Action Patterns of Sugarcane Acid Invertase

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INTRODUCTION

Sugarcane invertases have received much attention from the standpoint of number and properties (*2,3,14*),[2] physiological roles (*3,12,15*), and behavior in treated plants (*4,5*). Most assay techniques are based upon the ultimate appearance of fructose and glucose in a standarized reaction digest. Studies in Puerto Rico have all assumed that sucrose yields both fructose and glucose upon hydrolysis, or that the trisaccharides raffinose and melezitose yield free fructose and glucose, respectively (*2,3*). No attention was given to the reaction products other than to measure total reducing power.

More recent studies at this Station were centered upon individual sugars arising by invertase action. Techniques of paper chromatography proved to be of tremendous value. This paper summarizes the types of substrate hydrolyzed, number and behavior of products, and subsequent enzyme action upon initial invertase products.

MATERIALS AND METHODS

ENZYME PREPARATION AND ASSAY

Invertase was extracted with water from lyophilized meristem and immature storage tissues, in accordance with procedures described previously (*2*). The enzyme was precipitated from solution between 38- and 52-percent saturation by ammonium sulfate. Although invertase is inactivated by prolonged dialysis, a 2-hour treatment against distilled water was needed for removal of contaminant reducing sugar.

Enzyme assay was accomplished by measuring the amount of reducing sugar formed in a buffered solution of enzyme and substrate. The standard digest was composed as follows: 1.0 ml. of 0.1-M[3] acetate buffer, pH 5.5; 1.0 ml. of 1-percent substrate solution; and 0.5 ml. of enzyme preparation representing 10 to 15 units. The reaction proceeded for 1 hour at 37°C. and was then terminated by addition of 1.0 ml. of dinitro reagent (*23*). Upon development of color, reducing-sugar content was entimated by reference to a standard curve representing 0.01 to 0.25 mg. of *d*-glucose.

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[2] Italic numbers in parentheses refer to Literature Cited, pp. 152–3.

[3] The letter M signifies "molar" herein.

CHROMATOGRAPHY OF INVERTASE PRODUCTS

Most of the chromatography experiments employed enzyme digests which had been active for many hours. For such studies a digest of several times the standard volume was used. Aliquots of 0.5 ml. volume were taken periodically and inactivated by boiling for 5 minutes. A comparison of heat *vs.* chemical inactivation techniques is summarized in table 1. The heat treatment was considered much superior.

Enzyme products were identified by paper chromatography using a solvent mixture of butanol-pyridine-water (6:4:3, *v/v*). Each of the solvent components was redistilled under glass. Sheets of No. 1 Whatman filter paper were thoroughly washed with solvent and dried prior to receiving

TABLE 1—*Chemical and heat inactivation of sugarcane invertase*[1]

Treatment	Micromoles of additive per milliliter of digest—					
	0	0.0001	0.001	0.01	0.1	1.0
KI	23.5	25.0	24.5	25.0	21.5	16.0
$HgCl_2$	25.4	15.2	6.2	0	0	0
Treatment	Minutes in boiling water—					
	0	1	2	4	8	12
Heat	24.0	5.7	0	0	0	0

[1] Enzyme values are expressed as specific activity.

enzyme samples. Controls were established by spotting 12 λ of 1-percent sucrose, glucose, and fructose solution made up in boiled enzyme preparation. From 20 to 30 λ of inactivated digest were needed to obtain well-formed spots, although at times the digest samples were concentrated by freeze-drying prior to application.

The descending technique was employed in one dimension for all experiments. Resolution of sucrose, fructose, and glucose was obtained in as little as 12 hours, while additional time was needed for slower moving trisaccharides. Even after 48 hours the substrate stachyose was not clearly separated from one of its reaction products.

Sugars were revealed on dryed chromatograms by the silver nitrate "dip" method of Dube and Nordin (*9*). The sheets were dipped first in acetone saturated with silver nitrate. After drying, these were immersed in methanol containing 0.4-percent NaOH and allowed to remain until spots were clearly defined. Chromatograms were oven-dried following stabilization and wash-

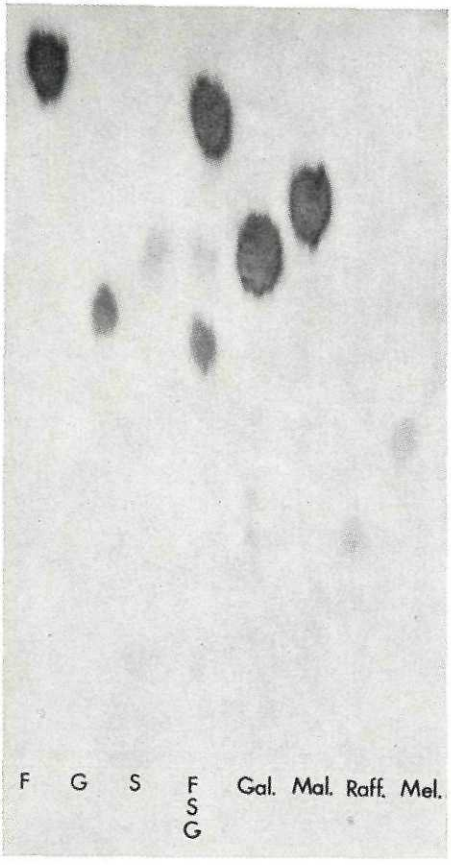


FIG. 1.—Relative chromatographic mobility and staining properties of several sugars studied during the present investigations. A 40-hour irrigation period was employed in accordance with procedures described under Materials and Methods. The letters G, S, and F refer to glucose, sucrose, and fructose, respectively. "Gal" and "Mal" stand for galactose and maltose. "Raf" and "Mel" signify raffinose and melezitose.

ing in 10-percent sodium thiosulfate solution. Figure 1 illustrates the mobility patterns of several sugars one might expect to find in cane extracts.

RESULTS AND DISCUSSION

PRODUCTS OF SUCROSE HYDROLYSIS

Initial chromatography experiments compared sucrose-enzyme digests against reference sugars made up in distilled water (fig. 2). It was soon evident that both sucrose and sucrose products trailed slightly in the presence of enzyme, indicating perhaps that a sugar-protein complex was

retained long after the enzyme was inactivated by boiling. However, it was clear that two sugars were produced within 20 minutes of invertase action (fig. 2,A), and that by 720 minutes all sucrose had disappeared in favor of the two products (fig. 2,B).

A significant observation was the fact that both reducing products had a mobility rate approaching that of fructose, *i.e.*, the fastest of the three reference sugars, whereas neither product appeared to resemble glucose, the

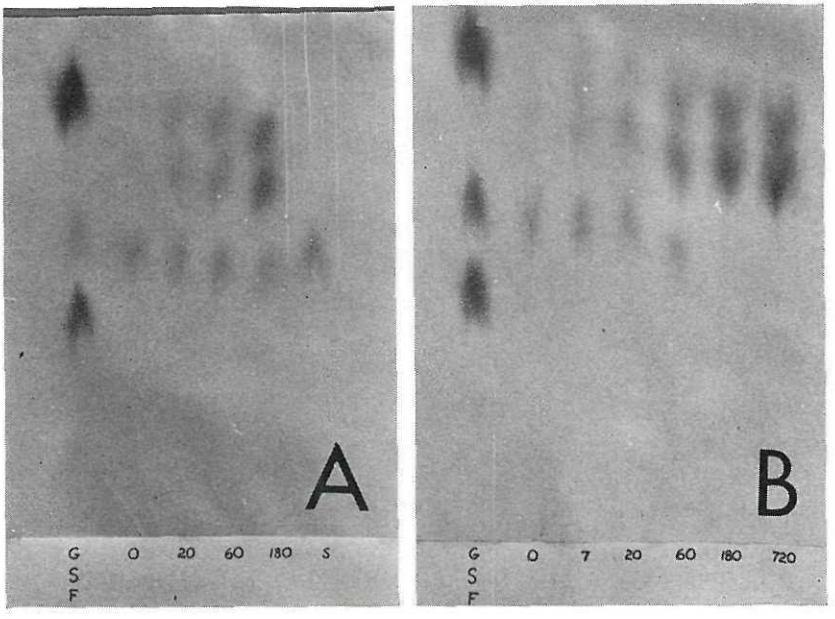


FIG. 2.—Two reducing sugars formed by cane invertase acting upon sucrose: A, Hydrolysis up to 3 hours; B, hydrolysis up to 12 hours. Reference sugars, at left side of chromatograms, appear from bottom to top as follows: Fructose (F), sucrose (S), and glucose (G).

trailing reference sugar. It was considered that the reaction products did not remain as free glucose and fructose but rather formed complexes with sucrose. One could thus anticipate such configurations as:

glucose-fructose-glucose
or
fructose-glucose-fructose

These exemplify "transosylase" reactions (*8,10*), in which water is the glucosyl acceptor only in special cases. Hehre (*11*) discovered a "dextran-sucrose," the presence of which in a solution of sucrose and dextran (n) yields dextran ($n+1$) plus fructose.

Such an enzyme was probably not active here. First, the products were obviously reducing sugars with relatively high mobility rates. The trisaccharides or polysaccharides such as those proposed above would be nonreducing and less mobile than sucrose. Second, upon hydrolysis of sucrose some glucose would likely have remained free, yet not a trace of glucose was evident on the chromatogram. A more plausible conclusion is that a chemical rearrangement of glucose took place concurrently with substrate hydrolysis to yield a sugar more nearly identical with fructose. This suggests that another catalyst was truly acting in conjunction with invertase.

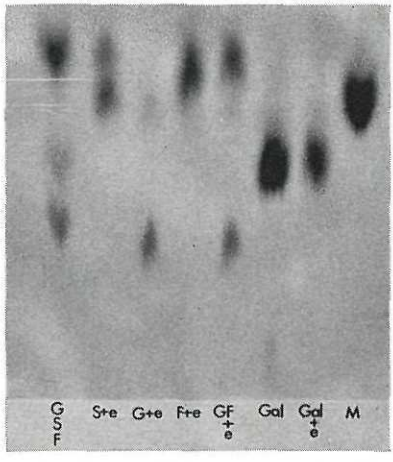


FIG. 3.—Chromatography of several free sugars, plus the same sugars exposed to cane invertase for 1 hour. The letter "e" signifies enzyme; "M" refers to maltose. Reference sugars, on left side of chromatogram, appear from bottom to top as follows: Fructose (F), sucrose (S), and glucose (G).

Figure 3 illustrates the reaction products of invertase when acting for an hour upon sucrose, glucose, fructose, and galactose. The inclusion of boiled enzyme preparation with reference solutions also permitted a close matching of control fructose with one of the invertase products. It seems clear that the cane invertase acts as a β-fructosidase.

ENZYMATIC CONVERSION OF GLUCOSE

Additional evidence of a glucose-modifying catalyst is illustrated by figure 3. Close examination reveals a trace of sugar produced when glucose was incubated with invertase preparation. No products were evident when fructose or galactose were exposed to the enzyme. Another experiment with fructose, glucose, and the trisaccharide melezitose is presented in figure 4.

Neither fructose nor melezitose was clearly affected, yet within 6 hours a second, more mobile sugar was appearing in the glucose-enzyme digest. Figure 5 illustrates the conversion of glucose in another experiment in which glucose, alone served as substrate. No change of digest reducing power was detected by the dinitrosalicylic acid technique (*23*), and only a very slight increase of ketose was given by the resorcinol method (*22*). From this

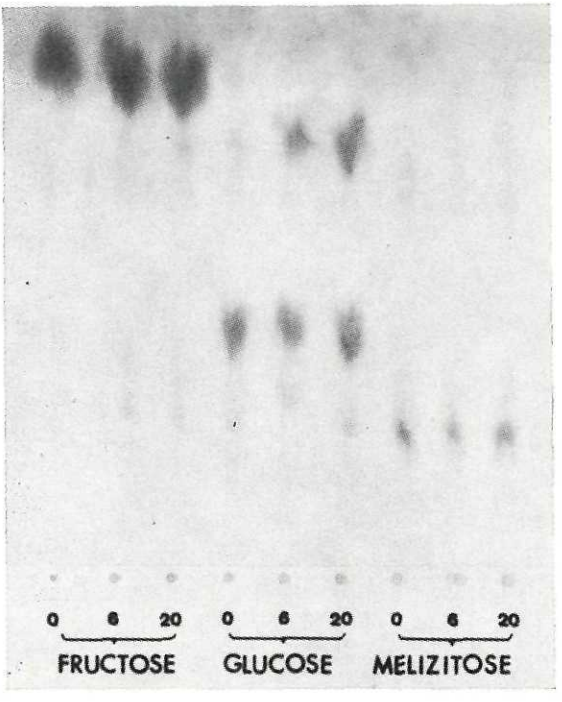


FIG. 4.—Action of sugarcane invertase on fructose, glucose, and melezitose. Numbers refer to reaction time in hours.

evidence we concluded that a contaminant enzyme converts glucose to a second reducing sugar, which is not a ketose, and which resembles fructose only with regard to mobility.

The identity of such an enzyme remains a mystery. Glucose oxidase, although known in cane (*1*), is ruled out, since the reaction is quite slow and produces gluconic acid rather than a reducing sugar. Hexose isomerase requires hexose-6-phosphate as substrate, and must also be ruled out on the basis that additional ketose would appear as a product. Glucose-6-phosphate dehydrogenase has long eluded our searching in cane preparations.

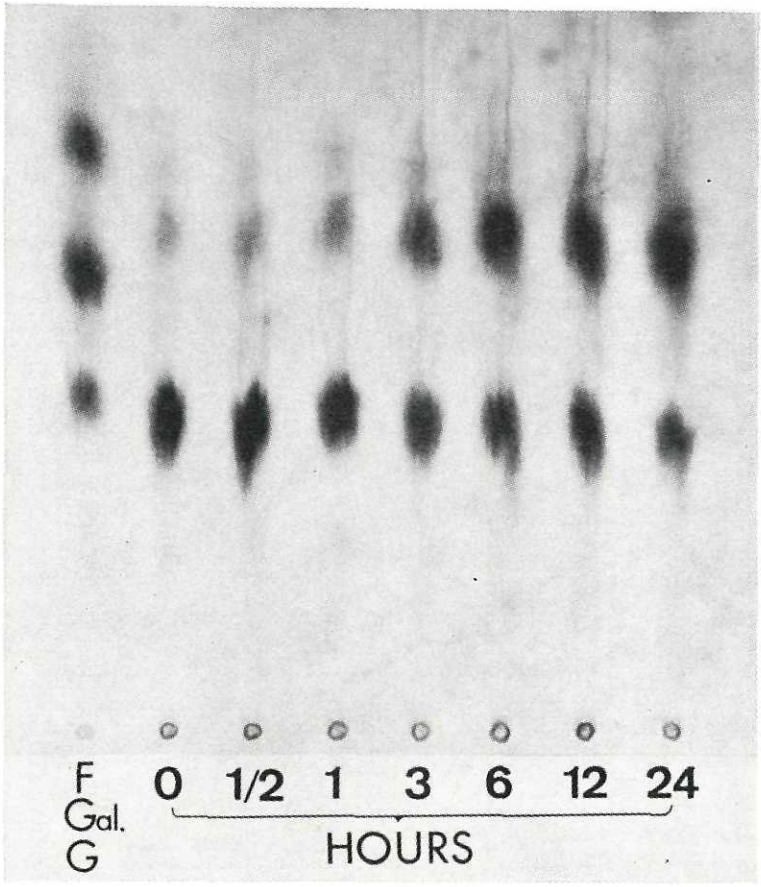


FIG. 5.—Action of a cane invertase preparation upon free glucose, illustrating the subsequent formation of an unidentified sugar with mobility intermediate between that of fructose and galactose. Numbers indicate reaction time.

The possibility of a nucleotide-derivative epimerase is the most logical suspect at the moment.[4] Anderson, *et al.* (*6*) and others (*16,17*) have shown that a deutero-labeled D—C—OH grouping can be enzymatically dehydrogenated to the corresponding —C=O with DPN+ as the transfer system. Thus an epimerase might catalyze the temporary alteration of

[4] A "galactowaldenase" was described in 1950 (*18*) which catalyzed the formation of UDP galactose from UDP glucose. More recent findings have shown the enzyme to be a nucleoside diphosphohexose-hydroxy epimerase (*6*).

a H—C—OH sugar moiety to a transient —C=O group, which, in turn, is specifically reduced to the HO—C—H group representing an opposite

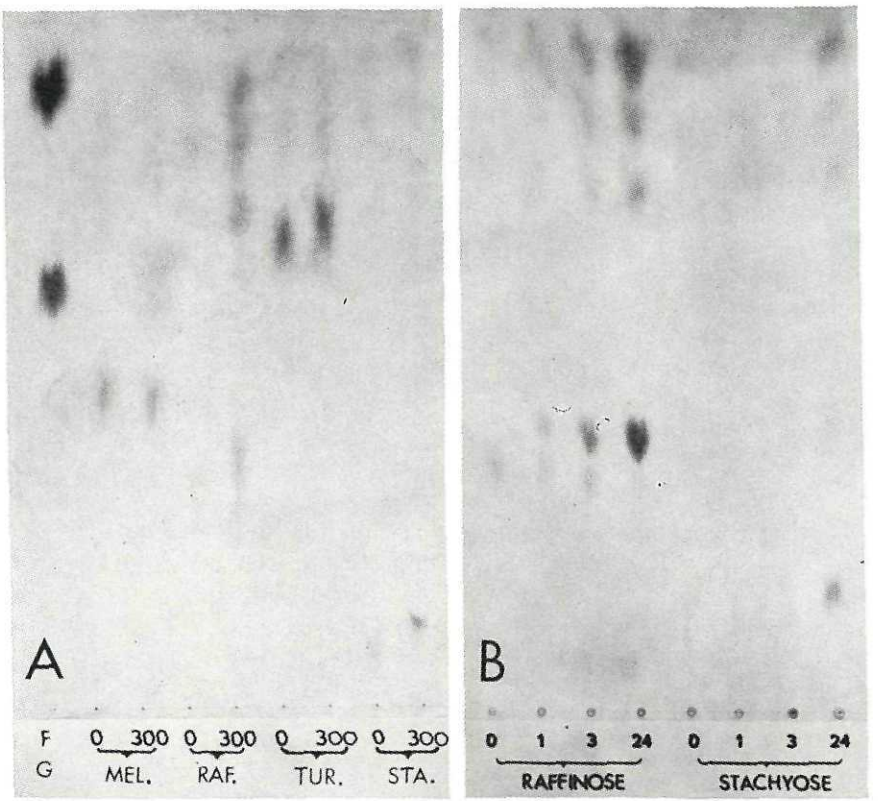


FIG. 6.—Sugarcane invertase action upon different substrates: A, Upon melezitose (MEL.), raffinose (RAF.), turanose (TUR.), and stachyose (STA.) for periods of 0 and 300 minutes; B, upon raffinose and stachyose, for periods up to 24 hours.

steric projection. Similar enzymes are known in plant extracts which promote the conversion of UDP xylose to UDP arabinose (*2*), and in liver which catalyze UDP *N*-acetylglucosamine synthesis from *N*-acetylgalactosamine (*21*). The nucleotide derivatives of cane meristem remain unexplored, yet preliminary studies of nucleotide constituents certainly do not rule out their possible roles in sugar transformation.[5]

[5] Personal communications with Dr. A. Marestsky, Physiology and Biochemistry Department, E.S.H.S.P.A., Honolulu, Hawaii.

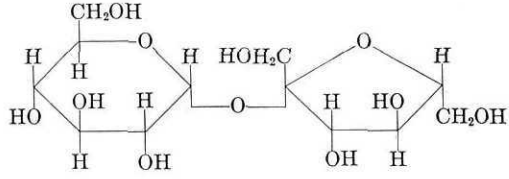
Our attempts to show that glucose is converted specifically to galactose proved inconclusive. Figure 5 represents a typical prolonged reaction in which the unidentified product lies midway between reference fructose and galactose. Notwithstanding, we feel that galactose is in fact produced, and the material on the chromatogram represents an unresolved galactose-enzyme or galactose-glucose complex.

SUBSTRATES HYDROLYZED BY CANE INVERTASE

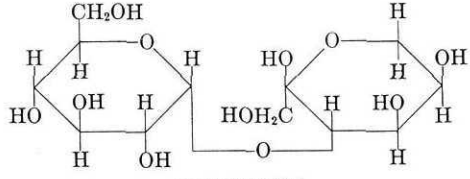
During previous studies we concluded that both α-glucosidase and β-fructosidase were represented in the cane invertase preparations (*2*). Invertases are known to attack raffinose, gentianose, stachyose, inulin, and irisin (*24*), in addition to melezitose and verbascose (*19*). Chromatography experiments were conducted here with sucrose, raffinose, melezitose, turanose, stachyose, and inulin acting as substrates.

It was found that besides sucrose only raffinose, and to a lesser extent stachyose, were readily hydrolyzed (fig. 6). Melezitose and turanose were not greatly affected (fig. 6,A), nor was inulin (not shown). A common property of sucrose, raffinose, and stachyose is their availability of a fructose component subject to release by hydrolysis. This again is evidence that β-fructosidase is the predominant invertase of cane meristem.

Failure to hydrolyze inulin, a starchlike polysaccharide composed solely of fructose residues, may be accountable to an unknown degree of branching. Turanose, on the other hand, more nearly resembles sucrose in that it is a disaccharide composed of a glucose and a fructose moiety. It is an α-*d*-glucoside, with the two monosaccharides connected by an oxygen bridge between carbon 3 of the *d*-fructose and carbon 1 of the *d*-glucose component (*13*):



SUCROSE

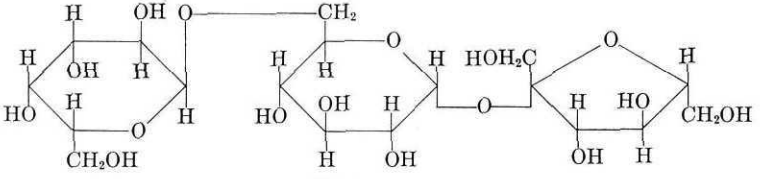


TURANOSE

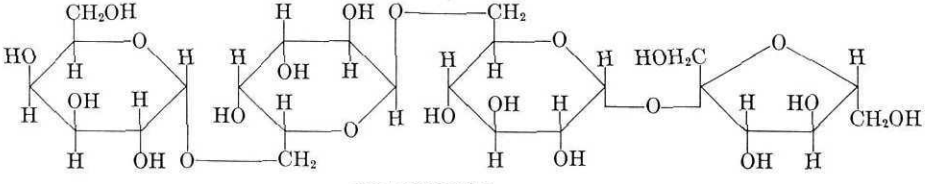
Altogether the 1 to 3 linkage, the pyranosyl ring structure of fructose, and

the steric projection of the fructose number 2 carbon atom present such a different configuration from sucrose that it is doubtful whether an enzyme-substrate complex can be formed.

A curious phenomenon is the number of products formed by action of invertase on both raffinose and stachyose (fig. 6,B). Within 24 hours each substrate yields four reducing sugars; one is closely aligned to the parent substrate, and three relatively mobile constituents behave much like the two products of sucrose. Four spots may be justified only if one assumes that the constituents fructose, glucose, and galactose each appear separately on the chromatogram, and that a glucose-altering enzyme is available to produce a secondary sugar. This is difficult to believe. A glance at raffinose and stachyose structural formulas shows that thorough hydrolysis of each glycosidic linkage would be required:



RAFFINOSE



STACHYOSE

This is asking a great deal from one preparation, and it has already been shown that invertase does not act upon free galactose (fig. 3). Thus we cannot adequately account for all of the products formed from raffinose and stachyose.

INVERTASE *VS.* SUGAR TYPE AND DISTRIBUTION IN CANE

A logical question arising from this work concerns the types of sugar which actually predominate among different cane tissues. One might wonder whether the invertase products discussed above are to be found only in the test tube. We therefore chromatogramed extracts from lyophilized meristem, leaves 2 to 3, sheaths 2 to 3, nodes 1 to 2, internodes 1 to 2 and roots.[6] The results, illustrated by figure 7, show that only sucrose, fructose, and an unidentified sugar are found in quantity. Sucrose was

[6] All tissue samples were obtained from 10-month-old plants of the variety Uba Marot.

quite predominant in nodes and internode extracts. It was almost nonexistent in meristem and root extracts, but its absence appears accountable to heavy reducing-sugar concentration. We were aware of high invertase concentration in meristem, but had never tested root preparations for the enzyme.

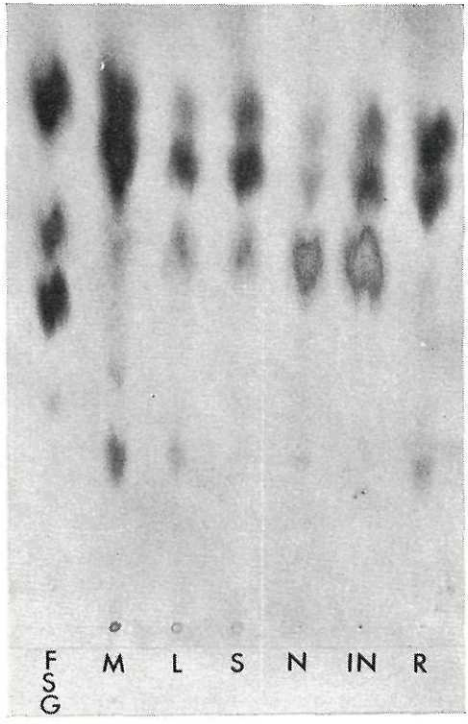


FIG. 7.—Chromatography of sugars in water extracts from different sugarcane tissues. Reference sugars, on the left side of the chromatogram, appear from top to bottom as follows: Fructose (F), sucrose (S), and glucose (G). The letters M, L, and S represent meristem, leaf, and sheath extracts. The letters N, IN, and R represent extracts of nodes (1–2), internodes (1–2), and roots, respectively.

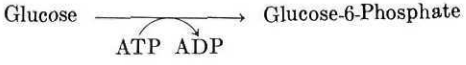
The same two reducing sugars already discussed in this paper as sucrose-hydrolysis products were found in varying degree among all tissues. One is obviously fructose, while the other more nearly resembles fructose or galactose in mobility and staining properties. The latter does not resemble glucose. Thus the contention that a glucose-modifying enzyme exists in cane is supported by the tissue-extract data.

It was interesting to note that a relatively immobile, low-staining sugar was present in both roots and meristem extracts (fig. 7). This corresponds

approximately to the trisaccharide raffinose. The possibility exists that, in areas of high invertase activity, some of the glucose product is linked with sucrose, or with other glucose molecules to form dextrans.

SIGNIFICANCE OF INVERTASE PRODUCTS

Prior to this study we had felt that a major reaction in sugarcane was the hexokinase-stimulated synthesis of glucose-6-phosphate:



By this reaction the glucose liberated from sucrose or starch might eventually find its way back into pools of phosphorylated metabolites. Our earlier studies actually showed that glucose would receive phosphate from ATP, whereas neither fructose nor galactose took part in the reaction. On the basis of present results we would deemphasize the importance of glucose and conclude that a fructokinase must in fact exist. This, in turn, may add significance to whatever enzyme is involved in conversion of free glucose.

SUMMARY

Chromatographic studies were made of the products of sugarcane acid invertase. The enzyme was precipitated from water extracts of lyophilized meristem tissues by ammonium sulfate. The 38- to 52-percent fraction was retained and dialyzed for 2 hours against distilled water. Substrates tested included sucrose, glucose, fructose, galactose, melezitose, raffinose, turanose, stachyose, and inulin. Samples from the digests were inactivated by boiling, spotted on Whatman No. 1 chromatography paper, and irrigated by the descending technique with butanol-pyridine-water (6:4:3 *v/v*). Sugars were revealed on chromatograms by the silver nitrate dip method.

Two reducing sugars were produced when invertase acted upon sucrose, but neither corresponded to reference glucose. Both products revealed staining and mobility properties similar to reference fructose. Evidence was obtained showing that glucose is acted upon by a second enzyme to yield a sugar similar to galactose. The possibility of a nucleotide-derivative epimerase is discussed.

Other sugars acted upon were raffinose and stachyose, thus confirming the invertase as a β-fructosidase. Four products appeared when raffinose and stachyose were hydrolyzed. Melezitose yielded no detectable reducing sugars, throwing doubt on the presence of an α-glucosidase. Turanose and inulin were not attacked. Significance of the invertase data is briefly discussed.

RESUMEN

Se llevaron a cabo estudios cromatográficos de los productos de la invertasa ácida de la caña de azúcar. La enzima se precipitó con sulfato de amonio de extractos acuosos de tejido meristemático liofilizado. Se retuvo la fracción de saturación entre 38 y 52 por ciento y se dializó durante 2 horas con agua destilada. Los sustratos que se probaron incluyeron la sacarosa, la glucosa, la fructosa, la galactosa, la melezitosa, la rafinosa, la turanosa, la estaquiosa y la inulina. Se inactivaron muestras de los digestos en agua hirviendo, luego se depositaron punteándose en papel cromatográfico Whatman Núm. 1 de filtrar y se bañaron mediante la técnica de la cromatografía descendente con butanol, piridina y agua (6:4:3 *v/v*). Se revelaron los cromatogramas de los azúcares mediante su inmersión en una solución de nitrato de plata.

Se formaron dos azúcares reductores cuando la invertasa actuó sobre la sacarosa pero ninguno de los dos correspondió a la glucosa de referencia. Ambos productos demostraron poseer propiedades de absorción de tintes y movilidad similares a las de la fructosa de referencia. La evidencia obtenida demuestra la acción de una segunda enzima sobre la glucosa para producir un azúcar parecido a la galactosa.

Se discute la posible existencia de una epimerasa derivada de los nucleótidos.

Otros azúcares sobre los cuales hubo acción fueron la rafinosa y la estaquiosa, identificando así la invertasa como una β-fructosidasa. Aparecieron cuatro productos al hidrolizarse la rafinosa y la estaquiosa. La melezitosa no produjo azúcares reductores cuya presencia pudiera ser determinada, haciendo dudosa la presencia de una α-glucosidasa. La turanosa y la inulina no fueron atacadas.

Se discute brevemente la importancia de la información obtenida acerca de la invertasa.

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