Contributions of Sucrose Synthase and Invertase to the Metabolism of Sucrose in Developing Leaves

ESTIMATION BY ALTERNATE SUBSTRATE UTILIZATION

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ABSTRACT

The relative contributions of invertase and sucrose synthase to initial cleavage of phloem-imported sucrose was calculated for sink leaves of soybean (Glycine max L. Merr cv Wye) and sugar beet (Beta vulgaris L. monohybrid). Invertase from yeast hydrolyzed sucrose 4200 times faster than 1'-deoxy-1'-fluorosucrose (FS) while sucrose cleavage by sucrose synthase from developing soybean leaves proceeded only 3.6 times faster than cleavage of FS. [14C]Sucrose and [14C]FS, used as tracers of sucrose, were transported at identical rates to developing leaves through the phloem. The rate of label incorporation into insoluble products varied with leaf age from 3.4 to 8.0 times faster when [14C]sucrose was supplied than when [14C]FS was supplied. The discrimination in metabolism was related to enzymatic discriminations against FS to calculate the relative contributions of invertase and sucrose synthase to sucrose cleavage. In the youngest soybean leaves measured, 4% of final laminar length (FLL), all cleavage was by sucrose synthase. Invertase contribution to sucrose metabolism was 47% by 7.6% FLL, increased to 54% by 11% FLL, then declined to 42% for the remainder of the import phase. In sugar beet sink leaves at 30% FLL invertase contribution to sucrose metabolism was 58%.

Phloem import and possibly control of phloem unloading are linked to the metabolism of rapidly growing sink organs as demonstrated by inhibitor studies (for review, see Refs. 14, 22). In addition, a pathway for phloem unloading through the symplast in developing leaves (19) and roots (7, 10) suggests that unloading is controlled by removal of the main osmoticum, often sucrose, from the phloem either by compartmentalization or breakdown in nontransport tissues. The initial step in sucrose metabolism is thus an important one in controlling both the movement of sucrose and its utilization. Two enzymes which catalyze the initial step in sucrose metabolism are invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) and sucrose synthase (UDPGlucose: D-fructose 2-α-β-glucosyltransferase, EC 2.4.1.13) (1, 2). Both invertase and sucrose synthase activities have been found in leaves of a variety of species though activities vary with extraction techniques and species (4, 9; for review, see Ref. 2).

A relationship between invertase activity and growth has been inferred from correlations of growth rate, reducing sugar levels and extractable invertase activity in leaves (4, 11, 16, 18) and stems (17). However, high invertase activities are also found in mature leaves (9, 18). Increases in sucrose synthase activity have also been correlated with growth rate and assimilate distribution in leaves of eggplant (4).

It is difficult to extrapolate in vivo enzyme activities from in vitro measurements because of the inability to recreate the chemical environment, in terms of concentration of substrates, modulators, inhibitors, etc. We have attempted to measure the relative in vivo activities of invertase and sucrose synthase in the breakdown of phloem-supplied sucrose by using an analog of sucrose, FS,3 which is a poor substrate for invertase but which is cleaved by sucrose synthase (3, 12). The ratio of metabolism of the two sugars was compared in developing leaves of soybean and sugar beet and related to the differential discrimination against FS by sucrose synthase and invertase in vitro.

MATERIALS AND METHODS

Plant Material. Soybean (Glycine max L. Merr cv Wye) and sugar beet (Beta vulgaris L. monohybrid) plants were grown under controlled conditions as previously described (15) with a light period of 14 h. Sugar beet plants were used 3 to 4 weeks after planting and soybean plants 8 to 13 days after planting.

Enzymic Discrimination between Sucrose and FS. Sucrose synthase activity in crude extracts was not stable at room temperature. Sucrose synthase was partially purified from immature first trifoliate leaves, 1.5 to 2 cm in length (17-22% FLL), from 11 d old soybean plants. Leaves were ground as described previously (12) in 100 mm Hepes-KOH (pH 7.0), 5 mm EDTA, 5 mm DTT, squeezed through Miracloth and centrifuged at 10 KxG for 10 min. The supernatant was centrifuged at 40 KxG for 30 min. The second supernatant, referred to as crude extract, was loaded onto a DEAE-cellulose (Cellex D, Bio-rad) column (37 ml volume) at 5 to 7°C (6). The column was previously equilibrated and eluted with 10 mm Hepes-KOH (pH 7.0), 1 mm EDTA, 1 mm DTT. Flow rate was 17 ml h-1 and 11 ml fractions were collected. Bound protein was eluted with a linear NaCl gradient from 0 to 0.25 M NaCl in equilibration buffer. Aliquots of fractions were assayed for sucrose synthase as described in “Enzyme Assays.” Protein was measured by absorbance at 280 nm. Fractions 10 to 14 were pooled and brought to 50% saturation with ammonium sulfate by stirring for 30 min with an equal volume of saturated ammonium sulfate in 10 mm Hepes (pH 6.0). Protein was pelleted by centrifugation at 40 KxG for

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3 Abbreviations: FS, 1'-deoxy-1'-fluorosucrose; FLL, final laminar length; UDPG, UDP-glucose.
30 min and resuspended in 0.5 ml 10 mM MES-KOH (pH 6.5), 1 mM DTT, 1 mM EDTA, 1 mM MgCl₂. The crude extract and partially purified extract had sucrose synthase activities of 0.2 and 2.4 mol h⁻¹ mg protein, respectively. Recovery of activity was 30%.

The ratio of \( V_{\text{max}} / K_m \) of sucrose to \( V_{\text{max}} / V_{\text{hfs}} \) for sucrose synthase was determined by measuring \( H^{14}C / H^{12}C \) ratio in UDPG after incubating partially purified extract with varying ratios of [U-\(^{14}\)C]-glucose-FS. The 0.1 ml reaction was begun by adding 0.01 ml of partially purified extract to 50 mM sucrose, 20 mM UDP, 14 mM MgCl₂, 1 mM DTT, in 10 mM MES-KOH (pH 6.5). The ratio of [U-\(^{14}\)C]-sucrose to [\(^{14}\)C]-glucose-FS ranged between 0.12 to 15 with amounts of radioisotopes from 33.7 to 2.8 kBq of [\(^{14}\)C]-glucose-FS and 8.6 to 56.1 kBq of [U-\(^{14}\)C] sucrose. The reaction was stopped after 2.5 h by freezing in dry ice followed by freeze-drying. Sugars were resuspended in 30% methanol and UDPG was separated from sucrose and FS by ion exchange chromatography with 0.5 ml of Bio-Rad AG 1X8 in formate form. Columns were washed in the following sequence: (a) twice with 11 ml H₂O, (b) 11 ml 50% methanol, (c) 6 ml 8 N formic acid, (d) 0.5 ml 2 N hydrochloric acid, (e) 1 ml 2 N hydrochloric acid. Eluant from the last hydrochloric acid wash contained 80% of the UDPG present and was counted by dual-isotope liquid scintillation counting. Background contamination in UDPG fractions from [\(^{3}\)H]-sucrose and [\(^{14}\)C]-FS ranged from 1.5% and 10% respectively, was subtracted. Initial \( H^{14}C / H^{12}C \) ratios were taken as the ratio of [\(^{14}\)C]-glucose-FS to sucrose, purified invertase from yeast (Sigma Chemical Co.) was used at concentration of 450 units (250 μl⁻¹) of reaction mix for FS and at 100-fold dilution for sucrose hydrolysis. Reactions contained 20 μM sucrose or 20 mM FS in 10 mM sodium acetate buffer (pH 5.0). Reaction was begun by adding 10 μl of appropriate invertase dilution. Glucose was measured either by absorbance at 340 nm of NADPH produced in coupled enzyme assay, or as \(^{14}\)C-glucose peracetyl produced by acetylation of the products from \(^{14}\)C]-sucrose or \(^{14}\)C]-FS. For coupled assays, 25 μl of the reaction was removed at 2, 5, and 10 min intervals, and added to 975 μl of assay medium consisting of 100 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM ATP, 1 mM NADP, 0.5 units of hexokinase and 2.5 units of glucose-6-phosphate dehydrogenase. There was no increase in absorbance when 20 mM stock sucrose or FS in sodium acetate buffer and invertase stock were added separately to assay medium. In radiolabeled assays, separate reactions were run with 123 kBq of either \(^{14}\)C]-sucrose or \(^{14}\)C]-FS. The 25 μl samples of the reaction mix were removed and added to 0.5 ml of pyridine-acetic anhydride (1:1, v/v) to stop the reaction and acetylate the sugars. Zero time samples for background subtraction were removed before addition of the extract. After 2 h acetylation, samples were dried under N₂, redissolved in ethyl acetate, and spotted on 0.25 mm silica gel plates. Thin layer chromatograms were developed with ethyl:hexane (4:1, v/v) and autoradiographed. Radioactive spots corresponding to acetylated hexoses and sucrose or FS were scraped from the plate after moistening with water, eluted in methanol, and counted by liquid scintillation counting.

Enzyme Assays. Sucrose synthase was measured in the cleavage direction by production of UDPG from either sucrose or FS. Continuous production of UDPG was assayed in a 1 ml reaction consisting of 100 mM MES-KOH (pH 6.5), 1 mM UDP, 5 mM MgCl₂, 2 mM inorganic pyrophosphate, 1 mM NADP, 3 units of phosphoglomutase, 1 unit each of UDPG pyrophosphorylase and glucose-6-phosphate dehydrogenase, crude extract and varying concentrations of sucrose or FS. Absorbance at 340 nm was measured continuously. Assay of invertase activity in crude extracts was done as previously described (16).

Transport and Metabolism of Sucrose and FS. All leaves but one sink and one source leaf were removed from sugar beet plants and both cotyledons and one primary leaf were removed from soybean plants the day prior to the experiment. All treatments were started 2 h after the beginning of the photoperiod. Radiolabeled sugars were supplied to source leaves of sugar beet via reverse vein flap feeding (19) and to soybean primary leaves acetone-acetylated leaf surface abraded with carborundum. Solution was held on abraded surfaces with Saran Wrap. Solutions were 10 μM sucrose in 25 mM MES-KOH (pH 5.5) with 1 mM CaCl₂. Initial experiments were done using [U-\(^{14}\)C]-sucrose and [\(^{14}\)C]-glucose-FS as tracers of sucrose supplied to the same plant, and subsequent experiments were done using [U-\(^{14}\)C]-sucrose or \([\(^{14}\)C]-glucose-FS) and [\(^{14}\)C]-glucose-FS supplied to separate plants. For sugar beet plants, 1.8 MBq of each radioisotope was used and for soybean plants, 0.37 MBq of \([\(^{14}\)C]-sugars and 1.1 MBq of [\(^{3}\)H]-sucrose was used. Arrival and accumulation of \(^{14}\)C in a sink leaf were monitored with a Geiger-Mueller tube clamped to the leaf (19). Sugar beet sink leaves were sampled by removing 4 cm diameter discs. Soybean leaves were sampled by removing either an entire leaflet or half of one leaflet of the first trifoliate leaf.

Leaf samples from single isotope experiments were frozen in dry ice and later extracted twice with 80% ethanol at 79°C for 30 min. Both the dried ethanol extracts and the extracted tissue were solubilized with 0.5 ml of NCS tissue solubilizer (Amersham), heated at 50°C for 2 h, cooled, neutralized with 30 μl acetic acid, and prepared for liquid scintillation counting. Dpm in ethanol extracts and extracted tissue were summed and the percent dpm in insolubles were calculated by dividing dpm in extracted tissue by total dpm.

Leaf samples from dual-isotope experiments were oxidized in a Biological Oxidizer OXZ300 (R. J. Harvey Instrument Corporation, Hillsdale, NJ). Leaf tissue was powdered at dry ice temperature and separate samples were either oxidized for total dpm or extracted in 80% ethanol and extracted tissue oxidized for dpm in insolubles. In several experiments ethanol extracts were further fractionated by ion exchange chromatography (12) and neutral fractions were analyzed for dpm in FS, sucrose, and hexoses by HPLC on a 4.6 mm x 25 cm Du Pont Zorbax amino column eluted with acetonitrile/water (7.3, v/v).

Sources and Identification of Radiolabeled Sugars. [U-\(^{14}\)C]-sucrose was purchased from American International and [U-\(^{14}\)C]-sucrose and \([\(^{14}\)C]-[\(^{14}\)C]-glucose-FS were purchased from New England Nuclear. \([\(^{14}\)C]-glucose-FS was synthesized from \([\(^{14}\)C]-glucose from New England Nuclear and 1-deoxy-1-fluorofructose (3) by the following procedure. \([\(^{14}\)C]-d-Glucose (0.5 μCi, 315 mCi mmol⁻¹) was purified by HPLC as described above to remove nonradioactive contaminants which inhibited subsequent enzymic reactions. Fractions containing \([\(^{14}\)C]-glucose were converted to [\(^{14}\)C]-glucose-6-phosphate in a reaction mix containing 4.8 mol of ATP, and two units of hexokinase in 0.5 ml of 50 mM HEPES buffer at pH 7.2 which was also 5 mM in MgCl₂. The reaction was allowed to proceed for about 1 h and was checked for completion by TLC of a 0.5 μl aliquot on cellulose using ethanol:1 μl ammonium acetate (pH 3.8) (5.2, v/v) as the developing system.
To the \([^{14}C]\)glucose-6-phosphate containing reaction mix was added 4 \(\mu\)mol of UTP and 3 to 4 units each of phosphoglucomutase, UDP-glucose pyrophosphorylase, and inorganic pyrophosphatase. The reaction was checked for completion using the TLC system described above. From 60 to 90% of the applied \(^{14}C\) co-migrated with UDP-glucose standards. The reaction mix was heated in a boiling water bath, centrifuged and used as the source of \([^{14}C]\)UDP-glucose in the sucrose synthase catalyzed synthesis of FS as described previously (3) except that defatted wheat germ (Sigma Chemical Co.) was used as the source of sucrose synthase.

**RESULTS**

Enzyme Discrimination Factors. Sucrose and FS were not identical substrates for sucrose synthase or invertase as measured by *in vitro* kinetic analysis. Approximate \(K_m\) values for sucrose and FS for sucrose synthase at 1 mM UDP were similar, 40 and 55 \(\mu\)M, respectively, and within the range of reported values for a variety of tissues and species (1, 2). However, the \(V_{max}\) with FS was 3 to 4 times lower than with sucrose (data not shown).

The discrimination factor for cleavage of the two sugars by sucrose synthase was calculated using low, tracer concentrations of \([^{3}H]\)sucrose and \([^{14}C]\)FS in varying ratios in a large, constant pool of \([^{13}C]\)sucrose. The slope of a plot of the initial ratio of \([^{3}H]\)sucrose to \([^{14}C]\)FS versus \(^{3}H/^{14}C\) in UDPG is equal to the ratio of the kinetic constant \((V_{max}/K_m)\) for the two substrates, provided the substrate concentrations are significantly lower than \(K_m\). Concentrations of radio-labeled sugars ranged from 3 to 24 \(\mu\)M, well below the \(K_m\) of 40 to 55 \(\mu\)M. The measured ratio of 3.6 (Fig. 1) agrees well with the ratio of \(V_{max}/K_m\) obtained by calculation of the four kinetic parameters for the two substrates in continuous assays, and is very similar to a factor of 3.1 calculated by the same method using crude extracts of wheat germ as the source of sucrose synthase (data not shown).

Attempts to determine the discrimination factor for hydrolysis of sucrose and FS by invertase using the method described for sucrose synthase were unsuccessful. Reaction conditions necessary for the production of measurable product from FS resulted in complete hydrolysis of sucrose. The reaction rates for hydrolysis of sucrose and FS at equal concentrations were therefore determined separately and compared. From the data of Figure 2, purified invertase from yeast at pH 5.0 and 20 mM substrate, hydrolyzed sucrose 4200 times faster than FS when product formation was followed either as appearance of \([^{14}C]\)glucose or by coupled enzyme assay of glucose.

**Transport and Metabolism of Sucrose and FS.** At tracer concentrations, FS showed the same translocation kinetics into leaves of both soybean (Fig. 3) and sugar beet (data not shown) as sucrose. \([^{14}C]\)FS was not cleaved during transport and accounted for 90% of translocated \(^{14}C\) in sugar beet leaves. FS was metabolized in sink leaves, but at a lower rate than sucrose. In sugar beet sink leaves, 140 min after arrival the percent of total \(^{14}C\) in FS had declined linearly to 60% (20). In the same sink leaf samples, \([^{1}H]\)sucrose from \([^{14}C]\)sucrose supplied to a source leaf was below detection.

The steady state rate of metabolism of phloem-imported sugar was measured by linear regression of the percent of total, translocated radiolabel incorporated into the ethanol insoluble fraction *versus* time (Fig. 4). Any labeled, ethanol insoluble product must be a product of either invertase or sucrose synthase and the further metabolism of the hexoses derived from those reactions. Although \([^{14}C]\)sucrose cleavage yielded label in both hexose moieties and cleavage of \([^{14}C]\)FS yielded label only in the glucose moity, experiments with either uniformly labeled or glucosyl-labeled sucrose gave the same rates of metabolism and both are
DISCUSSION

Sucrose synthase-catalyzed cleavage of phloem-imported sucrose was evident in both species tested and at all developmental stages tested. FS was metabolized 3.4 to 8 times slower than sucrose (Table 1).

If the initiation of metabolism were by sucrose synthase alone, the discrimination in metabolism would be that afforded by cleavage by sucrose synthase, i.e. 3.6 (Fig. 1). The lowest differential in metabolism of sucrose to FS, 3.4 (Table 1), occurred with the youngest leaves and cleavage of sucrose could be accounted for by sucrose synthase. As leaves developed, sucrose was metabolized 6 to 8 times faster than FS (Table 1), indicating that cleavage of sucrose was accomplished by both sucrose synthase and invertase.

Quantitative determination of the relative contributions of sucrose synthase and invertase to the breakdown of phloem-supplied sucrose can be made from measurements of the rates of metabolism of [$^{14}$C]sucrose and [$^{14}$C]FS, and the discrimination factors for the two sugars as substrates for sucrose synthase and invertase. The rate of breakdown of either sucrose or FS is the sum of the rates of cleavage by invertase and sucrose synthase so that:

$$R_s = I_s + SS_s$$  \hspace{1cm} (1)

and

$$R_{fs} = I_{fs} + SS_{fs}$$  \hspace{1cm} (2)

where $R$ is the total rate of metabolism for either sugar, $I$ is the contribution of invertase to that rate, and $SS$ is the contribution of sucrose synthase.

From the data of Figure 1, the ratio of rates of metabolism of sucrose to FS via sucrose synthase is equal to 3.6 times the ratio of the concentration of [\textsuperscript{14}C]sucrose to [\textsuperscript{14}C]FS. Although not measured directly as for sucrose synthase, the discrimination by invertase at tracer levels can be estimated at about 4200 from the data of Figure 2. Thus:

$$SS_s = 3.6 \frac{[\text{\textsuperscript{14}C-sucrose}]}{[\text{\textsuperscript{14}C-FS}]}$$  \hspace{1cm} (3)

and

$$I_s = 4200 \frac{[\text{\textsuperscript{14}C-sucrose}]}{[\text{\textsuperscript{14}C-FS}]}$$  \hspace{1cm} (4)

The concentration of unmetabolized [\textsuperscript{14}C]sucrose and [\textsuperscript{14}C]FS was not measured for each experiment and each leaf age. An estimate of the [\textsuperscript{14}C]sucrose to [\textsuperscript{14}C]FS ratio was calculated as the ratio of percent of radiolabel in insolubles from [\textsuperscript{14}C]sucrose versus [\textsuperscript{14}C]FS for each sampling time. The percent of $^4$C remaining in the soluble fraction was averaged for each time point for each leaf age group. The ratio of amounts of $^4$C-solubles from $^4$C sucrose to $^4$CFS is one when the $^4$C-sugars first arrive, but the ratio decreases with time due to the more rapid metabolism of sucrose compared to FS. The ratios of percent solubles from $^4$C sucrose to $^4$CFS ($Y$), calculated for the latest time used in...
the youngest leaves tested (4% FLL). Sucrose metabolism predominantly by sucrose synthase in these young leaves may be indicative of high sucrose synthase activity often reported in nonphotosynthetic tissue (1, 2). The contribution of invertase to sucrose metabolism increased abruptly with age such that metabolism initiated by invertase contributed 47 to 57% of total metabolic rate by 7.6% FLL. The increase in invertase contribution relative to sucrose synthase at 7.6% FLL occurred with and apparently accounted for an increased rate of sucrose metabolism. Sucrose metabolism peaked at 11% FLL and was paralleled by a peak in invertase relative to sucrose synthase. As leaves approached maturity, the contribution of invertase declined slightly and the two enzymes contributed fairly equally to total breakdown. Sugar beet leaves also exhibited both enzyme activities, with a sucrose synthase contribution of 42% and an invertase contribution of 58% in leaves at 30% FLL.

The metabolism of sucrose by invertase in sink leaves most likely represents part of a change in developmental state. The maximum contribution of invertase to sucrose metabolism occurred roughly at the end of the exponential phase of cell division and before the predominance of cell enlargement to leaf growth (5). This relationship between invertase and cell enlargement is similar to that suggested by correlations among soluble acid invertase activity, hexose concentrations, and cell enlargement in leaves (16, 11), and stems (for review, see Ref. 17).

Sucrose arriving via the phloem is not cleaved before or during uptake in sink leaves of sugar beet (8). Results here demonstrating that FS was transported through sink leaves of sugar beet and soybean at rates identical to sucrose, further support a lack of hydrolysis by cell wall invertase as an obligate step in phloem unloading. Invertase activity on phloem-imported sucrose is most likely intracellular. Schmitt et al. (21) reported alkaline invertase at half the activity of acid invertase in Wye soybean sink leaves but not source leaves, suggesting that intracellular hydrolysis may involve alkaline invertase.

In developing sugar beet leaves, the \textit{in vitro} contributions of invertase and sucrose synthase to the metabolism of sucrose were nearly equal, although the extractable specific activity of invertase exceeds that of sucrose synthase by about 18-fold (9). The discrepancy between our \textit{in vivo} measurements and activities in \textit{in vitro} extracts emphasizes the need for caution in extrapolating physiological function from \textit{in vitro} assays. The method used here to determine the \textit{in vivo} rates of throughput of parallel metabolic paths is analogous to the use of isotope discrimination to determine whether carbon fixation has occurred by C$_3$ or C$_4$ pathway of photosynthesis. Application of the method should be possible to any case for which differentially metabolized substrates can be found or developed, and in which these substrates can be supplied by the same route as the normal substrate.
LITERATURE CITED
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