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Carbon Partitioning during Sucrose Accumulation in Sugarcane Internodal Tissue[1]

Anne Whittaker and Frederik C. Botha*

Department of Biotechnology, South African Sugar Association Experiment Station, Mount Edgecombe, KwaZulu-Natal 4300, South Africa (A.W., F.C.B.); and the University of Natal, Natal, Durban 4001, South Africa (A.W., F.C.B.)

The temporal relationship between sucrose (Suc) accumulation and carbon partitioning was investigated in developing sugarcane internodes. Radiolabeling studies on tissue slices, which contained Suc concentrations ranging from 14 to 42% of the dry mass, indicated that maturation coincided with a redirection of carbon from water-insoluble matter, respiration, amino acids, organic acids, and phosphorylated intermediates into Suc. It is evident that a cycle of Suc synthesis and degradation exists in all of the internodes. The decreased allocation of carbon to respiration coincides with a decreased flux from the hexose pool. Both the glucose and fructose (Fru) concentrations significantly decrease during maturation. The phospho*enol*pyruvate, Fru-6-phosphate (Fru-6-P), and Fru-2,6-bisphosphate (Fru-2, 6-P_2) concentrations decrease between the young and older internodal tissue, whereas the inorganic phosphate concentration increases. The calculated mass-action ratios indicate that the ATP-dependent phosphofructokinase, pyruvate kinase, and Fru-1,6-bisphosphatase reactions are tightly regulated in all of the internodes, and no evidence was found that major changes in the regulation of any of these enzymes occur. The pyrophosphate-dependent phosphofructokinase reaction is in apparent equilibrium in all the internodes. Substrate availability might be one of the prime factors contributing to the observed decrease in respiration.

The maturation of sugarcane is characterized by the accumulation of Suc in developing internodes (Glasziou and Gaylor, 1972; for review, see Moore, 1995). In sugarcane Suc accumulation is suggested to be principally regulated at the level of sink and/or within the translocation system (for review, see Moore, 1995). However, despite numerous studies, the biochemical basis for the regulation of Suc accumulation in sugarcane is still poorly understood and requires further investigation (for review, see Moore, 1995).

Cycling of carbon between Suc and the hexoses, as a result of simultaneous synthesis and degradation of Suc, apparently occurs in all sugarcane Suc-storing cells and is believed to be primarily responsible for controlling Suc accumulation. This phenomenon was first described in young sugarcane internodal tissue (Sacher et al., 1963), and subsequently in young and older culm tissue (Batta and Singh, 1986), as well as in cell-suspension cultures (Wendler et al., 1990; Veith and Komor, 1993).

Such cycling of carbon between the hexose and Suc pools should result in a significant pool of UDP-Glc, which is not only the precursor for Suc synthesis, but also for cell wall polysaccharide synthesis and hexose and hexose phosphates, which could be respiratory substrates. However, despite the apparent carbon cycling, only 10% of the total radioactivity of compounds soluble in 70% ethanol was lost during a 10-h incubation period of sugarcane culm tissue (Sacher et al., 1963). This suggests a relatively small allocation of carbon to respiration and cell wall synthesis. In contrast to the culm tissue, twice as much carbon is respired than stored as Suc in sugarcane cell-suspension cultures (Wendler et al., 1990).

Knowledge about carbon partitioning between different cellular constituents in sugarcane culm tissues is limited to partitioning within the sugar pool (Sacher et al., 1963; Batta and Singh, 1986; Lingle, 1989). In sugarcane cell-suspension cultures, increased carbon partitioning into Suc is at the expense of both respiration and structural material under nitrogen-limiting conditions (Veith and Komor, 1993). In sugar beet, Suc accumulation in the developing root is associated with a decline in the partitioning of carbon into proteins, structural carbohydrates, amino acids, and organic acids (Giaquinta, 1979).

The aim of the present investigation was to characterize the partitioning of carbon into the different cellular constituents in internodal sugarcane tissues at different stages of development, and to look at possible control mechanisms. Here we report that Suc accumulation in culm tissue is associated with a decreased partitioning of carbon, derived from both [U-^{14}C]Suc and [U-^{14}C]Glc, into water-insoluble matter, amino acids, phosphorylated intermediates, and respiration.

MATERIALS AND METHODS

Biochemicals

All coupling enzymes, cofactors, and substrates used for metabolite determinations were from either Sigma or Boehringer Mannheim. The [U-^{14}C]Suc, [U-^{14}C]Glc, and [U-^{14}C]Fru were from Amersham International. Sep-Pak

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* Corresponding author; fax 31–595–406.

Abbreviations: FBPase, D-Fru-1,6-bisphosphatase (EC 3.1.3.11); PFK, ATP:D-Fru-6-phosphate 1-phosphotransferase (EC 2.7.1.11); PFP, pyrophosphate:D-Fru-6-phosphate 1-phosphotransferease (EC 2.7.1.90); PK, pyruvate kinase (EC 2.7.1.40); SuSy, Suc synthase (EC 2.4.1.13).

1-mL (100 mg) Alumina A cartridges and the Sugar-Pak 1 column (6.5 × 300 mm) were purchased from Waters. The Spherisorb 5 SAX column (250 × 4.60 mm) was obtained from Phenomenex (Torrance, CA). Cation- (Dowex AG 50W, 200–400 mesh, hydrogen form) and anion- (Dowex AG 1-X8, 100–200 mesh, chloride form) exchange resins were from Bio-Rad. All other solvents and biochemicals were of analytical grade.

Sample Collection

Mature, nonflowering stalks from field-grown sugarcane plants (NCo376 variety) were randomly selected from separate plants and cut in the field in the morning. Samples were taken from the top 10 internodes of plants with approximately 25 aboveground internodes. The internode attached to the leaf with the uppermost visible dewlap was defined as internode no. 1, according to the system of Kuijper (van Dillewijn, 1952).

^{14}C Labeling of Internodal Tissue

Transverse sections (1.0–2.0 g) of internodal tissue, spanning the core, mid-internodal, and peripheral internodal regions, were excised, sliced (approximately 0.5 mm in diameter), and washed according to the method of Lingle (1989). [U-^{14}C]Suc, [U-^{14}C]Glc, and [U-^{14}C]Fru (2.0 GBq mmol $^{-1}$) were independently supplied to tissues in 2 mL of 25 mM K-Mes (pH 5.7) containing 250 mM mannitol. To a center well, 500 μL of 12% (m/v) KOH was added. Prior to incubation, the labeled substrates were vacuum infiltrated into the tissues for 1 min. Tissue slices were then incubated at 28°C in airtight containers (500 mL) on a rotary-shaking incubator at 175 rpm for 5.5 h. The $^{14}CO_2$ evolved was sampled from the KOH in the center well. The tissue slices were then washed for 30 min in 15 mL of 25 mM K-Mes (pH 5.7) containing 250 mM mannitol and 1 mM $CaCl_2$ to remove apoplastic label as described by Lingle (1989). Thereafter, the tissues were frozen in liquid nitrogen. Preliminary trials indicated that the incorporation of label was linear over the uptake period. Tissues from separate plants were labeled, extracted, and fractionated in triplicate.

Fractionation of ^{14}C-Labeled Tissue Extracts

The tissues were ground to a fine powder in liquid nitrogen and extracted in 15 mL of methanol:chloroform: water, 12:5:3 (v/v) according to the work of Dickson (1979). After centrifugation at 4000*g* for 10 min, the supernatant was removed and further fractionated by the addition of 5 mL of chloroform and 4 mL of deionized H_2O to separate the lipid-soluble and H_2O-soluble components. The pellet fraction was thoroughly washed four times in 30 mL of H_2O to remove unincorporated label. The H_2O-soluble pool was dried down in a rotary evaporator at 40 to 50°C. Dried samples were dissolved in HPLC-grade H_2O and passed through Sep-Pak Alumina A cartridges, which were prepared by washing with 5 volumes of deionized, distilled H_2O. The resultant samples were filtered (0.22-μm Millex-GV4 filters, Millipore) and the sugars were fractionated by HPLC (model 8800 ternary pump, Spectra-Physics, San Jose, CA, and a model 410 differential refractometer detector, Waters) using a Sugar-Pak I column. Sugars were separated over 15 min with HPLC-grade H_2O containing 1.33 mM EDTA (disodium calcium salt) at a flow rate of 0.5 mL min^{-1}. Fractions (0.5 mL) were collected and the ^{14}C in Suc, Glc, and Fru was determined. Extraction efficiencies for [U-^{14}C]Suc, [U-^{14}C]Glc, and [U-^{14}C]Fru exceeded 92%. Labeling of the glucosyl and fructosyl moieties of Suc was determined by boiling the Suc fraction for 2 h in 75 mM citric acid and separating the hexoses by HPLC. In duplicate samples, the H_2O-soluble fraction was sequentially fractionated using both cation- (Dowex, AG 50W-X4) and anion- (Dowex, AG 1-X8) exchange chromatography to yield the neutral sugar, amino acid, organic acid, and sugar phosphate components as described by Dickson (1979). ^{14}C in the different chemical components was determined by liquid-scintillation spectroscopy (Tricarb 1900 TR, Packard, Meriden, CT).

Metabolite Analysis

After removing the rind from each internode, samples were prepared for metabolite analysis by rapidly slicing thin sections of tissue into super-cooled liquid nitrogen. Samples (2.5–4.0 g) were then powdered and extracted according to the work of Stitt et al. (1983). Samples were dried down in a rotary evaporator at 40 to 50°C, dissolved in 300 to 500 μL of deionized, distilled H_2O, and treated with activated charcoal to remove coloration. The following extracted metabolites were measured enzymatically at 340 nm, using a DU 7500 spectrophotometer (Beckman), as described previously: Suc, Glc, and Fru (Bergmeyer and Bernt, 1974); Glc-6-P and Fru-6-P (Lang and Michal, 1974); Fru-1,6-P_2 and triose phosphates (Michal and Beutler, 1974); PEP and pyruvate (Czok and Lamprecht, 1974); Fru-2,6-P_2 (van Schaftigen et al., 1982); and PPi (Botha and Small, 1987). UDP-Glc was determined by anion-exchange HPLC according to a modified method of Viola et al. (1994). Separation was performed on a 25-cm Spherisorb SAX (5 μm) column at a flow rate of 1.0 mL min^{-1} using a mobile phase of 10 mM ammonium phosphate pH 3.0 (A), and 450 mM ammonium phosphate pH 4.5 (B). The gradient employed was 100% (A) for 2 min, followed by a linear gradient to 20% (B) over 8 min, and finally to 65% (B) over a further 20 min. The gradient was maintained at 65% (B), 35% (A) for 5 min, and the column washed for 10 min with 100% (A) prior to application of the next sample. Pi and fiber were measured according to the method of Joyce and Grisolia (1960) and Anon (1987), respectively. Metabolites were extracted and measured in triplicate from separate plants. The extraction efficiency for all metabolites was higher than 80%.

Protein Determination

Protein content was measured according to the method of Bradford (1976) using gamma globulin as a standard.

RESULTS AND DISCUSSION

Suc Content and Suc Accumulation Rate

The number of cells per internode in the sugarcane culm reaches a maximum early in development. Internode expansion is therefore largely due to cell expansion (Komor, 1994). The storage parenchyma cells are highly vacuolated and the vacuole can make up to 90% of the total cellular volume (Komor, 1994). Total water-soluble protein and other cellular constituents expressed on an internodal basis are therefore also a reflection of the content per cell. Soluble protein content and Suc, on an internodal basis, increased between internode nos. 2 and 10 (Table I). The increase in protein content was similar to the observed change in total internodal or cellular volume (Botha et al., 1996), but the Suc increase was at least 3 times higher. This clearly suggests that the protein content per cell remains fairly constant but Suc content increases. The Suc content as a proportion of the total dry mass increased rapidly between internode nos. 2 and 10 (Fig. 1A). The water-soluble protein content, however, decreased as a result of the increased contribution of Suc to the total dry mass.

To accurately reflect changes in carbon partitioning, metabolite concentrations, and flux, all data in this paper are expressed on a protein basis. Since the protein content per cell remained fairly constant between internode nos. 2 and 10 (Botha et al., 1996), this probably is a close reflection of changes that occur per cell.

It is not evident from the available literature whether the higher Suc content in the older, more mature internodes is due to an increased rate of Suc accumulation or whether it is merely a result of the longer growth period. In the present study the rate of Suc accumulation was calculated by using the change in Suc content during the period in which a new internode was produced (dContent/dTime). From this there is evidently a very sharp increase in the rate of Suc accumulation between internode nos. 4 and 7 (Fig. 1B). The difference in the rate of Suc accumulation could be the result of higher uptake of carbon or it could be due to a redirection of incoming carbon toward Suc storage. The latter could be a result of reduced cleavage, higher synthesis, or a combination of both.

The rate of Suc accumulation also markedly changes during the growth cycle of sugarcane cell-suspension cultures (Wendler et al., 1990). In the latter case it was suggested that the rate of Suc synthesis is being controlled by cycles of synthesis and degradation, as was originally proposed by Sacher et al. (1963) for intact sugarcane culm tissue.

Table I. *Protein and Suc content in internodal tissue of NCo376*

Internode No.	Total Protein	Total Suc	Suc Content
	mg internode^{-1}	*mmol internode^{-1}*	*μmol mg^{-1} protein*
2	2.7 ± 0.5[a]	0.04 ± 0.01	15 ± 1
3	4.5 ± 0.6	0.17 ± 0.02	25 ± 3
4	6.2 ± 0.4	0.35 ± 0.06	56 ± 3
5	7.3 ± 1.0	0.73 ± 0.05	80 ± 5
6	8.8 ± 1.2	1.28 ± 0.20	145 ± 10
7	11.2 ± 0.9	2.32 ± 0.22	207 ± 17
8	12.4 ± 1.1	2.82 ± 0.21	227 ± 21
9	14.3 ± 1.2	3.51 ± 0.23	253 ± 19
10	15.6 ± 0.8	3.96 ± 0.31	255 ± 16

[a] Each value is the mean ± SD of three separate samples.

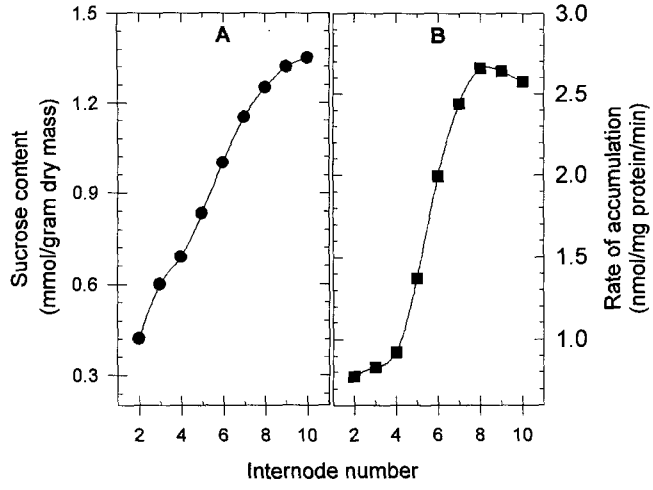

Figure 1. Changes in the Suc content (A) and the Suc accumulation rate (B) of internode nos. 2 to 10 in the culm of sugarcane.

Carbon Partitioning

Because of the large difference between the Suc accumulation rates between internode nos. 2 and 7 (Fig. 1B), those internodes were selected for carbon partitioning and metabolite analysis. Since both Suc (Lingle, 1989) and the two reducing monomers, Glc and Fru (Sacher et al., 1963; Glasziou and Gaylor, 1972; Batta and Singh, 1986), can be taken up by sugarcane culm tissue, the extent of carbon partitioning in the sink tissues was investigated by independently supplying tissue slices with all three of these sugars uniformly labeled.

The average recoveries of the ^{14}C taken up by the tissue slices was 103%, 99%, 95%, and 100% for internode nos. 2, 3, 5, and 7, respectively. No differences in recoveries were evident between the different labeling experiments.

After feeding the tissue ^{14}C-labeled Glc or Fru, less than 6% of the metabolized label remained in the endogenous specific hexose pool in all internodes investigated. The ^{14}C metabolized is defined as the total ^{14}C recovered in cellular components other than in the labeled substrate (Glc or Fru) at the end of the 5.5-h labeling period.

The percent distribution of label present in the endogenous Suc pool after feeding with labeled Suc increased from internode nos. 2 to 7 (Table II). Conversely, there was a decrease in the percentage of label partitioned into the H_2O-insoluble matter, the non-Suc H_2O-soluble component, and respiration. A similar distribution pattern was seen after labeling with Glc, with 31% of the label in Glc allocated to Suc in internode no. 2, and 66% in internode no. 7 (Table III).

The ^{14}C recovered in CO_2 (catabolic respiration), the amino acids, organic acids, and lipids (anabolic respiration) were summed as an indicator of the total carbon

Table II. *Incorporation and distribution of ^{14}C in internodal tissue slices of NCo376 supplied with [U-^{14}C]Suc and incubated for 5.5 h at 28°C*

Values in parentheses indicate the percentage of total label being allocated to the particular pool.

Component	Internode			
	2	3	5	7
	kBq mg^{-1} protein			
CO_2	1.67 ± 0.35 (6.8%)[a]	0.80 ± 0.26 (4.1%)	0.42 ± 0.02 (3.5%)	0.09 ± 0.03 (1.2%)
H_2O-soluble				
Suc	7.58 ± 1.12 (30.6%)	8.51 ± 3.89 (43.4%)	7.86 ± 0.35 (66.2%)	5.43 ± 1.60 (72.0%)
Glc	0.81 ± 0.09 (3.3%)	1.04 ± 0.26 (5.3%)	0.54 ± 0.07 (4.5%)	0.38 ± 0.04 (5.0%)
Fru	0.64 ± 0.06 (2.6%)	0.66 ± 0.16 (3.4%)	0.45 ± 0.07 (3.8%)	0.20 ± 0.05 (2.7%)
Other[b]	5.75 ± 0.5 (23.2%)	5.38 ± 0.4 (27.5%)	1.78 ± 0.2 (14.9%)	0.95 ± 0.1 (12.6%)
H_2O-insoluble	8.00 ± 0.86 (32.3%)	3.00 ± 0.52 (15.3%)	0.71 ± 0.17 (6.0%)	0.42 ± 0.26 (5.6%)
Lipid-soluble	0.30 ± 0.11 (1.2%)	0.21 ± 0.10 (1.1%)	0.12 ± 0.03 (1.1%)	0.07 ± 0.01 (0.9%)

[a] Each value is the mean ± SD of three separate samples. [b] The chemical component designated "Other" comprises amino acids, organic acids, and sugar phosphates.

partitioned into the respiratory pathway. Of the ^{14}C-Glc metabolized, 27, 19, and 15% entered respiration in internode nos. 2, 3, and 7, respectively. Catabolic respiration (CO_2 production) as a percentage of total respiration was comparable in internode nos. 2 (33%), 5 (24%), and 7 (27%).

Similar results were also obtained in tissues supplied with labeled Fru (results not shown). Combined, the insoluble and non-Suc H_2O-soluble components were the dominant competitors for carbon, since more than 90% of the increase in label in the Suc pool could be accounted for by a reduction in the labeling of these two pools (Tables II and III). The non-Suc H_2O-soluble component consists of hexose sugars, amino acids, organic acids, and phosphorylated intermediates. Fractionation of this component showed that there was a decrease in the allocation of carbon to the amino acids and phosphorylated intermediates during maturation (Tables II and III).

Hexose Mobilization

The specific activities of the endogenous hexose pools were calculated in two ways. First, we assumed that all the

Table III. *Incorporation and distribution of ^{14}C in internodal tissue slices of NCo376 supplied with [U-^{14}C]Glc and incubated for 5.5 h at 28°C*

Values in parentheses indicate the percentage of total label being allocated to the particular pool.

Components	Internode			
	2	3	5	7
	kBq mg^{-1} protein			
CO_2	3.82 ± 0.1 (8.6%)[a]	2.47 ± 0.1 (5.6%)	2.56 ± 0.3 (4.4%)	1.78 ± 0.3 (3.8%)
H_2O-soluble				
Suc	12.70 ± 1.4 (30.6%)	21.50 ± 7.2 (48.8%)	28.6 ± 9.8 (48.8%)	30.1 ± 6.1 (65.6%)
Glc	1.51 ± 0.7 (3.4%)	2.01 ± 0.9 (4.6%)	2.62 ± 0.8 (4.5%)	2.87 ± 1.0 (6.2%)
Fru	1.02 ± 0.3 (2.9%)	0.79 ± 0.3 (1.8%)	0.83 ± 0.4 (1.4%)	0.77 ± 0.4 (16.5%)
Other[b]	8.98 ± 1.2 (20.1%)	4.03 ± 0.9 (9.1%)	8.19 ± 1.3 (14%)	4.16 ± 1.0 (9.0%)
H_2O-insoluble	16.40 ± 0.2 (36.7%)	12.80 ± 2.4 (29.0%)	14.7 ± 2.4 (25.1%)	5.99 ± 2.0 (12.9%)
Lipid-soluble	0.32 ± 0.2 (0.7%)	0.49 ± 0.1 (1.1%)	0.83 ± 0.1 (1.4%)	0.94 ± 0.2 (2.0%)

[a] Each value is the mean ± SD of three separate samples. [b] The chemical component designated "Other" comprises amino acids, organic acids, and sugar phosphates.

hexoses were in the cytosol, and consequently would represent the lowest potential specific activity for those pools. This would represent the maximum potential flux and exemplify an extreme, since it is known that the sugars are probably equally distributed between the cytosol and the vacuole (for review, see Moore, 1995). The specific activities were then used to calculate the flux from the hexose pools (Table IV). From those data it is evident that the capacity to mobilize Glc and Fru is similar and that the flux is high enough to ensure a rapid turnover of the endogenous hexose pools, ranging between 12 min for the Glc pool in internode no. 2 and 32 min for the Fru pool in internode no. 7.

Second, we assumed that the measured cytosolic volume in sugarcane internodal tissue was 10% (Komor, 1994), and that the distribution of sugars in the cell was equal (Moore, 1995), and then assumed that all of the label was largely restricted to the hexoses in the cytosol. This probably represents the lower limit of the flux from the hexose pool (highest potential specific activity), since at least some labeled hexoses will be present in the vacuole. In the latter calculation the turnover time would range from 2 h for the Glc pool in internode no. 2 to 5.3 h for Fru in internode no. 7. However, regardless of the method of calculation, it is evident that the flux from both Fru and Glc decreases significantly between internode nos. 2 and 7 (Table IV).

Respiratory Flux

The percentage of radiolabeled carbon lost as CO_2 (fraction of total ^{14}C uptake) was twice as high in internode no. 2 than in no. 7 when fed with labeled Glc (Table III). This difference was even more marked when the tissue was fed with Suc (Table II). Although the decreased CO_2 release from Suc could be the result of the large difference in the in vivo pool sizes (and subsequent lower specific activity), this evidently was not the case after feeding labeled Glc (Tables IV and V). The latter therefore represents a real decrease in the respiratory carbon flux.

Using the internal specific activity of the Glc pool after labeling with Glc, and assuming that Glc was located exclusively in the cytoplasm and that the ^{14}C-Glc was equally distributed, the respiratory flux was calculated (Table V). It is evident that carbon flux from Glc into CO_2 and the combined pool of amino acids, organic acids, and lipids decreased. This could reflect a decrease in glycolysis, TCA cycle, and/or oxidative pentose phosphate pathway activity.

The decrease in the partitioning of carbon to the amino acids is probably a consequence of the decreased partitioning of carbon into respiration. It was previously shown that internal amino acid content is inversely related to the increase in Suc storage in sugarcane suspension cells (Wendler et al., 1990; Veith and Komor, 1993).

Carbon Cycling

If Suc is predominantly synthesized through Suc phosphate synthase (Wendler et al., 1990; Goldner et al., 1991), then both the fructosyl and glucosyl moieties of the synthesized Suc molecule would be labeled at more or less the same frequency. In all the internodes analyzed after feeding with labeled Glc, significant amounts of label were found in the Fru moiety of Suc. The ratio between labeled Glc and Fru varied from 1.34 in internode no. 3 to 1.0 in internode no. 7.

It is evident that substantial cycling between the hexose and Suc pools occurs. As was pointed out in a previous study (Wendler et al., 1990), label in the Glc pool following the feeding of labeled Fru probably could only come from hydrolyzed Suc. The amount of label present in the Glc in internode nos. 2 and 7 was 65 and 45%, respectively, of that present in the Fru pool after feeding labeled Fru (Table IV). In both internodes the label in Glc was approximately 9% of that in Suc. Since the mobilization capacity for the two hexoses apparently is similar (Table IV), this indicates that return of labeled Glc from Suc occurs at a rate similar to the rate of uptake of labeled Fru from the medium. Since the released Glc will be efficiently rephosphorylated (Tables III and IV), the label present in Glc will largely underestimate the true breakdown of Suc. These estimates are in close agreement to that determined in sugarcane cell-suspension cultures (Wendler et al., 1990).

Label in the Fru pool (Tables III and IV) after feeding labeled Glc to the tissue was more difficult to interpret, since it might indicate cleavage/hydrolysis of Suc, but could also merely reflect an equilibration of label between the free Fru and Fru in Suc due to the action of SuSy. The percentage label in Fru was higher in internode no. 7 than in internode no. 2, which is the same pattern as the label distribution in Suc (Table III). This could be consistent with

Table IV. *Endogenous Glc and Fru concentrations and specific activities of the two hexose pools after labeling of tissue slices with [U-^{14}C]Glc or [U-^{14}C]Fru*

The total fluxes from the hexose pools were calculated from the total radioactivity released as CO_2 and present in the tissue in forms other than that of the applied hexose.

Labeled Sugar	Internode No.	Total Activity		Endogenous Content	Specific Activity	Flux into Metabolism
		Glc	Fru			
		kBq mg^{-1} protein		*μmol mg^{-1} protein*	*kBq μmol^{-1}*	*nmol min^{-1} mg^{-1} protein*
Glc	2	1.51 ± 0.4[a]	1.02 ± 0.2	4.30 ± 0.56	0.35 ± 0.16	374
	7	2.87 ± 0.6	0.77 ± 0.2	5.31 ± 0.70	0.40 ± 0.15	245
Fru	2	0.71 ± 0.1	1.08 ± 0.3	4.64 ± 0.50	0.28 ± 0.08	421
	7	1.06 ± 0.3	2.38 ± 0.4	8.17 ± 1.80	0.29 ± 0.06	252

[a] Each value is the mean ± SD of three separate samples.

Table V. *The estimated carbon flux into CO_2 production, and the combined lipid, organic, and amino acid pool in internode nos. 2, 5, and 7*

The flux was calculated using the ^{14}C incorporation values from the ^{14}C-Glc labeling data.

Internode No.	Specific Activity of the Endogenous Glc Pool	Flux into CO_2 Production	Flux into Lipids, Organic, and Amino Acids
	kBq μmol^{-1}	*nmol min^{-1} mg^{-1} protein*	
2	0.35 ± 0.16[a]	33.1 ± 0.87	68.0 ± 22.4
5	0.44 ± 0.12	17.6 ± 2.07	55.0 ± 12.3
7	0.44 ± 0.15	12.3 ± 2.06	32.6 ± 6.19

[a] Each value is the mean ± SD of three separate samples.

a SuSy-catalyzed exchange of label between free Fru and Fru in Suc, provided that the specific activity of the cytosolic Suc pool was higher in internode no. 7 than in no. 3. A constant SuSy activity is present in internode nos. 2 and 10 (Botha et al., 1996). However, the higher amount of radioactivity present in Fru in internode no. 7 could also be due in part to the apparent decrease in the carbon flux from the Fru pool in the older internode (Table IV).

Although the carbon partitioning studies were conducted in vitro, we propose that this approach was adequate to assess the changes in symplastic metabolism associated with whole culm tissue. Precautions were taken to avoid most of the criticism previously leveled against the use of tissue slices in sugarcane (for review, see Moore, 1995). Precautions that were taken in our study included a prelabeling iso-osmotic wash buffer so as to not alter turgor (Lingle, 1989), variation in the duration of labeling experiments to ensure that the pattern of metabolism was not changing (for review, see Moore, 1995), an extensive postlabeling tissue wash to remove label associated with the apoplast (Lingle, 1989), and the extraction of tissues to investigate the partitioning only of the labeled substrates taken up by the cells.

Regulation of Carbon Flow

The reduction in the partitioning of carbon to respiration and total flux of carbon to CO_2 could be due to coarse control (gene expression), posttranslational regulation (e.g. Suc phosphate synthase phosphorylation status), substrate limitation, or fine control of one or more steps in the glycolytic pathway. In the present study we are only addressing the latter two possibilities.

Because of limited material in internode no. 2, metabolites were extracted from internode nos. 3 to 9. The metabolite content as well as the calculated cellular concentrations are presented in Table VI. It is evident that the concentrations of Glc, Fru, and UDP-Glc increased between internode nos. 3 and 5 and then very rapidly decreased to internode no. 9. Glc-6-P increased slightly between internode nos. 3 to 5 and then decreased toward internode no. 9. The triose-P level decreased between internode nos. 3 to 5, and then recovered to a level in internode no. 9 that was comparable with the level in internode no. 3. The Fru-2,6-P_2 level decreased significantly between internode nos. 3 to 9. No significant changes were evident in the other metabolites.

Although the Glc and Fru concentrations decreased significantly in the mature internodes (nos. 7 and 9), they are still an order of magnitude higher than the reported K_m values for hexokinase and fructokinase (Renz and Stitt, 1993a). However, it is evident from the flux analysis (Table IV) that mobilization of both Glc and Fru decreased in the more mature tissue and it might therefore imply that a more complex regulation than only hexose availability limits the in vivo activity of these enzymes. Hexokinases have previously been implicated in the regulation of glycolytic flux in plants (Renz and Stitt, 1993a, 1993b; Bouny and Saglio, 1996). In animal tissues hexokinases also play an important role in controlling glycolytic flux (German, 1993; Kashiwaya et al., 1994) and can have control coefficients as high as 0.7 to 0.9 (Rapoport et al., 1974). We are currently investigating this aspect in sugarcane.

The PEP concentration in all the internodes was within the K_m range (30–200 μM) for cytosolic and plastid isoforms of plant PK (Lin et al., 1989) and PEP specific phosphatase (Duff et al., 1989a, 1989b).

The calculated in vivo concentration of Fru-6-P in sugarcane lay within the lower limit of the concentration range (30–5000 μM) reported for other tissues (Botha and Small, 1987; Stitt, 1990; Wendler et al., 1990; Geigenberger and Stitt, 1991). The levels of Fru-6-P in sugarcane were within the lower limit required for half-maximal activity of both PFK_c and PFP from a range of other tissues (Garland and Dennis, 1980b; van Schaftigen et al., 1982; Kombrink et al., 1984; Botha and Small, 1987; Botha et al., 1988; Stitt, 1989). The PPi levels were similar to those in other tissues (Edwards et al., 1984; Botha and Small, 1987; Kesy and Kowakczyk, 1987) and higher than the K_m of PFP for PPi (van Schaftigen et al., 1982; Kombrink et al., 1984; Botha and Small, 1987; Stitt, 1989). Assuming that the Fru-2,6-P_2 extracted from sugarcane is not allosterically bound to enzymes, then the concentration was well in excess of the activation constant (Ka) (2.5–150 nM) required to fully activate PFP (van Schaftigen et al., 1982; Stitt, 1990).

Previous investigations have indicated that PEP is a strong allosteric inhibitor of plant PFK (Garland and Dennis, 1980a, 1980b; Botha et al., 1988; Cawood et al., 1988). The levels of PEP in sugarcane were within the I_{05} values reported for purified PFK isoenzymes (Garland and Dennis, 1980a; Isaac and Rhodes, 1986; Cawood et al., 1988). However, the in vivo inhibition of sugarcane PFK by PEP is likely to be significantly reduced by the prevailing intracellular Pi concentration (Table VI).

Table VI. *Levels of metabolites in internodes nos. 3 to 9 in the culm of NCo376*

Values in parentheses are the calculated metabolite concentrations in the internodes, assuming a 10% cytosolic volume. All the intermediates, with the exception of the sugars and Pi, were assumed to be confined to the cytosol.

Metabolite	Metabolite Concentration				
	Internode				
	3	4	5	7	9
	mmol mg^{-1} protein				
Glc	12 ± 0.1[a] (31)[b]	20 ± 0.6 (40)[b]	19 ± 6.1 (29)[b]	5 ± 1.8 (6)[b]	3 ± 1.1 (3)[b]
Fru	10 ± 0.3 (27)[b]	22 ± 0.3 (43)[b]	19 ± 1.5 (30)[b]	8 ± 0.9 (10)[b]	6 ± 2.2 (7)[b]
Pi	2.2 ± 0.6 (3.4)[b]	2.9 ± 0.5 (3.7)[b]	4.2 ± 0.3 (5.1)[b]	5.1 ± 0.5 (5.1)[b]	4.2 ± 0.3 (4.6)[b]
	μmol mg^{-1} protein				
UDP-Glc	8.6 ± 0.9 (187)[c]	17.5 ± 1.3 (275)[c]	23.5 ± 3.5 (302)[c]	12.9 ± 1.4 (196)[c]	13.9 ± 3.3 (158)[c]
Glc-6-P	13.0 ± 0.9 (301)[c]	14.0 ± 1.1 (263)[c]	15.0 ± 1.8 (211)[c]	11.5 ± 1.9 (130)[c]	11.3 ± 0.4 (112)[c]
Fru-6-P	7.9 ± 2.3 (178)[c]	8.4 ± 0.2 (153)[c]	9.8 ± 1.2 (123)[c]	7.9 ± 0.8 (85)[c]	9.6 ± 0.7 (104)[c]
Fru-1,6-P_2	1.8 ± 0.3 (42)[c]	2.5 ± 0.6 (46)[c]	3.1 ± 0.8 (40)[c]	3.5 ± 0.1 (37)[c]	2.9 ± 0.3 (32)[c]
Fru-2,6-P_2	0.4 ± 0.09 (3.1)[c]	0.4 ± 0.1 (3.0)[c]	0.3 ± 0.08 (2.3)[c]	0.1 ± 0.01 (1.0)[c]	0.1 ± 0.02 (1.0)[c]
Triose-P	10.3 ± 0.8 (239)[c]	8.5 ± 2.0 (158)[c]	5.2 ± 1.0 (72)[c]	8.0 ± 2.1 (93)[c]	9.1 ± 1.7 (94)[c]
PEP	6.3 ± 2.6 (139)[c]	5.9 ± 1.8 (110)[c]	6.6 ± 1.1 (85)[c]	8.4 ± 1.7 (88)[c]	7.1 ± 0.6 (86)[c]
Pyruvate	4.5 ± 1.0 (102)[c]	5.2 ± 0.9 (101)[c]	5.3 ± 0.6 (102)[c]	7.2 ± 1.8 (82)[c]	7.6 ± 0.7 (79)[c]
PPi	13.4 ± 1.7 (270)[c]	16.2 ± 2.0 (290)[c]	13.4 ± 0.8 (180)[c]	20.9 ± 4.8 (189)[c]	16.8 ± 3.9 (166)[c]

[a] Each value is the mean ± SD of four separate samples. [b] Calculated concentration as mM. [c] Calculated concentration as μM.

In contrast to PFK, Pi is a potent inhibitor of PFP activity in the forward (glycolytic) reaction (Mahajan and Singh, 1989; Stitt, 1990; Theodorou and Plaxton, 1996). The increased Pi in addition to the decreasing Fru-2,6-P_2 and Fru-6-P levels could therefore lead to a very significant decrease in the in vivo PFP activity.

To identify the potential reaction(s) subject to regulation, the in vivo product-to-substrate ratios for several of the key enzyme reactions involved in respiration were examined. Since fine control of plant glycolysis is primarily exerted at the reactions catalyzing PEP and Fru-6-P utilization, only the fine regulation of the PK, PFK, PFP, and FBPase reactions were considered (Table VIII). We also included the Glc-6-P to Fru-6-P ratio for a control, since the latter is presumably in equilibrium in all tissues.

Because we have not measured the ATP and ADP content of the different internodes and the subcellular distribution of metabolites cannot be determined, accurate cal-

Table VII. *Substrate ratios of substrates in internodes nos. 3 to 9 of NCo376 developing internodal tissue*

Ratios were calculated from the concentrations shown in Table VI.

Internode No.	Substrate Ratios				
	$\frac{[\text{Pyruvate}]}{[\text{PEP}]}$	$\frac{[\text{Fru-1,6-P}_2]}{[\text{Fru-6-P}]}$	$\frac{[\text{Fru-1,6-P}_2][\text{Pi}]}{[\text{Fru-6-P}][\text{PPi}]}$	$\frac{[\text{Fru-6-P}][\text{Pi}]}{[\text{Fru-1,6-P}_2]}$	$\frac{[\text{Fru-6-P}]}{[\text{Glc-6-P}]}$
3	0.7	0.2	3.0	1.4×10^4	0.6
4	0.9	0.3	3.9	1.2×10^4	0.6
5	1.2	0.3	9.2	1.6×10^4	0.6
7	0.9	0.4	11.7	1.2×10^4	0.7
9	1.0	0.3	8.6	1.5×10^4	0.9

culation of mass-action ratios for the PFK and PK reactions was not possible. However, using theoretical K_{eq} constants for the PK and PFK reaction steps, and the pyruvate/PEP and the Fru-6-P/Fru-1,6-P_2 ratios, we calculated the ATP/ADP ratios that would be needed in the cytosol for these reactions to be at equilibrium in vivo.

For the PK reaction ATP/ADP ratios ranging from 5.11×10^6 (internode no. 3) to 6.37×10^6 (internode no. 9) would be required. For the PFK reaction the required cytosolic ATP/ADP ratios would have to be in the range of 2.2×10^{-4} (internode no. 3) to 2.9×10^{-3} (internode no. 9). Since none of these ratios is likely to exist, and because the ratios required are at the opposing extremes, we conclude that both the PK and PFK reactions are tightly regulated in vivo. It is also evident that the Fru-1,6-P_2-to-Fru-6-P and the pyruvate-to-PEP ratios remained fairly constant (Table VII), indicating that regulation at these reaction steps did not change in the different internodes.

The metabolite ratios of the PK-catalyzed reaction in sugarcane are consistent with those published for other tissues (Leegood and ap Rees, 1978; Turner and Turner, 1980; Day and Lambers, 1983). Stimulation of carbon flux into the TCA cycle through activation of PK is traditionally depicted by a decrease in PEP and an attendant increase in pyruvate levels (Kobr and Beevers, 1971; Turner and Turner, 1980; Beaudry et al., 1989). In sugarcane, the ratio of pyruvate to PEP remained unchanged, indicating that there is no change in the regulation of PK activity associated with the decrease in respiration.

For the calculation of the mass-action ratios for the FBPase and PFP catalyzed reactions, we assumed that total PPi, Fru-6-P, and Fru-1,6-P_2 were only present in the cytosol and that Pi was equally distributed throughout the cell. The results indicate that the FBPase-catalyzed reaction is also far displaced from the expected value of 170 (Leegood and ap Rees, 1978) in all the internodal tissue. There was no indication that any change in the regulation of FBPase occurred between internode nos. 3 and 9.

These results are consistent with those published for other tissues and indicate that the PFK, PK, and FBPase reactions are tightly regulated in vivo (Leegood and ap Rees, 1978; Turner and Turner, 1980; Day and Lambers, 1983).

The range of these values calculated for the PFP-catalyzed glycolytic reaction (PPi-hydrolyzing direction) is close enough to the theoretical equilibrium value of 3.3 (Stitt, 1989) to suggest that the PFP-catalyzed reaction is in equilibrium at all stages in the sugarcane culm.

CONCLUSIONS

The maturation of sugarcane internodes coincides with a redirection of carbon from insoluble matter, amino acids, phosphorylated intermediates, and respiration to Suc. This occurs despite the observation that cycling of carbon between the hexose and Suc pools occurs in all of the internodes investigated.

The decreased allocation of carbon to respiration coincides with a reduction in the flux of hexoses into the respiratory pathway. No evidence was found to suggest that the decreased respiratory flux is caused by fine control of any of the reaction steps investigated. However, substrate availability could at least in part be responsible for the decreased flux. In addition, the prevailing Fru-6-P, Pi, and Fru-2,6-P_2 concentrations appear to be adequate to cause a significant reduction in the in vivo activity of PFP.

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