Sugar Accumulation Cycle in Sugar Cane. I. Studies on Enzymes of the Cycle[1]

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Introduction

From studies of sugar accumulation with tissue slices of sugar cane storage tissue (5, 6,13) the operation of a number of enzymes must be postulated. Included among these are enzymes for the synthesis and breakdown of sucrose, the formation and breakdown of hexose phosphates, and the interconversion of glucose and fructose. This paper describes the identification of many of these enzymes in preparations from sugar cane storage tissue.

Methods & Materials

Plant Materials. Most studies were carried out with enzyme preparations from cane varieties CO281 and NCO310 which are interspecific hybrids of Saccharum. Other cane varieties used in surveys for various enzymes are described in the text.

Reagents. The following reagents were obtained from commercial sources: sucrose-U-C^{14}, fructose-U-C^{14}, and glucose-U-C^{14} (Radiochemical Centre, Amersham,), UDP[3] and glucose-1-P (Sigma Chemical Co.), UDP-glucose and UTP (Pabst laboratories,), analytical invertase (Difco Laboratories,). 1-kestose (1^F-fructosylsucrose), and 6-kestose (6^F-fructosylsucrose) were kindly provided by Dr. Gagolski. Fructose-U-C^{14}-6-phosphate was prepared by incubating fructose-U-C^{14} with ATP, Mg^{++} and yeast hexokinase (Sigma Chemical Co.), and was purified by paper chromatography.

Preparation of Enzymes. Except where otherwise indicated the following procedure was employed for the extraction and partial purification of enzymes. The expressed juice of the rind-free tissue was immediately cooled to 0°, adjusted to pH 7.0 with 1 N KOH, then four volumes of saturated $(NH_4)_2SO_4$ (pH 7.0, 4°) was added. After standing for 2 hours at 0° the precipitated protein was obtained by centrifuging at 15,000 × *g*. The protein was dissolved in a small volume of 0.01 M potassium citrate buffer (pH 7.0) and dialysed against 2 liters of the same buffer for 8 hours with stirring. Insoluble material remaining after dialysis was removed by centrifugation at 20,000 × *g* and the supernatant fluid was stored at −15°. A similar procedure was used for leaf and sheath preparations. Protein was determined spectrophotometrically (17). Invertase associated with the cell debris was obtained by homogenizing the tissue for 2 minutes in a blendor, centrifuging the tissue homogenate at 2,000 × *g*, then washing the residue 4 times with 10 volumes of distilled water.

Assay of Enzymes. For studies with radioactive substrates, aliquots of reaction mixtures were co-chromatographed on Whatman No. 1 paper with unlabelled markers using ethyl acetate: pyridine: water (8:2:1) as the eluting solvent. Sucrose, glucose, and fructose were located on chromatograms by spraying with p-anisidine phosphate. The radioactivity in individual compounds was determined either by direct counting of the developed spots with a Geiger-Müller tube, or by passing the chromatograms through a recording strip-counter. All assays were at 30° and reaction mixtures which were incubated for more than 2 hours were saturated with toluene to prevent interference by microorganisms.

Two procedures were used in attempts to identify sucrose phosphorylase. These involved the measurement of sucrose-C^{14} formed from fructose-U-C^{14} and glucose-1-P, or of labelled hexoses from sucrose-U-C^{14} plus arsenate or phosphate (8). For sucrose synthesis reaction mixtures contained enzyme preparation, 3 to 7 mg of protein; glucose-1-P, 5 μmoles; fructose-U-C^{14}, 1.3 μmoles (3,500,000 dpm), and potassium citrate buffer (pH 6.8), 10 μmoles, in a total volume of 0.22 ml. For sucrose phosphorylysis or arsenolysis, reaction mixtures contained the enzyme preparation, 3 to 7 mg of protein; sucrose-U-C^{14}, 1.3 μmoles (1,300,000 dpm) and either 5 μmoles of phosphate or 2.5 μmoles of arsenate and potassium citrate buffer (pH 6.8), 10 μmoles, in a total volume of 0.2 ml.

UDP-glucose-fructose transglucosylase was assayed by measuring the incorporation of fructose-U-C^{14} into sucrose in the presence of UDP-glucose. Ex-

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[3] Abbreviations: UMP, uridinemonophosphate; UDP, uridinediphosphate; and UTP, uridinetriphosphate.

cept where otherwise indicated reaction mixtures contained the enzyme preparation, 2 to 3 mg of protein; fructose-U-C^{14}, 1.3 μmoles, (3,500,000 dpm); UDP-glucose, 0.75 μmole in 0.05 M tris-HCl buffer (pH 8.2). The total volume was 0.2 ml.

UDP-glucose pyrophosphorylase activity was detected by replacing UDP-glucose with UTP, 2.5 μmoles; glucose-1-P, 5 μmoles and $MgCl_2$, 2 μmoles, in reaction mixtures as described for UDP-glucose-fructose transglucosylase. Activity was measured by the formation of labelled sucrose.

Invertase activity was determined either by measuring radioactivity in glucose and fructose formed from sucrose-U-C^{14} or by measuring the formation of reducing sugars from unlabelled sucrose in a Technicon Auto-analyser using a modification of the method described by Hoffman (10). For the hydrolysis of 1-kestose by invertase, aliquots of reactions were chromatographed, and the sugars detected with p-anisidine phosphate or silver nitrate using the dip method. The chromatograms were then examined in a recording reflectance densitometer to determine the amounts of individual sugars formed. Standard sugar solutions, chromatographed and treated in the same manner, were used to quantitate the method.

Results

Sucrose Phosphorylase. In the present investigation we were unable to repeat observations that sucrose phosphorylase is present in sugar cane tissue (12, 14, 15). Preparations from immature storage tissue of two varieties, mature storage tissue of six varieties including canes from *S. officinarum*, *S. spontaneum* and hybrids, and young leaves of the *S. officinarum* variety, Badila, were tested. Assays were conducted both in the presence and absence of tris. It will be shown in a subsequent section that the invertase present in most preparations was completely inhibited by 0.025 M tris. Some preparations contained a phosphatase which hydrolyzed only a small proportion of the added glucose-1-P during the incubation periods used.

Enzymes Involved in Sucrose Synthesis. UDP-glucose-fructose transglucosylase is widely distributed in plant tissues (1). In this investigation, the enzyme was isolated from sugar cane storage tissue. Incorporation of fructose-U-C^{14} into sucrose was dependent upon added UDP-glucose (table I). When incubated for a sufficient period, UDP-glucose was almost quantitatively converted to sucrose. Several attempts to detect reversal of the reaction by supplying sucrose-U-C^{14} and UDP were unsuccessful. However, the enzyme preparation contained a phosphatase which rapidly hydrolyzed UDP to UMP, although UTP was hydrolyzed only slowly.

In separate experiments the pH optimum for sucrose synthesis and Michaelis constant for fructose were determined. The apparent pH optimum was 8.2 compared with 7.4 for the wheat germ enzyme. However, the sugar cane enzyme was active over a relatively broad pH range, showing more than 50 % of the maximum activity at pH 7.0 and pH 9.0. The Km for fructose was calculated to be 3×10^{-3} M. The Km for UDP-glucose was very low and could not be accurately determined; the enzyme was saturated with respect to UDP-glucose at 1×10^{-3} M (table I). The product, sucrose, was identified by co-chromatography with unlabeled sucrose on paper and by hydrolysis with yeast invertase followed by identification of the products.

When fructose was replaced by 0.3 μmole of fructose-U-C^{14}-6-P and 0.02 M KF in reaction mixtures of the composition described for UDP-glucose-fructose transglucosylase, only about 1 % of the label appeared in free sucrose in 1 hour. Approximately 3 % of the labeled fructose-6-P was converted to a compound which had the properties of sucrose phosphate. It remained at the origin of chromatograms

Table I

Sucrose Synthesis from UDP-Glucose & Fructose

Expt.	Reaction conditions*	Incubation time (min)	Sucrose formed (μmoles/reaction)
1.	Standard reaction	30	0.30
	"	60	0.55
	"	150	0.72
	" minus UDPG	60	0
	" but 0.2 μmole UDPG	15	0.14
	" but 1.4 μmoles UDPG	30	0.29
	" plus 0.025 M pyrophosphate	60	0.03
2.	Standard reaction	60	0.23
	" plus G-I-P, UTP, Mg^{++}	60	0.17
	" minus UDPG plus G-I-P, UTP, Mg^{++}	60	0.18
	" minus UDPG plus G-I-P, Mg^{++}	120	0
	" minus UDPG plus UTP, Mg^{++}	120	0.051**

* The composition of the standard reaction is described in the Methods and Materials section. Reactions of experiment 1 and 2 contained 3 mg and 1.5 mg of protein respectively. Enzyme was from mature storage tissue of variety CO281. The abbreviations UDPG for uridinediphosphoglucose and G-I-P for glucose-1-phosphate are used in the table.

** Calculated on the basis that sucrose was equally labeled in the glucose and fructose moieties.

Table II

UDP-Glucose-Fructose Transglucosylase in Storage Tissue from Different Canes

Source on enzyme* (cane variety)	Sucrose synthesized**	
	mμmoles/mg protein-hr	mμmoles/g fr wt-hr
CO281 (mature)	51	53
Badila (mature)	8	24
Chunnee (mature)	27	81
Fiji 28 (mature)	28	57
Fiji 10 (mature)	20	20
Mandalay (mature)	0	0
NCO310 (immature)	2	26
CO281 (immature)	8	18

* Mandalay is a *S. spontaneum* and Chunnee a *S. sinense*, the remainder being either *S. officinarum* or hybrids. The maturity of the storage tissue is indicated in brackets.

** Reaction mixtures were as described in Methods and Materials. Incubation time was 2 hours.

developed with the ethylacetate-pyridine-water solvent but moved with sucrose after treatment with a thymus alkaline phosphatase preparation. After phosphatase treatment the compound was hydrolyzed by invertase.

UDP-glucose-fructose transglucosylase was found in preparations from a mature and immature storage tissue of a number of varieties of *S. officinarum* and hybrid canes (table II). Activity per mg of protein was relatively low in immature tissue extracts but was similar to that for mature tissue when expressed on a fresh weight of tissue basis. Activity was absent in the only *S. spontaneum* preparation examined. Interference by invertase was overcome by conducting the assays in tris-HCl buffer.

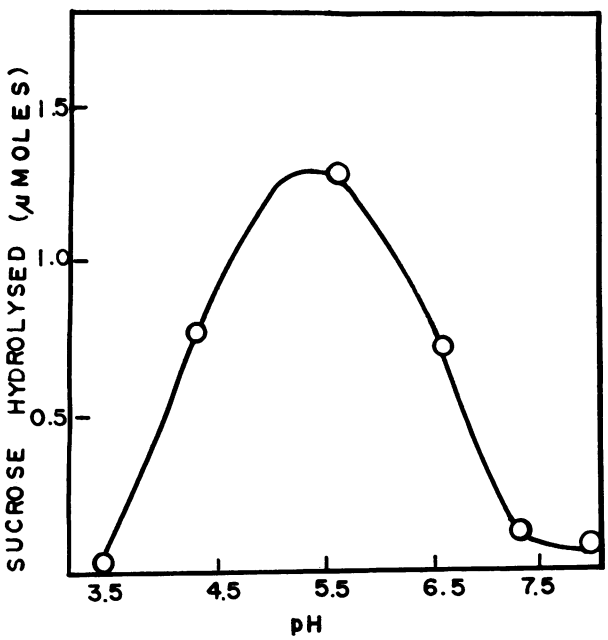
Evidence was also obtained for UDP-glucose pyrophosphorylase, the presence of which has been reported in several plant tissues (11, 16). Glucose-1-P, UTP and Mg^{++} could replace UDP-glucose for sucrose synthesis (table I). One or more of these components caused some inhibition of the incorporation of fructose-U-C^{14} into sucrose in the presence of UDP-glucose. Making allowance for this, the activity of UDP-glucose pyrophosphorylase in the extract was at least as high as that for UDP-glucose-fructose transglucosylase. Inhibition of UDP-glucose-fructose transglucosylase by pyrophosphate (table I) was probably due to breakdown of UDP-glucose via UDP-glucose pyrophosphorylase. When UTP was omitted no labeled sucrose was formed and activity was also very low in the absence of Mg^{++}. However sucrose synthesis was observed when UTP, Mg^{++} and fructose-U-C^{14} were supplied without glucose-1-P. The sucrose formed under these conditions was equally labeled in the glucose and fructose moieties. Apparently in this system fructose was phosphorylated to fructose-6-P and the fructose-6-P was then converted to glucose-1-P by the action of glucose-6-P isomerase and phosphoglucomutase. Other experiments showed that the preparation contained a hexokinase which utilized both ATP and UTP. When glucose-1-P was added in addition to UTP, Mg^{++} and fructose-U-C^{14}, there was no significant incorporation of radioactivity into the glucose moiety of sucrose. The added glucose-1-P would dilute any labeled glucose-1-P formed from fructose.

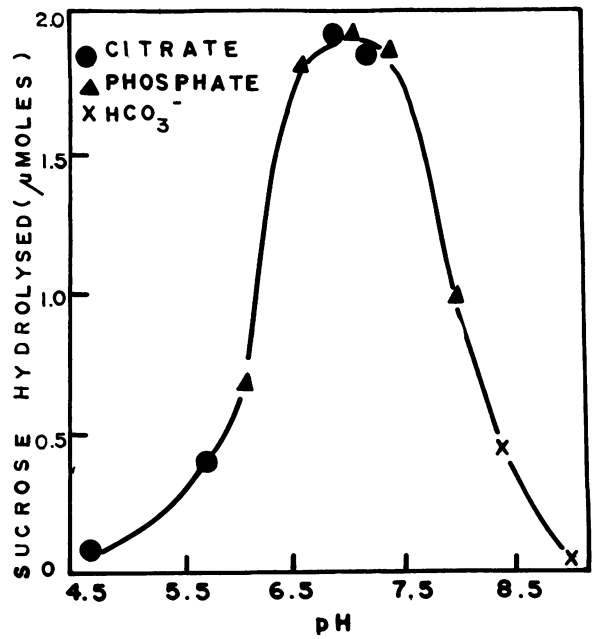
Metabolism of Hexose Phosphates. Some evidence for hexokinase, glucose-6-P isomerase, and phosphoglucomutase in mature tissue preparations has already been described. With preparations from mature and immature storage tissue of three varieties (CO281, NCO310, & Pindar), the label from glucose-U-C^{14} and fructose-U-C^{14} appeared in the hexose phosphates when ATP and Mg^{++} were supplied and labeled glucose was also formed from fructose-U-C^{14}. With the same preparations glucose and fructose were formed from fructose-U-C^{14}-6-phosphate. These results also indicate the presence of enzymes catalyzing the formation, interconversion, and breakdown of glucose-6-P and fructose-6-P in sugar cane storage tissue.

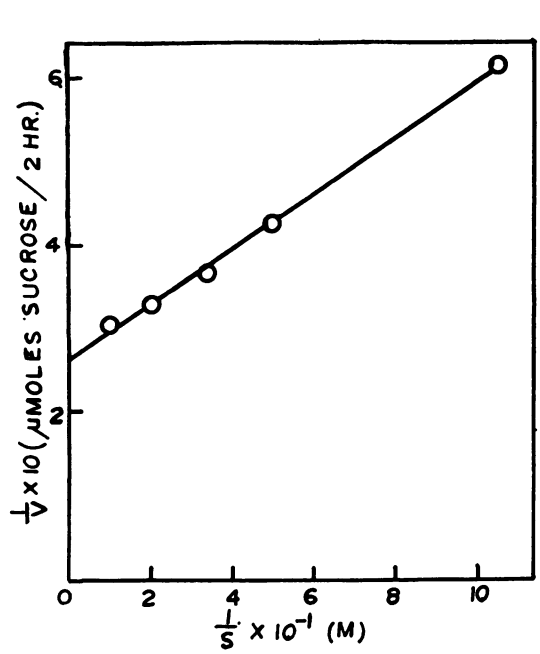
Invertases. Some of the properties of an invertase from immature tissue of sugar cane have been described in a preliminary report (7). This enzyme is optimally active between pH 5.0 and 5.5. Another invertase with a pH optimum of 7.0 has now been isolated from mature storage tissue. These enzymes are referred to subsequently as the acid invertase and the neutral invertase respectively. The pH activity curves for the two invertases are shown in figures 1 and 2. Both enzymes were active over a broad range of pH, each showing about 10 % of its maximum activity at the pH optimum for the other enzyme. Invertases with activity optima near pH 5.5 were also isolated from sugar cane leaf and sheath. The two invertases had similar km values for sucrose. Using a Lineweaver-Burk plot the values obtained were 2.5×10^{-2} M for the neutral invertase and 1.3×10^{-2} M for the acid invertase (fig 3 & 4).

The neutral invertase was completely inhibited by 0.05 M tris, and at 0.004 M inhibition was almost 80 %. At its pH optimum, the acid invertase was inhibited 25 % by 0.05 M tris. This difference was probably due to the effect of pH on the tris-enzyme interaction since the acid invertase was inhibited to a greater extent at pH values above its optimum.

Some invertases catalyze transfructosylation reactions to yield 1-kestose and other oligosaccharides (2). When incubated with 0.5 M sucrose-U-C^{14} both cane invertases catalyzed the formation of small quantities of labeled compound which co-chromatographed with 1-kestose. The radioactive area corresponding to 1-kestose was isolated free of sucrose by elution from chromatograms, then treated with yeast invertase. This treatment degraded most of the original compound, the radioactivity appearing in glucose and fructose in a 1 to 2 ratio.







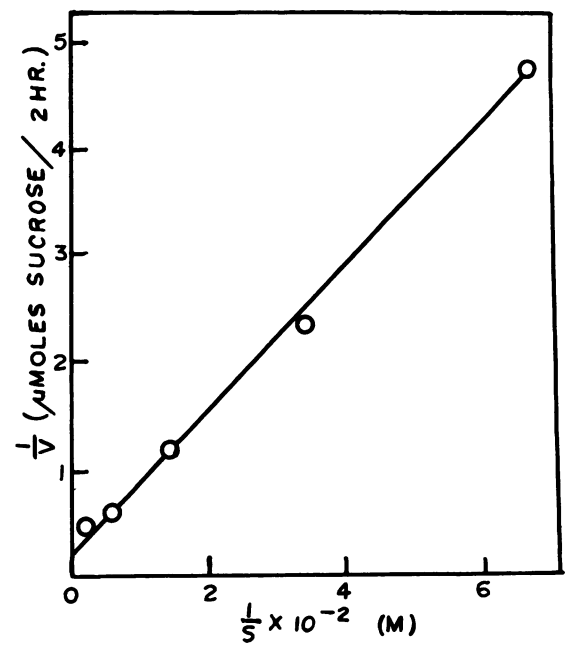


FIG. 1 (*upper left*). pH-activity curve for acid invertase from immature tissue of variety NCO310. Reaction mixtures contained enzyme (0.5 mg of protein), McIlwaine phosphate-citrate buffer adjusted to the desired pH and 15 μmoles of sucrose-U-C^{14} in a final volume of 0.6 ml. Incubation time was 1 hour. The reaction was followed by measuring the isotope in glucose and fructose.

FIG. 2 (*upper right*). pH-activity curve for neutral invertase from mature tissue of variety CO281. Reaction mixtures contained enzyme (6 mg of protein), 0.2 M sucrose and buffer in a total volume of 1.0 ml. Different buffers, as indicated in the figure, were added to a final concentration of 0.05 M. Reactions were incubated for 2 hours. Activity was measured by formation of reducing sugars.

FIG. 3 (*lower left*). Lineweaver-Burk plot for acid invertase from immature tissue variety NCO310. Reaction mixtures contained enzyme (0.8 mg of protein), sucrose and 0.05 M citrate buffer (pH 5.4) in a total volume of 1.0 ml. Activity was measured by the formation of reducing sugars after incubating for 1 hour.

FIG. 4 (*lower right*). Lineweaver-Burk plot for neutral invertase from mature tissue of variety CO281. Reaction mixtures contained enzyme (6 mg of protein) and sucrose in 0.03 M citrate buffer (pH 7.1). The total volume was 1.0 ml. Reactions were incubated for 2 hours then the formation of reducing sugars measured.

Both the acid and the neutral invertase preparations hydrolyzed 1-kestose to reducing sugars. The rate of hydrolysis was from 5 % to 10 % of that for sucrose. In a reaction mixture containing the acid invertase and 0.05 M 1-kestose the hydrolysis products were identified as a mixture of sucrose, glucose, and fructose after approximately 10 % of the kestose had been broken down. During these studies we found that 1-kestose inhibited the hydrolysis of sucrose by the acid invertase. At 5×10^{-2} M, 1-kestose inhibited the hydrolysis of 5×10^{-3} M sucrose-U-C^{14} by 62 %. Sucrose hydrolysis by the neutral invertase was not affected by this concentration of 1-kestose.

Fungal invertase (3) catalyzes the incorporation of labeled hexoses into sucrose in the presence of unlabeled sucrose. We incubated cane invertase preparations with either glucose-U-C^{14} or fructose-U-C^{14} in the presence of 0.25 M unlabeled sucrose but observed no incorporation of the label into sucrose.

Invertase was detected in washed cell residues of immature storage tissue, obtained by centrifugation of homogenates at 2,000 × *g*. With three varieties (CO281, NCO310, & Pindar) approximately 5 % of the total invertase of the tissue was associated with the cell fragments. The pH optimum and Km of sucrose for the bound invertase was 4.4 and 8×10^{-3} M respectively compared with values of 5.3 and 1.3×10^{-2} M for the soluble invertase obtained from the same preparations. At pH 3.4 the enzyme showed 85 % of its maximum activity.

Seasonal Variation of Acid Invertase. The acid invertase of immature storage tissue showed an interesting seasonal variation of activity (fig 5). The period of highest activity corresponded to the period of rapid growth. When growth declined as the result of cooler and drier conditions which prevail between May and October the enzyme was barely detectable. During this period the sucrose content of mature tissue increased rapidly. The correlation between acid invertase activity and growth rate of immature tissue has been examined more precisely using controlled environment facilities (9).

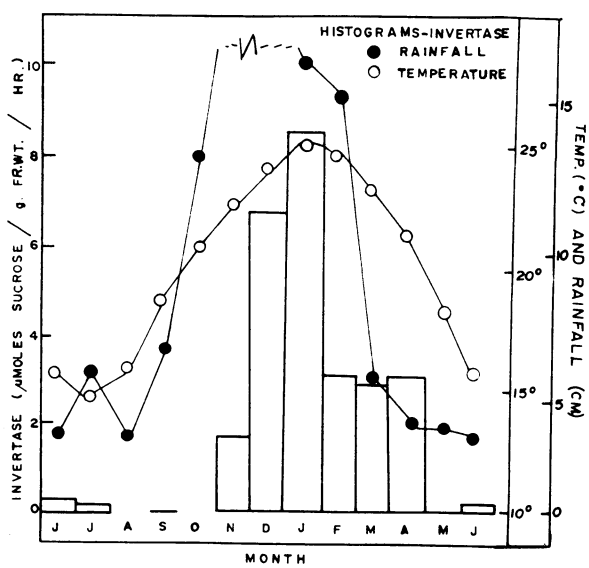


FIG. 5. Seasonal effect on acid invertase activity of immature storage tissue. Enzyme was obtained from immature tissue (variety NCO310) of plants grown in the same plot. The tissue was homogenized then the expressed juice was filtered, dialyzed for 16 hours at 2°, and assayed as described in the Methods and Materials section.

Discussion

Sucrose phosphorylase was originally isolated from Pseudomonas saccharophila by Hassid and coworkers (8). Shukla and Prabhu (14, 15) and Pandya and Ramakrishnan (12) have published brief descriptions of enzyme preparations from sugar cane juice and leaves, respectively, which apparently have sucrose phosphorylase activity. Attempts by other workers to demonstrate its occurrence in higher plant tissues were unsuccessful (4, 8). In the present investigation we could not demonstrate the operation of sucrose phosphorylase in a number of sugar cane tissue extracts, which however did contain UDP-glucose-fructose transglucosylase. In view of repeated failure to detect sucrose phosphorylase activity in sugar cane extracts, confirmatory work is required to prove the presence of this enzyme in higher plants.

A scheme depicting the metabolic transformation of sugars associated with sugar accumulation by sugar cane storage tissue has been proposed (13). In the present paper, enzymes which catalyze most of the reactions in this scheme were identified in preparations from sugar cane storage tissue. No precise information was obtained about the reactions leading to the formation of the proposed sucrose derivative, termed sucrose-X (13). However some storage tissue preparations converted UDP-glucose and fructose-6-P to a compound with the properties of sucrose phosphate. Because of the thermodynamic requirements for sugar accumulation against a concentration gradient we consider that UDP-glucose-fructose transglycosylase has no function in sugar accumulation (13). Support for this view comes from unpublished experiments in which we found that this enzyme is localized in the vascular tissue associated with the storage parenchyma.

Summary

I. Enzymes believed to be operative in the cyclic process of sugar storage in sugar cane have been isolated and described.

II. Uridinediphosphoglucose-fructose transglucosylase and uridinediphosphoglucose pyrophosphorylase were identified in preparations from mature and immature storage tissue. There was evidence for uridinediphosphoglucose-fructose-6-phosphate transglucosylase in some preparations. Contrary to previous reports sucrose phosphorylase could not be iso-

lated from sugar cane storage tissue or leaves.

III. Enzymes for the synthesis, interconversion, and breakdown of hexose phosphates were identified in mature and immature storage tissue.

IV. A soluble invertase with a pH optimum between 5.0 and 5.5 was isolated from immature storage tissue. A small proportion of the total invertase of immature tissue was associated with insoluble cell material. The properties of the insoluble invertase differed from those of the soluble invertase.

V. A soluble invertase with a pH optimum of 7.0 was present in mature storage tissue, but was not detected in immature tissue.

VI. Both soluble invertases were inhibited by tris (hydroxymethyl) aminomethane, the extent of inhibition being apparently related to pH. Each invertase formed 1-kestose from sucrose and also hydrolyzed added 1-kestose. The inversion of sucrose by the acid invertase was inhibited by added 1-kestose, but a similar effect was not observed with the invertase with a pH optimum of 7.0.

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