THE PHYSIOLOGY OF SUGAR-CANE

VIII. DIURNAL FLUCTUATIONS IN THE ACTIVITY OF SOLUBLE INVERTASE IN ELONGATING INTERNODES

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Summary

The activity of acid invertase in the storage compartment of elongating internode cells of sugar-cane stems fluctuates several fold during a single day, reaching a maximum early in the morning and falling to a minimum late in the afternoon. Parallel fluctuations in either protein content or hexokinase activity did not occur.

Diurnal fluctuations in invertase activity were observed both in field-grown plants and in plants grown in a controlled environment where the only environmental variable was a light–dark cycle. Exposure to low intensity red or far red light during darkness had no effect on invertase activity.

An explanation is presented in terms of the glucose-mediated repression of invertase synthesis, the degree of repression being dependent upon the cytoplasmic glucose concentration which fluctuates as the result of changes in the amount of sucrose in the translocation stream.

I. INTRODUCTION

The activity of soluble acid invertase in expanding internodes of sugar-cane stems changes by several orders of magnitude on a seasonal basis, and is positively correlated with the rate of internode expansion (Hatch and Glasziou 1963).

Studies with tissue slices on sugar movement in storage parenchyma cells indicated that soluble invertase is located in two cellular compartments—the outer space, a region in rapid diffusion equilibrium with sugar in a bathing medium, and the storage compartment which includes the cell vacuole (Sacher, Hatch, and Glasziou 1963*a*). Since the inversion of sucrose was found to be a prerequisite for its entry into metabolism, the outer space enzyme was considered to regulate the movement of sucrose into the tissue, and the storage compartment enzyme the return of sugars from storage (Sacher, Hatch, and Glasziou 1963*a*). Synthesis of the latter enzyme in tissue slices is apparently dependent upon a dual control system of hormone regulation and feedback repression by products of the enzyme's action (Sacher, Hatch, and Glasziou 1963*b*; Glasziou and Waldron 1964*a*, 1964*b*). In these tissue-slice studies the level of storage compartment invertase was found to change as much as 11-fold in a 6-hr period, suggesting that this enzyme may act to compensate for short-term fluctuations

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in the availability of reducing sugars for metabolic processes. The present investigation shows that similar short-term changes in the activity of storage compartment invertase occur in intact plants, and suggests that these changes are related to the fluctuations in the concentration of photosynthate in the translocation stream.

II. Materials and Methods

A commercial variety of sugar-cane (cv. Pindar) was grown in an irrigated field plot, or in vermiculite (Bull 1964) in either a glasshouse or artificially illuminated growth room. The latter two environments were maintained at constant temperature and 75% relative humidity. Light was supplied for 13 hr daily (0600-1900 hr) from six Philips 400-W HPL lamps supplemented by an incandescent source at a total intensity of approximately 2×10^5 erg cm^{-2} sec^{-1}. When required, red light was supplied from a single 400-W Philips HPL lamp covered with a red Perspex filter (I.C.I. 400) and far red light from a single 200-W incandescent bulb covered with both a red Perspex filter and a blue Perspex filter (I.C.I. 703).

At harvest approximately half fully elongated internodes of similar length were selected and the basal quarter of each assayed separately.

(*a*) *Sugar Determinations*

Reducing sugars produced in invertase assays and sucrose and reducing sugars in tissue extracts were determined as described by Hatch and Glasziou (1963).

(*b*) *Total Soluble Invertase Assays*

Soluble invertase was assayed in 1 ml of expressed juice as described by Glasziou and Waldron (1964*a*), and also in extracts concentrated by ammonium sulphate precipitation. For the latter method internode segments were split in half longitudinally, after removal of the rind. One half was used for sugar determinations. The other was weighed and ground with sand in an equal volume (w/v) of 0·1M potassium phosphate buffer, pH 6·9. Sodium diethyldithiocarbamate (final concentration 0·01M) was added to reduce polyphenol oxidase activity. The liquid was expressed through muslin, centrifuged at 25,000 *g* for 10 min, and four volumes (v/v) of saturated ammonium sulphate added to the supernatant. The precipitate, recovered by centrifugation, was dissolved in 0·005M potassium phosphate buffer, pH 6·9, and dialysed overnight against the same buffer. All operations to this stage were carried out at 3°C. Invertase was assayed by incubating 0·2 ml of the dialysed enzyme extract with 0·8 ml of 0·3M sucrose in 0·05M sodium acetate buffer, pH 5·5, for 2 hr at 30°C. The reaction was stopped by the addition of 2 ml absolute ethanol followed by heating at 70°C for 5 min.

(*c*) *Estimation of the Distribution of Soluble Invertase Activity between the Outer Space and Storage Compartment of the Tissue*

The activity of soluble invertase located in the tissue outer space and storage compartment was measured, as described by Sacher, Hatch, and Glasziou (1963*a*), using tissue disks (0·5 mm thick, 5·0 mm diameter) cut transversely from the basal 2 cm of elongating internodes.

(*d*) *Hexokinase Assay*

Hexokinase activity in the extracts concentrated by precipitation with ammonium sulphate was determined by incubating 0·05 ml dialysed enzyme extract, 4 μmoles ATP, 2 μmoles $MgCl_2$, 15 μmoles potassium phosphate buffer, pH 7·7, and 0·2 μmole [^{14}C]glucose (U) ($2{\cdot}2 \times 10^6$ counts/min) in a final volume of 0·2 ml at 30°C. Aliquots (10 μl) of the reaction mixture were co-chromatographed with unlabelled glucose and fructose on paper using ethyl acetate–pyridine–water (8 : 2 : 1 v/v) as the developing solvent. The sugars were located with *p*-anisidine phosphate and the

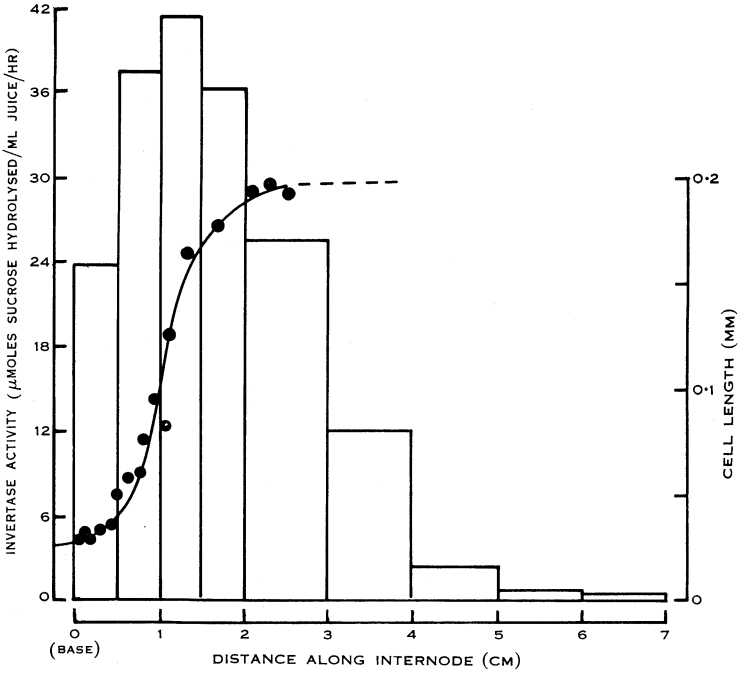

Fig. 1.—Distribution of invertase activity (histogram) and cell length (●) in a half fully elongated internode (length 8 cm). Invertase was assayed in expressed juice as described in Section II. Cell length was estimated from the number of cells across transverse sections (0·5 mm thick) taken at various positions along the internode.

radioactivity counted directly on the chromatograms with a Geiger–Müller counter (4·5% efficiency on paper). Hexokinase activity was determined from the distribution of counts between glucose and the phosphorylated compounds which remained at the origin of the chromatograms. The rate of glucose phosphorylation was linear during a 30-min assay. No radioactivity was detected in fructose.

(*e*) *Protein Determinations*

Protein was assayed by the Folin method (Lowry *et al.* 1951) using bovine serum albumin as an arbitrary standard.

(f) Determination of the Sugar Content of Leaf, Sheath, and Internode Tissue

The leaf and sheath attached to the top of each selected internode were cut into small pieces and subsamples extracted with three volumes (w/v) of boiling 75%

TABLE 1

INVERTASE ACTIVITY OF ELONGATING INTERNODES AT VARIOUS TIMES OF THE DAY

Stems were harvested at intervals during the day as indicated. Approximately half fully elongated internodes of similar length were selected and juice was expressed from the basal quarter of each internode. Invertase activity of dialysed juice was assayed as described in Section II

Growth Environment	Time of Harvest (hr)	No. of Internodes Used	Mean Invertase Activity (μmoles sucrose hydrolysed/ml juice/hr)
Field plot (sunrise 0600 hr)	0645	15	$55{\cdot}7 \pm 0{\cdot}9$*
	1600	12	$22{\cdot}4 \pm 1{\cdot}6$
Field plot (sunrise 0600 hr)	0600	12	$26{\cdot}6 \pm 2{\cdot}0$
	1130	12	$52{\cdot}0 \pm 2{\cdot}4$
	1600	11	$10{\cdot}8 \pm 1{\cdot}0$
Growth room, 30°C (lights on 0600 hr)	0630	3	$58{\cdot}4 \pm 1{\cdot}0$
	0930	4	$70{\cdot}0 \pm 1{\cdot}7$
	1230	4	$33{\cdot}5 \pm 3{\cdot}9$
	1630	4	$18{\cdot}3 \pm 1{\cdot}1$

* Standard error of mean.

ethanol. Juice expressed from each internode segment was placed directly into three volumes (v/v) of cold absolute ethanol. The ethanolic extracts were decolourized

TABLE 2.

PROTEIN CONTENT AND INVERTASE AND HEXOKINASE ACTIVITY OF ELONGATING INTERNODES AT VARIOUS TIMES OF THE DAY

Experimental detail as in Table 1 except that basal internode segments were extracted in an equal volume (w/v) of 0·1M KH_2PO_4–K_2HPO_4 buffer, pH 6·9, containing 0·02M sodium diethyldithiocarbamate and the protein precipitated with four volumes (v/v) of saturated ammonium sulphate. The protein content and invertase and hexokinase activity were assayed as described in Section II

Growth Environment	Time of Harvest (hr)	No. of Internodes Used	Mean Protein Content (mg/g fresh wt.)	Mean Invertase Activity (μmoles sucrose hydrolysed/g fresh wt./hr)	Mean Hexokinase Activity (μmoles glucose phosphorylated/g fresh wt./hr)
Growth room	0900	4	$1{\cdot}54 \pm 0{\cdot}08$*	$24{\cdot}2 \pm 1{\cdot}0$	$0{\cdot}33 \pm 0{\cdot}02$
26°C (lights on 0600 hr)	1600	3	$1{\cdot}56 \pm 0{\cdot}07$	$14{\cdot}4 \pm 1{\cdot}4$	$0{\cdot}34 \pm 0{\cdot}02$

* Standard error of mean.

with activated charcoal where necessary, and the sugar content determined as described above.

III. Results

(a) *Distribution of Soluble Invertase Activity within an Expanding Internode*

There were marked differences in the activity of soluble invertase in juice extracted from different regions of a half fully elongated internode (Fig. 1). Activity was highest in the basal quarter of the internode, which corresponds to the region of cell elongation, and decreased with the increasing maturity of fully elongated cells. Activity was lower in the first 0·5 mm section which includes the intercalary meristem.

Table 3

SOLUBLE OUTER SPACE AND STORAGE COMPARTMENT INVERTASE ACTIVITY OF WASHED TISSUE DISKS FROM ELONGATING INTERNODES AT VARIOUS TIMES OF THE DAY

Batches of 15 tissue disks (5·0 mm diameter, 0·5 mm thick) were randomly selected from disks cut from the basal 2 cm of each internode and incubated in 0·03M [^{14}C]sucrose (U) to enable determination of the outer space invertase activity. This activity was corrected for invertase activity associated with thoroughly washed tissue residues to give the soluble outer space invertase activity. Storage compartment invertase activity was calculated from the difference between the soluble invertase activity of juice expressed from tissue disks (assayed at 0·03M sucrose) and the soluble outer space invertase activity. For details see Section II

Growth Environment	Time of Harvest (hr)	No. of Internodes Used	Mean Soluble Outer Space Invertase Activity (μmoles sucrose hydrolysed/g fresh wt./hr)	Mean Storage Compartment Invertase Activity (μmoles sucrose hydrolysed/g fresh wt./hr)
Field plot (sunrise 0600 hr)	0930	4	1·21±0·08*	3·10±0·16
	1530	4	1·11±0·09	1·02±0·18

* Standard error of mean.

(b) *Soluble Invertase Activity in Elongating Internodes at Various Times of the Day*

The invertase activity in juice from the basal quarter of elongating internodes was higher shortly after sunrise than in the late afternoon, reaching a maximum early in the morning (Table 1). Similar results were obtained when a different method of invertase extraction was used (Table 2). With this method any possible effects of the low pH of the juice or the products of polyphenoloxidase activity on invertase activity were avoided by the use of buffer and sodium diethyldithiocarbamate during extraction. Although the invertase activity at the morning harvest was almost twice that at the afternoon harvest there was no change in the total protein content of the tissue nor evidence for any change in hexokinase activity during the day (Table 2).

These daily fluctuations in the activity of soluble invertase are attributable to variations in the activity of the storage compartment invertase, since no significant short-term changes were recorded for the outer space enzyme (Table 3). However, the activity of this enzyme certainly alters on a longer-term basis, becoming lower as the cells mature (Fig. 1), and disappears completely in fully elongated mature internodes (Hatch and Glasziou 1963). The lower invertase activity of washed tissue disks (Table 3) as compared with that of unwashed blocks of tissue (Tables 1, 2, and 4) can be attributed to the loss of enzyme from damaged cells.

(c) *Effect of Photosynthetic and Photomorphogenetic Light upon Changes in Invertase Activity*

The invertase activity in elongating internodes of plants exposed for 9 hr to photosynthetic light, low intensity red and far red light, and to darkness are shown in Table 4. Exposure to sunlight caused a 10-fold reduction in invertase activity. It appears that invertase activity is not affected by photomorphogenetic light since there was no significant difference between either the red, far red, or dark treatments. The fall in the invertase activity of the dark-grown plants between 0630 hr and 1530 hr may be due to a circadian rhythm not affected by light, or perhaps to substrate limitation of protein synthesis due to prolonged dark treatment.

TABLE 4

EFFECT OF SUNLIGHT, LOW INTENSITY RED AND FAR RED LIGHT, AND DARKNESS ON THE INVERTASE ACTIVITY OF ELONGATING INTERNODES

Plants, previously grown at 35°C in a glasshouse, were given one of the following light treatments: sunlight, darkness, red light, or far red light. Treatments were for 9 hr (0630–1530 hr) immediatly prior to harvest. Similar plants were harvested at 0630 hr. The invertase activity of elongating internodes was assayed as described in Table 1. Details of the red and far red light supplied are given in Section II

Treatment	No. of Internodes Used	Mean Invertase Activity (μmoles sucrose hydrolysed/ml juice/hr)
Harvested 0630 hr	5	51·6±3·2*
Sunlight (0630–1530 hr)	4	5·1±0·4
Darkness (0630–1530 hr)	3	25·0±3·4
Red light (0630–1530 hr)	5	28·2±2·1
Far red light (0630–1530 hr)	3	26·8±2·4

* Standard error of mean.

(d) *Sugar Content of Leaf, Sheath, and Internode Tissue at Various Times of the Day*

The sugar content of leaf, sheath, and internode tissue of the plants used in certain experiments was determined (Table 5). There was no accumulation of soluble sugars in either the leaf or sheath when plants were grown under the relatively low light intensity of a growth room (experiment B) nor in field-grown cane receiving full sunlight (Bull, personal communication). These observations together with the fact that the starch content of the leaves and sheaths of the cultivar Pindar remains at a low level throughout the day (<0·01% fresh weight—Bull, personal communication) suggests that little carbohydrate is stored in the leaves during photosynthesis. From the increase in sugar content of the elongating internode segments during the day (Table 5) it would appear that photosynthate accumulates in this tissue. It should be

emphasized that the sugar content of juice from the internode tissue is believed to reflect mainly the amount of sugar in the storage compartment, and as such, provides no information about the sugar content of either the cytoplasm or the tissue free space.

IV. Discussion

The results show that the activity of storage compartment invertase fluctuates diurnally in elongating cells of expanding internodes. The maximum activity occurs early in the morning and the minimum in the late afternoon or early evening. The diurnal changes in activity observed in field-grown plants were similar to those in plants grown in artificially lighted growth rooms where the only environmental variable was a light–dark cycle. No effect of photomorphogenetic light on invertase activity was observed and it appears, therefore, that the changes in activity were coupled in some way to photosynthetic illumination.

Table 5

SUGAR CONTENT OF LEAF, SHEATH, AND ELONGATING INTERNODES AT VARIOUS TIMES OF THE DAY

Plants used in the first experiment described in Table 1 and in the experiment described in Table 2 were analysed for soluble sugars. (They are referred to in this table as experiments A and B respectively.) Details of sugar extraction and analysis are given in Section II

Experiment	Time of Harvest	Reducing Sugar in Juice (% w/v)	Sucrose in Juice (% w/v)	Total Sugars in Leaf (% fresh wt.)	Total Sugars in Sheath (% fresh wt.)
A	6·45 a.m.	1·71±0·05	0·76±0·03	—	—
	4·00 p.m.	2·33±0·08	1·00±0·05	—	—
B	9·00 a.m.	2·85±0·18	0·42±0·04	0·72±0·12	0·87±0·20
	4·00 p.m.	3·71±0·15	0·56±0·07	0·89±0·07	1·06±0·09

* Standard error of mean.

From the results of tissue-slice studies, Sacher, Hatch, and Glasziou (1963*b*) proposed that the cytoplasmic glucose concentration has an overriding regulatory effect upon the level of invertase in the storage compartment, such that when the glucose concentration is low the invertase level is high and vice versa. If such a system is responsible for the diurnal changes in invertase activity in the intact plant then the cytoplasmic glucose concentration must decrease during darkness. This would be expected if the rate of supply of glucose to the cytoplasm falls disproportionately in relation to the rate of glucose utilization. The supply of glucose and fructose to the cytoplasm from translocated sucrose is mediated by outer space invertase (Sacher, Hatch, and Glasziou 1963*a*). No evidence was obtained for a diurnal change in the level of this enzyme and, therefore, a decrease in the production of glucose and fructose during the dark period could occur only when the sucrose available to the outer space invertase falls below its saturating concentration. It was shown that the leaves and sheaths of sugar-cane cv. Pindar store little of the photosynthate fixed during the day. Consequently the amount of sucrose available to the outer space invertase from the translocation stream would be expected to fall sharply with the onset of

darkness. The average rate of stalk extension during light and dark periods was similar in plants growing at constant temperature and humidity in a growth room (Slack, unpublished observation). From this it is tentatively assumed that the rate of carbohydrate utilization by the elongating internode is relatively similar during light and darkness under these environmental conditions.

It is concluded that the diurnal fluctuations in the activity of storage compartment invertase observed in intact plants are consistent with the proposal put forward by Sacher, Hatch, and Glasziou (1963*a*) that this enzyme is concerned with the regulation of sugar levels in the cytoplasm of expanding parenchyma cells. The coupling of invertase activity to light–dark cycles is thought to be related to the amount of sugar translocated from the leaves to the internode. There is some evidence that the glucose-mediated repression of invertase synthesis may be superimposed on a second regulatory system producing a circadian rhythm of invertase activity independent of light–dark cycles.

V. Acknowledgments

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VI. References

Bull, T. A. (1964).—*Aust. J. Agric. Res.* **15**: 77–84.

Glasziou, K. T., and Waldron, J. C. (1964*a*).—*Aust. J. Biol. Sci.*, **17**: 609–18.

Glasziou, K. T., and Waldron, J. C. (1964*b*).—*Nature, Lond.* **203**: 541–2.

Hatch, M. D., and Glasziou, K. T. (1963).—*Plant Physiol.* **38**: 344–8.

Lowry, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J. (1951).—*J. Biol. Chem.* **193**: 265–75.

Sacher, J. A., Hatch, M. D., and Glasziou, K. T. (1963*a*).—*Plant Physiol.* **38**: 348–54.

Sacher, J. A., Hatch, M. D., and Glasziou, K. T. (1963*b*).—*Physiol. Plant.* **16**: 836–42.