Hydrolytic Proteins of Sugarcane: The Acid Invertases

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INTRODUCTION

Few enzymes have been studied so long and extensively as invertase, a catalyst the history of which dates from the year 1828, when sucrose fermentation was shown to require water.[2] The enzyme has been studied most frequently in yeasts, but it is also known in green algae, bacteria, invertebrates such as the honey bee and jellyfish, and numerous higher plants and animals.

Invertase is no longer considered a single enzyme, but rather, a group of glycosidases[3] is recognized whose common property is to catalyze the hydrolysis of sucrose to α-D-glucose and β-D-fructose. The subsequent change in optical rotation of the reaction mixture, from a positive to a negative value, gives rise to the name "invertase", although some workers still prefer "sucrase" and "saccharase", or more specific names such as "*alpha-glucosidase*" and "*beta-fructosidase*". A logical grouping of the invertases is based on the portion of the sucrose molecule attacked, *i.e.*, the glucose end (glucosidase), or the fructose end (fructosidase). Other substrates reportedly attacked by invertase include raffinose, gentianose, stachyose, inulin, and irisin (*30*),[4] in addition to melezitose and verbascose (*23*).

Contrary to the meticulous study given yeast invertase by workers in many nations, the sucrose-hydrolyzing systems of sugarcane have received scant attention. Hartt (*15*) reported relationships between cane invertase and potassium supply in 1934. More recent studies include the work of Glasziou (*14*), and those of Hatch and coworkers (*17,18*). Initial studies at this laboratory showed very low yields of invertase in leaf tissues, as com-

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[2] The reader is referred to the thorough review by Neuberg and Mandl (*23*). For those interested in the potential nonhydrolytic reactions of invertase, we recommend the article by Dedonder (*12*), who treats carbohydrases as transosylases, *i.e.*, catalyzing reactions in which water is the acceptor of the glycosyl radicle only in special cases.

[3] The glycosidases comprise 2 broad groups: those that catalyze the hydrolysis of glycosidic bonds in simple glycosides or in oligosaccharides, and those that catalyze the hydrolysis of polysaccharides. Some workers prefer to call only the first group "glycosidases," and employ "polysaccharidases" for the second group.

[4] Italic numbers in parentheses refer to Literature Cited, pp. 306–7.

pared with amylase (*2*). However, this work was not immediately followed up and remained inconclusive with regard to invertase.

Among the many factors that alter the sucrose content of cane, invertase must undeniably bear a major responsibility. Considerable effort is being expended here to isolate and characterize those sucrose-hydrolyzing systems which are significantly active in sugarcane. This paper reviews the behavior and properties of acid invertases extracted from sugarcane meristem.

EXPERIMENTAL PROCEDURE

PREPARATION OF ENZYME

Enzyme preparations were made with distilled-water extracts of lyophilized tissues from 4- and 10-month-old sugarcane of the variety M. 336. Meristem tissue was obtained by peeling away all true leaf and sheath material in the area of the growing point. The tissue discussed herein as "meristem" consisted of a small, undifferentiated cylinder, somewhat less than a lead pencil in thickness and about 3 inches long. The tissue is extremely tender and brittle, exhibits strong polyphenol oxidase activity (browning) immediately after being cut, and generally passes through all aspects of freezing, drying, grinding, and extraction with great ease.

Other samples included the basal 12 to 14 inches of leaves −1 and 0, leaves +2 and +3, leaves +5 and +6, the entire sheaths +5 and +6, 8 to 10 internodes, and 8 to 10 node tissue. Each node and internode sample represented a complete cross-section of the cane, that of the node being a slice about ¾ inch in thickness, and the internode a 2-inch section taken midway between the respective nodes. All samples were inserted in large pyrex culture tubes and frozen immediately in a mixture of Dry Ice (solid carbon dioxide) and acetone. These were later stored at −20° C. The frozen tissues were lyophilized, ground with a Wiley mill to pass a 60-mesh screen, and stored at −20° C. in sealed sample jars until needed for extraction.

Invertase was extracted from the powdered tissues with distilled water. Much use has been made of the process of self-digestion (autolysis) and plasmolyzing agents to increase invertase yields from yeast, presumably through increased mechanical disruption of cell walls and simultaneous degradation of substances loosely combined with invertase. This procedure has not been widely used in separating the enzyme from tissues of higher plants. However, during the present study it was found advantageous to pretreat suspensions of powdered cane tissue by ultrasonic disintegration prior to extraction with water. This was accomplished with a Branson Model S-75 sonifier, equipped with transistorized power supply and sonic-converter probe. The only difficulty encountered was a rapid heating of the suspension during treatment. A procedure was adopted by which samples were alternately chilled to 2° C. and then sonified until the temperature rose to 18° C. The suspensions were again chilled and the treatment re-

peated until each sample had been treated for 5 minutes. Ultrasonic disintegration made possible superior enzyme yields from leaf and sheath powders. Apparently, a fair percentage of leaf and sheath cells pass unscathed through the Wiley mill and 60-mesh screen. The sonifier had a less pronounced effect upon meristem, node, and internode tissues.

After treatment by the sonifier, each sample was extracted for 1 hour at 22° C. with the aid of a mechanical shaker operating at slow speed. Tissue debris was removed by expressing the mixture through four layers of Curity absorbent gauze. The suspension was clarified by centrifuge at 3,500 r.p.m. The supernatant, pH 5.5 to 5.6, was now ready for fractionation with ammonium sulfate, the details of which will be described in a later section.

ENZYME ASSAY

Invertase was measured colorimetrically by determining the amount of reducing sugar formed from sucrose under standardized conditions. The standard reaction mixture was composed of 0.5 ml. of acetate buffer (pH 4.65 or 5.50), 0.5 ml. of substrate solution, sufficient to make 30 μmoles of sucrose per milliliter of digest, 0.25 ml. of water or additive, and 0.25 ml. of undialyzed enzyme preparation. Control vessels received distilled water in place of sucrose. An additional set of controls was necessary when dialyzed enzyme concentrates were diluted with sugar solutions. The standard reaction was run for 20 minutes at 30°C.

Reducing sugars were measured by a modification of Sumner's dinitrosalicylic acid technique (*28*) as described in an earlier report (*2*). No reagent was employed specifically to stop the invertase reaction, but this was effectively accomplished by immediately adding dinitro reagent to the reaction mixture and inserting the tubes in boiling water. After the color had been developed, optical density of the solutions was measured at 525 μ and reducing-sugar content was determined by reference to a standard curve representing 0.05 to 0.250 mg. of D-glucose.

Invertase activity was recorded in terms of specific activity, units per milligram of protein. The activity unit was arbitrarily defined as the amount of enzyme catalyzing the formation of 0.10 mg. of reducing sugar under the prescribed conditions of the assay. Protein content of the enzyme preparations was measured by the microtechnique of Sutherland *et al.* (*31*).

RESULTS AND DISCUSSION

LOCALIZATION AND pH-DEPENDENCE OF INVERTASE

Hatch and coworkers (*14,17,18*) have reported an acid invertase (pH 5.0 to 5.5) in immature internodes, and a neutral invertase (pH 7.0) in mature internodes of sugarcane. Previous work at this laboratory revealed only traces of invertase activity in leaf and sheath tissue. In order to test for the presence and distribution of acid and neutral invertase in M. 336, a series

of experiments were run with meristem, leaf, sheath, node, and internode preparations at pH 4.65 and 7.0. Typical results are summarized in table 1.

In general agreement with Hatch *et al.* (*14,17,18*) invertase was detected under both acidic and neutral conditions. However, activity was predominant in meristem and node preparations rather than internodes. Furthermore, both the acidic and neutral reactions were detected in 10-month-old cane, in contrast to immature tissue for acid invertase and mature tissue for neutral invertase.

The specific activity values presented in table 1 are qualitative and somewhat misleading as to the quantitative importance of each tissue. Protein

TABLE 1.—*Specific activity of invertase extracted from different tissues of 10-month-old sugarcane and measured in the presence of acidic and neutral buffers*[1]

Buffer	Data for enzyme source indicated—						
	Meristem	Leaves −1 and 0	Leaves +2 and +3	Leaves +5 and +6	Sheaths +5 and +6	Nodes 8 to 10	Internodes 8 to 10
Acetate (pH 4.65)	8.9	3.8	3.3	2.8	3.9	7.8	2.7
Phosphate (pH 7.0)	7.1	3.8	3.1	2.9	4.5	8.0	2.9

[1] Each reaction mixture was composed of 0.5 ml. of buffer, 0.5 ml. of 5-percent sucrose solution, and 0.5 ml. of crude enzyme preparation. Control tubes received distilled water in place of enzyme. All reactions were run for 20 minutes at 30° C.

content of meristem was 2 to 3 times that of the leaves, and 10 to 12 times greater than for nodes and internodes. Consequently, the sucrose-inverting power per gram of meristem far exceeded that of any other tissue analyzed. Meristem, therefore, was utilized as an enzyme source throughout the remainder of the study.

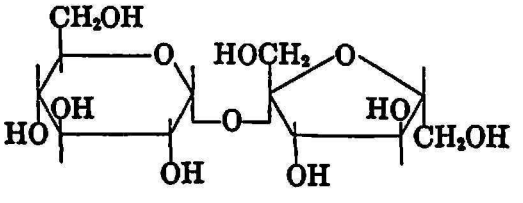
SALT FRACTIONATION

The enzyme-localization tests were run with undialyzed protein, precipitated with ammonium sulfate from clarified extracts between 0 and 95-percent saturation at pH 5.5 and 22°C. Further fractionation tests showed that practically all acid invertase was precipitated below 55-percent saturation. The 0 to 60-percent fraction was then divided into 2-percent increments to determine the saturation range of greatest invertase yield (fig. 1).

Although some invertase was precipitated at as low as 12-percent saturation, the greatest yield was obtained between 30- and 52-percent saturation. This fraction was adopted for analysis during the remainder of the study. Representing slightly more than 60 percent of the total acid invertase of the crude extract, the 30- to 52-percent fraction was nearly free of Q enzyme, P enzyme, and α-amylase, but contained strong β-amylase and acid-phosphatase activity.

SUBSTRATE SPECIFICITY

Two distinct but isodynamically equal invertases are known to exist (*23*), and these may be classified according to the end of the sucrose molecule being attacked. Sucrose is so constructed that hydrolysis may occur either between the glycosidic oxygen and carbon 2 of the fructofuranosyl group, or



Sucrose
(α-D-glucopyranosyl - β-D-fructofuranoside)

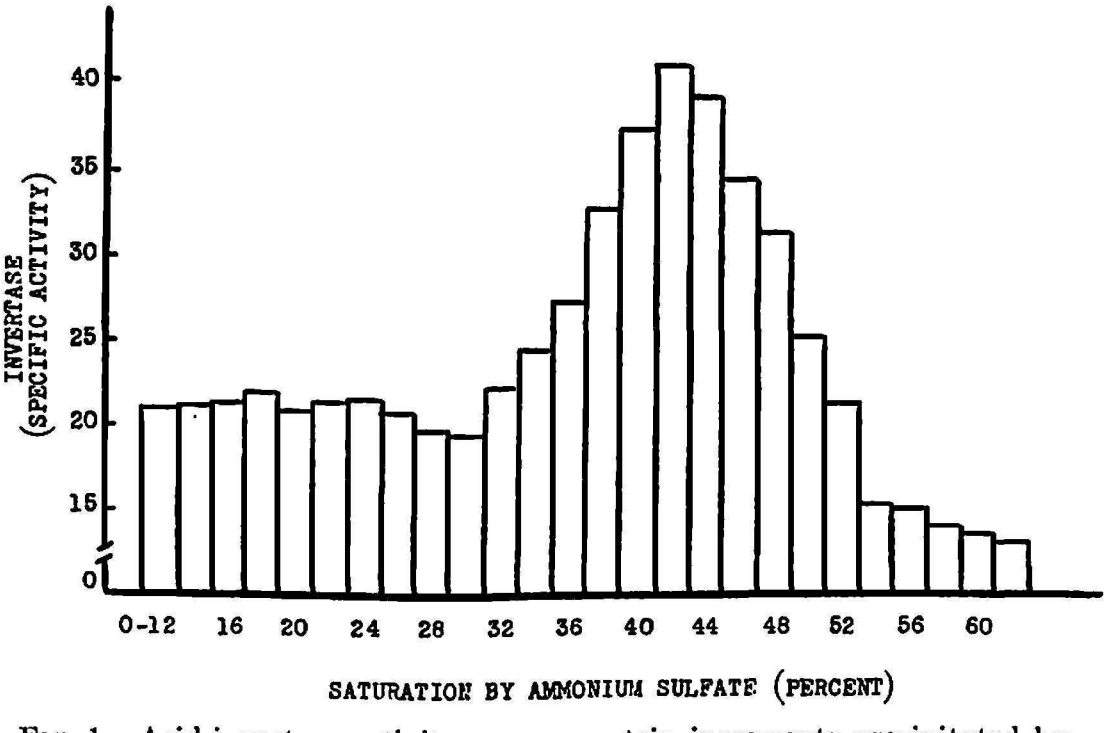
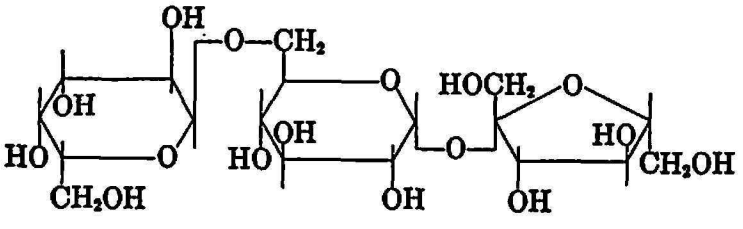


FIG. 1.—Acid-invertase activity among protein increments precipitated by ammonium sulfate from cane-meristem extracts.

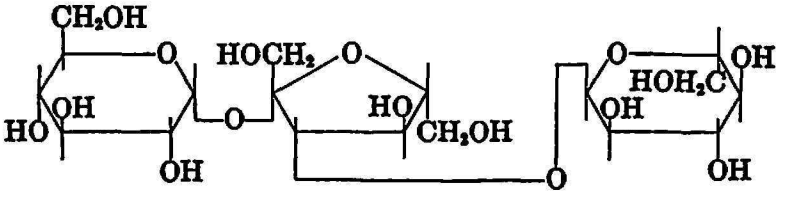
between the glycosidic oxygen and carbon 1 of the glucopyranosyl group. When yeast invertase (β-fructofuranosidase) acts upon sucrose in water labeled with O^{18}, the isotope does not appear in the resulting glucopyranose, indicating that the fructose moiety has been cleaved from sucrose (*19*). Conversely, the glucose portion is attacked by a different invertase, α-glucopyranosidase, or "taka"-invertase, the action of which is to catalyze the cleavage between the glycosidic oxygen and carbon 1 of the glucopyranosyl group.

The existence of two invertases can be demonstrated by use of substrates in which one or the other potential reactive site is blocked by linkage with

a third factor. For example, the substrate raffinose may be attacked only by the yeast-type of invertase because the glucose end is blocked by linkage with galactose. By the same principle, the tetrasaccharide stachyose is also attacked only by β-fructosidase, its three glucose components being



Raffinose
(α-D-galactopyranosyl-α-D-glucopyranosyl-
β-D-fructofuranoside)



Melezitose
(α-D-glucopyranosyl-β-D-fructo-
furanosyl-α-D-glucopyranoside)

united by 1 → 6 linkages, and only the terminal fructose is subject to invertase action (*16*). On the other hand, the trisaccharide melezitose is immune to yeast invertase, *i.e.*, the fructose moiety of its sucrose component is linked with a second glucose molecule to form the disaccharide turanose. The glucopyranosyl unit of melezitose is open to attack by taka-invertase (α-glucosidase). Thus, a pure preparation of taka-invertase would not affect raffinose and stachyose, and yeast invertase would not affect melezitose, even though both enzymes would readily hydrolyze sucrose.

The cane-meristem preparation was tested repeatedly against sucrose, raffinose, stachyose, and melezitose. Each sugar was supplied at the rate of 30 μmoles/ml. of digest. Assigning the sucrose reaction an arbitrary value of 100 percent, the corresponding averages for all reactions against raffinose and stachyose were 63.1 and 66.7 percent, respectively, and that of melezitose was 34.3 percent. In other words, both the taka and yeast types of invertase were present, with the fructose-cleaving enzyme dominant by approximately 2 to 1.

The existence of taka-invertase in cane is of more than academic interest. This system should theoretically take part in the final degradation of oligosaccharides[5], comprised of glycosidic-linked glucose residues, resulting

[5] The oligosaccharides comprise a large group of carbohydrates consisting of relatively few monosaccharide units (Greek *oligos* = a few) joined through glycosidic linkages. Only simple sugars remain after complete acid hydrolysis. Two sugar units joined in this manner yield a disaccharide, 3 yield a trisaccharide, etc. The mode of

from the hydrolysis of polysaccharides such as amylose and amylopectin. Of particular interest are the products of α-amylase, which, acting upon glycosidic linkages in the interior and branched areas of the starch polysaccharides, apparently forms the trisaccharide maltotriose (*8*), and (1 → 6)-linked oligosaccharides as small as pentasaccharides (*13*, p. 434). Maltose might also be hydrolyzed by taka-invertase, and, according to Neuberg and Mandl (*23*, p. 543), evidence obtained by Weidenhagen has led this investigator to believe that α-glucosidase is identical with maltase.

Sacher *et al.* (*27*) suggested that invertases take part in regulating the movement of sucrose from conducting tissue, and its subsequent utilization for growth and storage. They have also shown that rate of internode elongation can be correlated with acid invertase, and that neutral invertase may regulate movement of sucrose from vascular to storage tissue in mature internodes (*18*). It can now be suggested that within the "acid invertase" group there exist at least two invertases, based upon the portion of sucrose being attacked. It is further suggested that the α-glucosidase might serve as a constituent of the polysaccharide-degrading mechanisms of cane. The proposed roles of α-glucosidase may be placed in perspective by Scheme I (p. 294).

With the exception of phosphoglucomutase, each of the enzymes represented above has been encountered in cane-leaf or meristem tissue (*2,6, 4,5,3*). It would be interesting to learn the ratios of β-fructosidase to α-glucosidase in cane varieties and specific tissues which vary distinctly in their ability to form amylose and amylopectin.

OPTIMUM pH

Meristem invertase preparations were tested at pH values ranging from 3 to 8. Sucrose and raffinose were used as substrates at 30 μmoles/ml. of digest. All reactions were run for 20 minutes at 30° C.

Maximum activity lay between pH 4.75 and 5.5 against sucrose, and at pH 5.5 against raffinose (fig. 2). However, invertase was quite active against both substrates over a broad pH range of 4.0 to 6.5. The results coincide fairly well with those of Glasziou (*14*) who found acid invertase of immature cane tissue to be optimally active between pH 5.0 and 5.5.

OPTIMUM SUBSTRATE CONCENTRATION

Both sucrose and raffinose were subjected to invertase at concentrations ranging from 1 to 200 μmoles/ml. of digest. The pH was maintained at 5.5 with acetate buffer, and all reactions were run at 30° C. for 20 minutes.

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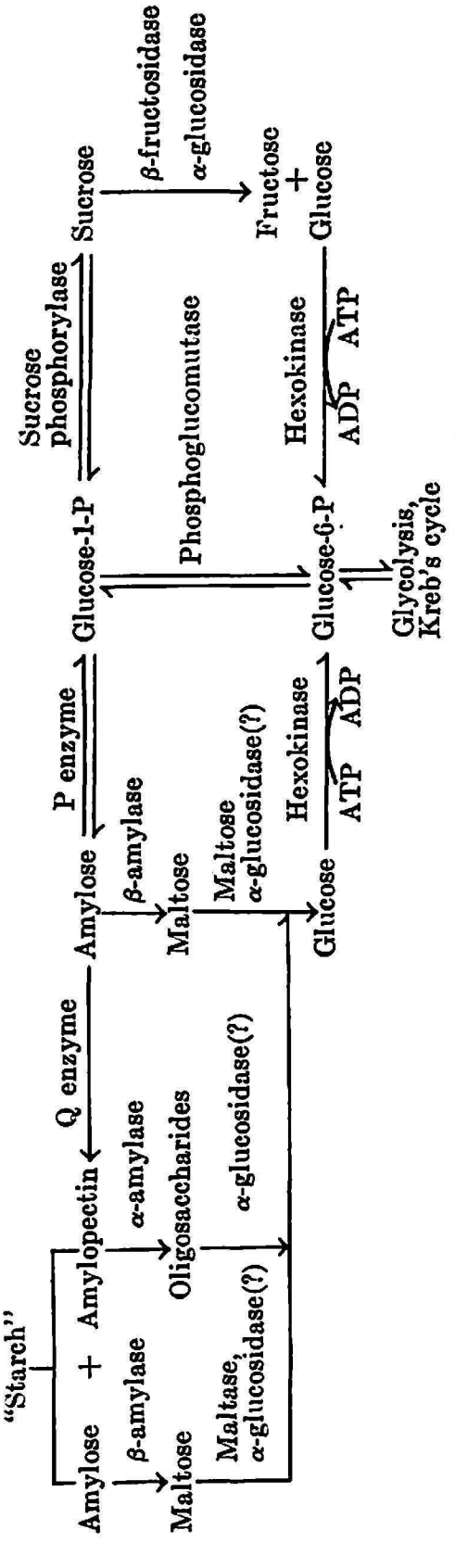
linkage resembles that of polysaccharides, and no sharp distinction can be drawn between the 2 groups. Hassid and Ballow (*16*) arbitrarily assigned the oligosaccharides 10 or less monosaccharide residues per molecule, whereas certain polysaccharides have several thousand.


"Starch"
Amylose + Amylopectin
β-amylase
α-amylase
Maltase, α-glucosidase(?)
Maltose
Oligosaccharides
α-glucosidase(?)
Q enzyme
P enzyme
Amylose
Amylopectin
β-amylase
α-amylase
Maltose
Oligosaccharides
Maltose
α-glucosidase(?)
α-glucosidase(?)
Hexokinase
ATP ADP
Glucose
Glucose
Sucrose phosphorylase
Glucose-1-P
Phosphoglucomutase
Glucose-6-P
Hexokinase
ADP ATP
Glucose
Sucrose
β-fructosidase
α-glucosidase
Fructose + Glucose
Glycolysis, Kreb's cycle

SCHEME I.

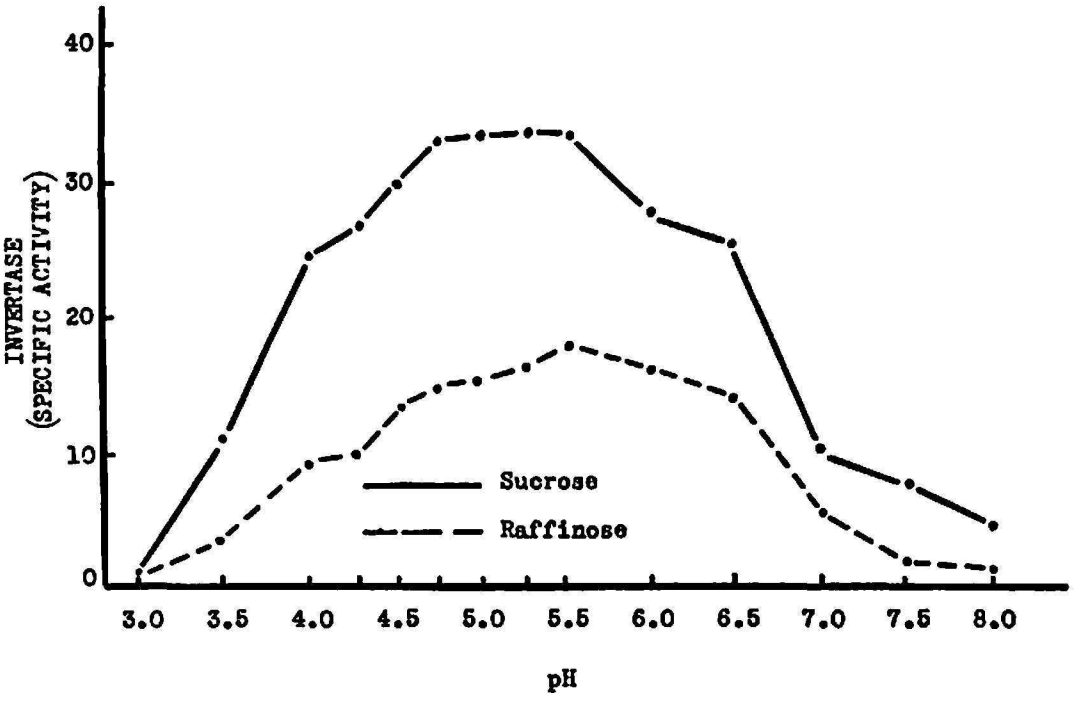


FIG. 2.—Effects of pH upon invertase of sugarcane meristem.

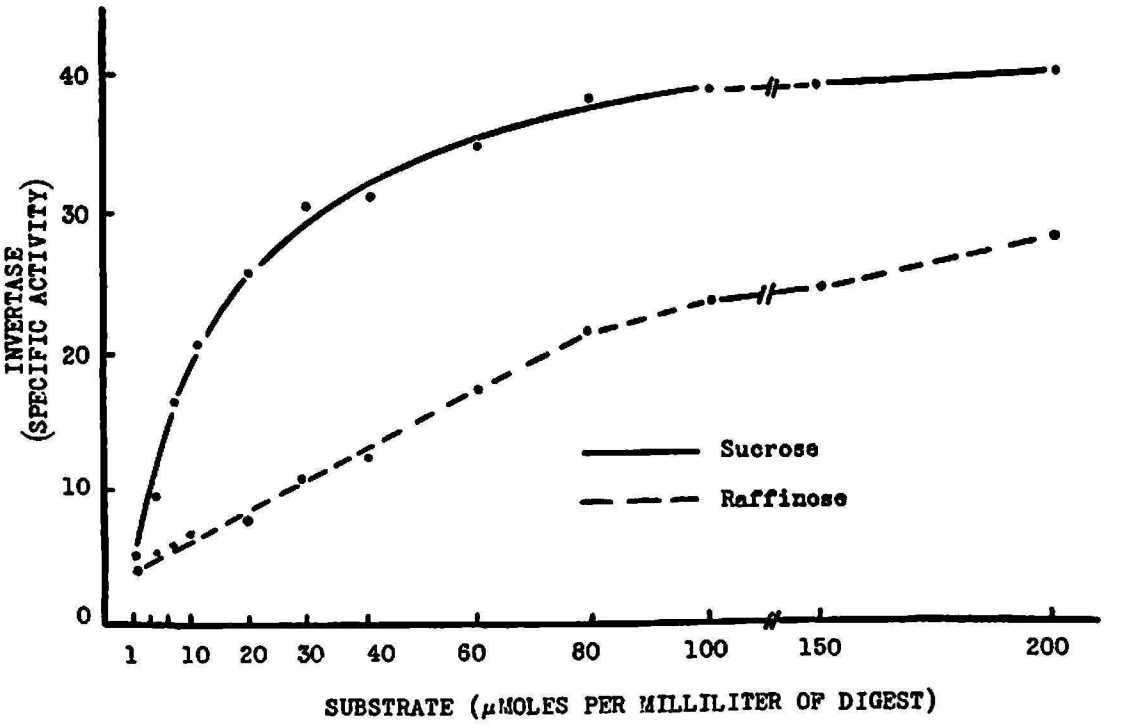


FIG. 3.—Effects of variable substrate concentrations upon acid invertase from sugarcane meristem.

Sucrose hydrolysis was linear to about 10 μmoles/ml. of digest (fig. 3), but activity increased moderately to 80 μmoles/ml. Quite curiously, the raffinose reaction was linear to 80 μmoles/ml. of digest. Since raffinose could be hydrolyzed only by β-fructosidase, we believe that the discrepancy between optimum substrate concentrations was due to the competitive effects of

α-glucosidase acting upon sucrose. In other words, each time a sucrose molecule was hydrolyzed by α-glucosidase, another site of action was effectively removed from β-fructosidase, even though the glucosidase had acted upon the opposite end of the sucrose molecule. It is also possible that α-glucose produced during sucrose hydrolysis, but not during raffinose hydrolysis, was acting as an inhibitor of the sucrose-cleaving reactions, in accordance with the inhibitory properties of glucose discussed by Neuberg and Mandl (*23*, p. 540).

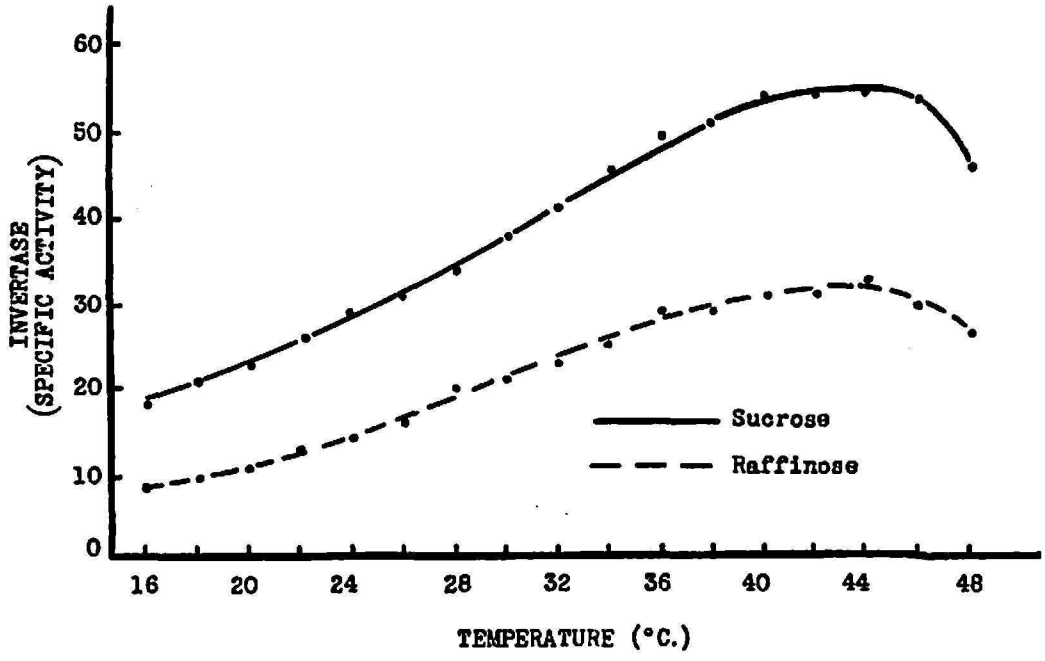


FIG. 4.—Effects of temperature upon acid invertase of sugarcane meristem.

OPTIMUM TEMPERATURE

Again using both sucrose and raffinose as substrate (30 μmoles/ml. of digest), temperature trials were conducted between 16 and 48° C., employing acetate buffer (pH 5.5), and 20-minute reaction periods. Both the sucrose and raffinose reactions were markedly restricted below 20° C., and achieved maximum velocity at about 44° C. (fig. 4). Acid invertase of cane appears to be quite tolerant of heat.

Hatch and Glasziou (*18*) reported that the acid-invertase content of immature storage tissue, roughly equivalent to the "meristem" of our studies, increased nearly tenfold as environmental temperature of the growing cane was increased from 18 to 34° C. Adequate water was essential for the invertase increase. Our own experiments showed that invertase specific activity was slightly more than doubled between 18° and 34° C., so that with a tenfold rise in invertase content, one might have expected a twentyfold increase in absolute inverting power per gram of tissue.

STABILITY OF INVERTASE

When dealing with any enzyme preparation the investigator must know how long it can be used reliably, and which storage conditions will best preserve the active catalyst. Invertases measured during the present study had already survived the processes of quick-freezing, lyophilization, grinding by metallic blades, storage within dried powders, sonic disintegration of their native cells, extraction by water, and salt fractionation. Further experiments were conducted to learn the enzymes' stability in aqueous solution, when maintained under toluene, and exposed to room, laboratory, and refrigerator temperatures.

Invertase samples stored at 2° and 22° C. showed little or no decline after 9 days (table 2). Only at room temperature (28 to 29° C.) did appreciable

TABLE 2.—*Specific activity of invertase preparations following variable treatments of time and temperature*[1]

Temperature (°C.)[2]	Data for days following preparation indicated					
	0	1	3	5	7	9
2	37.9	44.8	46.3	44.4	43.1	43.1
22	37.9	41.5	40.3	37.6	36.3	35.2
28–29	37.9	36.3	34.0	33.1	31.1	28.2

[1] Each reaction mixture was composed of 0.5 ml. of acetate buffer (pH 5.5), 0.5 ml. of sucrose solution (sufficient to make 30 μmoles/ml. of digest) and 0.5 ml. of enzyme preparation. Reactions were run for 20 minutes at 30° C.

[2] The values 2, 22, and 28–29° C. represent refrigerator, laboratory, and room temperature, respectively. All samples were stored under toluene to prevent destruction by micro-organisms.

activity loss occur. It is evident from table 2 that samples refrigerated at 2° C. increased activity within 3 days. Possibly, some of the invertase was combined with an endogenous inhibitor at the time of extraction, and a gradual dissociation of the enzyme-inhibitor complex took place thereafter. This stimulation was not apparent among samples maintained at room temperature, although the adverse effects of higher temperatures might simply have masked any such tendencies as were noted at 2° C.

INHIBITORS

Preliminary tests with potential invertase inhibitors, at concentrations of 10 μmoles/ml. of digest, revealed that iodide (I), arsenic (As), lead (Pb), Tungsten (W), and mercury (Hg) all severely inhibited invertase. Little or no effect was recorded for bromide, fluoride, calcium, molybdenum, iron, copper, zinc, and boron, each of which was tested at the rate of 10 μmoles/

ml. of digest. Potassium cyanide and manganese interferred with the colorimetric reaction at this concentration and their effects were not immediately ascertained.

The determination of cane-invertase inhibitors is not entirely an academic problem, for the day is not far distant when serious attempts will be made to inhibit invertase for the betterment of sucrose yields. The ideal inhibitor, in addition to being specific for invertase, would need to be effective in very minute quantities. No economically feasible method of foliar application will ever guarantee more than partial coverage of a commercial planting, hence, the more effective an inhibitor is in small amounts, the easier will be the grower's task of supplying sufficient quantities to a stand of cane.

TABLE 3.—*Influence of inhibitors upon specific activity of meristem invertase*[1]

Inhibitor	Influenced inhibitor concentration (μmoles/ml. of digest) indicated—							
	0	0.01	0.05	0.10	0.50	1.0	5.0	10.0
As	36	37	38	37	36	35	9	3
I	36	34	30	26	16	12	2	0
Pb	34	32	26	19	4	1	0	0
W	36	37	36	36	36	35	22	11
Hg	35	0	0	0	0	0	0	0

[1] Each reaction mixture was composed of 0.5 ml. of acetate buffer (pH 5.5), 0.5 ml. of sucrose solution (sufficient to make 30 μmoles/ml. of digest), 0.25 ml. of inhibitor solution or distilled water, and 0.25 ml. of enzyme preparation. All tests were run for 20 minutes at 30° C.

Further inhibitor tests were run with As, I, Pb, W, and Hg at concentrations ranging from 0.01 to 10.0 μmoles/ml. of digest (table 3). Neither As nor W was effective below 5 μmoles/ml., whereas Pb and I began to inhibit invertase in the range 0.05 to 0.10 μmole/ml. of digest. Tungsten, in an earlier study, was found to inhibit cane phosphatase in extremely minute concentration, *i.e.*, in the area of 0.00005 μmole/ml. of digest (7). However, there is little hope that W, as a phosphatase inhibitor, could serve a dual purpose by also inhibiting invertase, since the *in vitro* quantity needed to effectively retard invertase is roughly 100,000 times greater than that needed to inhibit acid phosphatase.

Mercury completely inhibited invertase at 0.01 μmole/ml. (table 3). The element was later shown to exert its main inhibitory effects within the unusually narrow range of 0.0001 to 0.0003 μmole/ml. of digest (fig. 5). Mercury was the most effective of the five inhibitors revealed by this study.

The inhibitors I, Pb, and Hg were studied under conditions of increasing

substrate concentration in an effort to determine whether any one of them acted noncompetitively (irreversibly). The type of inhibition, competitive, noncompetitive, or uncompetitive, is of particular importance from the standpoint of discovering an effective invertase inhibitor for use in the field. The high incidence of endogenous substrate in cane would counteract a competitive, reversible inhibitor, no matter how effective that inhibitor might appear in the test tube.

Figure 6 illustrates the effects of I, Pb, and Hg on invertase acting in the presence of 20 to 200 μmoles of sucrose per milliliter of digest. Inhibition by

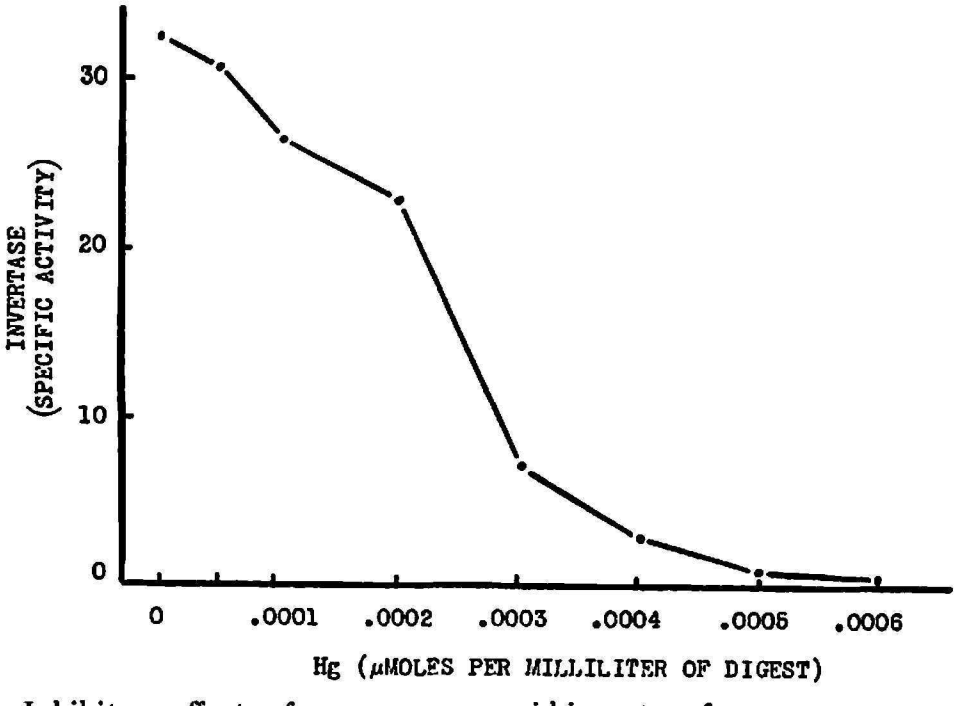


FIG. 5.—Inhibitory effects of mercury upon acid invertase from sugarcane meristem.

I was definitely reversible, and Pb inhibition was partly so. The Hg reaction was changed but little by increasing substrate, and was practically unaffected above 80 μmoles, which again suggests that Hg would be the most promising element for trials with growing cane. The limited reversibility of the Pb reaction is not clear, since 200 μmoles of sucrose should have been adequate for complete reversal, if this were at all possible. Perhaps only one of the two invertase types was competitively inhibited, while the other remained inactive in the presence of increasing substrate.

ACTIVATORS

As mentioned earlier, both potassium cyanide and manganese (Mn) interferred with the reducing reaction at 10 μmoles/ml. While testing these reagents at lower concentrations, it was learned that Mn exerted a stimulatory effect upon invertase. Additional experiments showed that as little as

0.0001 μmole of Mn per milliliter of digest caused a measurable increase in the standard invertase reaction, and that the activating effect of Mn continued up to 0.5 μmole/ml. of digest (fig. 7). The standard invertase reaction, without added Mn, proceeded at about half of its potential rate.

Preliminary experiments with invertase preparations revealed that 12 hours of dialysis against distilled water reduced activity by 60 to 70 percent. Prolonged dialysis for 36 hours removed all but some 5 percent of the original activity (fig. 8).

All of the additives studied as potential inhibitors were again tested at

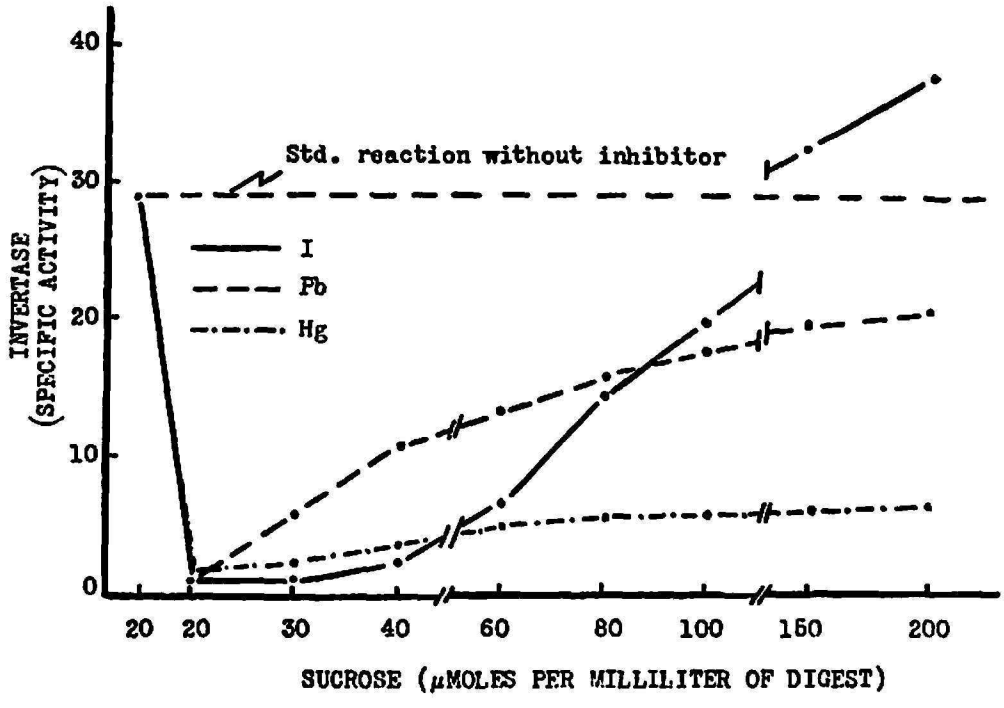


FIG. 6.—Effects of increasing substrate concentration upon the invertase-inhibitory action of iodide, lead, and mercury (5, 0.5, and 0.0003 μmoles/ml. of digest, respectively).

concentrations ranging from 0.001 to 1.0 μmole/ml., in an effort to identify the essential factor lost during dialysis. Only Mn served to reactivate invertase. Figure 9 illustrates the reviving influence of Mn, nearly half of which was brought about by 0.005 μmole of Mn per milliliter. The activating effects of Mn continued up to 0.5 μmole, at which level the dialyzed invertase was far exceeding the activity of undialyzed enzyme. Manganese thus appears essential not only for maximum activity of the acid invertases, but possibly it is an essential cofactor needed for any appreciable activity at all. More thorough dialysis experiments are needed to confirm this.

The possibility that a nonmetallic factor was lost during dialysis was also considered. In particular, the presence of sugars in a capacity other than substrate may be essential for normal invertase activity. According to

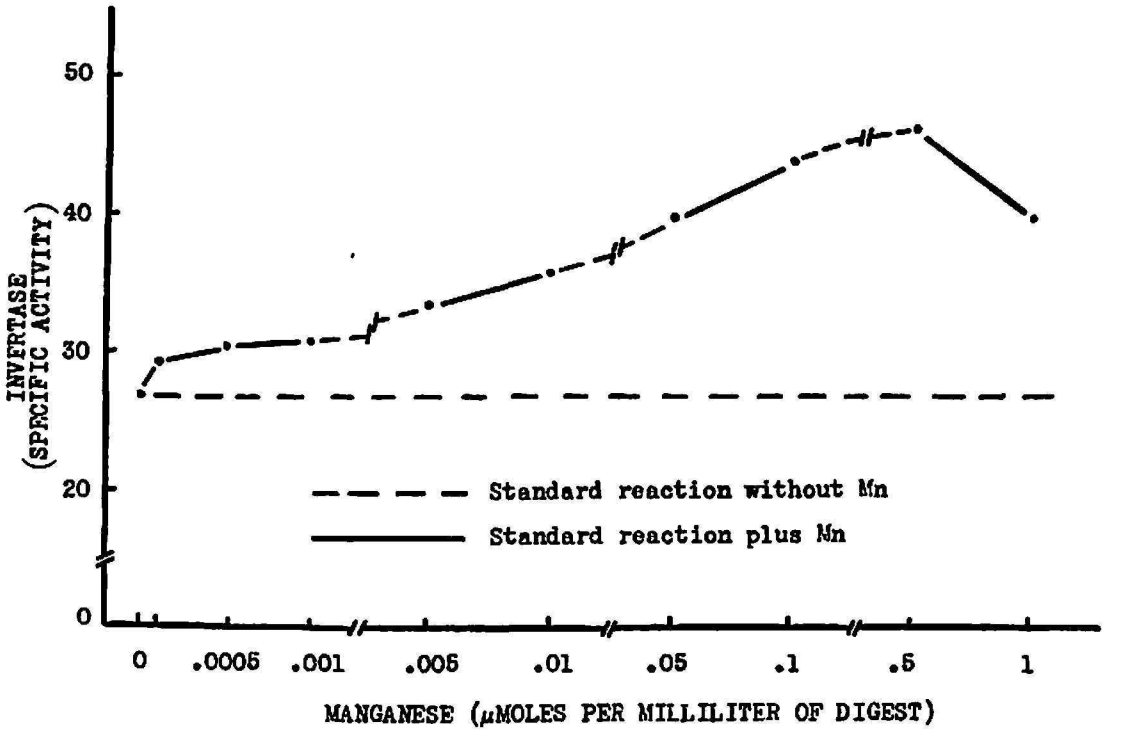


FIG. 7.—Stimulatory effects of manganese upon acid invertase of sugarcane meristem.

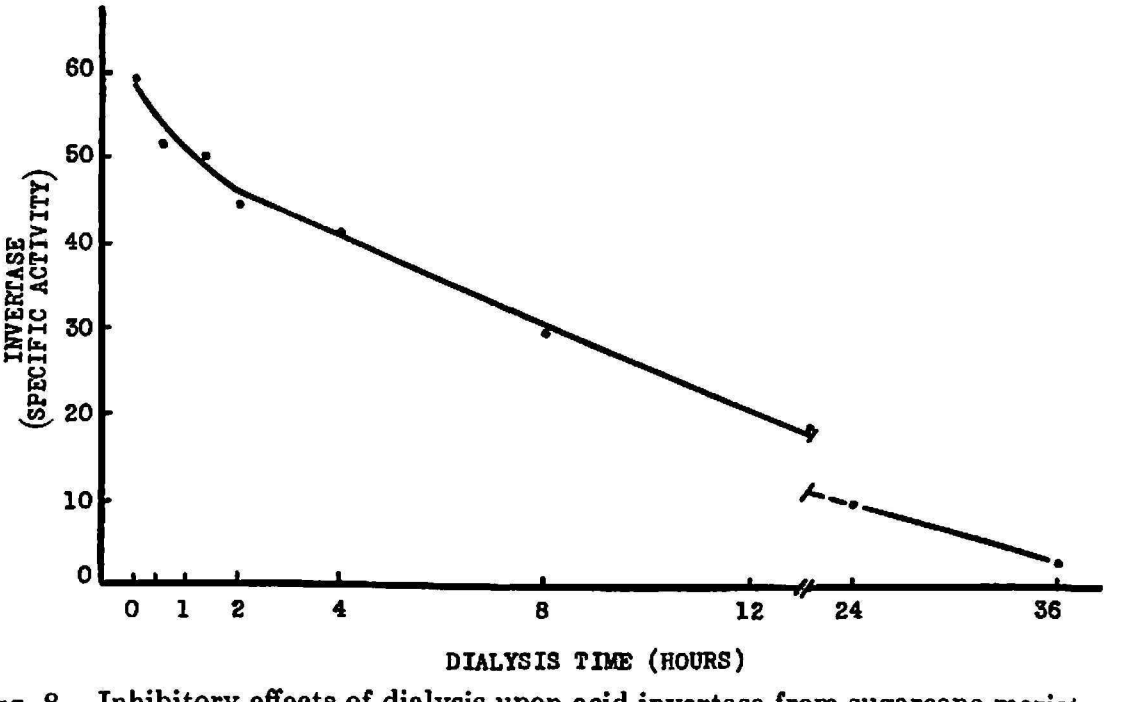


FIG. 8.—Inhibitory effects of dialysis upon acid invertase from sugarcane meristem.

Neuberg and Mandl (*23*), preautolysis treatment of yeasts with sucrose may cause as much as threefold increases in enzyme content. Invertase accumulation is also brought about by stimulation of yeasts with small amounts of sucrose during fermentation. Sumner and O'Kane (*29*) concluded that invertase was a polysaccharide-protein, while Adams and

Hudson (*1*) reported the enzyme as a carbohydrate-protein. It was suggested by Quastel and Yates (*24*) that the supposed zwitterion property of invertase permitted oppositely charged groups to be bridged by sucrose, the glucose portion being attached to the anion, and the fructose moiety to the cation.

The question of a sugar-invertase complex was investigated by the following procedure: Protein concentrate was dialyzed against distilled water for 36 hours at 2° C. Just prior to conducting the invertase assays, six

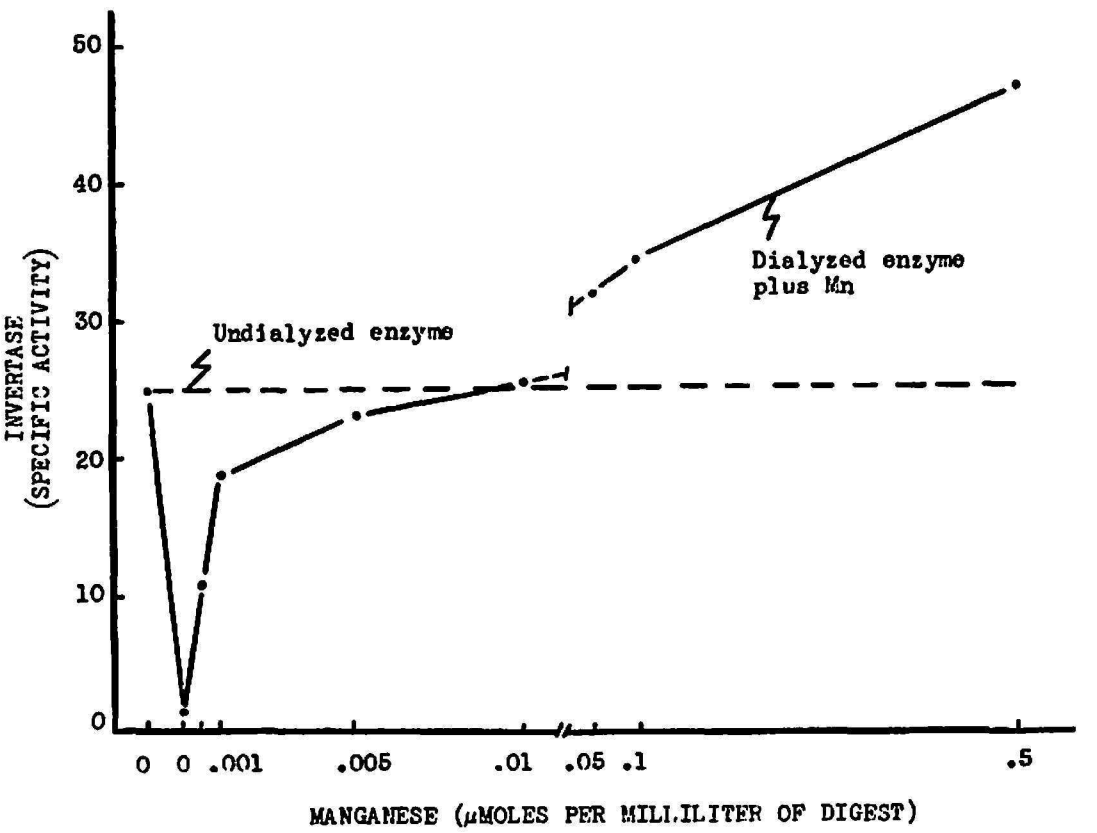


FIG. 9.—Stimulatory effects of manganese upon dialysis-inactivated invertase from sugarcane meristem.

equal samples were withdrawn from the concentrate, and one sample was diluted to the standard concentration with distilled water. The remaining five samples were diluted with sugar solutions of sufficient strength to yield a final concentration of 1 μmole of sugar per milliliter of protein solution. Sugar-protein solutions were made up with sucrose, maltose, galactose, glucose, and fructose.

Comparison of the protein-water and protein-sugar solutions with undialyzed preparations showed that 93 to 96 percent of the standard invertase reaction was lost via dialysis, and that traces of sugar additives in the dialyzed enzyme preparations sufficed to revive some of the activity.

Doubling the sugar concentrations to 2 μmoles/ml. further increased the activity of dialyzed invertase (table 4). Sucrose was least effective of the added sugars, yet it returned all of the activity lost via dialysis, while maltose, galactose, glucose, and fructose each stimulated the dialyzed enzyme well above control (undialyzed) invertase values. These experiments, as did those with added Mn, showed that the standard invertase preparation was acting considerably below its full potential.

Our conclusions from the dialysis, Mn-, and sugar-additive experiments are that the active, acid invertases of cane are protein-sugar-Mn complexes. The protein alone is nearly inactive, and either a sugar or Mn (possibly both) must be present for maximum activity.

TABLE 4.—*Inhibition of invertase by dialysis and reactivation by traces of sugar additives*[1]

Sugar concentration of enzyme solution (μmoles/ml.)	Specific activity of invertase prepared as follows—						
	Undialyzed + H_2O	Dialyzed + H_2O	Dialyzed + sucrose	Dialyzed + maltose	Dialyzed + galactose	Dialyzed + glucose	Dialyzed + fructose
1	29.2	1.3	2.9	16.9	20.2	18.5	15.9
2	29.7	2.1	30.0	43.3	41.3	43.8	41.3

[1] Each reaction mixture was composed of 0.5 ml. of acetate buffer (pH 5.5), 0.5 ml. of sucrose solution (sufficient to make 30 μmoles/ml. of digest), and 0.25 ml. of enzyme preparation made up in distilled water or dilute sugar solution.

SIGNIFICANCE OF SUGARCANE INVERTASES

The invertases of cane may be considered both beneficial and detrimental, depending upon what viewpoint (the plants' or the growers') is being expressed. The work of Hatch and coworkers has assigned truly positive roles to the invertases involved in sucrose movement and accumulation, and in internode elongation. The α-glucosidase encountered during the present studies could play a part in the degradation of unwanted polysaccharides. Invertases may also be regarded as necessary catalysts during the transfer of sucrose constituents to the metabolic pathways. In this respect, however, the hydrolytic reaction of invertase would be an inferior one to phosphorolysis, *i.e.*, the reverse reaction of sucrose phosphorylase, which would yield glucose-1-phosphate. Each of the hexoses resulting from the hydrolysis of sucrose would need to be phosphorylated before being metabolized, thus involving expenditure of ATP.

One should not overlook the potential transglycosylation reactions, in which invertases may catalyze the transfer of fructofuranosyl groups to

various alcohols and sugars, as well as to water[6]. Invertase will undoubtedly assume greater significance when these reactions have been clarified in sugarcane.

The very presence of invertase in cane might seem an unnecessary evil to the sugarcane grower. Undoubtedly, there are combinations of climatic factors and soil types, cultural practices and plain bad luck which will permit invertase to reduce his sugar yield below a level he might otherwise have obtained. A specific example is the delay of one to several days which is unavoidably granted to cane between the time it is cut and the time it is milled. Cross (*11*) reported in 1926 that delays in milling caused important losses to the Java sugar industry. Decline of cane-juice quality during storage has concerned workers in Hawaii (*25*), Louisiana (*20*), Egypt (*26*), and Puerto Rico (*9*). Being unusually stable in the unpurified form[7], invertase probably plays a major role in postharvest sucrose losses.

SUMMARY

Invertase has been extracted from meristem, leaf, sheath, node, and internode tissue of sugarcane. The meristem was the richest source for invertase acting under both acidic (pH 4.65) and neutral (pH 7.0) conditions. Acid invertase was extracted from meristem with water after the samples had been frozen, lyophilized, ground to a fine powder, and sonified in a powder-water suspension. Virtually all invertase was precipitated from solution with ammonium sulfate below 55-percent saturation. Acid invertase was precipitated primarily between 30- and 52-percent saturation.

Within the acid-invertase preparation, two distinct enzymes were demonstrated, one, α-glucosidase, "taka-invertase", which attacks the glucose end of the sucrose molecule, and the other, β-fructosidase, "yeast invertase", attacking the fructose end. β-fructosidase is predominant by about 2 to 1. The possibility that α-glucosidase takes part in the degradation of glucosidically linked oligosaccharides, or products of polysaccharide hydrolysis, is discussed.

Optimum pH for the acid-invertase preparation lay between 4.75 and 5.5. Optimum temperature was 44° C., and substrate concentration about 80 μmoles of sucrose per milliliter of digest.

Invertase was inhibited by iodide, lead, and mercury at concentrations of 1.0, 0.5, and 0.0003 μmole/ml. of digest, respectively. Iodide inhibition was completely reversed by increasing substrate concentration, and lead

[6] The enzymatic synthesis of sucrose from uridine diphosphate glucose, as elucidated by Leloir and coworkers (*10,21,22*), is an example of a transglycosylase reaction in which the donor molecule is a sugar nucleotide (*12*).

[7] Crude invertase preparations are known to have retained their activity for 19 years (*23*, p. 535).

inhibition was partly reversed. The inhibitory effects of mercury were not reversible.

Arsenic and tungsten also inhibited invertase, but at relatively high concentrations, 5.0 and 10.0 μmoles/ml. of digest, respectively.

Manganese doubled invertase activity at 0.5 μmole/ml. of digest, and as little as 0.005 μmole markedly stimulated the reaction.

Prolonged dialysis (36 hours) against distilled water reduced invertase activity by about 95 percent. Added manganese revived the activity and stimulated the enzyme beyond predialysis levels. Activity was also revived by sucrose, maltose, galactose, glucose, and fructose, when these were added to the dialyzed enzyme. It was concluded that the active, acid-invertases are protein-sugar-manganese complexes, in which the protein constituent is virtually inactive in the absence of either manganese or sugar.

RESUMEN

Se extrajo invertasa del tejido del meristemo, de la hoja, de la yagua, del nudo y del entrenudo de la caña de azúcar. El meristemo fue la fuente más rica de invertasa bajo condiciones tanto ácidas (pH 4.65) como neutrales (pH 7.0). La invertasa ácida se extrajo del meristemo con agua, después que las muestras se congelaron, liofilizaron, redujéronse a polvo fino y sonificaron en una suspensión de polvo y agua. Virtualmente, toda la invertasa se precipitó de la solución con sulfato de amonio, a una saturación menor de 55 por ciento. La invertasa ácida se precipitó principalmente a una saturación de 30 a 52 por ciento.

En la preparación de invertasa ácida, se estableció la presencia de dos enzimas distintas: una (glucosidasa-α, "taca-invertasa") que actúa sobre el extremo glucosado de la molécula de sacarosa y la otra (fructosidasa-β, "invertasa de levadura") que actúa sobre el extremo fructosado. La fructosidasa-β predomina en proporción de 2 a 1. Discútese también la posibilidad de que la glucosidasa-α participe en la reducción de las oligosacaridas ligadas a los glucosidas, o sea, los productos de la hidrólosis de las polisacaridas.

El pH óptimo para la preparación de invertasa ácida osciló entre 4.75 y 5.5. La temperature óptima fue de 44° C. y la concentración de substrato aproximadamente de 80 μmoles de sacarosa/ml. de digesto.

La invertasa fue inhibida por el yoduro, el plomo y el mercurio a concentraciones de 1.0, 0.5 y 0.0003 μmole/ml. de digesto, respectivamente. La acción inhibidora del yoduro se revertió completamente al aumentarse la concentración del substrato y la de plomo se revertió parcialmente. Los efectos inhibitorios del mercurio no fueron reversibles.

También el arsénico y el tungsteno ejercieron una acción inhibitoria sobre

la invertasa, pero a concentraciones relativamente altas (5.0 y 10.0 μmoles/ml. de digesto, respectivamente).

El manganeso redobló la actividad de la invertasa a una concentración de 0.5 μmole/ml. de digesto, y una cantidad tan pequeña como 0.005 μmole estimuló marcadamente la reacción.

Una diálisis prolongada (36 horas) en presencia de agua destilada redujo la actividad de la invertasa en alrededor de un 95 por ciento. Al agregársele manganeso, se reactivó y estimuló la enzima superando los niveles que existían antes de la diálisis. También la reactivaron vestigios de azúcares (sacarosa, maltosa, galactosa, glucosa y fructosa a 2 μmoles/ml. de digesto) añadidos a la enzima dializada. Se concluyó que las invertasas ácidas y activas son complejos de proteína, azúcar y manganeso, en los que la proteína se inactiva, virtualmente, en la ausencia lo mismo de manganeso que de azúcar.

LITERATURE CITED

1. Adams, M., and Hudson, C. S., New methods for the purification of invertase and some properties of the resulting products, *J. Amer. Chem. Soc. 65:* 1359–68, 1943.
2. Alexander, A. G., Sucrose-enzyme relationships in immature sugarcane as affected by varying levels of nitrate and potassium supplied in sand culture, *J. Agr. Univ. P.R. 48* (3): 165–231, 1964.
3. ——, The biosynthesis of sucrose in sugarcane, *J. Agr. Univ. P.R. 48* (4): 265–83, 1964.
4. Alexander, A. G., and González-Ibáñez, J., Residual effects of Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] on the sugar content and sugar-metabolizing enzymes of ratoon sugarcane 25 months following variable soil treatment, *J. Agr. Univ. P.R. 48* (4): 284–303, 1964.
5. Alexander, A. G., Behavior of enzymes governing starch and sucrose-forming pathways in two sugarcane varieties supplied with variable nitrate and phosphate in sand culture, *J. Agr. Univ. P.R. 49* (2): 153–75, 1965.
6. ——, Hydrolytic proteins of sugarcane: The Q enzyme, *J. Agr. Univ. P.R. 49* (2): 176–203, 1965.
7. ——, Hydrolytic proteins of sugarcane: The acid phosphatases, *J. Agr. Univ. P.R. 49* (2): 204–28, 1965.
8. Bird, R., and Hopkins, R. H., The action of some α-amylases on amylose, *Biochem. J. 56:* 86–99, 1954.
9. Boneta-García, E., and Lugo-López, M. A., Losses in cut cane kept under shade or sun for different periods, *J. Agr. Univ. P.R. 46* (3): 189–94, 1962.
10. Cardini, C. E., Leloir, C. F., and Chiriboga, J., The biosynthesis of sucrose, *J. Biol. Chem., 214:* 149–55, 1955.
11. Cross, W. E., The most important loss in making sugar from Java sugarcane, *Int. Sugar J. 28:* 245–8, 1926.
12. Dedonder, R. A., Glycosidases and transglycosidation, *Ann. Rev. Biochem. 30:* 347–82, 1961.
13. Fruton, J. S., and Simmonds, S., General Biochemistry, John Wiley & Sons, Inc., New York, N.Y., 1959.

14. Glasziou, K. T., Accumulation and transformation of sugars in sugarcane stalks: Mechanism of inversion of sucrose in the inner space, *Nature 193:* 1100, 1962.
15. Hartt, C. E., Some effects of potassium upon the amounts of protein and amino forms of nitrogen, sugars, and enzyme activity of sugarcane, *Plant Physiol. 9:* 453–90, 1934.
16. Hassid, W. Z., and Ballou, C. E., in Pigman, W., The Carbohydrates, chap. 9, Academic Press Inc., New York, N.Y., 1957.
17. Hatch, M. D., Sacher, J. A., and Glasziou, K. T., Sugar accumulation cycle in sugarcane; I, Studies on enzymes of the cycle, *Plant Physiol. 38:* 338–43, 1963.
18. Hatch, M. D., and Glasziou, K. T., Sugar accumulation cycle in sugarcane; II, Relationship of invertase activity to sugar content and growth rate in storage tissue of plants grown in controlled environments, *Plant Physiol. 38:* 344–48, 1963.
19. Koshland, D. E., and Stein, S. S., Correlation of bond breaking with enzyme specificity; Cleavage point of invertase, *J. Biol. Chem. 208:* 139–48, 1954.
20. Lauritzen, J. I., Losses of sucrose in mill cane during the interval between cutting and milling in Louisiana, *Sugar J. 10* (6): 8–13, 1947.
21. Leloir, L. F., and Cardini, C. E., The biosynthesis of sucrose, *J. Amer. Chem. Soc. 75:* 6084, 1953.
22. Leloir, L. F., and Cardini, C. E., The biosynthesis of sucrose phosphate, *J. Biol. Chem. 214:* 157–65, 1955.
23. Neuberg, C., and Mandl, I., in Sumner, J. B., and Myrback, K., The Enzymes, Chap. 14, Academic Press Inc., New York, N.Y., pp. 527–550, 1950.
24. Quastel, J. H., and Yates, E. D., The action of dyestuffs on invertase. The nature of the union between yeast invertase and sucrose, *Enzymologia 1:* 60–80, 1936.
25. Report of the Expt. Sta. Committee of the Hawaiian Sugar Planters Ass'n., pp. 45–6, 1919.
26. Rosenfeld, A. H., Deterioration of harvested sugarcane in Egypt, *Trop. Agr. 19:* 133–68, 1942.
27. Sacher, J. A., Hatch, M. D., and Glasziou, K. T., The sugar accumulation cycle in sugarcane; III, Physical and metabolic aspects of the cycle in immature storage tissue, *Plant Physiol. 38:*348–54, 1963.
28. Sumner, J. B., Dinitrosalicylic acid: A reagent for the estimation of sugar in normal and diabetic urine, *J. Biol. Chem. 47:* 5–9, 1921.
29. Sumner, J. B., and O'Kane, D. J., The chemical nature of yeast saccharase, *Enzymologia 12:* 251–3, 1948.
30. Sumner, J. B., and Somers, G. F., Chemistry and Methods of Enzymes, Academic Press Inc., New York, N.Y., 1953.
31. Sutherland, E. W., Cori, C. F., Haynes, R., and Olsen, N. S., Purification of the hyperglycemic-glycogenolytic factor from insulin and from gastric mucosa, *J. Biol. Chem. 180:* 825–37, 1949.