Plant Physiol. (1972) 49, 82-86

Sugar Transport in Immature Internodal Tissue of Sugarcane

I. MECHANISM AND KINETICS OF ACCUMULATION[1]

Received for publication June 7, 1971

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ABSTRACT

Transmembrane sugar transport into immature internodal parenchyma tissue of sugarcane (*Saccharum officinarum* L.) is a metabolically regulated process as evidenced by its sensitivity to pH, temperature, anaerobiosis, and metabolic inhibitors. All sugars studied—glucose, fructose, galactose, sorbose, glucose 6-phosphate, 3-O-methylglucose, and 2-deoxy-D-glucose—were apparently transported via the same carrier sites since they competed with each other for uptake. External concentrations of these sugars at one-half V_{max} were in the range of 3.9 to 8.4 nM. Preliminary data indicated that phosphorylation may be closely associated with glucose transport. The dominant intracellular sugar after 4-hours incubation was sucrose when glucose, glucose-6-P, or fructose was the exogenously supplied sugar; but when galactose was supplied, only 28% of intracellular radioactivity was in sucrose. Sorbose, 3-O-methylglucose, and 2-deoxy-D-glucose were not metabolized. Thus, by using these analogs, transport could be studied independently of subsequent metabolism, effectively eliminating a complicating factor in previous studies.

Transport of sugars into "inner spaces" of immature internodal parenchyma of sugarcane is considered to be metabolically mediated (1–3, 6–10). Invariably, however, these studies (1–3, 6–10) have employed glucose, fructose, and sucrose as the transported sugars. Therefore, interpretation of data has been complicated by a difficulty inherent in the use of sugars that are metabolized by internodal tissue; *i.e.*, it has not been possible to study transport independently of subsequent metabolism.

Another problem associated with studies of transport of metabolically utilized sugars is that these sugars may undergo interconversions outside of the diffusion barrier in which the transport system is located, a possibility suggested by Reinhold and Eilam (14) as a result of their work on respiratory utilization of exogenous sugars by sunflower hypocotyl segments. In the case of internodal tissue of sugarcane, it has been reported that the first, and often the rate-limiting, step in sucrose transport into the tissue is hydrolysis by an invertase external to the cytoplasmic membrane (10), although Bieleski's (3) data do not support this contention.

A goal of the present work was to find a hexose or hexose derivative that was actively transported, but that was not metabolized by sugarcane storage tissue. It was also important that this sugar be transported via the glucose uptake mechanism. Reinhold and Eshbar (15) successfully applied this rationale in earlier studies with carrot roots. Three sugars were found that fulfilled these criteria; sorbose, 3-O-methylglucose, and 2-deoxy-D-glucose. By using these inert sugars, it was possible to study transmembrane sugar movement independently of subsequent metabolism. Four readily metabolized sugars were included in the present experiments for comparative purposes.

An abstract of work along this line was recently published (5). The data reported therein were obtained with tissue aged for 24 hr. An important difference was observed later between aged tissue and the fresh tissue used in this phase of the study. Whereas galactose had no effect on transport of glucose, fructose and sorbose in aged tissue, transport of each of the latter three sugars was strongly inhibited by galactose in fresh tissue. The independent galactose transport system proposed for aged tissue could not be demonstrated in freshly cut tissue. The basis for this anomaly is not understood at present.

MATERIALS AND METHODS

Plant Tissue. Immature internodes were cut from field-grown sugarcane (*Saccharum officinarum* L., var. H49-5) approximately 1 yr old and brought into the laboratory. Tissue discs, 6 mm diameter × 0.5 mm thick, were cut from these internodes with a sharp cork borer and a microtome, and washed in flowing tap water for 60 min prior to the start of each experiment. The tap water, assayed daily, contained at least 70 μM Ca^{2+}, thus precluding the possibility of depleting the tissue of this essential ion during washing. Total time lapse from removal of plants from the field until start of an experiment was always less than 2 hr.

Measurement of Sugar Uptake. Tissue discs (0.5 g fresh weight) were placed in ^{14}C-sugar solutions (9 nc/μmole sugar) in 50-ml beakers, covered with aluminum foil, and placed on a reciprocal shaker at 75 oscillations per min. The volumes of sugar solutions were such that the sugar concentration decreased less than 10% during an experiment. All solutions also contained 0.5 mM $CaSO_4$. All experiments were run at 28 C and at pH 6.5 for 2 hr except in those cases where one of these parameters was the variable. After the absorption period, the tissue was rinsed three times for 1 min each in distilled water, and then rinsed for 45 min in flowing tap water. Occasional exceptions to these experimental conditions are noted in the text.

Extraction and Separation of Sugars and Sugar Phosphates. Sugars were extracted by crushing the tissue in 3 ml of hot 95% ethanol, and the extracts were filtered through 14 μ-pore

[1] Journal Series No. 1330 of the Hawaii Agricultural Experiment Station.

diameter Millipore filters. When total sugar uptake was to be measured, 1-ml aliquots of the filtered extract were assayed for radioactivity without further preparation.

For identification of individual ^{14}C-sugar components of the extract, free and phosphorylated sugar fractions were separated as follows: 1-ml aliquots of the filtered extract were applied to columns (6 × 0.5 cm) of Bio-Rad AG1-X2 anion exchange resin (100–200 mesh, formate form). Free sugars were eluted from the column with three 1-ml portions of distilled water, and the fractions were collected, combined, and saved for radioassay and for further separation by paper chromatography. Sugar phosphates were next eluted with six 0.5-ml aliquots of 0.5 M ammonium formate in 0.2 M formic acid, and the eluates were combined and assayed for radioactivity.

Identification of Free Sugars in Tissue Extracts. Free sugars in the water effluent from the anion exchange column were concentrated in a vacuum desiccator and separated by ascending chromatography on Whatman No. 1 paper using the multiple development technique (4). Five solvent systems were used in the course of this work: (*a*) 1-butanol-acetic acid-H_2O (5:1:4, v/v, upper layer; (*b*) 1-butanol-ethanol-H_2O (5:1:4, v/v, upper layer); (*c*) 1-butanol-pyridine-H_2O (6:4:3, v/v); (*d*) ethyl acetate-pyridine-H_2O (8:2:1, v/v); and (*e*) 1-propanol-ethyl acetate-H_2O (7:1:2, v/v). Sugars were eluted from the chromatograms with water and assayed for ^{14}C-activity.

No effort was made to identify specific sugar phosphates in any experiments.

Radioactivity Measurement. One milliliter aliquots of tissue extract or other ^{14}C-sugar preparations were plated on flat stainless steel planchets and dried under an infrared lamp. Radioactivity was counted with either a thin end-window Geiger-Müller tube or with a scintillation probe equipped with a 2.5-cm diameter anthracene crystal and corrected for self-absorption.

Kinetic Analysis. The graphical methods of Lineweaver and Burk (12) and of Hofstee (11) were used to estimate K*m* and K*i* values for the absorption processes.

Possible Metabolism of Sugars Within the Tissue. Two methods were used to check whether or not a given sugar was utilized by sugarcane internodal tissue. First, tissue slices were incubated in solutions of the ^{14}C-sugar for 4 hr, after which intracellular sugars were extracted, chromatographed, and radioautograms prepared. Absence of multiple spots on the radioautograms was accepted as evidence that the sugar was not metabolized.

Secondly, respiratory CO_2 from tissue discs bathed in ^{14}C-sugar solutions was trapped in $Ba(OH)_2$, and the radioactivity of precipitated $BaCO_3$ was assayed. Both methods indicated that L-sorbose, 2-deoxy-D-glucose, and MeG[2] were not metabolized. Glucose, fructose, galactose, and glucose-6-P were readily metabolized, however.

Chemicals. The following ^{14}C-sugars were obtained from Amersham/Searle Corp., Arlington Heights, Illinois: D-glucose-^{14}C(U); D-fructose-^{14}C(U); L-sorbose-^{14}C(U); D-galactose-1-^{14}C; D-glucose-^{14}C(U)-6-phosphate; 3-O-methyl-D-glucose-^{14}C (U). The 2-deoxy-D-glucose-1-^{14}C was a product of New England Nuclear Corp. The ^{12}C-sugars were purchased from Calbiochem and Nutritional Biochemicals Corp. All sugars were checked chromatographically for purity before use. SITS, NEM, NPM, and PCMB were also products of Nutritional Biochemicals Corp. PCMBS and NDS were obtained from Sigma Chemical and Distillation Products Industries, respectively. Diazo-NDS was prepared by the method of Pardee and Watanabe (13) Nembutal was the product of Abbott Laboratories, North Chicago, Illinois.

RESULTS AND DISCUSSION

Sugar Uptake as a Function of Time and External Concentration. Under the experimental conditions employed, the uptake of glucose, fructose, sorbose, galactose, glucose-6-P, and MeG from 1 mM solutions by immature internodal tissue of sugarcane remained linear for at least 4 hr (Fig. 1). Accumulation actually continued for 24 hr, but linearity of uptake was not checked beyond 4 hr. When uptake of each of these six hexoses was measured as a function of external concentration over the 1 to 15 mM range, the resultant curves were hyperbolic (Fig. 2). Curves such as these are characteristic of many absorption processes and have been accepted as evidence for the mediation of uptake by hypothetical membrane-bound "carriers." With the hexoses studied in these experiments, the absorption systems were not saturated even when the external sugar concentration was 15 mM.

Kinetics of Sugar Absorption. Values of K*m* (mM) for transport of hexoses in immature sugarcane parenchyma tissue were as follows: for glucose, 6.7; fructose, 8.4; sorbose, 5.1; galactose, 7.4; glucose-6-P, 4.0; and MeG, 3.9. The value of 6.7 mM for glucose compares well with that of 7.0 mM reported earlier by Glasziou (7). In more mature internodal tissue, Bieleski (3) found the K*m* for glucose absorption to be 1.7 mM.

Effect of Temperature on Sugar Uptake. Absorption of glucose, fructose, sorbose, and galactose was strongly temperature-dependent (Table I). Tissue was pretreated for 30 min at

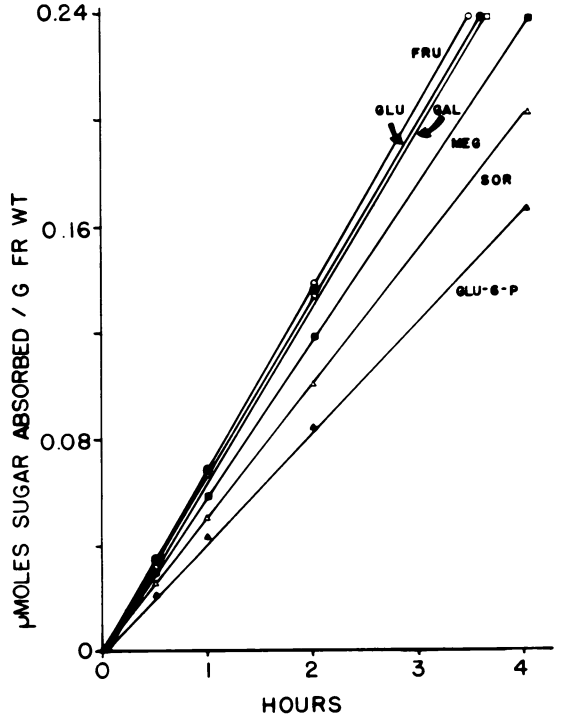

FIG. 1. Transport of fructose, glucose, galactose, MeG, L-sorbose, and glucose-6-P into "inner space" of immature internodal parenchyma tissue of sugarcane. External sugar concentration was 1 mM; pH was 6.5; 28 C.

[2] Abbreviations: MeG: 3-O-methylglucose; PCMB: *p*-chloromercuribenzoate; PCMBS; *p*-chloromercuriphenylsulfonate; NEM: N-ethyl maleimide; NPM: N-phenylmaleimide; NDS: 7-amino-1,3-naphthalenesulfonate; diazo-NDS: 7-diazonium-1,3-naphthalenedisulfonate; SITS: 4-acetamido-4′-isothiocyanostilbene-2′,2′-disulfonate; Nembutal: 5-ethyl-5-(2-pentyl)-barbituric acid, sodium salt.

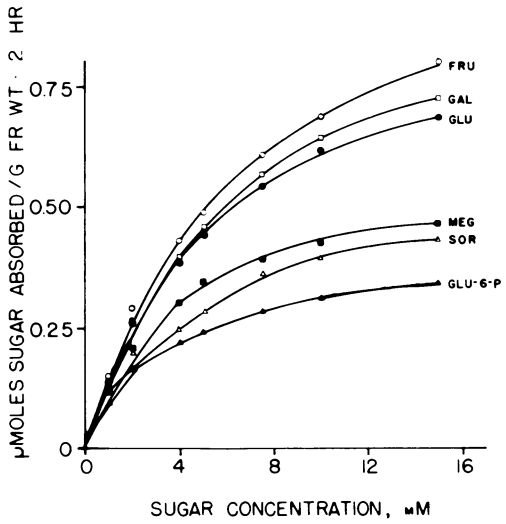

FIG. 2. Sugar accumulation by immature storage tissue as a function of external sugar concentration. Absorption period was 2 hr; pH was 6.5; 28 C.

Table I. *Effect of Temperature on Sugar Uptake and its Reversibility*

External sugar concentration was 1 mM; pH was 6.5.

Temperature (C)		Glucose	Fructose	Sorbose	Galactose
Pre-treatment for 30 min	Incubation	*nmoles sugar absorbed/g fr wt·2 hr*			
2	2	27	29	20	24
7	7	36	56	27	32
12	12	59	66	46	58
16	16	74	80	50	74
20	20	101	131	71	96
24	24	132	136	99	130
28	28	134	138	98	128
32	32	123	100	90	107
39	39	47	42	41	34
50	50	2	1	4	2
2	28	131	135	99	128
28	28	133	137	100	129
39	28	89	89	46	84
50	28	4	10	1	1

the temperature at which sugar uptake was to be measured subsequently. For each sugar tested, maximum absorption occurred at 24 to 28 C (Table I). At 2 C and at 50 C, uptake rates were sharply reduced (Table I).

To determine whether or not the temperature-induced reduction in sugar absorption was reversible, the following experiment was performed. Tissue sections were pretreated for 30 min in aerated 0.5 mM $CaSO_4$ solution at 2 C, 28 C, 39 C, or 50 C. Absorption of each sugar was measured after readjustment of the temperature to 28 C. Statistically there was no difference in absorption of each sugar by tissue pretreated at 2 C after readjustment to 28 C (Table I). Therefore, it must be concluded that the reduction in sugar uptake by low temperature during the absorption period is essentially a reversible inhibition.

In contrast, sugar uptake by tissue pretreated at 50 C was not restored by readjusting the temperature to 28 C (Table I), indicating that heat inactivation of sugar transport is irreversible.

Effect of pH on Sugar Transport. Hawker and Hatch (10) reported that the rate of glucose uptake by mature sugarcane storage tissue was relatively unaffected by pH over the 4.0 to 7.0 range, although sucrose storage varied 10-fold over this range. Since glucose and sucrose are metabolized rapidly by this tissue, the effect of pH on an actively accumulated, but non-metabolized, sugar, *i.e.*, sorbose, was determined. The optimum pH for sorbose uptake was about 6.5 (Fig. 3).

Sugar Transport Under Anaerobic Conditions. Anaerobiosis greatly reduced absorption of sugars by immature internodal tissue of sugarcane, with inhibition ranging from 69.6% for galactose to 80.7% for glucose-6-P (Table II). Failure of anaerobiosis to block sugar absorption completely was probably due to provision of requisite metabolic energy through glycolysis, since N_2 was passed through a vanadyl sulfate-amalgamated Zn gas train to remove residual O_2 before introduction into the sugar solutions.

Effect of Metabolic Inhibitors on Sugar Transport. Accumulation of sugars by internodal parenchyma cells of sugarcane is apparently a metabolically mediated process in that it is sensitive to pH, temperature and anaerobiosis. Further, the absorption mechanism is characterized by saturation kinetics. Therefore, effects of respiratory inhibitors, uncouplers of oxidative phosphorylation, and non-specific SH inhibitors on sugar accumulation were ascertained.

Sugar absorption was strongly inhibited by 0.1 mM NaN_3, 1 mM CN^-, 10 mM arsenate, and 10 mM NaF and was completely inhibited by 10 μM Nembutal (Table III).

An uncoupler of oxidative phosphorylation, DNP, was also

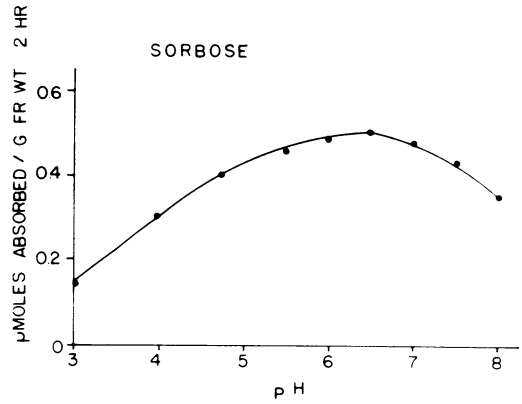

FIG. 3. Uptake of L-sorbose by immature sugarcane storage tissue as a function of pH. Absorption period was 2 hr; external sugar concentration was 1 mM; 28 C.

Table II. *Effect of Anaerobic Conditions on Sugar Transport in Immature Internodal Tissue of Sugarcane*

External sugar concentration was 1 mM; pH was 6.5; 28 C.

Sugar	Air	N_2
	nmoles sugar absorbed/g fr wt·2 hr	
Glucose	134	37
Fructose	136	32
Sorbose	96	21
Galactose	135	41
Glucose-6-P	88	17
3-O-Methylglucose	114	25
2-Deoxy-D-glucose	116	25

inhibitory to sugar transport, as evidenced by the decreased uptake in the presence of 10 and 100 μM DNP (Table III).

Inhibition of uptake of glucose, fructose, sorbose, and galactose by SH inhibitors was studied also. Uptake of each sugar was inhibited by 73% or more by 1 mM PCMB, 1 mM PCMBS, 2 mM iodoacetate, 2 mM iodoacetamide, 1 mM NEM, and 1 mM NPM (Table III).

The protein reactive reagents NDS and SITS did not significantly alter the rates of sugar uptake, but diazo-NDS reduced uptake of glucose, fructose, sorbose, and galactose by 32, 31, 33, and 33%, respectively (Table III).

Interactions in Sugar Uptake. Possible mutual interactions in the absorption of hexoses and hexose derivatives by immersed immature internodal parenchyma sections of sugarcane were studied in a series of factorially designed experiments. Each bathing solution contained 1 mM ^{14}C-sugar, and one nonradioactive sugar, also at a concentration of 1 mM. Controls contained only the ^{14}C-sugar. All sugars used in these experiments—glucose, fructose, sorbose, galactose, glucose-6-P, 2-deoxy-D-glucose and MeG—mutually inhibited uptake of the others (Table IV). The inhibitions were competitive, as determined from double reciprocal plots, implying that each sugar was absorbed via the same carrier sites. There were, however, wide differences in the affinities of the carrier sites for different sugars. Inhibitor constants (K*i*) for the competing sugars, measured against glucose as the substrate, were as follows (mM): for fructose, 14.8; sorbose, 50.0, galactose, 6.9; glucose-6-P, 26.0; and MeG, 21.7. As an example of the differences in carrier site affinities, consider the K*m* value of 6.7 mM for glucose as the substrate and the K*i* value for the competing sugar, *e.g.*, sorbose, respectively, which indicate that affinity of these sites for glucose is seven times greater than that for sorbose. Estimates of other relative affinities may be calculated from these data also.

Table III. *Effect of Metabolic Inhibitors on the Absorption of Glucose, Fructose, Sorbose, and Galactose by Immature Internodal Tissue of Sugarcane*

External sugar concentration was 1 mM; pH was 6.5; 28 C.

Additive	Glu	Fru	Sor	Gal
	nmoles absorbed/g fr wt·2 hr			
Control (no additive)	136	142	94	133
0.1 mM NaN_3	34	33	19	29
1 mM CN^-	42	37	19	38
10 mM Arsenate	48	31	26	26
10 mM NaF	39	44	21	41
10 μM Nembutal	0	0	0	0
10 μM DNP	106	96	40	88
100 μM DNP	43	31	16	27
1 mM PCMB	21	13	12	15
1 mM PCMBS	37	35	25	28
2 mM Iodoacetate	17	9	9	13
2 mM Iodoacetamide	11	2	12	8
1 mM NEM	9	12	7	9
1 mM NPM	6	9	9	10
0.2 mM SITS	132	146	96	132
0.2 mM NDS	128	130	87	119
0.2 mM Diazo-NDS	93	98	63	89

Table IV. *Mutual Effects of Hexoses and Hexose Derivatives in their Absorption by Immature Internodal Tissue of Sugarcane*

External sugar concentrations was 1 mM; pH was 6.5 and the temperature was 28 C.

Nonlabeled Sugar Present	Glucose	Fructose	Sorbose	Galactose	Glucose 6-P	MeG	2-Deoxy-D-glucose
1 mM	*nmoles absorbed/g fr wt·2 hr*						
Control (none)	137	139	94	131	92	118	114
Glucose	...	84	21	58	31	46	52
Fructose	77	...	27	50	24	41	53
Sorbose	124	130	...	119	65	101	96
Galactose	64	78	27	...	30	57	57
Glucose-6-P	110	118	47	104	...	78	80
3-O-Methylglucose	79	90	19	64	27	...	61
2-Deoxy-D-glucose	83	79	29	68	33	55	...

Table V. *Contribution of ^{14}C from Supplied Sugars to Components of the Alcohol-soluble Neutral Fraction of the Tissue Extract*

One g fr wt of tissue was incubated for 4 hr at pH 6.5 and 28 C in 10 ml of 1 mM ^{14}C-hexose.

^{14}C-Hexose Supplied	Percentage of ^{14}C in Alcohol-soluble Neutral Fraction of Tissue Extracted after 4-hr Incubation with ^{14}C-Hexose						
	Sucrose	Glucose	Fructose	Sorbose	Galactose	Unidentified	P-Sugars
Glucose	81	4	6	...	...	8	1
Fructose	78	4	8	...	...	8	2
Sorbose	...	...	...	97	...	3	...
Galactose	28	2	3	...	59	7	1
Glucose-6-P	66	3	5	...	...	12	14

Table VI. *Distribution of ^{14}C from Exogenously Supplied ^{14}C-Glucose in Intracellular Sugars as a Function of Time*

The pH was 6.5 and the temperature was 28 C.

Minutes	Percentage of Total Intracellular ^{14}C			
	Glucose	Fructose	Sucrose	Sugar phosphates
0.25	16	2	0	82
0.50	18	4	2	76
0.75	13	3	12	72
1	8	4	29	59
2	9	4	51	36
3	11	3	56	30
4	3	6	70	21
5	4	3	74	19

Intracellular Conversions of Absorbed Sugars. Sucrose was the predominant sugar found in the tissue after 4-hr incubation in glucose, fructose, or glucose-6-P (Table V). Glasziou (6) previously reported that sucrose accumulated in young internodes, regardless of whether glucose or sucrose was supplied in the bathing solution. When galactose-^{14}C was the exogenous sugar in the present study, 59% of the radioactivity in the extract remained in galactose and 28% was in sucrose (Table V). Less than 12% of the ^{14}C-activity of the extract was in free glucose or fructose when the external solution contained either glucose, fructose, glucose-6-P, or galactose (Table V). Exogenously supplied glucose-6-P resulted in recovery of 14% of the radioactivity in the sugar-P fraction (Table V).

The metabolic inertness of sorbose was confirmed in this experiment. When tissue discs were bathed in ^{14}C-sorbose, 97% of the intracellular ^{14}C-activity was recovered in free sorbose (Table V).

Preliminary Studies of Phosphorylation Associated with

Sugar Transport. Tissue discs used in these experiments were cut thinner (6 mm diameter × 75 μ thick) than in other studies reported herein to facilitate equilibration with ^{14}C-sugar solutions in short term uptake measurements. Tissue discs (0.5 g fresh weight) were incubated in 1 mM ^{14}C-glucose at 28 C and pH 6.5. Tissue samples were removed at 15-sec intervals for 1 min and at 1-min intervals for an additional 4 min, rinsed for 15 sec, and frozen in Dry Ice-acetone for later extractions. There was thus a 15 to 20 sec lapse between sampling and cessation of metabolic activity. The data in Table VI have been corrected for lapsed time, *i.e.*, tissue samples were actually removed from the bathing solution 15 sec prior to the time stated. Intracellular sugars were extracted from the frozen tissue, separated into free and phosphorylated sugar fractions, and the free sugar fraction was chromatographed.

As seen from Table VI, ^{14}C-sugar phosphates accumulated in the cells within 15 sec. Sucrose constituted a major portion of intracellular sugars after the first minute of incubation, increasing rapidly through 5 min. The initial high level of ^{14}C in phosphorylated sugars indicates that phosphorylation may play a role in glucose transport. However, it is not possible at this stage to ascertain whether phosphorylation occurs prior to, during, or subsequent to the theoretical carrier-sugar complex formation. This aspect of sugar absorption will be dealt with in detail in a paper now in preparation.

CONCLUSIONS

Sugar transport in immature internodal parenchma of sugarcane is an active process, as judged by its sensitivity to pH, temperature, anaerobiosis, metabolic inhibitors, and presence of competing sugars in the medium. Inhibitions of transport by respiratory inhibitors and an uncoupler of oxidative phosphorylation indicate that the energy required for transport may be derived from oxidative phosphorylation. The seven sugars studied in these experiments—glucose, fructose, galactose, sorbose, glucose-6-P, MeG, and 2-deoxy-D-glucose—are mutually competitive for uptake and so appear to be transported via the same carrier sites.

Metabolism of transported sugars has posed a problem in interpreting results from previous sugar transport studies conducted over periods as long as 24 hr and was likewise a difficulty in these experiments. Extensive interconversions of glucose, fructose, galactose, and glucose-6-P occur within 4 hr and, in the case of glucose, some ^{14}C appeared in fructose and unidentified sugar phosphates within 15 sec.

Acknowledgments—I thank the personnel of Mauna Kea Sugar Company for providing the sugarcane tissue used in these studies. I am also indebted to Dr. Andrew Maretzki and Miss Margaret Thom of the Hawaiian Sugar Planters' Experiment Station, Honolulu, Hawaii, for many helpful discussions.

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