

Transport of Stachyose and Sucrose by Vacuoles of Japanese Artichoke (*Stachys sieboldii*) Tubers¹

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ABSTRACT

Vacuoles are the stores for large amounts of stachyose [α gal (1,6) α glc (1,2) β fru] in tubers of Japanese artichoke (*Stachys sieboldii*). The uptake of stachyose by these vacuoles was examined and compared with that of sucrose. The uptake mechanisms of both sugars were quite similar. The kinetics showed a single saturable response to increasing external concentrations of ¹⁴C-sugars with similar apparent K_m values of about 50 and 30 millimolar for stachyose and sucrose, respectively. The uptake rates, however, were always higher for stachyose than for sucrose. Stachyose and sucrose uptake was inhibited by fructose and raffinose, and, reciprocally, by sucrose and stachyose, but not by glucose or galactose. The main structural feature common to all sugars recognized by the uptake systems seems to be a terminal fructosyl residue. The uptake of both sugars was stimulated by Mg-ATP and inorganic pyrophosphate, suggesting a proton-sugar antiport system. The possibility that stachyose and sucrose might be transported by the same carrier is discussed.

Japanese artichoke (*Stachys sieboldii*), a member of the Lamiaceae, is a perennial that overwinters with storage carbohydrates in small elongated tubers. The main storage carbohydrate of these tubers is the tetrasaccharide stachyose, a sucrosyl galactan of the raffinose family [α gal (1,6) α gal (1,6) α glc (1,2) β fru]. Large amounts of stachyose are stored in the vacuoles of the tuber parenchyma during the resting period (up to 80% of the dry weight, about 180 mM) and are remobilized during sprouting (7).

The biochemical processes leading to stachyose accumulation in parenchyma vacuoles of *Stachys* tubers are currently not understood. Obviously, they must be complex as they involve a series of events such as stachyose synthesis in the leaves, loading, translocation and unloading in the phloem, and finally accumulation in the parenchyma. The aim of this study was to investigate the last step of this series of events, the passage of stachyose across the tonoplast. As compartmentation studies had indicated a steep concentration gradient of stachyose from cytosol to vacuole in cells of *Stachys* tuber parenchyma (7), stachyose transport was anticipated to proceed actively in a proton-antiport fashion, maybe similar to the sucrose/H⁺-antiport systems found in vacuoles of other storage tissues such as redbeet (5, 13) and sugarbeet (1). This paper presents evidence for such a carrier-mediated stachyose/

H⁺-antiport system in vacuoles of *Stachys* tuber parenchyma. Apart from its particular importance in sugar accumulation in *Stachys* tubers, this finding is somewhat unique in more general terms as it contradicts the common assumption that biological membranes are impermeable for sugars larger than disaccharides. Although stachyose uptake into whole plant tissue has been described before (leaf discs of *Coleus blumei*) (8, 12), the present report is, to my knowledge, the first direct demonstration of a stachyose carrier of a plant membrane.

MATERIALS AND METHODS

Plant Material

Tubers of Japanese artichoke (*Stachys sieboldii* Miq.) were imported from Peveragno (Cuneo, Italy) in November and were stored in moist sand in the dark at 5°C for up to 4 months.

Isolation of Protoplasts and Vacuoles

Protoplasts and vacuoles from the storage parenchyma of resting tubers were prepared essentially as described previously (7). Briefly, the chopped and washed tissue was incubated with 1% Cellulase Y-C, 1% Driselase, and 0.1% Pectolyase Y-23 (all w/v) in 0.8 M glycinebetaine, 25 mM Mes-Tris, pH 5.5, 0.2 mM PEG-4000, 1 mM CaCl₂, 1 mM DTT, 0.002% rifampicin, 0.1% BSA (both w/v) in a Petri dish lined with a layer of 4% agar, 4% gelatin, and 1% activated charcoal (all w/v). After 16 h at 27°C with gentle rotary shaking, the protoplasts were filtered through a double layer of cheesecloth and washed twice with a medium containing 0.8 M glycinebetaine, 5 mM Mes-Tris, pH 5.5, 1 mM CaCl₂, 1 mM DTT, and 0.1% BSA by sedimentation at 1g.

The purified protoplasts were lysed in a 10-fold volume of 0.5 M glycinebetaine, 50 mM Tricine (KOH), pH 8.0, 5 mM EGTA, 1 mM DTT, and 0.1% BSA. The released vacuoles were purified by filtering through one layer of cheesecloth and washing with the transport medium containing 0.7 M glycinebetaine, 25 mM Hepes (KOH), pH 7.6, 50 mM KCl, 0.1% BSA, and 1 mM each of CaCl₂, MgCl₂, DTT, and EGTA. After sedimentation at 1g, the supernatant was withdrawn and the vacuoles were washed three times with transport medium.

Sugar Uptake

Sugar uptake was performed at 23 ± 2°C in a reaction mixture composed of transport medium supplemented with

¹ This work was supported by the Swiss National Foundation.

0.8 $\mu\text{Ci}/\text{mL}$ of $\text{U-}^{14}\text{C}$ -sugars at the desired concentrations, 0.4 $\mu\text{Ci}/\text{mL}$ [^3H] H_2O , and chemicals as indicated in the text. At selected times, vacuoles were separated from the incubation medium by silicone oil centrifugation essentially as described (10). Briefly, 100 μL samples were pipetted into 400 μL polypropylene microtubes that contained (from bottom to top) 50 μL 50% Percoll in 0.6 M sorbitol and 200 μL silicone oil (Wacker AR 20:AR 200 = 1:3, v/v). After centrifugation in a Beckman Microfuge E at 14,000g for 15 s, the microtubes were snapfrozen in liquid N_2 . The tips containing the vacuoles were excised and the radioactivity was counted in 3 mL of Kontrogel (Kontron, Zürich, Switzerland) in a Beckman LS 7800 scintillation spectrometer. The radioactivity of [^3H] H_2O in the tips served for indirect quantification of the recovered vacuoles (4) and the uptake rates, therefore, are expressed on a per microliter vacuole basis. In control experiments, a density of about 5 to 7 $\times 10^3$ vacuoles/ μL was determined. The data are the medians of six replicates. The confidence intervals (95%) were consistent at about $\pm 10\%$ of the medians. The experiments were repeated at least twice.

Preparation of ^{14}C -Labeled Stachyose

The youngest fully expanded leaves (2–3 g fresh weight/leaf) of pumpkin (*Cucurbita maxima* cv gelber Zentner) were photosynthetically labeled with $^{14}\text{CO}_2$ (25 $\mu\text{Ci}/\text{g}$ fresh weight) in large jam jars (750 mL) for 3 h. The leaves were ground in liquid N_2 and extracted twice with 30% methanol in an ultrasonic bath. The combined extracts were rotary evaporated, taken up in H_2O (1.5 mL/g fresh weight), and desalted. The neutral fractions of extracts of five leaves were separated on a Fractogel TSK HW-40(S) column (26 \times 1000 mm, Merck) eluted with H_2O (0.6 mL/min), and monitored with a preparative refractive index detector (Knauer, Lausanne, Switzerland). The [^{14}C]stachyose-containing fractions were pooled, and purity and specific activity was determined by radio-HPLC. The HPLC separation took place on a Dionex PA1 column using 150 mM NaOH (1 mL/min) as the eluant, and an EG & G model 400 pulsed amperometric detector (Princeton Applied Research, Princeton, NJ) and a Radiomatic Flo-One radiodetector (Canberra Packard, Zürich, Switzerland) were used as monitors. Routinely, a specific activity of [^{14}C]stachyose of about 1 mCi/mmol was obtained.

RESULTS AND DISCUSSION

Time Course and Product Analysis

Yield and purity of the isolated vacuoles were similar to those of our earlier report (7). We routinely obtained a yield of vacuoles of about 25 to 30% of the original protoplasts used for lysis. Cytoplasmic contamination of the vacuolar preparations as determined by malate dehydrogenase (7) was about 10%. The removal of external stachyose in the vacuolar preparations needed special attention. Only after three washings of the isolated vacuoles had the stachyose concentration in the uptake medium reached the lowest possible steady-state level of about 0.2 mM at the dilution used in the standard assay. Because the specific activity of our [^{14}C]stachyose preparations was relatively low (1 mCi/mmol), the lowest possible external stachyose concentration in an assay was 1 mM.

Sugar uptake into vacuoles from 1.5 mM external solutions was linear with time for about 12 min for stachyose and at least 15 min for sucrose (Fig. 1). The leveling off in the uptake rate of stachyose after 12 min most probably reflects an increase of the external concentration due to burst vacuoles. We repeatedly observed that vacuoles started to lyse after about 12 min of incubation, increasingly so toward the end of dormancy of the tubers. Because the endogenous concentration of stachyose in the vacuoles is very high (about 180 mM) as compared with that of sucrose (about 4 mM), it is obvious that lysis of vacuoles would primarily affect the uptake of stachyose. Figure 1 shows that the initial uptake rates of stachyose and sucrose are of the same order of magnitude. In 10 separate experiments, throughout the whole resting period of the tubers the uptake rates of stachyose were always higher (by a factor of 1.3–3) than those of sucrose, despite considerable day-to-day variability of uptake rates (data not shown). Differences of sugar uptake rates as a function of distinct developmental stages (importing *versus* resting *versus* exporting), however, is not ruled out but has not been studied.

The relatively inert sugar alcohol mannitol was not taken up by the vacuoles during the first 15 min of incubation (Fig. 1), indicating that the uptake of stachyose and sucrose was not by simple diffusion.

The positive y axis intercept represents the amount of residual medium cosedimenting with the vacuoles through the silicone oil. Correction for this carryover was not needed as uptake rates were derived from the slope of the curves.

Analysis of the neutral fractions of the transport medium and the vacuoles after 12 min incubations by radio-HPLC (3) revealed the sole presence of the original substrates ([^{14}C]stachyose and -sucrose, respectively), indicating that sugar uptake had taken place without chemical conversion.

Concentration Dependence

Figures 2 and 3 show the concentration dependence of stachyose and sucrose uptake into vacuoles, respectively. The

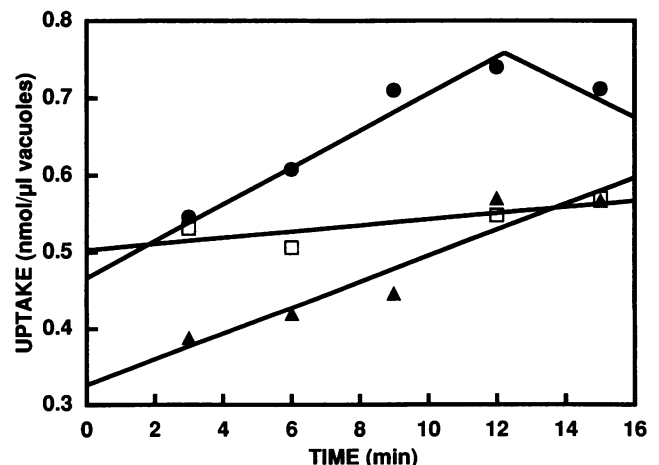


Figure 1. Time course of uptake of [^{14}C]stachyose (\bullet), -sucrose (\blacktriangle), and -mannitol (\square) by vacuoles isolated from Japanese artichoke tubers. Incubations were performed in the transport medium supplemented with 1.5 mM external carbohydrates.

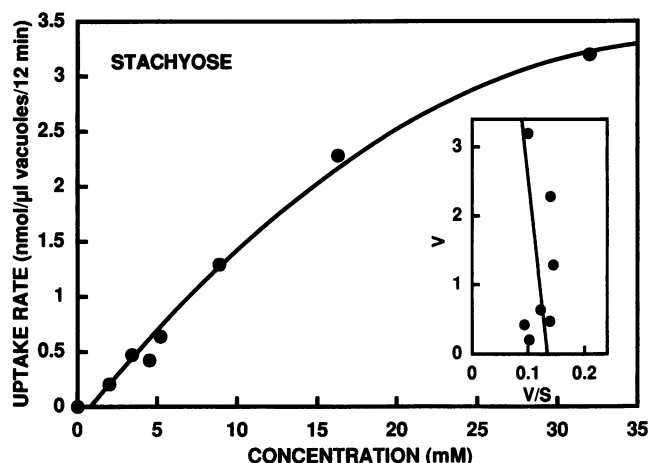


Figure 2. Concentration dependence of the uptake of [^{14}C]stachyose by vacuoles isolated from Japanese artichoke tubers. Incubations were performed for 12 min in the transport medium supplemented with the desired concentrations of the external sugars.

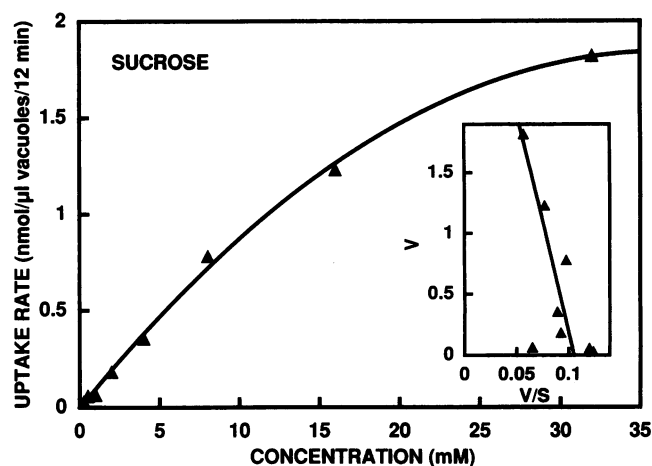


Figure 3. Concentration dependence of the uptake of [^{14}C]sucrose by vacuoles isolated from Japanese artichoke tubers. Incubations were performed for 12 min in the transport medium supplemented with the desired concentrations of the external sugars.

uptake of both sugars seems to display Michaelis-Menten-type saturation kinetics for the concentration range tested (1.5–32 mM and 0.25–32 mM for stachyose and sucrose, respectively), suggesting carrier-mediated transport. Eadie-Hofstee plots confirm the monophasic nature of sugar uptake (Figs. 2 and 3, insets). Lineweaver-Burk plots revealed apparent K_m values of 53 and 32 mM and V_{max} values of 45 and 19 nmol/ μL vacuoles/h for the uptake of stachyose and sucrose, respectively. These relatively high K_m values are not unusual for vacuolar carriers of storage sugars such as sucrose in redbeet taproot (22 mM) (13) and barley mesophyll (20–30 mM) (6, 9). Simple saturation kinetics for vacuolar sugar transport has been reported before, e.g. for sucrose in sugarbeet taproot (sucrose/ H^+ -antiport) (1) and barley mesophyll (facilitated diffusion) (9). Biphasic uptake kinetics with a linear concentration range above 32 mM cannot be ruled out. However, it was not tested, as such high sugar concentrations

cause the density of the transport medium to rise above that of the silicone oil, making a filtration of the vacuoles by the standard procedure impossible.

Inhibition

To further substantiate the carrier nature of sugar uptake by *Stachys* vacuoles, inhibition studies with putative competitors were performed by measuring ^{14}C -sugar uptake at 1.5 mM external concentrations in the presence of competitors at 10 mM external concentrations. These relatively low concentrations were chosen to circumvent the technical problems inherent in using too high sugar concentrations in the transport medium (see above). Table I shows that stachyose uptake was completely inhibited by fructose and sucrose and strongly inhibited by raffinose (69% inhibition). Sucrose uptake was completely inhibited by fructose and strongly inhibited by

Table I. Effect of Various Solutes on the Uptake of [^{14}C]Stachyose and [^{14}C]Sucrose by Vacuoles Isolated from Japanese Artichoke Tubers

Incubations were performed for 12 (stachyose) and 15 (sucrose) min in the transport medium supplemented with 1.5 mM external sugars. Values are medians \pm confidence intervals (95%) of six replicates. n.d., Not determined.

Treatment	Concentration mM	Uptake Rate			
		Stachyose		Sucrose	
		nmol/ μL vacuoles/12 min	%	nmol/ μL vacuoles/15 min	%
Control		0.140 \pm 0.024	100	0.113 \pm 0.012	100
Mg-ATP	5	0.266 \pm 0.037	190	0.175 \pm 0.029	155
PPi	2	0.231 \pm 0.023	165	0.139 \pm 0.007	123
PCMBs	2	0.123 \pm 0.014	88	0.108 \pm 0.011	96
D-Glucose	10	0.122 \pm 0.016	87	0.139 \pm 0.009	123
D-Galactose	10	0.151 \pm 0.012	108	0.081 \pm 0.012	72
D-Fructose	10	0.011 \pm 0.001	8	0	0
Sucrose	10	0	0	n.d.	
Raffinose	10	0.043 \pm 0.006	31	0.038 \pm 0.008	34
Stachyose	10	n.d.		0.041 \pm 0.011	36

raffinose and stachyose (66 and 64% inhibition, respectively). No inhibition was observed by the two aldohexoses tested, glucose and galactose. These results indicate that the specificity of the stachyose and sucrose carriers are quite similar. The main structural feature common to all sugars recognized by the uptake systems (fructose, sucrose, raffinose, stachyose) is a terminal fructosyl residue. It is tempting to speculate that the observed inhibition is due to competitive inhibition and that, in *Stachys* vacuoles, stachyose and sucrose are transported by the same carrier. However, definite conclusions have to await the results of the determination of proper K_i values for a larger variety of structurally related competitors. Competitive inhibition of sucrose uptake by raffinose has been demonstrated for redbeet vacuoles (13). In that system, fructose was only slightly inhibitory and the effect of stachyose was not tested. Likewise, the sucrose carrier of barley mesophyll vacuoles was also inhibited by raffinose, but not by fructose (6, 9). Stachyose neither inhibited sucrose uptake, nor was it transported by barley vacuoles (F. Keller, E. Martinoia, unpublished). Stachyose transport by *Stachys* vacuoles, therefore, seems not to be a general phenomenon but is most likely typical of vacuoles from cells specialized in stachyose accumulation.

Sugar transport systems have often been observed to depend on sulfhydryl groups for proper functioning (for a review, see ref. 2). The sulfhydryl reagent PCMBs,² however, did not show any considerable inhibition of stachyose and sucrose uptake, respectively (Table I). Although insensitivity of sugar carriers to PCMBs has been reported before (e.g. glucose carrier in barley vacuoles [9]), it seems to be rather the exception than the rule. Carrier-mediated uptake of stachyose and sucrose in leaf discs of *Coleus blumei* was reported to be moderately to strongly inhibited by PCMBs (8, 12). As in these discs the sugar uptake most likely reflected plasmalemma transport, it may be speculated that the PCMBs-insensitivity observed in *Stachys* may be a typical property of tonoplast sugar carriers.

Energy Requirement

Vacuolar sugar carriers have been reported to be either energy-independent (facilitated diffusion; barley mesophyll [6, 9]; sugarcane [14]) or energy-dependent (active; redbeet root [5, 13]; sugarbeet root [1]). A facilitated diffusion system at the tonoplast allows the equal distribution of a solute between vacuole and cytosol, whereas an active system allows a specific sequestration into the vacuole against a concentration gradient. In *Stachys* vacuoles, an active uptake mechanism had been suggested for stachyose (7). This suggestion was based on indirect calculations of the local concentrations in the cytosol (low) and vacuole (high), respectively, from compartmentation studies. For sucrose, the reverse situation seemed to be indicated (7). Therefore, it was of special interest to test the energy requirement for transport of these two

sugars. As tonoplast energization may be achieved by two different proton-transporting enzymes, ATPase and PPase (for review, see ref. 11), the effect of the two corresponding substrates on sugar transport by *Stachys* vacuoles was studied. Table I shows that the uptake of both sugars was stimulated. Mg-ATP stimulated transport of stachyose and sucrose by 90 and 55%, respectively, whereas PPi showed a 65 and 23% stimulation, respectively.

The stimulation by PPi and Mg-ATP of sugar uptake by *Stachys* vacuoles forms a good basis for more systematic work to further substantiate the presence of sugar/H⁺-antiport systems in *Stachys*. As *Stachys* tubers are seasonal and only available in the fall, its elucidation is planned but currently dormant.

ACKNOWLEDGMENTS

I would like to thank Helen Greutert for her technical assistance and Philippe Matile, Enrico Martinoia, and Nick Carpita for their critical readings of the manuscript.

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² Abbreviation: PCMBs, *p*-chloromercuribenzenesulfonic acid.