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Sucrose Phosphate Is Not Transported into Vacuoles or Tonoplast Vesicles from Red Beet (*Beta vulgaris*) Hypocotyl[1]

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ABSTRACT

Tonoplast vesicles and vacuoles isolated from red beet (*Beta vulgaris* L.) hypocotyl accumulated externally supplied [^{14}C]sucrose but not [^{14}C]sucrose phosphate despite the occurrence of sucrose phosphate phosphohydrolytic activity in the vacuole. The activities of sucrose synthase and sucrose phosphate synthase in whole cell extracts were 960 and 30 nanomoles per milligram protein per minute, respectively; whereas, no sucrose synthesizing activity was measured in tonoplast preparations. The results obtained in this investigation are incompatible with the involvement of sucrose phosphate synthase in the process of sucrose synthesis and accumulation in the storage cells of red beet.

The accumulation of sucrose into the vacuole of plant cells is thought to be mediated by an electrogenic tonoplast bound sucrose/H^+ antiport system (2, 3, 19). In photosynthetic cells, sucrose is synthesized *de novo* from UDP-Glc and Fru-6-P in a sequence of two reactions catalyzed by SPS[2] (EC 2.4.1.14) and SPP (EC 3.1.3.24). In contrast, it is generally believed that sucrose moves symplastically into the cytosol of sink cells before its accumulation into the vacuole (6, 7, 11, 12, 20). For some storage tissues, however, including sugar beet hypocotyl (4), muskmelon fruit (9), rice grain (16), and bananas (10), evidence has been presented implicating the involvement of *de novo* synthesis of sucrose via SPS during sucrose accumulation.

Based on observations from a number of studies, it is generally concluded that SPS activity is localized exclusively in the cytosol of both photosynthetic and nonphotosynthetic cells (1, 5, 10, 17, 18). Although much less attention has been paid to SPP, its activity has been localized in the vacuole of storage tissues such as red and sugar beet hypocotyl and sugarcane stem (8). The occurrence of SPS in the cytosol and SPP in the vacuole of storage cells carries the inherent supposition that sucrose-P must be transported across the tonoplast in order to be dephosphorylated.

The capacity of the tonoplast-bound sucrose/H^+ antiport system to transport sucrose-P has not been examined with membrane preparations competent in active sucrose uptake. In the present study, the capacity for sucrose-P transport was determined with intact vacuoles and tonoplast vesicles from red beet hypocotyl. Also, the intracellular distribution of SPP was reexamined in this tissue. The results of the uptake studies and enzyme localization were inconsistent with the role of SPS in sucrose accumulation in red beet hypocotyl.

MATERIALS AND METHODS

Plant Material

Red beet (*Beta vulgaris* L.) storage roots were purchased from a local grocery store. Care was taken in selecting the beets to ensure maximum freshness.

Isolation of Tonoplast Vesicles

Sealed tonoplast vesicles were prepared as follows. Storage hypocotyl (100 g) was peeled, cut into small segments, and homogenized for 1 min in 180 mL of a solution containing 250 mM sucrose, 70 mM Tris-HCl, 4 mM DTT, 3 mM EDTA, 0.5% PVP-40, and 0.1% BSA at pH 8.0 (buffer A). After 20 min at 4°C (to allow the foam to dissipate), the homogenate was filtered through four layers of cheesecloth and centrifuged at 10,000*g* for 15 min. The pellet was discarded and the supernatant centrifuged at 80,000*g* for 45 min. The resulting pellet, containing the microsomal fraction, was resuspended in 5 mL of a solution containing 250 mM sucrose, 10 mM Tris/Mes, and 2 mM DTT at pH 7.0 (buffer B). To the resuspended pellet, an additional 5 mL of 50% buffer B and 300 mM KCl were added and centrifuged at 80,000*g* for 45 min. The microsomal pellet was resuspended in 2 mL of buffer B and layered on a discontinuous sucrose gradient of 40, 34, 26, and 16% sucrose in 10 mM Tris/Mes (pH 7.0), and 2 mM DTT. After 2 h at 80,000*g*, the tonoplast vesicles were collected from the 16 to 26% sucrose interface. This fraction was diluted five times with a solution of 250 mM sorbitol, 5 mM BTP/Mes (pH 7.2), and sonicated for 5 s (to free the vesicles from entrapped sucrose and any other cytoplasmic components). After a final centrifugation at 80,000*g* for 45 min, the tonoplast vesicle pellet was resuspended in 0.25 mL of the same solution as above.

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[2] Abbreviations: SPS, sucrose phosphate synthase; SPP, sucrose phosphate phosphatase; BTP, Bis-Tris propane; SS, sucrose synthase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Vacuole Isolation

The procedure described by Giannini and Fishbeck (personal communication) was used to isolate intact vacuoles from red beet hypocotyl. Beet hypocotyl (≈150 g) was peeled and finely chopped in 150 mL of 1 M sorbitol, 5 mM EDTA, 0.5% PVP-40, 40 mM Tris/Mes (pH 8.0), and 5 mM mercaptoethanol (buffer A). The homogenate was filtered through a metal grid (0.3 mm) and centrifuged at 2,000*g* for 10 min. The pellet was suspended in 10 mL of 35% Percoll in buffer B containing 1 M sorbitol, 1 mM EDTA, and 25 mM Tris/Mes (pH 7.0). Ten milliliters of buffer B were layered on top of the 35% Percoll solution containing the vacuoles and centrifuged at 15,000*g* for 30 min. The vacuoles and other cellular debris floated to the 0 to 35% Percoll interface. To further purify the vacuoles, the 0 to 35% Percoll interface was diluted with equal volume of buffer B and layered on top of a 34% sucrose solution. After 15 min at 2,000*g*, a highly purified vacuole fraction was collected from the top of the 34% sucrose layer.

Radiolabeled Compounds

[^{14}C]Sucrose was obtained from New England Nuclear and supplied at a specific activity of 1.4 μCi/μmol. UDP-[^{14}C]Glc was prepared from [U-^{14}C]Glc as described previously (14). [^{14}C]Sucrose-P was prepared from UDP-[^{14}C]Glc by reaction with Fru-6-P in the presence of purified spinach leaf SPS (14) in a 700 μL reaction mixture containing 70 mM Hepes-KOH (pH 8.0), 15 mM $MgCl_2$, 3 mM DTT, 12.4 mM UDP-[^{14}C]Glc (2 mCi/mmol), 0.47 mM Glc-6-P, 12.4 mM Fru-6-P, 3.5 mg BSA, and 1 unit spinach leaf SPS. After 90 min at 25°C, [^{14}C] sucrose-P was isolated from the reaction mixture by chromatography on a 20 mL column of benzyl-DEAE cellulose using a linear gradient from 0.05 to 0.3 M NH_4HCO_3. [^{14}C] Sucrose-P was taken to dryness *in vacuo* and stored as a potassium salt in a frozen aqueous solution at −80°C.

Uptake Experiments

Tonoplast vesicles (≈8 μg protein/mL) and vacuoles (≈16 μg protein/mL) from red beet hypocotyl were energized by preincubating in 250 mM sorbitol, 50 mM KI, 50 mM $MgCl_2$, 5 mM ATP, and 25 mM Tris/Mes (pH 7.0) for 5 min at 30°C (2). The uptake of [^{14}C]sucrose, [^{14}C]sucrose-P, and UDP-[^{14}C]Glc was initiated by the addition of radiolabeled substrate to the energized vesicles or vacuole mixture. Samples for the time course of sucrose uptake into tonoplast vesicles at 30 mM sucrose were taken at 0, 5, 10, 15, and 30 min of incubation. All other samples were taken at 30 min. For some assays, [^{14}C]sucrose-P was generated during uptake by including 7 mM UDP-[^{14}C]Glc, 3 mM Fru-6-P, 15 mM Glc-6-P, and purified spinach leaf SPS in the uptake reaction mixture. The production of [^{14}C]sucrose-P in these assays was verified by anion-exchange HPLC (14). The reaction mixtures were incubated at 30°C for the appropriate times. Uptake was stopped by applying the reaction mixture to a 0.22 μm Millipore filter attached to a mild vacuum. The filter was subsequently washed five times with 1 mL of 250 mM sorbitol and 5 mM Tris/Mes (pH 7.0) and the radioactivity present in the filter determined by a liquid scintillation spectroscopy.

Enzyme Assays

The activities of SPS and SS were determined at 30°C by a modified radiometric assay (14). Red beet hypocotyl was homogenized at 4°C in a solution containing 50 mM Hepes-KOH (pH 7.2), 5 mM $MgCl_2$, 1 mM EDTA, 25 mM mercaptoethanol, 5 mM DTT, 1% (w/v) PVP-40, 1 mM PMSF and 10 μM leupeptin. The homogenate was centrifuged for 2 min at 13,000*g* and aliquots of the supernatant were desalted through a 2 mL column of Sephadex G-50–80, equilibrated with 50 mM Hepes-KOH (pH 7.2), 5 mM $MgCl_2$, 1 mM EDTA, and 25 mM mercaptoethanol. Desalted red beet hypocotyl extracts were used to initiate 10 min reactions containing 50 mM Hepes-KOH (pH 7.5), 15 mM $MgCl_2$, 1 mM EDTA, 6 mM UDP-[^{14}C]Glc, and either 3 mM Fru-6-P and 15 mM Glc-6-P for SPS or 10 mM fructose for SS. Protein was determined as in Hubbard *et al.* (14).

Sucrose-P phosphohydrolase activity was assayed in a reaction mixture containing 100 mM Hepes-KOH (pH 7.0), 1 mM sucrose-P, and 20 mM $MgCl_2$ as described by Hawker *et al.* (8). Betanin was measured spectrophotometrically. One unit of betanin is defined as the concentration which gave an increase in A of 0.01 at 550 nm.

RESULTS

The rates of sucrose uptake into tonoplast vesicles and vacuoles from red beet hypocotyl are shown in Table I. For tonoplast vesicles, the rates of sucrose uptake at 30 mM remained relatively constant for time periods up to 30 min (Fig. 1). On a protein basis, the uptake of sucrose into vesicles was approximately 15 times higher than into intact vacuoles. It is likely that the differences in the uptake rates were due to the higher specific activity of the tonoplast carrier protein in the membrane vesicles than in vacuoles (which also contain internal soluble proteins) and to the greater vesicle surface area. The decreased uptake of sucrose in the presence of CCCP and/or valinomycin was consistent with sucrose transport being driven by a proton motive force (2).

Table I. *Uptake of [^{14}C]Sucrose and [^{14}C]Sucrose-P into Tonoplast Vesicles and Vacuoles Isolated from Red Beet Hypocotyl*

Results are the average of at least two experiments. SE ≤ 10%.

Additions	Uptake	
	Vesicle	Vacuole
	nmol·min^{-1}·mg protein	
Sucrose (30 mM)	18.3	1.31
+ Valinomycin (5 μM)	13.9	0.72
+ CCCP (1 μM)	12.9	0.68
+ Valinomycin, CCCP	10.8	0.65
+ Sucrose-P (1 mM)	19.8	NA
Sucrose (1 mM)	8.6	NA
+ Sucrose-P (1 mM)	7.9	NA
Sucrose-P (1 mM)	0	0
+ Alkaline Phosphatase	7.9	NA
Sucrose-P (7 mM)	0	0
7 mM UDPG, 3 mM Fru-6-P 15 mM Glc-6-P + SPS	0	NA

[a] Not assayed.

When included at a final concentration of 7 or 1 mM, sucrose-P was not transported into either tonoplast vesicles or vacuole preparations. Similarly, no uptake of sucrose-P occurred when sucrose-P was generated *in situ*. Uptake of sucrose at 1 and 30 mM was unaffected by the presence of 1 mM sucrose-P; and uptake, at rates comparable to the rate with 1 mM sucrose, was observed when 1 mM sucrose-P was pretreated with alkaline phosphatase (Table I).

The distribution of sucrose-P phosphohydrolytic activity in red beet hypocotyl was determined by comparing the specific activity of the enzyme in crude preparations to that in purified vacuolar samples using betanin as vacuolar marker (Table II). The ratio of the specific activity of the enzyme in the vacuole as compared with the whole cell homogenate was ≥1. These results are in agreement with those of Hawker *et al.* (8) using similar tissues and indicate that sucrose-P phosphohydrolytic activity was located within the vacuole.

The activities of SPS and SS were determined in desalted extracts from red beet hypocotyl and in tonoplast preparations. Sucrose synthase activity was 960 nmol/mg protein·min compared to only 30 nmol/mg protein·min for SPS. Neither SS nor SPS activity was detected in tonoplast vesicle preparations.

Table II. *Activity of Sucrose-P Phosphohydrolytic Activity in Whole Cellular Homogenates and in Vacuole Preparations*

Values are the average of three experiments.

	Sucrose-P Phosphohydrolytic Activity
	pmol/min·unit of betanin
Homogenate	25.8 ± 5.2
Vacuole	27.9 ± 4.3
Percentage enzyme activity in vacuole	108 ± 23

DISCUSSION

The results of the present study demonstrated that under the conditions tested, sucrose-P was not transported across the tonoplast of red beet storage cells that were otherwise capable of accumulating sucrose. This conclusion is supported by the lack of sucrose-P uptake either when supplied externally or synthesized *in situ* and by the uptake of sucrose when supplied directly or produced by the action of phosphatase treatment on sucrose-P (Table I). Our data also support the concept that sucrose transport into the vacuole is, at least partially, electrogenic (2, 3). It is noteworthy that in an earlier report (12) sugarcane tonoplast vesicles did not accumulate sucrose-P when supplied at 200 μM. These vesicles, however, were also unable to accumulate sucrose which raises questions about the integrity of the membrane system.

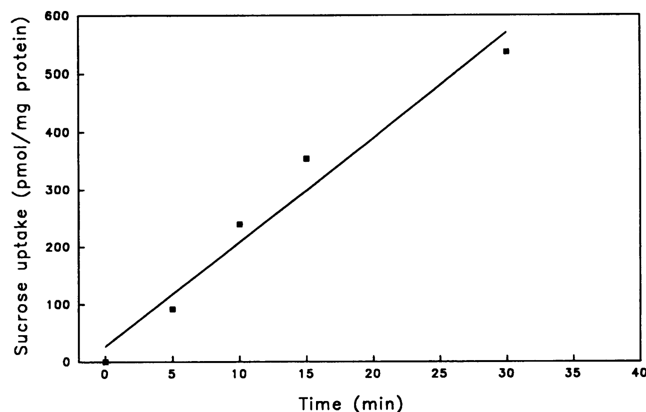

Figure 1. Time course of [^{14}C]sucrose uptake into red beet tonoplast vesicles. Uptake was initiated by the addition of [^{14}C]sucrose at a final concentration of 30 mM. Data points are the average of three experiments. SD ≤ 5%.

Although consistent with the results of a previous study (8) the localization of SPP activity in the vacuole of red beet hypocotyl poses a mechanistic problem. From the intracellular distribution of SPP and SPS, cytoplasmically synthesized sucrose-P must be transported into the vacuole in order to be dephosphorylated. However, the inability of tonoplast vesicles and intact vacuoles to transport sucrose-P indicated that sucrose-P must be dephosphorylated before accumulation into the vacuole. Given these mechanistic constraints, it is not possible for sucrose synthesis to proceed through the SPS-SPP system in the storage tissue of red beet.

The experimental limitations of the localization and uptake experiments presented here make it difficult to totally exclude the involvement of SPS in *de novo* synthesis of sucrose in red beet hypocotyl. For example, localization of SPP activity reported here and previously (8) relied on activity measurements which could be misleading if inactivation of a cytosolic form of SPP occurred during extraction or if nonspecific phosphatases were present in the homogenate. Similarly, the inability of vacuoles and tonoplast vesicles to accumulate sucrose-P may have been due to the conditions used for the uptake experiments rather than to the absence of a transport mechanism. However, accumulation of sucrose occurred under the same conditions used for the uptake experiments with sucrose-P, which indicated that conditions were adequate for the activity of the sucrose antiporter.

It is of interest to note that the activity measurements presented here and elsewhere (4) showed that, in red beet hypocotyl and in other types of storage tissue (13, 15), the capacity for synthesis of sucrose via sucrose synthase was markedly higher than the capacity for sucrose-P synthesis via SPS. An involvement of sucrose synthase in *de novo* synthesis of sucrose would be consistent mechanistically with our current understanding of intracellular compartmentation of the enzymes of sucrose metabolism and the transport properties of the tonoplast membrane in red beet hypocotyl and probably other storage tissues. An involvement of SPS in the *de novo* synthesis of sucrose in storage tissues of some species is supported by the relatively high activities of the enzyme in these tissues. However, enzyme localization and transport studies need to be conducted in these tissues to determine if the mechanistic details of sucrose metabolism are consistent with a role of SPS in sucrose accumulation.

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