions: 80 μCi [^{14}C]phenylalanine; 12·10^7 protoplasts in 15 ml medium B; light intensity 6 W/m^2. At the time marked by the arrow protoplasts were transferred to a medium containing 0 mM (- - - - - -) or 5 mM benzylamine (· · · · · ·). Vacuolar pH was determined in parallel experiments (but without [^{14}C]phenylalanine), using [^{14}C]nicotine (300000 dpm per assay) as a probe. Radioactivity in *o*-coumaric acid glucoside per 10^6 protoplasts (closed symbols) or per 10^6 vacuoles (expressed on an α-mannosidase basis) (open symbols).

trans / cis isomerisation of o-coumaric acid glucoside in light and dark conditions

Protoplasts were incubated in the presence of [^{14}C]phenylalanine for 4 h (Table III). The *trans* and the *cis* isomer then represented 60 and 40% of the synthesized *o*-coumaric acid glucoside, respectively. After longer incubation (10.5 h) in continuous light synthesis and vacuolar transport of *o*-[^{14}C]coumaric acid glucoside continued as described in the previous experiment (Fig. 3) and no significant changes affected the relative propor-

TABLE III

IN VIVO ISOMERISATION OF *o*-COUMARIC ACID GLUCOSIDE (*o*-CG) IN THE LIGHT AND IN THE DARK

$1 \cdot 10^8$ protoplasts were incubated in the presence of 60 μCi of [^{14}C]phenylalanine. Light intensity was 22 W/m^2.

Incubation time	4 h in the light	4 h in the light + 6.5 h in the light	4 h in the light + 6.5 h in the dark
Total *o*-CG synthesized (dpm)	9900	18900	9380
Recovery in the vacuoles (dpm) [a]	5433	11605	4670
% vacuolar	55	61	50
% *trans* isomer in the vacuoles	60	57	28
% *cis* isomer in the vacuoles	40	43	72

[a] Results expressed on an α-mannosidase basis.

tions of the two isomers. In contrast, if protoplasts were transferred to the dark after 4 h of illumination, the synthesis and transport of *o*-coumaric acid glucoside ceased and a drastic *trans* to *cis* isomerisation occurred inside the vacuole. These results show that (i) *trans* to *cis* isomerisation of *o*-coumaric acid glucoside is not light-dependent and (ii) *trans* to *cis* isomerisation of *o*-coumaric acid glucoside occurs independently of the transport of the compound inside the vacuole.

Selective permeability of the tonoplast to the trans and cis isomers of o-coumaric acid glucoside

Tonoplast vesicles were prepared from isolated vacuoles and loaded with different radioactive molecules. When the vesicles were loaded with [^{3}H]inulin and/or [^{14}C]dextran (two molecules which can not permeate biological membranes), the radioactivity trapped inside the vesicles could be easily separated from the free radioactive molecules by gel filtration on Sepharose 4B. In the eluate the first peak of radioactivity (Fig. 6) corresponds to the polymers trapped in the intravesicular space and the second peak corresponds to the untrapped molecules. The fact that both inulin and dextran are eluted in the same way reduces the probability of artifacts caused by impurities of high molecular weight in the commercial radioactive polymers or caused by the adsorption of the markers on membrane fragments.

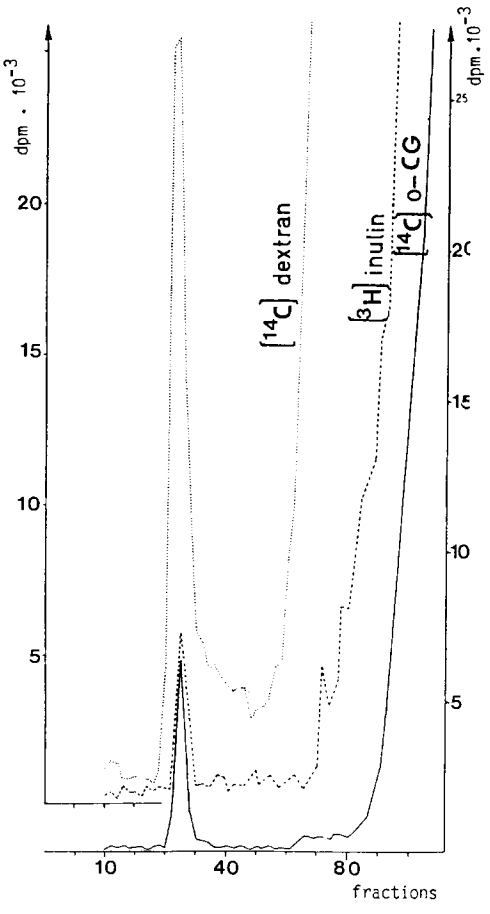

Fig. 6. Elution profile of reconstituted vesicles loaded with different radioactive molecules, on a Sepharose 4B column. [^{14}C]*o*-CG, *o*-[^{14}C]coumaric acid glucoside.

When vesicles were loaded with *o*-[^{14}C]coumaric acid glucoside radioactivity appeared in the eluate at the same position as did [^{3}H]inulin and [^{14}C]-dextran. Furthermore, the simultaneous loading of tonoplast vesicles with both *o*-[^{14}C]coumaric acid glucoside and [^{3}H]inulin gave rise to only one peak in the high molecular weight range; as for inulin and dextran, free *o*-coumaric acid glucoside was eluted later from the column.

In further experiments, vesicles were loaded with both [^{3}H]inulin and either the *trans* or the *cis* form of *o*-[^{14}C]coumaric acid glucoside. Comparison of the ^{14}C/^{3}H ratio in the initial suspension and in the collected fractions corresponding to the vesicles, after the Sepharose sieving, is described in Table IV. For the *cis* isomer, the ratio is essen-

TABLE IV

PERMEABILITY OF TONOPLAST VESICLES TO *trans* AND *cis* ISOMERS OF *o*-COUMARIC ACID GLUCOSIDE (*o*-CG)

Freeze-dried tonoplast corresponding to $2 \cdot 10^7$ vacuoles was homogenised in 25 mM Tris-Mes buffer (pH 6.5), 0.3 M mannitol, 5 mM β-mercaptoethanol containing about 5 μCi of *o*-[^{14}C]coumaric acid glucoside (*trans* or *cis* isomer) and 5 μCi of [^{3}H]inulin. In Expt. 1 vesicles were directly passed through sepharose. In Expt. 2 vesicles were first diluted 2-fold with the vesiculisation medium before sieving on sepharose.

[^{14}C] Isomer	$^{14}C/^3H$		*o*-CG remaining in the vesicles (in %)
	In the initial suspension	In vesicles after molecular sieving	
Expt. 1			
trans	1.12	0.66 ± 0.06	58
cis	1.06	1.06 ± 0.09	100
Expt. 2			
trans	0.99	0.37 ± 0.01	37
cis	1.49	1.62 ± 0.04	108

tially the same before and after molecular sieving; in contrast, the ratio decreases dramatically in isolated vesicles loaded with the *trans* isomer. The decrease is even more pronounced if the initial suspension is firstly diluted by the vesiculisation medium before sieving. This indicates that after dilution and during the migration of the vesicles through the column, the *cis* *o*-coumaric acid glucoside is entirely retained inside the vesicles while a large part of the *trans* *o*-[^{14}C]coumaric acid glucoside is lost, probably because of its efflux through the vesicular membrane.

Discussion

As recently emphasised by Gallun [34] and by Fowke and Gamborg [35], isolated protoplasts are useful tools for metabolic studies in plants. However, a number of authors have demonstrated that the metabolism of isolated protoplasts is often disturbed or even changed when compared to the original cells. For example, uptake [36] excretion [37,38] and synthesis of proteins and nucleic acids [39,40], are strongly modified in protoplast suspensions. Therefore, caution is in order when using this simplified model. For this reason we first examined the ability of *Melilotus alba* protoplasts to synthesize *o*-coumaric acid glucoside.

It was shown that sweet clover protoplasts, like intact leaves [41,42], were able to form *o*-coumaric acid glucoside from phenylalanine supplied in the incubation medium.

It should be noted that protoplasts of a given population are physiologically heterogeneous. For example phenolic metabolism (like other secondary metabolisms) is not expressed in a similar manner in all cells (see for example Ref. 43). So it was important to determine whether the protoplast population is homogeneous with respect to *o*-coumaric acid glucoside metabolism. The obtained results show that this is indeed the case. Moreover, in our experimental conditions, no significant degradation of *o*-coumaric acid glucoside occurred in isolated protoplasts, as demonstrated also by Kosuge and Conn [44] in intact plants. For these reasons, we considered that isolation of protoplasts does not seriously modify *o*-coumaric acid glucoside metabolism.

We then studied the intracellular transport of *o*-coumaric acid glucoside in the isolated protoplasts. The results of Conn's group [22,31], have shown that at least two steps of the biosynthetic pathway are extravacuolar: synthesis and hydroxylation of cinnamic acid. Nevertheless, recent work of Oba et al. [23] has shown that *o*-coumaric acid glucoside is almost completely vacuolar. the question arises as to what molecular species crosses the tonoplast: (i) *o*-Coumaric acid synthesized in plastids might be glucosylated either at the tonoplast or in the vacuolar sap; (ii) *o*-Coumaric acid glucoside might be transported into the vacuole after complete synthesis in the cytoplasmic spaces of the cells.

In accordance with other authors [45,46] we found the UDPG/*o*-coumaric acid glucoside transferase activity to be absent from isolated vacules. Although this seems to exclude the first hypothesis, the possibility remains that the transferase dissociates from the tonoplast during isolation of the vacuoles. However, the kinetics of the glucoside appearance in the vacuole unambiguously demonstrate that the compartment of synthesis (extravacuolar) is distinct from the storage compartment (vacuole). Thus *o*-coumaric acid glucoside is the molecular species carried through the tonoplast.

Moreover, while *o*-coumaric acid glucoside is synthesized in the cytoplasm in the form of the *trans* isomer it accumulates mainly in the *cis* form in the vacuole (similar results were obtained in intact leaves [44]). Thus after the complete synthesis of *o*-coumaric acid glucoside, an isomerisation step occurs which could be related to its vacuolar transport or accumulation.

We tried to determine what energisation processes are involved in the transport of *o*-coumaric acid glucoside into the vacuole. A possible explanation could be developed considering the results discussed above. Indeed, a continuous *trans / cis* isomerisation of *o*-coumaric acid glucoside inside the vacuole could induce a passive flux from the extravacuolar compartment (with a high *trans o*-coumaric acid glucoside content) to the vacuolar sap (with a low *trans o*-coumaric acid glucoside content). However, the results in Table IV are not in accordance with such a possibility because of the lack of constant correlations between transport and *trans* to *cis* isomerisation of *o*-coumaric acid glucoside.

Other experiments have shown that:

(1) synthesis and transport of *o*-coumaric acid glucoside is related to photosynthesis,

(2) photosynthesis induces acidification of the vacuolar sap,

(3) vacuolar transport of *o*-coumaric acid glucoside is dependent on the vacuolar pH value (a rise in the vacuolar pH inhibits the transport).

Thus we may assume that the transport of *o*-coumaric acid glucoside into the vacuole would be drive by the ΔpH between the cytoplasm and the vacuole (vacuole interior more acidic) which in turn would be related to photosynthesis.

The relationship between photosynthesis and the vacuolar acidification could be explained by one the following alternatives:

(1) photosynthesis may lead to the extrusion of protons out of the chloroplasts [47], and the protons may then be immediately transferred into the vacuole (as demonstrated in some algae by Davis [48] and Guyenes et al. [48])

(2) photosynthesis may be necessary for the fueling of a proton-translocating ATPase located at the tonoplast.

In both cases, an electrochemical proton gradient across the tonoplast is established which may provide energy for the transport of *o*-coumaric acid glucoside into the vacuole in vivo. A similar model has been proposed for malate accumulation in vacuoles of CAM plants [50,51] and for alkaloid transport into vacuoles in *Catharnathus roseus* cells [52].

If we now consider the case of isolated vacuoles, it has been shown in CAM plants that malate transport occurs in the absence of an energy source and proceeds even in the presence of uncouplers which dissipate the electrochemical proton gradient [13]. Similarly, we found uptake of *o*-[^{14}C]coumaric acid glucoside by isolated *Melilotus* vacuoles in the absence of an identifiable energy source. The transport of malate and of *o*-coumaric acid glucoside into vacuoles isolated from *Bryophyllum* and *Melilotus* respectively may represent an exchange of the ^{14}C-labelled substrate in the incubation medium against the preexisting unlabelled malate or *o*-coumaric acid glucoside in the vacuolar sap. A similar process has been unambiguously demonstrated for arginine transport in isolated yeast vacuoles [53]. Thus, isolated vacuoles do not appear to be well suited to the study of metabolite transport. Therefore, studies of metabolite transport in intact protoplasts, followed by the rapid isolation of vacuoles, as described in the present publication, are more appropriate.

As configurational changes of phenolic compounds may be involved in the trapping of molecules inside the vacuolar sap [54], we studied such a possibility in the case of *o*-coumaric acid glucoside which has been demonstrated to undergo *trans* to *cis* isomerisation.

When tonoplastic vesicles were loaded with either the *trans* or the *cis* isomer of *o*-coumaric acid glucoside, a marked difference appeared as far as the permeability of the tonoplast to these compounds is concerned. *trans-o*-Coumaric acid glucoside flows out easily form the vesicles when the external concentration is lowered while, under the same conditions, *cis-o*-coumaric acid glucoside is firmly retained inside the vesicles.

The lack of tonoplastic markers does not enable us to determine whether the vesicles are inside out or not but we can logically consider that a mixed-population is obtained during vesiculisation. This is of minor importance for interpreting the results

because *cis-o*-coumaric acid glucoside is completely trapped inside the vesicles indicating a total impermeability of the tonoplast to this isomer, whatever the direction of movement of the molecule across the membrane.

We have demonstrated that *o*-coumaric acid glucoside accumulates in the vacuole of protoplasts mainly under the *cis* form, as it does in intact leaves [27]. So the selective permeability of the tonoplast toward the two glucoside isomers may provide the vacuole of sweet clover with an interesting trapping mechanism, different to those identified in other plants [15–18].

Haskins et al. [27] and Edwards and Stocker [55] have demonstrated the involvement of ultraviolet light in *trans/cis* isomerisation of *o*-coumaric acid glucoside. Careful examination of the results in Table IV suggest the involvement of other mechanisms, since *trans* to *cis* conversion does occur under conditions of darkness. However, like these authors, we were unable to demonstrate an enzymatic (isomerase) conversion of *trans* to *cis-o*-coumaric acid glucoside. A proton-dependent isomerisation suggested by Matern et al. [54] in the case of flavonoids does not seem to occur, since no changes in the *trans/cis* ratio of chemically synthesized *trans o*-coumaric acid glucoside was noticed on incubation for 12 h in buffered medium ranging from pH 5 to pH 8. Thus, with *o*-coumaric acid glucoside, this mechanism does not seem to be involved.

The overall results described in this publication emphasise the relationships occurring between external conditions (light/dark) and the transport of *o*-coumaric acid glucoside which is representative of naturally stored glucoside in plants. In addition, a new hypothesis for explaining the vacuolar retention of secondary compounds is presented. Work is now in progress to obtain a better understanding of the coupling between plastids and vacuoles, as far as vacuolar sap acidification is concerned, and to clarify the processes leading to *trans/cis* isomerisation of *o*-coumaric acid glucoside in *Melilotus*.

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