THE REGULATION OF INVERTASE SYNTHESIS IN SUGAR-CANE: EFFECTS OF SUGARS, SUGAR DERIVATIVES, AND POLYHYDRIC ALCOHOLS

By K. T. Glasziou* and J. C. Waldron*

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Summary

The acid invertase content of tissue slices from immature internodes of sugar-cane decreases rapidly when glucose is supplied in the bathing medium, maximum effectiveness occurring at about 0·03M.

The extracted enzyme is unstable in solution at pH values below 5·0, but glucose (0·1M) does not affect stability. The pH of juice was not altered by incubation of tissues in glucose for 8 hr. Treatments which induced a sixfold change in invertase content gave no significant changes in protein nitrogen content of the tissue.

The effectiveness of a large number of sugars and related compounds in changing the acid invertase content of the tissue has been examined. An interpretation is given in terms of the hypothesis that occupation of a controller site by D-glucose or suitable structural analogues regulates synthesis of the enzyme.

I. Introduction

The "glucose effect" on suppression of many catabolic enzymes in micro-organisms has long been known (Epps and Gale 1942; Monod 1947). Formation of both inducible (inositol dehydrogenase, histidase of *Aerobacter aerogenes*) and constitutive enzymes (invertase, α-glucosidase in yeast) may be suppressed by addition of glucose to the culture medium (Magasanik 1961). Depending on the organism, compounds related to glucose such as gluconic acid, mannitol, and galactose also repress the formation of catabolic enzymes. All glucose-sensitive enzymes give products which are readily synthesized from glucose. From these and other observations Magasanik has proposed that metabolites which are formed readily from glucose accumulate in the cell and repress the formation of enzymes whose activity would augment the already large intracellular pools of these compounds.

In immature storage tissue of sugar-cane availability of carbohydrate in the cell cytoplasm appears to be regulated by the level of two acid invertases, one located in the outer space (which includes the cell wall) and the second in the storage compartment (Sacher, Hatch, and Glasziou 1963*a*). Control of invertase level appears to be mediated through auxin which may increase or decrease the level depending on concentration, and a feedback control involving the level of sugar present in the metabolic compartment (cell cytoplasm) (Sacher and Glasziou 1962; Sacher, Hatch, and Glasziou 1963*b*). Sugars such as glucose, fructose, sucrose, and galactose will

* David North Plant Research Centre, The Colonial Sugar Refining Co. Ltd., Toowong, Qld.

reduce the invertase content of immature cane tissue. Thus this system has analogies with the glucose effect in microorganisms. It is the purpose of this paper to describe in more detail the properties of the sugar-mediated control over invertase level in immature cane storage tissue.

II. Materials and Methods

(*a*) *Sugar-cane Tissue*

Immature internodes from a *Saccharum officinarum*-type hybrid, cv. Pindar, were used throughout. Tissue was taken from the basal 2 cm of rapidly elongating internodes from field-grown cane. Tissues were cut into slices approximately 1·0 mm in thickness, washed thoroughly in tap water to remove cell debris, randomized, and weighed into 2·0-g lots. Treatments were carried out in 5·0 ml solution contained in 50-ml conical flasks. Solutions were changed at 2–3-hr intervals. The flasks were shaken at 120 strokes per minute in a water-bath at 30°C. After treatment, the tissues were ground with pestle and mortar, the juice squeezed out through cheesecloth, and 1·0-ml aliquots transferred to dialysis sacks for dialysis at 3°C. No loss of invertase activity was incurred for periods up to 24 hr during dialysis at 3°C.

(*b*) *Enzyme Assays*

After dialysis, the contents of the sacks were washed out with 10% sucrose buffered at pH 5·5 with 0·05M acetate, and made to a final volume of 4·0 ml with this solution. Toluene was added to all assay mixtures which were incubated at 30°C for 1–3 hr depending on the amount of enzyme present. Aliquots (1·5 ml) were removed and added to 3·0 ml absolute ethanol to stop the reaction. Reducing sugars were measured in the ethanolic solution as previously (Hatch, Sacher, and Glasziou 1963).

(*c*) *Reagents*

1-[^{14}C]glucosamine was obtained from New England Nuclear Corporation; all other sugars were obtained from Nutritional Biochemicals Corporation.

III. Results

(*a*) *Changes in Invertase Levels in Tissue Slices Kept in Water*

In the experiments described in this paper cane grown under field conditions has been used in preference to cane from constant artificial environment as we feel that in the exploratory stages the use of variable source material has helped to avoid overemphasizing aspects of the control system which are apparent only under certain closely specified conditions. This procedure has lead to some difficulty in expression of results as the tissues placed in water behave in a variable manner. In some experiments the invertase level in tissues so treated may remain virtually constant for 18 hr; in others there may be an immediate and continuing increase in enzyme level or an initial decrease, usually followed by a rapid increase after about 4–6 hr. Hence where enzyme content of control in water varied from the initial level, results

are expressed in terms of percentage of control or the changes in the control are indicated. Possible reasons for the variable behaviour of tissue in water are discussed in a later section.

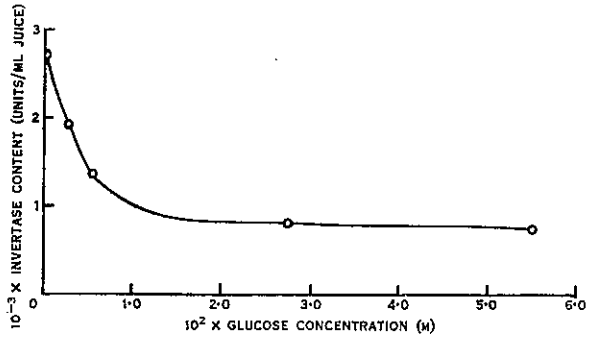

Fig. 1.—Effect of D-glucose on levels of acid invertase. 2-g lots of tissue were incubated for 8 hr at 30°C in 5 ml glucose solution following which the tissues were ground, the juice squeezed through muslin, dialysed at 2–3°C overnight, then assayed for invertase activity. Initial level of invertase activity was 3060 units.

(*b*) *Characteristics of Glucose Suppression*

The curve for effects of different concentrations of glucose on the levels of acid invertase in immature tissue following an 8-hr incubation period (Fig. 1) showed the effect reached a maximum with a concentration of approximately 0·03M glucose.

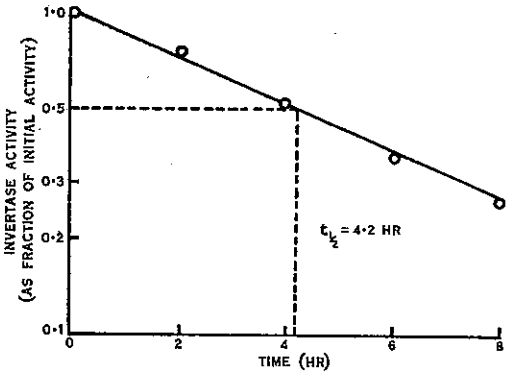

Fig. 2.—Time course for loss of invertase activity in tissues treated with 0·055M D-glucose. Experimental detail as described for Figure 1 except that tissues were harvested at the time intervals shown.

The time course for loss of enzyme in tissue placed in 0·055M glucose gave the relationship expected for a first-order reaction (Fig. 2). In this experiment the time for loss of half the enzyme present initially was little more than 4 hr. In other experiments a lag of several hours occurred before loss of invertase became appreciable.

TABLE 1

EFFECTS OF SUGARS, SUGAR DERIVATIVES, AND POLYHYDRIC ALCOHOLS ON LEVELS OF ACID INVERTASE IN IMMATURE STORAGE TISSUE

2-g lots of 1·0-mm tissue slices were incubated for 7–9 hr at 30°C with shaking in 5·0 ml test solution containing 0·03 mM $CaCl_2$. When necessary the pH was adjusted to 6·0. At the end of the incubation period the tissues were ground and the juice squeezed out through muslin, dialysed at 2–3°C overnight, and then assayed for invertase activity. Glucose was included for comparison purposes in each experiment

Substance	Molarity	Invertase Content as Percentage of Water Control	Invertase Content Relative to Glucose*
2-Deoxy-D-glucose	0·006	10	0·14
	0·06	10	0·20
D-Mannose	0·055	25	0·55
2-Amino-D-glucose	0·028	40	0·97
	0·055	29	0·89
D-Galactose†	0·055	67	0·94
L-Ascorbic acid	0·06	36	1·1
D-Arabinose	0·06	52	—
	0·12	47	—
D-Galacturonic acid	0·06	39	1·2
Melibiose	0·03	72	1·4
Maltose	0·06	53	1·7
Raffinose	0·02	108	—
	0·04	84	3·8
	0·20	21	2·0
Lactose	0·03	108	—
	0·04	95	4·3
	0·20	59	5·4
α-Methyl-D-mannoside	0·06	106	3·1
	0·12	83	3·2
L-Arabinose	0·04	152	6·9
	0·077	109	—
	0·20	84	7·7
i-Inositol	0·06	96	2·8
	0·12	99	3·9
Mannitol	0·055	145	2·9
Glycerol	0·06	137	2·8
α-Methyl-D-glucoside	0·005	109	1·6
	0·05	151	4·4
D-Arabitol	0·06	160	4·4
	0·12	179	7·6
Ribitol	0·06	150	4·4
	0·12	208	8·1
D-Sorbitol	0·06	186	5·1
	0·12	214	9·1
Galactitol	0·06	183	5·0
	0·12	254	11·0

* Expressed as relative to invertase content for glucose-treated tissue in the same experiment and at the same concentration.

† Incubation time 4½ hr.

(c) Effects of Sugars, Sugar Derivatives, and Polyhydric Alcohols on Acid Invertase Levels

Many sugars and related compounds affected the invertase level of the tissue when compared with tissues in water or glucose at the same concentration as the test substance (Table 1). Some, such as 2-amino-D-glucose (Fig. 3) and 2-deoxyglucose reduce invertase levels below controls in water at all concentrations used. Others increased invertase levels when supplied in low concentration but suppressed at high concentrations. Yet a third group increased the invertase level at both low and high concentrations. One substance, i-inositol, appeared to have no effect.

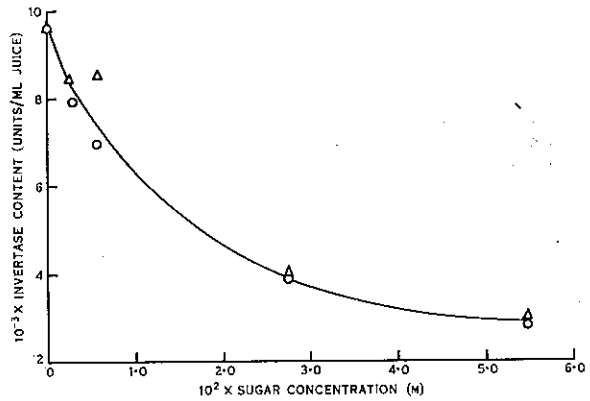

Fig. 3.—Comparison of effects of D-glucose (△) and 2-amino-D-glucose (○) on levels of acid invertase. Experimental detail as described for Figure 1, except that solutions were buffered at pH 5·5 with 0·005M acetate buffer.

(d) Comparison of Effects of D-Glucose and 2-Amino-D-Glucose on Acid Invertase Levels

2-Amino-D-glucose was selected for further study as a substance which suppressed invertase levels and which is not normally found in higher plants. Comparison with glucose on the basis of concentration in the external solution showed virtually identical effects (Fig. 3). No significant differences were obtained between the two sugars when supplied at 0·055M in media adjusted to pH values of 4·5, 5·5, and 6·7.

Studies were made using ^{14}C-labelled 2-amino-D-glucose at a concentration of 0·055M to determine whether this sugar was metabolized by the tissues. After an 8-hr incubation period, tissues were either killed immediately with 3 volumes of hot ethanol or washed in running tap water for 1 hr to remove substances in the outer space (Glasziou 1960) prior to placing in ethanol. Controls were tissues killed in ethanol and ^{14}C-labelled 2-amino-D-glucose added subsequently. The extracts were examined using paper-chromatographic techniques. Radioactivity was found in two zones other than the 2-amino-D-glucose area in both washed and unwashed tissue but not in controls. One zone ran in the same area as sugar phosphates on single- and two-dimensional chromatograms and contained 69% of the total radioactivity in extracts

from water-washed tissues. After elution with water the sugar phosphate zone was treated with alkaline phosphatase, then applied to Whatman No. 54 paper, overspotted with authentic non-radioactive 2-amino-D-glucose, and the chromatogram developed in one direction with n-propanol–ammonia–water (6 : 3 : 1 v/v) and in a second direction in n-propyl acetate–formic acid–water (11 : 5 : 3 v/v). A radioautogram was prepared after which the chromatogram was sprayed with ninhydrin to detect the 2-amino-D-glucose marker. The area showing blue with ninhydrin corresponded in every detail to the radioactive zone detected by radioautography and accounted for 74% of the radioactivity in the chromatogram. Hence we conclude that one of the substances produced from 2-amino-D-glucose by sugar-cane tissue was 2-amino-D-glucose phosphate.

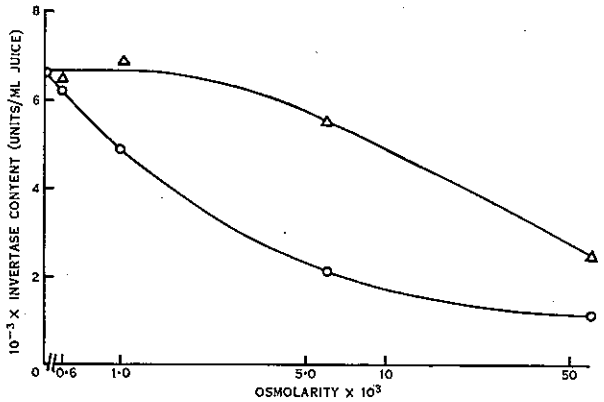

Fig. 4.—Comparison of effects of D-glucose (○) and potassium chloride (△) on levels of acid invertase. Experimental detail as described for Figure 1. Control was tissue in water. Osmolarity was taken as 1 × molarity for glucose and 2 × molarity for potassium chloride.

A second radioactive substance produced from 2-amino-D-glucose was present in the initial extracts and was also detected when the sugar phosphate zone was rechromatographed following treatment with alkaline phosphatase. This substance has not yet been identified. It was not detected in extracts from tissues treated with radioactive glucose.

(*e*) *Osmotic Effects on Invertase Levels*

A comparison of effects of glucose and potassium chloride showed that glucose was much more effective in decreasing invertase content than potassium chloride (Fig. 4). Other potassium and also calcium salts, expressed on an osmolar basis, gave similar results to potassium chloride. The effects of inorganic salts may be unrelated to the effect of glucose and other sugars. In support of this premise, the results given in Table 1 show that several substances had no marked effect on invertase content even though present at higher osmolarities than the highest used for potassium chloride in this experiment.

(*f*) *Changes in Invertase Levels and Lack of Correlated Changes in the Total Protein Content of the Tissue*

The changes in protein nitrogen content and invertase content of tissues incubated in various media (Table 2) showed that while invertase content varied sixfold, no significant change occurred in the total protein nitrogen content of the tissue. This result would be expected for an enzyme which performs a regulatory function by virtue of changes in the level of enzyme in the tissue; hence support is given to the proposal of Sacher, Hatch, and Glasziou (1963*a*) that acid invertase is a key regulatory enzyme in immature stalk tissue of sugar-cane.

TABLE 2

CHANGES IN INVERTASE CONTENT AND PROTEIN NITROGEN

Tissues for invertase assays were treated as described for Table 1. For protein nitrogen, 2 g fresh weight tissue was extracted, once for 10- and twice for 30-min periods, with 5% trichloroacetic acid. After removal of trichloroacetic acid, two extractions were made with 1 : 1 (v/v) ethanol–diethyl ether, each for 1 hr. Protein nitrogen was determined on the residues

Treatment	Invertase Content (units/ml juice)	Protein Nitrogen (mg/g fresh wt.)
Fresh tissue	2700	0·80
Water, 8 hr	6500	0·81
0·1M glucose, 8 hr	1050	0·85

(*g*) *Properties of Extracted Acid Invertase*

The Michaelis constant and pH activity curve were given previously (Hatch, Sacher, and Glasziou 1963). We found no change in stability of the extracted enzyme in the presence of 0·1M glucose or mannitol. The enzyme in juice is unstable at 30°C and low pH. For an enzyme preparation partially purified by ammonium sulphate fractionation, the time for loss of half the initial activity was 30 hr at pH 5·2, and and 3·5 hr at pH 4·2. The pH of juice extracted from immature tissue of the variety in use was 5·0 and was not altered after incubation of tissue for 8 hr in 0·05M glucose, indicating that acid production will not account for the glucose effect on invertase levels.

IV. DISCUSSION

Compared with tissue pretreated with glucose, previous results (Sacher, Hatch, and Glasziou 1963*b*) showed that a much higher rate of increase of invertase content occurred when the tissues were transferred to water if pretreatment was with glycine plus glucose. This observation was taken to indicate that a pool of precursors for enzyme synthesis accumulated during the glycine and glucose pretreatment and were

incorporated when glucose was removed. Our present results show that glucose has no effect on the stability of the extracted enzyme, that stability decreased as the pH was lowered, but that incubation of tissues in glucose did not change the pH of the juice. The simplest hypothesis which accords with these results is that glucose reduces the rate of synthesis of acid invertase.

Many paper chromatograms have been run in this laboratory over a period of years on extracts of a large number of sugar-cane varieties. No free sugars other than glucose, fructose, and sucrose have been observed except in one isolated instance when a trace amount of kestose was detected in a wild cane (*S. spontaneum*). If a sugar and not a derived metabolite is the natural suppressor for invertase synthesis, we probably need only consider glucose, fructose, and sucrose. Since they are readily interconverted in the tissue (Glasziou 1960), the observation that each of these sugars suppresses invertase synthesis when supplied in the medium bathing tissue slices (Sacher and Glasziou 1962) does not indicate which one is effective.

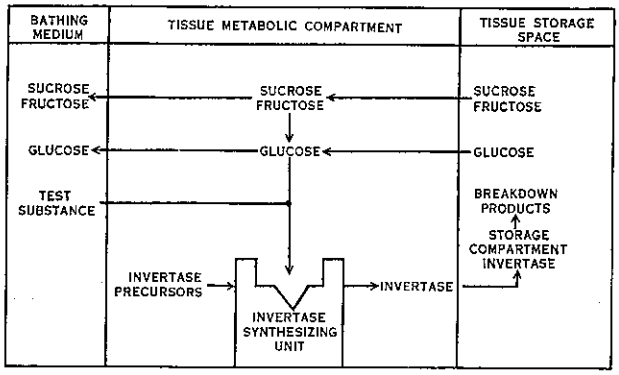

Fig. 5.—Schematic representation of system for measuring effects of test substances on invertase synthesis. A substance added to the bathing medium competes with endogenous glucose for the repression site. Substances which fit well stop synthesis. Poorly fitting substances may prevent endogenous glucose reaching the repressor site, and may thus act as promotors.

The effect of D-glucose on invertase synthesis in tissue slices is closely simulated by 2-amino-D-glucose, these two sugars giving equivalent effects over a 20-fold concentration range and also at three different pH values. Metabolism of 2 amino-D-glucose yields 2-amino-D-glucose phosphate and an unidentified substance and its phosphorylated derivative. However, the latter substance has not been detected as a product of glucose metabolism. Since it is unlikely that glucose and 2-amino-D-glucose gave rise to their phosphorylated derivatives with equal efficiency, we draw the tentative conclusion that glucose is the natural feedback controller of invertase synthesis in sugar-cane stalks and that 2-amino-D-glucose acts as a structural analogue.

The simplest model system which accords with present knowledge is shown in Figure 5. The subsequent discussion offers an explanation for our results in terms of this model.

When immature internodal tissue is placed in water, sucrose, glucose, and fructose leak from the storage compartment to the medium (Glasziou 1960). Hence, we expect the endogenous glucose concentration at the site for suppression of invertase synthesis to be a function of glucose concentration in the storage compartment, the permeability of cell membranes, the rate at which glucose is utilized in the metabolic compartment, and the rates at which sucrose and fructose are converted to glucose during efflux from the tissue. Since the medium bathing the tissues is changed frequently during an experiment it may be considered to have infinite volume. Thus our control system (tissue in water) is one in which the full potential for invertase synthesis is partially suppressed by endogenous glucose.

We next consider three classes of substances which may compete with glucose for the "controller site":

A: Substances which fit the site and have the appropriate configuration to effectively halt invertase synthesis.

B: Substances as in A, but because of poor configurational fit, are not very effective in halting enzyme synthesis.

C: Substances which have affinity for the controller site but do not have the structural characteristics necessary to halt invertase synthesis.

The first seven substances listed in Table 1 probably belong to class A, and have additive effects to endogenous glucose. Thus they inhibit enzyme synthesis at all concentrations.

Substances in class B would behave as suppressors of invertase synthesis at all concentrations only in the complete absence of endogenous glucose. If the control tissue is partially suppressed by a low level of endogenous glucose, class B substances would give apparent promotion when added at low levels and suppression when added at high levels. If the endogenous glucose level was very high, this class would act as apparent promotors of invertase synthesis in our test system. Examples may be L-arabinose and lactose.

Class C substances will act as competitive inhibitors of the endogenous glucose effect in our test system, that is as promotors of invertase synthesis. The polyhydric alcohols listed at the end of Table 1 are probably representatives of this class.

The data at present available are inadequate to define exactly the configurational requirements for prevention of invertase synthesis. Some of the substances shown in Table 1 may owe part of their effect to entry into metabolism and conversion to glucose. If we accept that the first seven substances of Table 1, the α-methyl-D-glycosides, and the polyhydric alcohols act as such it would seem that the configuration about carbon atoms 1 and 2 and both the oxygen atom attached to carbon 1 and the ring oxygen of the glucopyranose structure may be of primary importance. A carbonyl oxygen at carbon 1 is apparently satisfactory (ascorbic acid), but substitution of $-OCH_3$ for –OH virtually destroys activity (α-methyl glycosides). The –OH group on carbon 2 is unnecessary for activity and may be replaced by –H or $-NH_2$ (2-deoxy-D-glucose, 2-amino-D-glucose). Activity is improved if the –OH group on carbon atom 2 is on the opposite side of the ring from that in D-glucose (as in D-mannose). Work on structural requirements for activity is being continued.

V. References

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