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SUGAR TRANSFORMATIONS ASSOCIATED WITH HEXOSE TRANSPORT IN IMMATURE INTERNODAL TISSUE OF SUGAR CANE*

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Key Word Index—*Saccharum officinarum*; Gramineae; sugar cane; phosphorylated sugars; hexoses; membrane transport.

Abstract—After a 15 sec incubation in D-glucose-^{14}C(U), 53–70% of the intracellular radioactivity in immature internodal tissue of sugarcane was in glucose-6-phosphate, and the remainder was in free glucose. Two unmetabolized glucose analogs, 2-deoxy-D-glucose and 3-*O*-methyl-D-glucose, were transported at rates comparable to glucose but neither of these analogs was phosphorylated. Doubly-labeled D-glucose-1-^{14}C-6-phosphate-^{32}P was dephosphorylated prior to deposition in the inner space, and ^{14}C was transported into this tissue twice as rapidly as ^{32}P. It was also shown that ^{32}P in exogenously supplied glucose-6-^{32}P was not the source of phosphate for the intracellular synthesis of glucose-6-P. Galactose transport was similar to that of glucose in that the first major product recovered intracellularly was a phosphorylated sugar, i.e. ^{14}C-galactose-1-P, when the tissue was incubated in D-galactose-^{14}C(U). Although fructose, glucose, and galactose competed for transport into this tissue, free fructose and glucose predominated in the tissue extract after a 15-sec incubation in D-fructose-^{14}C(U). This contrasted sharply with the products of ^{14}C-glucose transport which were comprised largely of phosphorylated sugars after 15 sec.

INTRODUCTION

MUCH evidence has accumulated pertaining to the possible physiological significance of phosphorylation associated with transmembrane transport of sugars in bacteria,[1–4] actinomycetes,[5] and yeast.[6,7] Contrarily, Brown and Romano[8] presented evidence against the necessity for phosphorylation of deoxyglucose analogs during transport into germinating spores of *Aspergillus nidulans*.

Sugar phosphorylation and transformation during transport in higher plant tissues has received comparatively little attention in recent years. Hatch[9] proposed that sucrose phosphate (phosphorylated at the C-6 position of the fructose moiety) may be an intermediate

* Part III in the series "Sugar Transport in Sugarcane Internodal Tissue". For Part II see BOWEN, J. E. and HUNTER, J. E. (1972) *Plant Physiology* **49**, 789.

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in the sugar accumulation process in sugarcane storage tissue. Evidence against this hypothesis has been advanced recently, however.[10] Kursanov *et al.*[11,12] studied the intracellular distribution of hexokinases in sugar beet conducting tissue and concluded that a non-specific membrane-bound hexokinase functioned in the transport of hexoses into those cells.

In the first paper of this series[10] it was observed that one or more sugar phosphates were among the early intracellular products of glucose transported into immature internodal tissue of sugarcane, suggesting that glucose may have been phosphorylated during transport. Maretzki and Thom[13] reached a similar tentative conclusion from their work with cell suspensions of sugarcane. This paper is the result of a detailed study of transformation of sugars prior to, during, and immediately subsequent to transport into the inner space. However, a specific answer to the question of whether or not phosphorylation is an integral function of transport has not been obtained. A method for studying transport in isolated membrane vesicles, such as that used by Kaback[14] in his bacterial studies, would be most helpful in resolving this question.

RESULTS

Transport of glucose and glucose analogs

After a 15-sec incubation in ^{14}C-glucose, sugarcane internodal tissue discs contained, on 1 g fr wt basis, 0·16 nmol ^{14}C-glucose-6-P and 0·14 nmol ^{14}C-glucose (Table 1). Since it was reported previously[10] that as much as 82% of the intracellular radioactivity was in the sugar phosphate fraction under similar conditions, the reason for this discrepancy

TABLE 1. ^{14}C DISTRIBUTION AMONG SUGARS IN IMMATURE INTERNODAL PARENCHYMA TISSUE OF SUGARCASE AS A FUNCTION OF TIME AND ^{14}C-SUGAR SUPPLIED EXOGENOUSLY

Sec	Concentration of intracellular ^{14}C-sugars and ^{14}C-sugar phosphates, nmol/g fr. wt						
	Glu	Fru	Suc	Glu-6-P	Glu-l-P	Fru-6-P	Fru-l,6-DP
			Incubated in D-glucose-^{14}C(U)				
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 D-fructose-^{14}C(U)				
15	0·15	0·11	0	0·02	0	0	0
30	0·32	0·17	0	0·06	0	0·03	0·04
60	0·49	0·28	0·24	0·14	0	0·06	0·04
			Incubated in D-glucose-l-^{14}C-6-^{32}P				
15	0·07	0	0	0·13	0	0	0
30	0·19	0·04	0	0·13	0	0·02	0
60	0·24	0·03	0·013	0·14	0	0·11	0·09

pH 6·5; temp., 28°; exogenous sugar concn. was 1 mM; exogenous Ca^{2+} concn was 0·5 mM.

was investigated. The size of the sugar phosphate fraction varied from one tissue preparation to another, but was reproducible within ± 5% in replicate experiments with tissue discs from the same plant. The extract containing 82% of the intracellular radio-activity

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in ^{14}C-glucose-6-P represented an extremely high proportion in this fraction. In all subsequent experiments the ^{14}C-glucose-6-P concn ranged from 53 to 70% of intracellular ^{14}C-activity. The only other radioactive compound present in the tissue after a 15-sec incubation in ^{14}C-glucose was free ^{14}C-glucose, with one exception reported earlier[10] in which a small quantity (less than 0·03 nmol) of ^{14}C-fructose was detected.

Further metabolic transformations of exogenously supplied ^{14}C-glucose were manifested after incubation periods of 30 and 60 sec (Table 1). After 30 sec, radioactivity was detected in fructose, fructose-6-P, and fructose-1,6-DP. After 60 sec, ^{14}C-sucrose and ^{14}C-glucose-1-P were present, in addition to the above free sugars and sugar phosphates.

Immature internodal tissue of sugarcane actively transports 3-*O*-methyl glucose (MeGlc) and 2-deoxyglucose (2-dGlc) into the cells, but MeGlc and 2-dGlc are not metabolized by this tissue.[10] This was confirmed in the present short-term experiments. When tissue discs were incubated in 1 mM ^{14}C-MeGlc or 2-dGlc for 60 sec, all intracellular radioactivity was recovered in free MeGlc and 2-dGlc, respectively. No evidence of any MeGlc-P or 2-dGlc-P was found. The transport rate for MeGlc and 2-dGlc was 0·95 and 0·97 nmol/g fr. wt/min, respectively, which compared well with the glucose transport rate of 1·12 nmol/g fr. wt/min.

Transport of glucose 6-phosphate

Glucose-6-P is actively transported into immature internodal parenchyma tissue of sugarcane.[10] Further, it is apparently transported via the same sites as glucose, MeGlc, 2-dGlc, galactose, and fructose since each of these compounds mutually competitively inhibited transport of the others.[10] The question to be answered, however, is whether glucose-6-P is transported as such, or whether it is dephosphorylated extracellularly and the glucose and phosphate moieties transported via separate systems. Tissue discs (0·5 g fr. wt) were incubated in D-glucose 1-^{14}C-6-^{32}P-phosphate. The rates of ^{14}C and ^{32}P uptake were both linear with time over the 5-min incubation period, but ^{14}C was transported into the tissue twice as rapidly as ^{32}P, thus demonstrating that glucose-6-P was likely dephosphorylated external to the plasmalemma.

Internodal tissue discs were incubated in 1 mM D-glucose 1-^{14}C-6-^{32}P for up to 1 min to ascertain the distribution of ^{14}C in the intracellular products. The ^{14}C distributional pattern was in every respect similar to that obtained when glucose-^{14}C(U) was supplied exogenously (Table 1). As with glucose, the first products of glucose-6-P transport were free glucose and glucose-6-P, with a glucose-6-P/glucose ratio of approx. 2 being observed after a 15-sec incubation. The ^{14}C was present in fructose, fructose-6-P, fructose-1,6-DP, and sucrose after 60 sec (Table 1).

Tissue sections were incubated for 60 sec in D-glucose-1-^{14}C-6-^{32}P, after which the doubly-labeled endogenous metabolites were extracted, separated, and the sp. act. of glucose-1-^{14}C-6-^{32}P determined. The specific activity of the exogenous doubly-labeled glucose-6-P was adjusted to 1 μC/μmol with respect to ^{32}P. However, the sp. act. of the endogenous glucose-1-^{14}C-6-^{32}P was only 0·07 μC/μmol with respect to ^{32}P. This indicates that the ^{32}P supplied exogenously as D-glucose-1-^{14}C-6-^{32}P was not the source of phosphate for the intracellular resynthesis of glucose-6-P.

Fructose transport

Although fructose and glucose compete for uptake into this tissue,[10] the early intracellular products differed greatly. After a 15-sec incubation in ^{14}C-fructose, free glucose and

fructose predominated in the tissue extract (Table 1), comprising 54 and 39% of the total radioactivity, respectively. In addition, a small quantity of glucose-6-P was found. This contrasts sharply with glucose transport in which case glucose-6-P constituted a large proportion of the labeled compounds after 15 sec. Of particular interest in the case of fructose transport was the large amount of free ^{14}C-glucose in the extract throughout the first minute that the tissue was exposed to exogenous ^{14}C-fructose. If glucose-6-P is also considered, after a 15-sec incubation in ^{14}C-fructose, 61% of the ^{14}C activity in the tissue has been converted to glucose and glucose-6-P (Table 1). Only after 30 sec was radioactivity found in fructose-6-P and fructose-1,6-DP.

Galactose transport

Transport of galactose, which competes with glucose and fructose for uptake, was similar to that of glucose in that the first major labeled compound in the tissue was a phosphorylated sugar; i.e. galactose-1-P (Table 2). In fact, galactose-1-P accounted for approx. 60% of the total intracellular radioactivity throughout the 1-min incubation period. Although ^{14}C-labeled glucose, fructose, glucose-1-P, and glucose-6-P appeared in the tissue within 1 min as products of ^{14}C-galactose transport, the rate of appearance of ^{14}C in these compounds was much slower than when ^{14}C-glucose or ^{14}C-fructose was supplied. Only a small amount of free ^{14}C-galactose was detected in the tissue during the 1-min exposure of the tissue to exogenous ^{14}C-galactose. No conversion of galactose to fructose phosphates was found under the conditions of these experiments.

TABLE 2. ^{14}C DISTRIBUTION AMONG SUGARS IN IMMATURE INTERNODAL TISSUE OF SUGARCANE AS A FUNCTION OF TIME WHEN D-GALACTOSE-^{14}C(U) IS SUPPLIED EXOGENOUSLY

	Concentration of intracellular ^{14}C-sugars and ^{14}C-sugar phosphates, nmol/g fr wt					
Sec	Glu	Fru	Gal	Gal-1-P	Glu-1-P	Glu-6-P
15	0·05	0	0·04	0·19	0	0·04
30	0·08	0·05	0·06	0·34	0·02	0·07
60	0·07	0·09	0·07	0·74	0·07	0·18

pH 6·5; 28°; exogenous sugar conc was 1 mM; Ca^{2+} conc was 0·5 mM.

DISCUSSION

A basic question underlying studies of transmembrane transport of sugars is whether or not vectorial phosphorylation[14] may be the driving force. As discussed in the Introduction an unequivocal answer to this question has not yet been obtained from studies in which whole cells or intact tissues are employed as the experimental material. Much indirect evidence against transport-associated phosphorylation of sugars has been presented herein, however.

It has been emphasized in this and an earlier report[10] that the glucose-6-P/glucose ratio varied from approximate unity to greater than five with different tissue preparations after a 15-sec incubation in D-glucose-^{14}C(U). It should be emphasized also, however, that despite these wide variations in the glucose-6-P/glucose ratio, total ^{14}C accumulation in tissue discs cut from different plants varied by less than 10%. Thus, the rate of glucose-6-P accumulation can vary greatly without an accompanying variation in the ^{14}C transport rate. This observation may mean either that glucose transport is independent of phosphorylation or that the rate of intracellular dephosphorylation of glucose-6-P varies. When the

rate of glucose-6-P accumulation in the tissue discs was compared with the levels of activity of soluble hexokinase in the tissue, a strong positive correlation ($r = 0.798$, 1% level) was observed. Contrarily, there was no statistically significant correlation between free glucose accumulation and phosphatase activity ($r = 0.097$, not significant). These facts are interpreted as evidence that free glucose is transported into the tissue and that it is phosphorylated subsequent to its deposition in the inner spaces.

Other evidence against the necessity for phosphorylation in transport comes from the fact that MeGlc and 2-dGlc are transported into the tissue at rates comparable to glucose,[10] but no evidence could be found that either MeGlc or 2-dGlc is phosphorylated or otherwise metabolized by the immature internodal tissue.[10] Since these glucose analogs are transported readily, yet they are not phosphorylated, phosphorylation does not appear to be prerequisite to transport.

EXPERIMENTAL

Tissue discs (6 mm dia. × 75 μ thick) were cut from immature internodal parenchyma tissue of 12-month-old sugar cane (*Saccharum officinarum* L., var. H49-5). Discs were cut to these dimensions to facilitate rapid equilibration of the "free spaces" with ^{14}C-sugar solutions and so that a 15-sec rinse in flowing tap H_2O would remove 98% of the reversibly-absorbed sugars. Methods of cutting and preparing tissue discs and measuring radioactivity were described earlier.[10] When both ^{14}C and ^{32}P were present in the incubation medium, the tissue was extracted as described below. Ten ml PCS Solubilizer (Amersham/Searle Corp.) was added to an aliquot of each aq. fraction, and the ^{14}C and ^{32}P activities were measured with a liquid scintillation counter. Tissue discs were immersed in 1 mM soln of a ^{14}C-sugar, ^{14}C-sugar phosphate, or sugar ^{32}P-phosphate for up to 1 min to accumulate labeled sugars in the inner space. All solns also contained 0·5 mM $CaSO_4$ to maintain structural integrity of the membranes. Discs (0·5 g fr. wt) were removed from the radioactive sugar solns at intervals, rinsed for 15 sec, and frozen for later extraction. Thus there was a 15-sec lapse between sampling and cessation of metabolic activity. The data have been corrected for the lapsed time; i.e. tissue samples were actually removed from the bathing soln 15 sec prior to the time stated. In Tables 1 and 2, the 15-sec period thus means that the tissue was placed in the ^{14}C-sugar solution and removed immediately, and then rinsed for 15 sec and frozen. A change was made in the earlier methodology in that tissue was frozen immediately in liquid N_2 after rinsing, and was extracted subsequently by grinding in 50% ethanol. After ethanol was removed under N_2, the aq. extract was adjusted to pH 8·0 and applied to a 1 × 30 cm anion exchange column (AGl × 4, Cl^-, 200–400 mesh). The column was washed with 100 ml of 0·001 M NH_4OH to remove the free sugars that were present. This effluent was concentrated and the free sugars were separated by paper chromatography.[10] Sugars were eluted from the chromatograms with H_2O and assayed for ^{14}C-activity. Sugar phosphates were eluted from the column with an ammonium chloride–potassium borate gradient.[15] Flow rate was 50 ml/hr and 10-ml fractions were collected. Radioactivity was determined on an aliquot of each fraction.[10] Galactose l-phosphate and glucose l-phosphate were not separated completely by this method, so when ^{14}C-galactose was supplied exogenously, the fractions containing galactose-l-P and glucose-l-P were combined and concentrated. After borate was removed with methanol, the combined fractions were passed through a 1 × 8 cm column of AG 50 × 8 cation exchange resin. The sugar phosphates were hydrolyzed with HCl and free galactose and glucose separated by paper chromatography.[10]

The ^{14}C-labeled compounds in extracts of tissue incubated in ^{14}C-2dGlc or ^{14}C-MeGlc were separated by addition of the Ba–Zn reagent,[6,16] a procedure that has been shown to separate free sugars and sugar phosphates quantitatively.[6] Radioactivity of each fraction was then determined, and it was found that neither 2-dGlc-6-P nor MeGlc-P was present, indicating that 2-dGlc and MeGlc were not metabolized by this tissue. Hexokinase and phosphatase were extracted by grinding 20 g of immature internodal tissue with distilled water in a Ten Broeck tissue homogenizer. The extract was filtered through 8 thicknesses of cheesecloth, and cellular debris removed by centrifugation at 1500 × g for 10 min. Protein was precipitated with $(NH_4)_2SO_4$ and the 35–60% saturation fraction was redissolved in 0·05 M Tris buffer, pH 7·5 and used for all enzyme assays. This entire procedure was conducted at 4°. Protein concentrations in these preparations were measured by a modification of the Lowry method.[17]

Hexokinase activity was assayed with a system based on dephosphorylation of ATP to ADP in the presence of hexose whereby ADP was coupled with pyruvate kinase and lactic dehydrogenase, and the rate of NADH

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oxidation measured at 340 nm.[8] The hexokinase reaction mixture contained 120 μmol Tris buffer, pH 7·5; 60 μmol $MgCl_2$; 250 μmol KCl; 20 μmol phosphoenolpyruvate; 1·0 μmol NADH; 20 μmol ATP; 30 μmol glucose; 60 μg lactic dehydrogenase containing pyruvate kinase (Sigma Type I); and enzyme preparation containing 0·25 mg of protein, in a total volume of 3 ml. Reactions were run for 5 min at 30°.

The phosphatase reaction mixture contained 75 μmol acetate buffer, pH 5·5; 30 μmol glucose-6-P; and enzyme preparation containing 0·25 mg of protein in a total vol. of 3 ml. Reactions were run for 5 min at 30°, and were terminated by adding 1 ml of 10% trichloroacetic acid to the reaction system. Liberated phosphate was measured with the phosphomolybdic acid method.

The following radioactive sugars were obtained from Amersham/Searle Corp., Arlington Heights, Illinois: D-glucose-^{14}C(U), D-glucose-^{14}C(U) 6-phosphate, D-galactose-^{14}C(U), fructose-^{14}C(U), 3-*O*-methyl-D-glucose-^{14}C(U). The 2-deoxy-D-glucose-1-^{14}C was a product of New England Nuclear Corp. Glucose-6-^{32}P was prepared with yeast hexokinase (Nutritional Biochemical Corp.), ATP (α, β, γ -^{32}P) (Schwarz/Mann) and D-glucose, and purified by paper chromatography. The D-glucose-1-^{14}C 6-^{32} Phosphate was similarly prepared with D-glucose-1-^{14}C (Amersham/Searle Corp.).

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