Plant Physiol. (1972) 49, 789–793

Sugar Transport in Immature Internodal Tissue of Sugarcane

II. MECHANISM OF SUCROSE TRANSPORT[1]

Received for publication December 6, 1971

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ABSTRACT

The mechanism by which sucrose is transported into the inner spaces of immature internodal parenchyma tissue of sugarcane (*Saccharum officinarum* L. var. H 49-5) was studied in short term experiments (15 to 300 seconds). Transport of sucrose, glucose, and fructose was each characterized by a V_{max} of 1.3 μmoles/gram fresh weight·2 hours, and each of these three sugars mutually and competitively inhibited transport of the other two. When ^{14}C-glucose was supplied exogenously, ^{14}C-glucose 6-phosphate and ^{14}C-glucose were the first labeled compounds to appear in the tissue; no ^{14}C-sucrose was detected until after 60-second incubation. After 15-second incubation in ^{14}C-sucrose, all intracellular radioactivity was in glucose, fructose, glucose 6-phosphate, and fructose 6-phosphate; trace amounts of ^{14}C-sucrose were found after 30 seconds and after 5 minutes, 71% of the intracellular radioactivity was in sucrose. Although it was possible that sucrose was transported intact into the inner space and then immediately hydrolyzed, it was shown that the rate of hydrolysis under these conditions was too low to account for the rate of hexose accumulation. Pretreatment of the tissue with rabbit anti-invertase antiserum eliminated sucrose transport, but had no effect on glucose transport. Since the antibodies did not penetrate the plasmalemma, it was concluded that sucrose was hydrolyzed by an invertase in the free space prior to transport. The glucose and fructose moieties, or their phosphorylated derivatives, were then transported into the inner space and sucrose was resynthesized. No evidence for the involvement of sucrose phosphate in transport was found in these experiments.

Hydrolysis external to the plasmalemma appears to be a prerequisite for metabolic utilization of exogenously supplied sucrose in some higher plant cells (12, 17, 18) and fungi (6, 15). Conversely, other higher plant tissues store sucrose but have low or undetectable invertase activity (16), implying that hydrolysis of sucrose prior to uptake is not necessary in these tissues.

Sugarcane presents an enigma insofar as sucrose transport is concerned. In early studies of sucrose absorption and accumulation in immature storage parenchyma tissue of sugarcane, Bieleski (3) reported that sucrose was absorbed without prior hydrolysis and that its uptake was characterized by a K_m value distinct from that of glucose and fructose. However, Sacher *et al.* (19) reported that sucrose was inverted in passing from the external solution to the storage compartment where it reappeared as sucrose. Support for this contention was gained from the observation of Hatch and Glasziou (11) that when (^{14}C-fructosyl)-labeled sucrose moved from the vascular tissue to the parenchyma, it initially was broken down and subsequently resynthesized with random labeling of the glucose and fructose moieties. To explain these observations, Sacher *et al.* (19) proposed that sucrose was accumulated via a sucrose derivative that could be formed from sucrose only via the hexose moieties. This derivative, thought to be sucrose phosphate (phosphorylated at the C-6 position of fructose), then moved across the limiting membrane, presumably the tonoplast, and ultimately into the vacuole (9, 19).

In previous studies of transmembrane transport of sucrose into immature parenchyma tissue of sugarcane, uptake measurements and identification of the sugar components of the tissue were made after 4 hr (7–9, 19), and in one case after 24 to 36 hr (3). Extensive metabolism of sugars transported and accumulated during these time periods precludes any conclusions about the initial reactions in the transport process.

Another facet of sucrose transport was studied recently by Maretzki and Thom (14) with cell suspension cultures of sugarcane. Specifically, they did not observe any transmembrane movement of sucrose into these cells (14). However, these cells typically have very low levels of invertase activity external to the plasmalemma (Maretzki, personal communication), a fact that may be interpreted as evidence that sucrose is transported only after being hydrolyzed.

Direct evidence for prerequisite extracellular hydrolysis of sucrose before transport into immature sugarcane storage tissue is reported herein. Furthermore, the glucose and fructose moieties, or their phosphorylated derivatives, apparently are transported into the cells, rather than sucrose or sucrose phosphate as proposed earlier (3, 9, 19).

MATERIALS AND METHODS

Tissue discs (6 mm diameter × 75 μ thick) were cut from immature internodal parenchyma tissue of 12-month-old sugarcane (*Saccharum officinarum* L. var. H49–5). The discs were

[1] Journal Series No. 1400 of the Hawaii Agricultural Experiment Station.

cut to these dimensions to facilitate rapid equilibration of the intercellular spaces with ^{14}C-sugar solutions. After incubation of 0.5 g fresh weight of discs for 15 to 300 sec, the tissue was rinsed for 15 sec which removed 98% of the "free space" sugars. Methods of cutting and preparing tissue discs, incubating in ^{14}C-sugar–0.5 mM $CaSO_4$ solutions, and measuring ^{14}C-activity were described previously (4). To determine the distribution of ^{14}C among the intracellular sugars, the tissue was frozen in liquid nitrogen immediately after rinsing and was extracted subsequently by grinding with 50% ethanol. After ethanol was removed under N_2, the aqueous extract was adjusted to pH 8 and applied to a 1 × 30 cm anion exchange column (AG 1 × 4 (Cl), 200–400 mesh). Free sugars and sugar phosphates were eluted from the column with an NH_4Cl/$K_2B_4O_7 \cdot 8H_2O$ gradient (2). Flow rate was 50 ml/hr, and 10-ml fractions were collected. Radioactivity was determined on an aliquot of each fraction (4). Fractions containing free sugars were pooled, concentrated, and separated by paper chromatography (4).

A protein preparation of high invertase activity was obtained from immature storage parenchyma by the methods of Hatch *et al.* (10) and Alexander (1). This preparation was partially purified by $(NH_4)_2SO_4$ precipitation. The 40 to 50% $(NH_4)_2SO_4$ fraction manifested the highest invertase activity and thus was used throughout this study. Invertase activity was determined by measuring colorimetrically the glucose formed from sucrose by the "Glucostat" method (Worthington Biochemical Corporation). The reaction mixture contained 20 μmoles of sucrose, 10 μmoles of phosphate-citrate buffer, pH 5.5, and 0.5 mg of enzyme protein in a total volume of 1 ml. Reactions were initiated by adding sucrose and were run for 2 hr at 28 C.

Protein was measured by the method of Lowry *et al.* (13).

For production of anti-invertase antiserum, a preparation of sugarcane protein in phosphate-buffered 0.86% NaCl solution was injected into the marginal ear vein of a rabbit. Six, 12, 18, 24, 24, and 24 mg of protein were injected on day 1, 4, 6, 8, 11, and 13, respectively. On day 15, an intraperitoneal booster injection of 100 mg of protein was given. The injections on days 13 and 15 were given with 0.1 ml of antihistamine (Fortamine solution, Fort Dodge Laboratories, Fort Dodge, Iowa) to prevent anaphylaxis. The rabbit was bled by cardiac puncture 10 days after the last injection.

The precipitin titer of the antiserum was determined as the highest 2-fold dilution that produced discernible lines of precipitation in a precipitin ring test. The tubes were incubated at 37 C for 4 hr and final readings were made after refrigeration overnight. Immunodiffusion assays were conducted at room temperature in 60 × 15 mm Petri dishes containing a 4-mm layer of 0.7% agar supplemented with 15 mM sodium azide and 15 mM NaCl.

For enzymic reactions with the crude protein preparation the antiserum was added to the reaction system 3 min before sucrose was added and was present during the incubation period. In sugar transport experiments, tissue sections were incubated in the antiserum for 10 min, after which the tissue was rinsed in 0.5 mM $CaSO_4$ solution three times for 1 min each, and transferred to ^{14}C-sugar solutions.

Normal serum taken from the rabbit before it was immunized served as a control in these experiments. In no instance was there a significant effect attributable to the presence of the control serum when tested at the same dilutions as the antiserum, nor was there any evidence of protease activity in the incubation medium under these experimental conditions.

D-Glucose-^{14}C (U), D-fructose-^{14}C (U), sucrose-^{14}C (U) and UDP-glucose-^{14}C (U) were products of Amersham/Searle Corp. The (^{14}C-glucosyl)-labeled sucrose was synthesized enzymically using a UDP-glucose fructose transglucosylase preparation from sugarcane (19). The sucrose was purified by paper chromatography (4). A sample of the sucrose was treated with yeast invertase and the products rechromatographed. Only the glucose moiety was labeled. The sucrose preparation contained no other labeled compounds.

All experiments were replicated at least three times, and the data were generally reproducible within ±5% unless stated otherwise.

RESULTS AND DISCUSSION

Kinetics of Sugar Transport. The concentrations of glucose and fructose which gave one-half the maximum ^{14}C uptake were 6.7 and 8.4 mM, respectively, and the V_{max} value for both hexoses was 1.3 μmoles/g fresh weight·2 hr (4). Sucrose uptake also is characterized by an apparent V_{max} of 1.3 μmoles/g fresh weight·2 hr, as determined from a double reciprocal plot in the present study. However, the *Km* for sucrose transport varied widely among different tissue preparations, making impossible a meaningful estimation of this parameter, although the *Km* values for glucose and fructose were readily reproducible. Thus, it was hypothesized that one or more rate-limiting steps may precede transmembrane transport of sucrose into this tissue, and that the rate of this limiting reaction may vary with different tissue preparations. Since Hatch and co-workers (9, 19) found acid invertase activity in the free space to vary with different tissue preparations from 18 to 69% of the total invertase activity in the tissue, and moreover, since invertase has been implicated in sucrose transport into sugarcane tissue (9, 19), it was considered that activity of this enzyme may be the rate-limiting factor in sucrose transport.

Interactions in Sugar Transport. Glucose and fructose are transported into the inner spaces of immature sugarcane parenchyma tissue slices via the same pathway (3, 4, 7). However, agreement has not been reached upon whether sucrose also is transported by this system (3, 9, 19). Transport differs in sugarcane cellular suspension cultures in that glucose and fructose do not compete for uptake (14).

The possible occurrence of interactions and mutual competitions in the transport of glucose, fructose, and sucrose was investigated in a series of factorially designed experiments. Each solution contained 0.5 mM $CaSO_4$; 1 mM ^{14}C-glucose, ^{14}C-fructose, or ^{14}C (U)-sucrose; and except in the controls, one or more of the above three ^{12}C-sugars at 1 mM.

When the uptake of glucose, fructose and sucrose after 5 min was examined in the presence of each of the other sugars, each sugar mutually interfered with uptake of the others (Table I). Additional experiments demonstrated that each of these three sugars competitively inhibited the transport of the others as determined from double reciprocal plots. Therefore, it was concluded that the transport of glucose, fructose, and sucrose apparently is effected through the same mechanism, *i.e.*, the same carrier sites, thus supporting the findings of Bieleski (3). No answer can be advanced from these data to the question of the form in which sucrose is transported, *i.e.*, whether hydrolytic cleavage occurs prior to or during transport.

Distribution of ^{14}C in Intracellular Sugars as a Function of Time and Exogenous Sugar Supplied. Tissue discs (0.5 g) were cut, washed, and immersed in 1 mM ^{14}C-glucose for up to 1 min, or in 1 mM ^{14}C (U)-sucrose or 1 mM (^{14}C-glucosyl)-labeled sucrose for up to 5 min to accumulate labeled sugars in the inner space. Discs were removed from the ^{14}C-sugar solutions at intervals, rinsed for 15 sec, and frozen for later extractions. Thus there was a 15-sec lapse between sampling and cessation of metabolic activity. The data in Table II have been corrected for lapsed time, *i.e.*, tissue samples were actually removed

from the bathing solution 15 sec prior to the times stated. Intracellular sugars were extracted from the frozen tissue, separated into free and phosphorylated sugar fractions, and individual components of the two fractions were separated and quantitatively estimated by radioassay.

After 15-sec incubation in ^{14}C (U)-glucose, the tissue contained, on a gram fresh weight basis, 0.16 nmole ^{14}C-glucose-6-P and 0.14 nmole ^{14}C-glucose (Table II). Earlier it was reported (4) that as much as 82% of the intracellular radioactivity was in the phosphorylated sugar fraction under similar conditions. In the present detailed studies, however, it was observed that the size of the phosphorylated ^{14}C-sugar fraction varied from 53 to 70% of the total intracellular radioactivity with different tissue preparations, although in replicate experiments with tissue discs from the same preparation, the magnitude and composition of the free sugar and sugar phosphate fractions were very reproducible (within ±5%). The glucose-6-P/glucose ratio in the experiment reported in Table II is the lowest found in any experiment. After 30 and 60 sec the intracellular concentrations of ^{14}C-glucose and ^{14}C-glucose-6-P had increased and radioactivity also began to appear in fructose, sucrose, glucose-1-P, fructose-6-P, and fructose-1,6-diP (Table II).

When ^{14}C (U)-sucrose was supplied exogenously, most of the intracellular radioactivity was concentrated in glucose, glucose-6-P, and fructose after 15 sec (Table II). No radioactivity was detected in sucrose until after 30 sec, when 6% of the total ^{14}C appeared in this sugar. The ^{14}C-sucrose concentration increased sharply through 5 min when 71% of the intracellular ^{14}C-activity was in this sugar (Table II). The absence of radioactivity in sucrose until after a 30-sec incubation may indicate that sucrose is not transported as the disaccharide, or at least that the transport rate for sucrose is considerably less than that of glucose and fructose.

When (^{14}C-glucosyl)-labeled sucrose was supplied to the tissue discs, after 15 sec, 43 and 57% of the intracellular radioactivity was contained in glucose and glucose-6-P, respectively (Table II). After 30 sec ^{14}C-fructose and ^{14}C-fructose-6-P were detected, indicating that glucose is converted readily to fructose by this tissue. The experiment with ^{14}C-glucose further supports this conclusion. In other respects, the distribution of ^{14}C from exogenously supplied (^{14}C-glucosyl)-labeled sucrose was quite similar to that from ^{14}C (U)-sucrose and ^{14}C-glucose (Table II).

The possibility that sucrose may be hydrolyzed after transport into the storage compartment, *i.e.*, the vacuole, was considered in the following experiment. Tissue discs were incubated in 1 mM ^{14}C (U)-sucrose for 5 min and rinsed. A tissue sample was removed for analysis, and the remainder was placed in 0.5 mM $CaSO_4$–1 mM ^{12}C-sucrose solution to accumulate sugar for an additional 2 hr. Tissue samples were taken at intervals during this period. After a 5-min incubation in ^{14}C (U)-sucrose, 71% of the intracellular radioactivity was in sucrose, 3% in glucose, 6% in fructose, and 20% in phosphate derivatives of glucose and fructose (Table II). After a 2-hr incubation in ^{12}C-sucrose, 64% of the total intracellular radioactivity still remained in sucrose. The radioactivity of glucose and fructose had increased slightly after 2 hr, comprising 7% and 9% of the total radioactivity, respectively (Fig. 1). Over the 2-hr period the total intracellular radioactivity decreased by 6%.

Glasziou (8) calculated the half-time ($t_{1/2}$) for inversion of ^{14}C-sucrose to glucose and fructose in the inner space to be about 6 hr, and the turnover time about 8.6 hr. Similar estimates calculated from the present data using Glasziou's formulae (8) were a $t_{1/2}$ of 5.4 hr and a turnover time of 7.8 hr. These values can be utilized to demonstrate that intracellular hydrolysis of sucrose occurred too slowly to account for the rate of ^{14}C-glucose and ^{14}C-fructose accumulation in this tissue. The rate of apparent sucrose transport into this tissue was 3.1 nmoles/g fresh weight·5 min (Table I). Since $t_{1/2}$ for inversion of ^{14}C-sucrose was 5.4 hr, the 5-min incubation period was equivalent to approximately 0.02 $t_{1/2}$. During a time period equal to 0.02 $t_{1/2}$, during which sucrose was transported apparently at a rate of 3.1 nmoles/g fresh weight, the loss of ^{14}C from sucrose solely from turnover of the intracellular sucrose pool would be equivalent to (0.02) (0.5) (3.1 nmoles), or 0.03 nmole of ^{14}C-sucrose. Therefore, the maximum amounts of ^{14}C-glucose and ^{14}C-fructose that could arise from intracellular hydrolysis of ^{14}C-sucrose after transport would be 0.03 nmole of each, assuming that no interconversions of glucose and fructose occurred. Such interconversions do occur, however, so assuming for example that all ^{14}C-fructose derived from ^{14}C-sucrose was converted to ^{14}C-glucose, the maximum ^{14}C-glucose concentration derived from ^{14}C-sucrose would be 0.06 nmole/g fresh weight·5 min.

When tissue sections were incubated in ^{14}C (U)-sucrose for

Table I. *Mutual Effects of Glucose, Fructose, and Sucrose on their Absorption by Immature Internodal Tissue of Sugarcane*

The concentration of each sugar was 1 mM; $CaSO_4$, 0.5 mM; pH 6.5; and the temperature was 28 C. The absorption period was 5 min, and the tissue was 0.5 g fresh weight.

Sugars Present	^{14}C Absorption in Terms of ^{14}C-Sugar Supplied		
	Glucose	Fructose	Sucrose
	nmoles/g fresh wt·5 min		
Glucose	6.3	—	—
Fructose	—	6.5	—
Sucrose	—	—	3.1
Glucose + fructose	3.2	4.4	—
Glucose + sucrose	5.7	—	1.0
Fructose + sucrose	—	5.4	2.5
Glucose + fructose + sucrose	2.7	4.2	0.7

Table II. *^{14}C Distribution Among Sugars in Immature Internodal Parenchyma Tissue of Sugarcane as a Function of Time and ^{14}C-Sugar Supplied Exogenously*

The exogenous sugar concentration was 1 mM; 0.5 mM $CaSO_4$; pH 6.5; and the temperature was 28 C.

Seconds	Concn of Intracellular Sugars and Sugar Phosphates						
	Glucose	Fructose	Sucrose	G-6-P	G-1-P	F-6-P	F-1,6-diP
	nmoles/g fresh weight						
	Incubated in ^{14}C (U)-glucose						
15	0.14	0	0	0.16	0	0	0
30	0.25	0.03	0	0.27	0	0.06	0.02
60	0.23	0.05	0.36	0.38	0.06	0.11	0.06
	Incubated in ^{14}C (U)-sucrose						
15	0.05	0.05	0	0.05	0	0.02	0
30	0.05	0.07	0.02	0.09	0	0.09	0
60	0.09	0.06	0.15	0.13	0.02	0.22	0.02
300	0.10	0.20	2.30	0.23	0.07	0.16	0.20
	Incubated in (^{14}C-glucosyl)-labeled sucrose						
15	0.06	0	0	0.08	0	0	0
30	0.08	0.04	0	0.11	0	0.02	0
60	0.10	0.07	0.16	0.14	0.03	0.08	0.03
300	0.15	0.24	2.08	0.12	0.06	0.24	0.15

5 min, 3% of the total intracellular ^{14}C appeared in free glucose and 6% in fructose at the end of the incubation period. These values are equivalent to 0.10 nmole of ^{14}C-glucose and 0.20 nmole of ^{14}C-fructose. As seen above, the maximum ^{14}C-hexose concentration derivable from ^{14}C-sucrose was 0.06 nmole, so intracellular hydrolysis of ^{14}C-sucrose could account for only 20% of the total intracellular free ^{14}C-hexoses. If the phosphorylated ^{14}C-hexoses in the inner space are considered, less than 7% of the ^{14}C-hexoses can be attributed to ^{14}C-sucrose hydrolysis. Thus, it may be concluded that ^{14}C-glucose, ^{14}C-fructose, and their phosphorylated derivatives in the inner space cannot be derived totally from hydrolysis of ^{14}C-sucrose by invertase subsequent to transport. These observations render unlikely the contention that sucrose is transported intact into the inner space (3). Rather, a more plausible explanation would be that sucrose is hydrolyzed extracellularly, the glucose and fructose moieties transported across the membrane, and sucrose is then resynthesized in the inner space. This hypothesis explains the present data as well as the earlier findings of other investigators (9, 11, 19).

Effect of Rabbit Anti-invertase Antiserum on Sucrose and Glucose Transport. The rabbit antiserum yielded a single band in the immunodiffusion assay, a surprising result since the sugarcane protein preparation, *i.e.*, the antigen, was not pure invertase. It can only be assumed that the contaminant proteins were either nonantigenic or were present in concentrations too low to elicit an antibody response. One-half milliliter of the rabbit antiserum to sugarcane protein diluted 1:32 almost completely precipitated 100 μg of the protein preparation and also inactivated its invertase. Normal serum had no effect on the invertase reaction. A titer of 1024, expressed as the reciprocal of the antiserum dilution, was obtained with the antiserum. Purified γ-globulin isolated from this antiserum (5) reacted in the same manner as the crude antiserum, except that the former was more active.

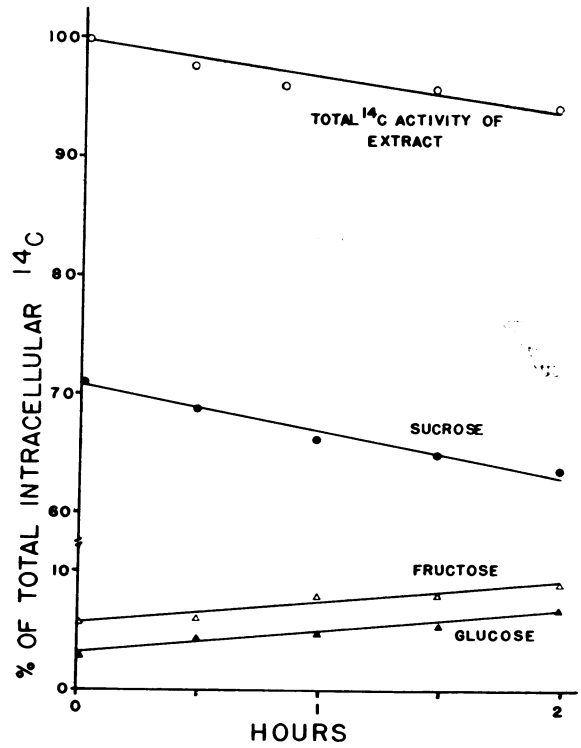

FIG. 1. Hydrolysis of ^{14}C sucrose in the inner space of immature storage parenchyma of sugarcane. Tissue discs were incubated in ^{14}C (U)-sucrose for 5 min and rinsed to remove ^{14}C-sugars from the free space. Tissue was then transferred to 0.5 mM $CaSO_4$–1 mM ^{12}C-sucrose solution for 2 hr. Temperature was 28 C, pH 6.5.

Table III. *Effect of Rabbit Antisera to Sugarcane Proteins on Sucrose and Glucose Absorption by Immature Storage Parenchyma Tissue of Sugarcane*

External sugar concentration was 1 mM; $CaSO_4$, 0.5 mM; pH 6.5; and the temperature was 28 C. The absorption period was 5 min.

Sugar Supplied	Other Additives	^{14}C Activity in Terms of Sugar Supplied
		nmoles/g fresh wt·5 min
^{14}C (U)-Glucose	None	6.2
	1 mM Sucrose	5.7
	Antibody	6.0
	1 mM Sucrose + antibody	6.2
^{14}C (U)-Sucrose	None	3.2
	1 mM Glucose	1.0
	Antibody	0.1
	1 mM Glucose + antibody	0
(Glucosyl-^{14}C)-labeled sucrose	None	3.1
	1 mM Glucose	1.1
	Antibody	0.1
	1 mM Glucose + antibody	0.1

To test the crude antiserum for nonspecific binding of glucose and fructose, the antiserum, diluted 1:32, was placed in dialysis tubing and suspended in either 10 μM glucose or fructose. After 12 hr at 4 C, the sugar concentrations inside the dialysis tube and in the external solution were measured. For both sugars the concentrations were similar on both sides of the membrane, indicating that neither glucose nor fructose was bound to the components of the antiserum.

Antiserum to the sugarcane protein preparation was added to intact immature sugarcane parenchyma tissue discs which contained acid invertase activity in the free space. The antibody was removed readily from the bathing solution, as demonstrated by the diminished ability of the solution to precipitate protein. No reaction of normal rabbit serum was observed *in vitro* or with intact tissue.

The effects of rabbit anti-sugarcane-protein antiserum upon transport of ^{14}C-glucose, ^{14}C (U)-sucrose, and (^{14}C-glucosyl)-labeled sucrose are summarized in Table III. The experiment was thrice replicated with no significant variation in results. As would be expected if hydrolysis of sucrose by invertase was a prerequisite to transport, pretreatment of the tissue discs with the antiserum reduced accumulation of ^{14}C from ^{14}C (U)-sucrose and (^{14}C-glucosyl)-labeled sucrose by 95% or more in the 5-min absorption period. The antiserum had no significant effect upon ^{14}C-glucose transport.

CONCLUSIONS

According to the scheme developed by Glasziou (8) and Sacher *et al.* (19), exogenously supplied sucrose is hydrolyzed by an invertase in the free space of immature storage parenchyma of sugarcane prior to accumulation in the storage compartment of the cell. It was proposed that the glucose and fructose moieties from sucrose were subsequently phosphorylated and converted to sucrose phosphate (8, 19), and that it was sucrose phosphate which was transported ultimately into the vacuole. While the present data support the finding that sucrose is hydrolyzed prior to transport, no evidence for sucrose phosphate transport was found after 5-min incubation.

Rather, data are presented to indicate that after inversion of sucrose, glucose, and fructose or their phosphorylated derivatives are transported into the cellular storage compartment before sucrose is resynthesized. It is probable that this disparity in results is attributable to the great difference in time that the tissue was incubated in exogenous ^{14}C-sucrose, *i.e.*, 15 sec to 5 min in the present study *vs.* 4 hr in the earlier ones (8, 19). Since glucose, fructose, and sucrose are metabolized readily by the sugarcane tissue, the premise was accepted that the shorter incubation period would provide more meaningful data relative to the initial reactions at the transport sites.

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