

**THE METABOLIC FATE OF SUCROSE IN INTACT
SUGARCANE INTERNODAL TISSUE**

by

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Preface

The experimental work described in this thesis was carried out from January 1997 to December 1998 under the supervision of Prof. FC Botha and co-supervision of Dr. BI Hockett in the Biotechnology Department of the South African Sugar Association Experiment Station (SASEX), Mt Edgecombe, Kwazulu-Natal. The facilities of the Institute of Plant Biotechnology (IPB), Stellenbosch were also made use of during the course of the study

These studies represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

Ngem Ronald

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ABSTRACT

The study was aimed at determining the metabolic fate of sucrose in intact sugarcane internodal tissue. Three aspects of the fate of sucrose in storage tissue of whole plants formed the main focus of the work. These were the rate of sucrose accumulation in the developing culm, the characterisation of partitioning of carbon into different cellular organic fractions in the developing culm and the occurrence of sucrose turnover in both immature and mature stem tissues. Specific attention was paid to confirming the occurrence of sucrose turnover in both immature and mature internodal tissue. This sucrose turnover has been described previously in both tissue slices and cell suspension cultures. However, certain results from previous work at the whole plant level have indicated that sucrose turnover does not occur in mature internodal tissue.

Radiolabeled carbon dioxide ($^{14}\text{CO}_2$) was fed to leaf 6 of sugarcane culms of a high sucrose storing variety (*Saccharum* spp. hybrid cv. Nco376). All plants were of similar age (12 months) and were grown under similar conditions. The movement and metabolic fate of radiolabeled sucrose was determined at four time points, (6 hours, 24 hours, 7 days and 6 weeks) during a 6 week period. The metabolic fate of sucrose was determined in internodes number 3, number 6 and number 9. Internode 3 was found to have a relatively high hexose sugar content of 42 mg glc&fruc fw g⁻¹ and a low sucrose content of 14 mg suc fw g⁻¹. In contrast the sucrose content of internode 9 was much higher at 157 mg suc fw g⁻¹ and the hexose sugar content much lower at 4.3 mg glc&fruc fw g⁻¹. Based on previous work, the sugar content of internode 3 and internode 9 are characteristic of immature and mature tissues respectively. Internode 6 occupies an intermediary position between internode 3 and 6 with its sucrose content higher than its hexose sugar content, but with the hexose sugar content still being notable at 15 mg glc&fruc fw g⁻¹.

Although the metabolic fate of sucrose within sink tissue was the focal point of the study, the experimental design also allowed for certain aspects of sucrose production in the source to be investigated. The average photosynthetic rate for leaf 6 in full sunlight was estimated at $48 \text{ mg CO}_2 \text{ dm}^{-2} \text{ s}^{-1}$. During photosynthesis, only 30% of the fixed carbon was partitioned into the storage carbohydrate pool while the remaining 70% was partitioned into sucrose for immediate export from the leaf. This high rate of carbon fixation combined with a high rate of carbon export is characteristic of C_4 plants such as sugarcane.

On entering the culm, translocation of radiolabeled sucrose was predominantly basipetal with relatively little acropetal translocation. The majority of the radiolabeled carbon was found to be stored in mature internodes. No significant loss of radiolabeled carbon was observed in mature and elongating internodes over the study period. A 22% loss of total radiolabeled carbon was observed in immature internodes over the same period. This can probably be attributed to the higher rates of cellular respiration known to occur in immature tissues.

There appear to be three phases of sucrose accumulation in the developing culm. Initially, the accumulation rate in rapidly growing tissue, as internode 3 develops into internode 6, is relatively low. This is followed by a rapid increase in the rate of sucrose accumulation during internode elongation, as internode 6 becomes internode 9. Finally, a decrease in the rate of sucrose accumulation is observed during late maturation, as internode 9 becomes internode 12. Determination of the sucrose content in internodes 3, 6 and 9 revealed that there is a notable increase in sucrose content during internode maturation. It is proposed that the higher sucrose content of mature tissue is not merely a consequence of the longer growth period of mature tissue, but is due to the increased rate of sucrose accumulation observed during internode elongation.

Short-term (24 hours) analysis of carbon partitioning revealed that internodal maturation was associated with a redirection of carbon from non-sucrose cellular

organic fractions to sucrose storage. In immature internodes only 20% of the total radiolabeled carbon was present in the sucrose pool 24 hours after feeding. In elongating internodes the figure increased to 54% while in mature internodes as much as 77% of the total radiolabeled carbon was retained in the sucrose pool. Concomitant with the increased carbon partitioning into stored sucrose down the developing culm is a decrease in carbon partitioning into the hexose sugar pool. In immature tissue, 42 % of the total radiolabel is present in the hexose sugar pool, while in mature tissue the percentage drops to 11%. This decrease is probably indicative of decreased levels of carbon cycling between the sucrose and hexose sugar pool as a result of internode maturation. Internode maturation was also found to be associated with a decrease in the amount of carbon in the water insoluble matter pool and the amino acid/ organic acid/ sugar phosphate pool. Thus, internode maturation is associated with a redirection of carbon from total respiration to sucrose storage. Long-term (6 weeks) analysis of carbon partitioning confirmed that sucrose storage in mature tissue is greater than that in immature tissue. From the 6 hour time point to the 6 week time point, an 87% reduction in the stored radiolabeled sucrose content was observed in immature internodes. During the same period only a 25% reduction in the stored radiolabeled sucrose was observed in mature internodes. Radiolabel loss from the radiolabeled sucrose pool in both mature and immature internodes was accounted for by relative radiolabel gains in other cellular organic fractions.

At all time points during the study, and in all three tissues studied, radiolabel was found in the sucrose pool, the hexose sugars pool, the ionic pool and the water insoluble matter pool. The occurrence of radiolabel in the non-sucrose tissue constituents suggests that sucrose turnover is occurring in both immature, and mature internodal tissue.

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LIST OF ABBREVIATIONS

ADP	Adenosinediphosphate
Bq	Bequerels
^{14}C	Radiolabeled carbon
CO_2	Carbon dioxide
$^{14}\text{CO}_2$	Radiolabeled carbon dioxide
CuCl_2	Copper (II) chloride
d	days
DPM	Disintegrations per minute
Etoh	Ethanol
fw	Fresh weight
Fru	D-fructose
Glc	D-glucose
G/F	Glucose/Fructose
H^+	Hydrogen cation
h	Hour
hrs	Hours
H_2O	Water
HCL	Hydrochloric acid
H.P.L.C	High performance liquid chromatography
insol	insoluble
KBq	Kilobequerels
Km	Michaelis-Menton constant

KOH	Potassium hydroxide
Mg ²⁺	Magnesium
mg	milligrams
mM	millimole
mCi	millicurie
mL	millilitre
M	Molar
min	minutes
NaHCO ₃	Sodium bicarbonate
NAI	Neutral Acid Invertase
P	Phosphate
RPP	Reductive Pentose Phosphosphate
SAI	Soluble Acid Invertase
SPP	Sucrose Phosphatase
SPS	Sucrose Phosphate Synthase
SuSy	Sucrose Synthase
UDP	Uridine diphosphate
wks	weeks
μL	microlitre
μmol	micromole

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CHAPTER 1

INTRODUCTION

The development of higher sucrose yielding sugarcane varieties through conventional breeding is proving increasingly difficult. With advancements slowing to a trickle, it is presently believed that the limits of conventional breeding are being reached. Increasing sucrose yields through genetic modification appears to be a way to transcend these limits. Essentially this would entail the manipulation of the genes associated with key enzymes involved in the sucrose accumulation process. However, in order for these key enzymes to be identified and successfully manipulated, a sound understanding of the sucrose accumulation process is imperative.

Work on the characterisation of sucrose accumulation specifically for sugarcane was begun in the early 1960's. Initially work was carried out on whole sugarcane plants (Hartt et al., 1963), but the complexity of this organisational level prompted many workers to adopt a simpler experimental system, namely the tissue slice system. In this system, tissue slices are excised from internodal tissue and incubated in a medium containing radiolabeled sugars. Based on radiolabeling patterns of stored sugars and enzyme studies, a model describing the sucrose accumulation pathway was proposed by Sacher and co-workers (1963a). The two salient features of the model are the occurrence of a 'futile' cycle of sucrose degradation and re-synthesis during sucrose accumulation and the compartmentalisation of the sucrose accumulation process. The model has been refined in the last three decades, but 'futile' cycling and compartmentalisation are still considered to be important factors in the sucrose accumulation process.

Significant sucrose cycling has been noted in tissue slices from both immature and mature sugarcane culm tissue (Batta and Singh, 1986; Whittaker and Botha, 1997). It has been postulated that cycles of sucrose synthesis and degradation play an important role in regulating sucrose accumulation in sugarcane internodes (Sacher et al., 1963a; Komor, 1994). Consequently, the enzymes involved in the sucrose cycle have become the focal point of much research into the regulation of sucrose accumulation (Sacher et al., 1963a; Glasziou and Gaylor, 1972; Veith and Komor, 1993; Komor, 1994). These enzymes include the main sucrose degraders, acid invertase and neutral invertase, and the sucrose synthesizers, sucrose phosphate synthase and sucrose synthase. Research into regulation is still in its infancy and although certain enzymes appear to play important roles, there is no clear picture of how sucrose accumulation is regulated. In an attempt to further understand regulation, research has also been conducted into the link between internode maturation and carbon partitioning in tissue slices. Results indicate that internode maturation coincides with a re-direction of carbon from respiration and biosynthesis into sucrose. This redirection of carbon is seen to explain the observed gradient in sucrose accumulation down the sugarcane culm (Whittaker and Botha, 1997), but the exact link between internode maturation and sucrose accumulation is still unclear. Sucrose accumulation has also been investigated in sugarcane cell suspension cultures (Komor et al., 1982; Thom et al., 1982; Wendler et al., 1990; Veith and Komor, 1993). To date, results confirm the presence of a futile cycle (Wendler et al., 1990) and a re-direction of carbon, similar to that found in tissue slices, was also noted in batch cultured cell suspensions (Wendler et al., 1990).

Thus, our current understanding of the sucrose accumulation process in sugarcane is a result of work done not on whole sugarcane plants, but on slices of stem tissue and on cell suspension cultures. This raises important questions, as it has not been conclusively confirmed that either system is truly representative of the whole plant system.

The sugarcane stem has an unusually large free space volume. Cell destruction due to slicing alters the relative apoplastic and symplastic volumes and concentrations of cellular components (Moore, 1995). The extent of this alteration is dependent on the thickness of the slices and on tissue preparation. Tissue preparation, especially pre- and post- radiolabeling water washes, could result in alterations in turgor pressure (Moore, 1995). These alterations, combined with the possible complications caused by tissue oxidation and the tissue's wound response, could result in significant changes in the metabolism of the sliced tissue. In light of these concerns, the use of cells from sugarcane cell suspension cultures was seen as a more accurate method to characterise *in vivo* sucrose metabolism. However, the similarity between cells in culture and stem parenchyma cells of sugarcane culm has been questioned. Suspension cells were found to differ from parenchyma cells in that they contained both soluble acid invertase and soluble neutral invertase in the cytoplasm, but no acid invertase in the vacuole. Further, when compared to mature parenchyma cells, the cytoplasm of suspension cells was found to occupy much less of the total cell volume (Hawker, 1985) and the maximal sucrose concentration obtained was less than a half of that in mature sugarcane culm (Komor, 1994). It has also been suggested that cells in cell suspension cultures are more stressed than cells in normal parenchyma cells (Hawker, 1985). It is therefore possible that the sucrose accumulation process described in tissue slices and in cell suspension cultures is not reflective of the sucrose accumulation process in intact stem tissue.

Attempts have been made to improve the tissue slice system (Whittaker, 1997; Whittaker and Botha, 1997), but without any comparison with *in vivo* experiments, the success of these attempts cannot be evaluated objectively. Scepticism surrounding the use of tissue slices is also strengthened by previous work on sucrose translocation in whole plants. Although not studied in detail, the fate of sucrose in intact stem tissue was briefly investigated by Hartt and co-

workers(1963). Results obtained suggest that significant turnover of incoming radiolabeled sucrose occurs only in immature stem tissue and not in mature tissue. Similar results were obtained from translocation work on whole plants presented by Hatch and Glasziou (1964). These findings imply that in mature tissue sucrose moves directly into storage without breakdown or re-synthesis. This is in contradiction to subsequent work on tissue slices, which shows that significant sucrose turnover does occur in mature tissues (Batta and Singh, 1986; Whittaker, 1997).

Doubts concerning work on tissue slices can only be confirmed or allayed by comparison with work on whole plants. The study of sucrose accumulation at the whole plant level is, however, complicated by the intractable nature of the stem tissue as an experimental system. The stem is a complex organ composed of several distinct tissue types and divided into internodal segments at different stages of development. It is not only the region of sucrose accumulation but is also an integral part of the sugarcane plant. Thus, the destructive sampling that most physiological studies necessitate, makes long-term study on the same tissue impossible. Further, repeatability is elusive in a rapidly changing system, where the dynamics of growth and development are intricately linked to those of physiology and metabolism. Taking the intricacies involved in whole plant study into consideration, the present investigation focuses on only three aspects of sucrose metabolism in intact sucrose in storage tissue. These aspects include the rate of sucrose accumulation in the developing culm, the characterisation of partitioning of carbon into different cellular organic fractions in the developing culm and the occurrence of sucrose turnover in both immature and mature stem tissues. Specific focus will be placed on the occurrence of sucrose turnover in the culm tissue. The hypothesis is that only the total absence of sucrose turnover will discount the occurrence of cycling in the culm tissue at the whole plant level.

One of the main challenges associated with the study of sucrose metabolism in whole plants is the creation of a labeled sucrose pool within plant tissue, without compromising the integrity of the tissue. In a previous study by Hartt and co-workers (1963), the approach adopted was to introduce radiolabeled carbon into the sucrose pool by feeding radiolabeled carbon dioxide to photosynthesizing sugarcane leaves. The movement and metabolic fate of the radiolabeled sucrose could then be monitored by isotope detection. In the present study the same experimental approach has been adopted, but the focus of the present study is more on the metabolic fate of sucrose in the culm than on the translocation of sucrose throughout the culm.

CHAPTER 2

LITERATURE REVIEW

The review is divided into three main sections. In the first, the properties of sucrose and the enzymes associated with its metabolism in green plants are introduced. Secondly, the topic of sucrose accumulation is reviewed specifically for sugarcane. Finally, our present understanding of sucrose accumulation in other plant systems is discussed relative to sucrose accumulation in sugarcane.

2.1 Sucrose

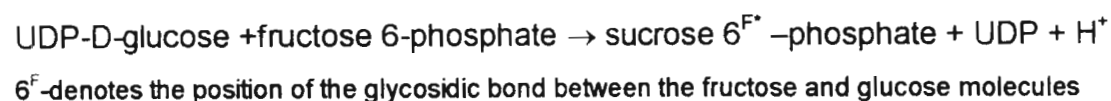
Sucrose is a non-reducing disaccharide of an α -D-glucopyranose and β -D-fructofuranose joined by a (1 \rightarrow 2) linkage. It is hydrolysed by dilute acids, but is stable in the presence of alkalis (Hawker, 1985). As a result of the glycosidic linkage between an α -glucopyranosyl moiety in the chair conformation and a β -D-fructofuranosyl moiety in the envelope conformation sucrose is relatively labile (Akazawa and Okamoto, 1980). It has been proposed that sucrose, as a relatively unreactive derivative of glucose, allows glucose to be stored without attacks from enzymes which catalyse its metabolism (Pontis, 1977). Another physicochemical advantage of sucrose is that it is highly soluble and can attain considerable concentrations without an apparent inhibitory effect on most biochemical reactions in the cell (Avigad, 1982). In most green plants sucrose is both the primary photoassimilate and primary translocate (Hawker, 1985). It is found in nearly all organs of plants and accumulates to high concentrations in organs of plant species in which sucrose is the principle storage carbohydrate (Edelman, 1971).

2.1.1 Sucrose synthesis

In higher plants three enzymes, namely, sucrose phosphate synthase (SPS), sucrose phosphatase (SPP) and sucrose synthase (SuSy) are associated with sucrose synthesis (Avigad, 1982). SPS and SPP were first isolated and characterized by Cardini and co-workers (1955). All three enzymes are known to occur in the cytoplasm of cells (Hawker, 1985).

2.1.1.1 Sucrose phosphate synthase (SPS)

SPS catalyses the following reaction:

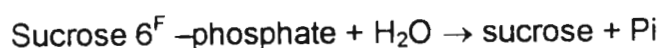


SPS is found in most plants in which sucrose is synthesised and is associated with the cytoplasm (Milner and Avigad, 1964). The majority of the work done on characterising SPS has been carried out on SPS isolated from leaf extracts of spinach, wheat, bean, peanuts, peas, tobacco and barley (Hawker, 1985). SPS has a high specific requirement for its two substrates, UDP-glucose and fructose 6-P, and reaction towards sucrose synthesis is almost irreversible (equilibrium constant of 3250) (Pontis, 1977). There are conflicting reports as to the nature of the saturation curves for UDP-glucose and for fructose-6-P, with both sigmoidal (Hawker, 1985) and hyperbolic curves (Harbron et al., 1981; Doehlert and Huber, 1983) being reported. It is unclear whether these differences are due to interfering reactions in the media or to species or varietal differences in SPS (Harbron et al., 1981; Hawker, 1985). The difficulty associated with the isolation of SPS as a result of its lability (Pontis, 1977; Harbron et al., 1981; Amir and Preiss, 1982), is seen as the main cause for the conflicting reports on its properties (Avigad, 1982). SPS is inhibited by its products, UDP and sucrose-P, and by inorganic phosphate (competitive with UDP-glucose) and by fructose 1,6-bisphosphate (competitive with fructose 6-P). The level to which SPS is inhibited

by each of these metabolites is dependent on the species and on the presence of Mg^{2+} (Pontis, 1977).

2.1.1.2 Sucrose phosphatase (SPP)

SPP catalyses the following reaction:



The discovery of SPS pointed to the presence of a specific sucrose phosphate phosphatase in plants. Evidence for SPP has been found in extracts from both lower and higher plants (Hawker, 1985). The enzyme has been partially purified in sugarcane (Hatch, 1964) and carrot roots (Hawker and Hatch, 1966). SPP is relatively unstable and requires Mg^{2+} for activity (Hawker and Hatch, 1975). It is inhibited by sucrose but not by glucose and fructose. Inhibition by sucrose has been shown to be partially competitive (Hawker, 1967). SPP has been detected in vacuolar preparations from sugar beet roots and immature sugarcane stem (Hawker, 1985).

2.1.1.3 Sucrose synthase (SuSy)

SuSy catalyses the following reaction:



The enzyme is ubiquitous in tissues of all higher plants. It was the first enzyme capable of sucrose synthesis discovered (Cardini et al., 1955). However, radiolabeling experiments, the properties of the enzyme and the distribution of the enzyme in tissues indicate that the enzyme is more involved in sucrose breakdown than in sucrose synthesis. The enzyme is much more stable than SPS or SS and has been purified to homogeneity from maize (Su and Preiss, 1978) and partially purified from many other plant systems. The reaction catalysed by the enzyme is easily reversible (Avigad, 1964, Avigad and

Milner, 1964) with an equilibrium constant value of about 5 (Goodwin and Mercer, 1983). The enzyme is inhibited by nucleotides, sugars and sugar phosphates (Delmer, 1972; Pontis et al., 1972; Wolosiuk and Pontis, 1974). Unfortunately, due to the differing results obtained from different plant species it is not possible to draw general conclusions about the physiological significance of these effectors.

2.1.2 Sucrose degradation

The enzymes responsible for sucrose degradation include the invertases and sucrose synthase. Both acid and neutral invertases occur in plants, with pH optima of about 5 and 7.5 respectively. Acid invertase is found in the free space and vacuoles of plant cells, while neutral invertase and sucrose synthase are thought to be confined to the cytoplasm (ap Rees, 1974).

2.1.2.1 Sucrose synthase (SuSy)

SuSy catalyzes the following reaction:

Sucrose + UDP \leftrightarrow UDP-glucose + fructose (Adenosinediphosphate can replace UDP)

As mentioned previously SuSy is capable of sucrose degradation. The enzyme exhibits high K_m values for sucrose (20-50 mM) and shows sigmoidal saturation kinetics with sucrose (Hawker, 1985). It is one of the major soluble proteins in many growing plant tissues. SuSy has been implicated in sucrose degradation in pathways for starch synthesis and other reserve polysaccharides (Su, 1982). SuSy has been found in fruits including, grapes, bananas, apples, pears, and citrus fruits and in eggplant, carrot, sugar beet and sugarcane (Hawker, 1985). The possible roles of SuSy in sucrose accumulation in these systems will be dealt with in later sections.

2.1.2.2 Invertases

Invertases catalyze the following reaction:



Two invertases are present in higher plants, namely neutral invertase (NAI) and soluble acid invertase (SAI). Neutral invertase is usually associated with the cytoplasm while most of the acid invertase in many species, including sugar beet, sugarcane, grape berries and maize scutellum, is found as soluble acid invertase in the vacuole (Glasziou and Gaylor, 1972). In certain species, e.g., sugarcane, acid invertase has been found in the apoplast (ap Rees, 1974). Acid invertase in the apoplast may take the form of soluble acid invertase or cell wall bound acid invertase. Acid invertases have K_m values for sucrose between 2 and 13 mM and alkaline invertases between 9 and 25 mM. Glucose is known to inhibit the production of invertases in sugarcane and it is postulated that cytosolic glucose concentrations play an important role in regulating synthesis of intracellular invertase in many species. However, glucose is known to have little effect on invertase activity in carrot or lentil tissues (ap Rees, 1974; Avigad, 1982). Naturally occurring protein inhibitors of invertase have been found in several plant tissues (Pressey, 1968). The physiological significance and binding properties of these inhibitors are still to be determined.

2.2 Sucrose accumulation in sugarcane

An extensive body of work is to be found on the transport and accumulation of sucrose in the sugarcane culm (refer to reviews by Glasziou and Gaylor, 1972; Moore, 1995). Work has been carried out using whole plants, stem tissue slices and more recently using cell suspension cultures. This section of the review describes how work at all these levels has contributed to our current understanding of sucrose accumulation in the sugarcane plant.

2.2.1. Culm development

The culm is a complex organ composed of several distinct tissue types and divided into internodal segments at different stages of development. During growth and development of the sugarcane shoot, the shoot apical meristem successively blocks off phytomeric units. Each phytomer consists of a leaf primordium associated with a subtending intercalary meristem that gives rise to the internode. (Moore, 1995) Internodes subtending non-expanded leaves are just initiating development. Once full leaf expansion is obtained, elongation in the subtending internodes becomes apparent. During elongation, the internodes accumulate increasing amounts of sucrose. In immature internodes the incoming sucrose is used for growth while in older internodes incoming sucrose is stored (Glasziou and Gaylor, 1972; Moore, 1995). This results in a gradient of maturation and sucrose accumulation down the culm to a point where full maturity and a stable, high sucrose concentration is reached (Moore, 1995). Stored sucrose can account for up to 50% of the total dry weight, reaching a concentration of 500mM (Glasziou and Gaylor, 1972). Internode maturation is also associated with a decrease in hexose sugar content and an increase in insoluble matter content ((Whittaker and Botha, 1997; Moore, 1995).

2.2.2 Source to sink movement of sucrose

To date, work at the whole plant level has been most productive in characterizing the source to sink movement of sucrose in the sugarcane plant. All experimental work in this area has been based on the feeding of radiolabeled carbon dioxide ($^{14}\text{CO}_2$) to photosynthesizing sugarcane leaves and the subsequent time-course analyses of radiolabeled carbohydrates. The following is a summary of a fifteen-year study on the translocation of sucrose in the sugarcane culm as presented by Hartt and co-workers (1963). No other work challenging the conclusions drawn from this comprehensive study could be found in the available literature.

The study was carried out on a high sucrose storing member of the *Saccharum* complex. Sucrose was found to be the principal photoassimilate translocated throughout the plant. The percentage of newly formed sucrose leaving the fed part of the leaf within the first 90 minutes was dependent on both climatic and edaphic (soil related) factors and on the rank of the fed leaf. Values ranged between 30 and 73%. Translocation rates were found to be highest from the middle rank leaves (leaves 5-8). On entering the culm, translocation of radiolabeled sucrose was predominantly downwards. Radioactive photosynthate was found to move down the culm more than one internode but less than nine internodes before finding its way into an upward-moving system. The majority of the radiolabeled photosynthate was found to be stored in ripening internodes situated below the fed internode.

The form in which photosynthate was found varied with internode number. At five hours after radiolabeling, the radiolabeled photosynthate in the fed internode was 93% sucrose, 4% phosphates and amino acids and 3% hexose sugars. In internodes above the fed internode the amount of radiolabel in sucrose was found to decrease while the amount of radiolabel in hexose sugars increased. The converse was found to be true in internodes below the fed internode, with radiolabeled sucrose constituting 100% of the radiolabel in certain internodes. This suggests that there is no sucrose breakdown in mature internodes.

While the study of sucrose translocation at the whole plant level is relatively easily tackled, the detailed study of sucrose accumulation is made difficult by the complexity of this organisational level. To gain a better understanding of sucrose accumulation, a simpler experimental system was adopted by research workers, namely, the tissue slice system.

2.2.3 The sugar accumulation cycle and associated enzymes

Early radiolabeling, kinetic and tracer studies on storage parenchyma tissue slices revealed that there were two spatially distinct sugar pools, one occurring in

the cytosol and one in the vacuole (Glasziou, 1960; Glasziou, 1961). The presence of a third sugar pool is described in work presented by Sacher et al (1963a). Collectively, this work has led to the recognition of a sugar accumulation pathway through three distinct compartments, namely the 'outer space' (apoplast and cell walls), the metabolic compartment (cytosol) and the storage compartment (vacuole).

To establish whether or not sucrose is hydrolysed and re-synthesized during accumulation, Hatch and co-workers (1963) performed a series of experiments using asymmetrically radiolabeled sucrose. The rationale behind these experiments is that if the asymmetrically radiolabeled sucrose is hydrolyzed by extracellular invertase prior to uptake, and the hexoses re-synthesized to sucrose in the cytoplasm, randomisation of the radiocarbon among the hexose moieties of the sucrose molecule would be introduced as a result of the isomerase activity during re-synthesis. When feeding ^{14}C (fructosyl)-sucrose, the resulting radiolabeled glucose to radiolabeled fructose ratio is usually about 0.3 in sugarcane tissue (uniformly radiolabeled sucrose results in a value of 1). This indicates that significant randomisation of the radiocarbon does occur. Using asymmetrically radiolabeled sucrose, sucrose was shown to be hydrolysed prior to membrane transport into immature sugarcane storage tissue (Hatch and Sacher, 1963; Hatch and Glasziou, 1963) and into mature sugarcane storage tissue (Hawker and Hatch, 1965).

Sacher and co-workers (1963a) proposed that the sucrose accumulation pathway in sugarcane is cyclic in nature, with sucrose being simultaneously synthesized and degraded within the cell. This cycling of carbon between sucrose and the hexose sugars is proposed to occur in immature (Sacher et al., 1963a) and mature internodal tissue (Batta and Singh, 1986; Whittaker and Botha, 1997). Figure 2.1 illustrates the sugar accumulation cycle as proposed by Sacher and co-workers (1963). Sucrose is initially cleaved in the apoplast. The

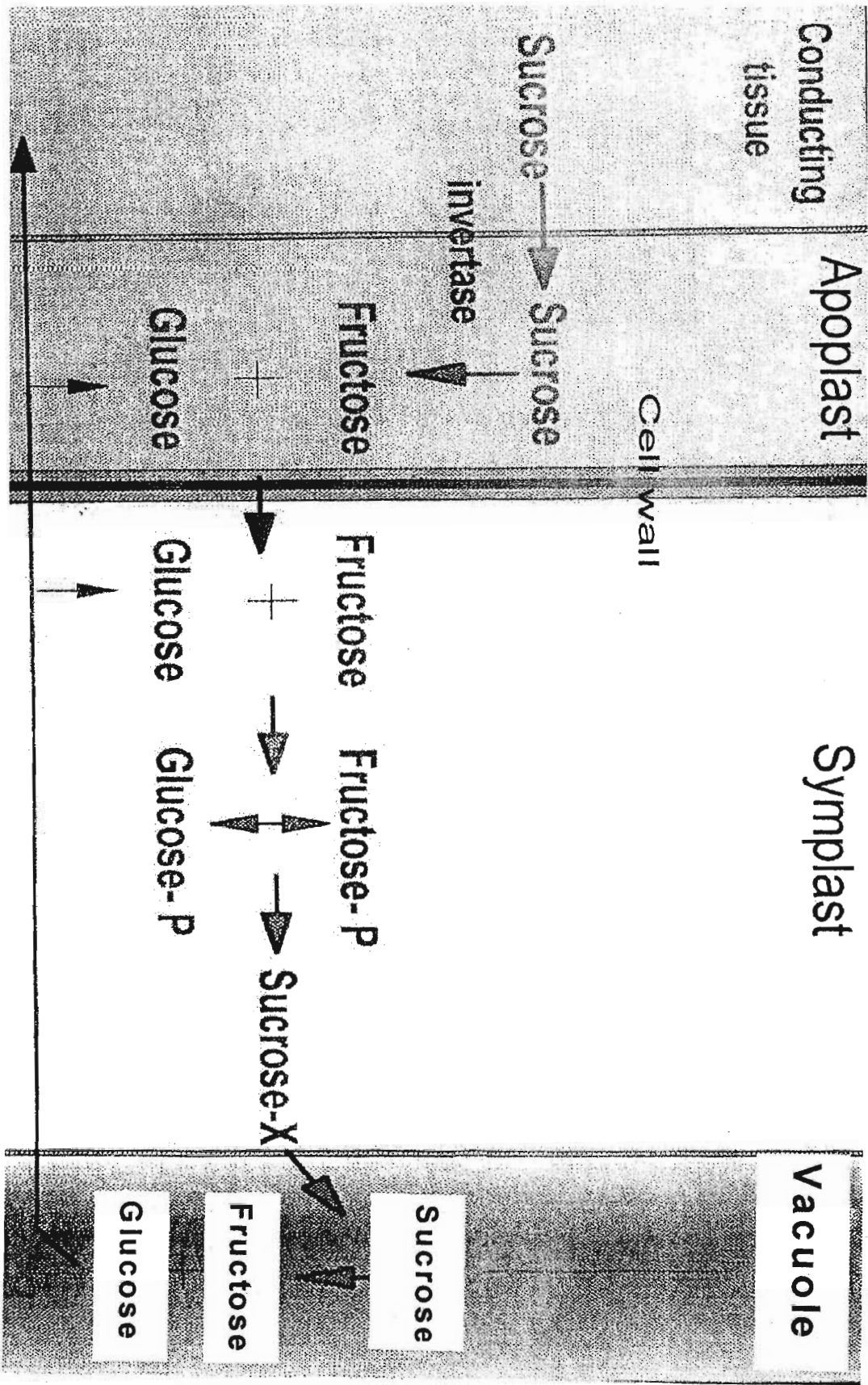


Figure 2.1 Sucrose accumulation cycle in sugarcane tissue (adapted from Sacher et al., 1963)

resultant hexose sugars then cross the cell membrane via a hexose transporter. Within the metabolic compartment an activated sucrose derivative, termed 'sucrose-X' by Glasziou (1960), is synthesized from phosphorylated hexose intermediates (Sacher et al., 1963a). The breakdown of this activated derivative is postulated to provide the energy required for active accumulation of the re-synthesized sucrose in the storage compartment (Glasziou, 1961). By following the movement of radiolabel in sugars in intact tissue slices (compartmentation present) and crushed tissue slices (no compartmentation) Sacher and co-workers (1963) determined that there is movement of radiolabeled sucrose both into and out of the vacuole. Thus, carbon is not only being cycled between the sucrose and hexose sugars in each compartment, but is also being cycled between the compartments themselves. The extent of this cycling was found to be dependent on the activity of specific enzymes expressed within these compartments. Figure 2.2 shows an updated version of the sucrose accumulation cycle proposed by Sacher and co-workers (1963a). In this figure the proposed positioning and roles of the sucrose related enzymes, as currently understood, are more apparent.

In storage parenchyma tissue, sucrose can be catabolised by sucrose synthase (SuSy) or one of three invertases, namely soluble acid invertase (SAI), cell wall bound acid invertase and neutral invertase (Hawker, 1985). The activity of SuSy was found to be low in both immature (Hatch and Sacher, 1963; Hatch and Glasziou, 1963; Slack, 1965) and mature (Hawker and Hatch, 1965) storage parenchyma cells, suggesting that it plays a nominal role in sucrose breakdown. Rather, it was proposed that sucrose hydrolysis is achieved by one of the invertases.

Cell wall bound acid invertase is responsible for sucrose cleavage in the apoplast, neutral invertase in the metabolic compartment and soluble acid invertase in the storage compartment. The activity levels of cell-wall-bound acid invertase were found to be similar in both immature and mature internodes

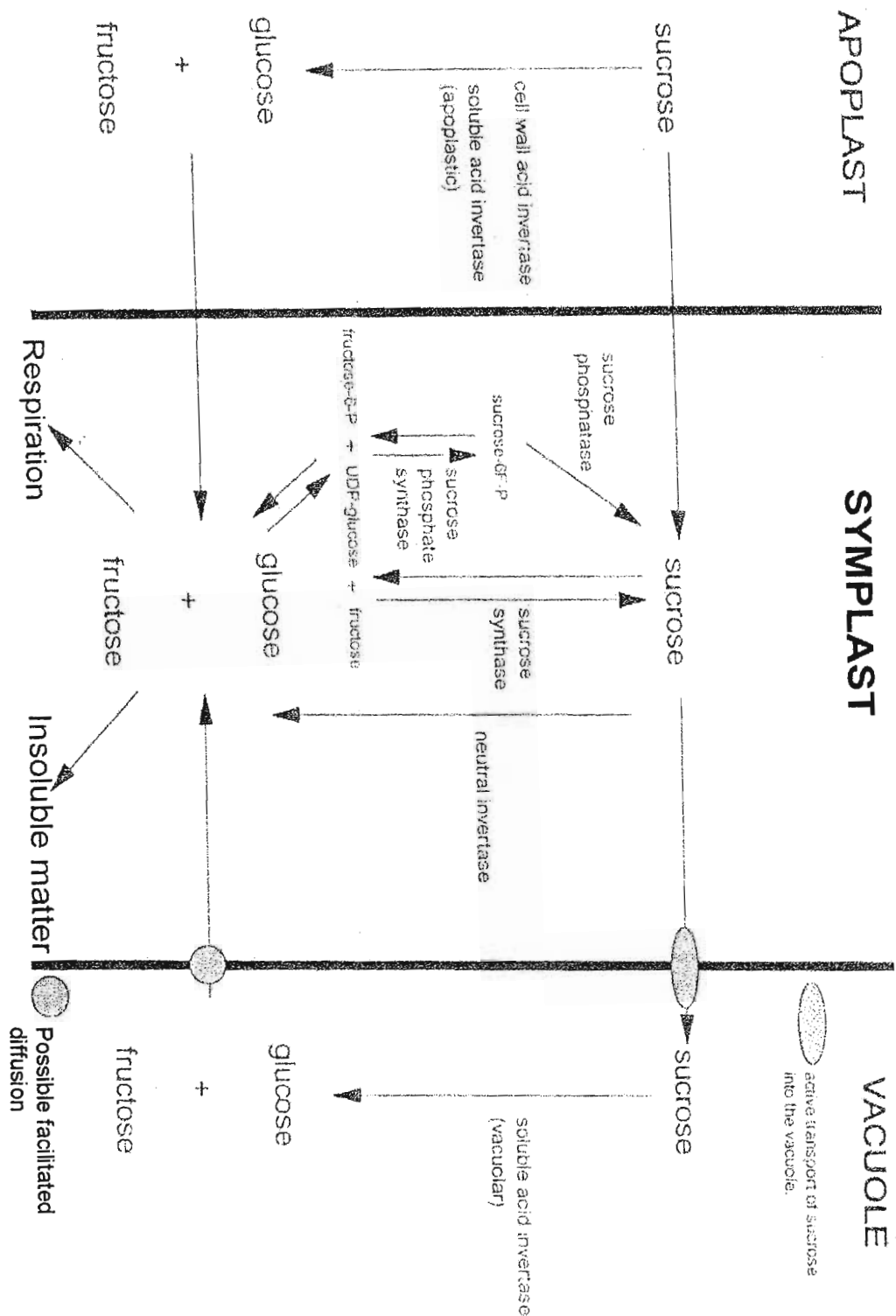


Figure 2.2 Sucrose accumulation cycle with associated enzymes.

(Hatch and Glasziou, 1963; Hawker and Hatch, 1965; Botha et al., 1996). Neutral invertase activity was found to be low in immature tissue and slightly higher in mature tissue (Sacher et al., 1963a; Hawker and Hatch, 1965; Gaylor and Glasziou, 1972; Botha et al., 1996; Zhu et al., 1997).

Conclusions drawn from work done on soluble acid invertase activity are more ambiguous. Initially SAI activity was found to be high in the apoplast and vacuole of immature tissue and very low or absent in mature tissue (Hatch and Glasziou 1963; Sacher et al., 1963a). More recent work has shown that while SAI activity does decline sharply from immature to mature tissue, activity is still significant in mature tissue (Botha et al., 1996). A possible reason for these contradictory results could be the presence of naturally occurring acid invertase inhibitors (Avigad, 1982; Hawker, 1985). Although little is known about the physiological significance and binding properties of invertase inhibitors, it is plausible that differing tissue handling and assay techniques may have resulted in the alteration of the binding properties of these inhibitors. These alterations could then have manifested themselves in the observed differences in SAI activity.

The re-synthesis of sucrose in the metabolic compartment is proposed to be carried out by sucrose phosphate synthase (SPS) and sucrose phosphatase (SPP) in immature and mature tissue (Hatch and Sacher, 1963; Hatch and Glasziou, 1963; Hatch, 1964; Hawker et al., 1991; Komor, 1994). The importance of SuSy in sucrose biosynthesis still remains to be determined (Moore, 1995).

To determine which enzymes are important in regulating sucrose storage, work has been carried out at both the whole plant and tissue slice levels. These studies suggest that acid invertase may play an important role in the regulation of photosynthate partitioning between growth and storage in internodal tissues. In whole plant studies SAI activity levels in elongating internodes were found to increase with increasing growth rates and decrease as growth slowed down (Hatch and Glasziou, 1963; Glasziou and Gaylor, 1972). SAI activity levels were

also found to be high in low sucrose storing immature internodal tissue and low in high sucrose mature tissues (Hatch and Glasziou, 1963; Glasziou and Gaylor, 1972; Zhu et al., 1996; Zhu et al., 1997). The important regulatory role of SAI is also supported by a correlation found between SAI activity levels and the ability of different members of the *Saccharum* complex to accumulate sucrose. SAI activity was found to be low in the mature internodes of high sucrose storing members and relatively high in mature internodes of low sucrose storing varieties (Hatch and Glasziou, 1963). More recently Zhu and co-workers (1997), showed that there was a critical threshold of SAI activity above which high concentrations of sucrose did not accumulate. This finding adds to the growing pool of evidence which indicates that SAI is of key importance in determining sucrose accumulation.

Work carried out on tissue slices also found that high SAI activity was associated with low sucrose accumulation while low SAI activity was associated with high sucrose accumulation. (Hawker and Hatch, 1965; Sacher et al., 1963c; Su et al., 1992; Botha et al., 1996). It was proposed that SAI activity is regulated in part by an auxin-sugar control mechanism, with glucose repressing activity and auxin promoting activity (Sacher et al., 1963b; Glasziou and Waldron, 1964). Based on the finding that auxin concentrations diminish with increasing distance from the shoot apex (Glasziou and Waldron, 1964), it was hypothesized that the reduction in invertase activity during maturation is due to reduced auxin supply. This reduction in activity then leads to a reduction in sucrose turnover which ultimately results in more sucrose being accumulated in the storage compartment (Hawker and Hatch, 1965; Su et al., 1992).

Although the body of evidence supporting SAI's key role in determining sucrose accumulation is notable, there is also evidence that suggests that while SAI is important, it is not the only determinant of sucrose accumulation. In the work carried out by Zhu and co-workers(1997), it was found that while SAI activity needed to be low to allow for sucrose accumulation, the low SAI activity alone

was not sufficient to account for the level of sucrose accumulation in high sucrose storing varieties. Thus, there must be some other determinant of sucrose accumulation. Zhu and co-workers (1997) proposed that the ultimate determinant of sucrose accumulation was the difference between SPS activity and SAI activity. However, from the data presented in the work it cannot be conclusively stated that this difference is responsible for observed levels of sucrose accumulation. In addition to evidence which questions SAI role as a sole determinant of sucrose accumulation, there is also evidence which suggests that SAI's importance may be overestimated. In the work carried out by Hatch and Glasziou (1963) the data presented show that there is no difference in the levels of hexose sugars in mature internodes of low storing sucrose varieties and high sucrose storing varieties. This implies that the extractable SAI activities might not represent the *in vivo* SAI activity.

It has been proposed that in mature tissue, neutral invertase and not SAI was responsible for controlling sugar flux (Hawker and Hatch, 1965). Support for a possible regulatory role of neutral invertase in mature tissue is also found in more recent work. Due to increased lignification and suberisation in mature tissue (Jacobsen et al., 1992) it has been suggested that a large proportion of sucrose in mature tissue occurs symplastically and is not compartmentalized in the vacuole (Welbaum and Meinzer, 1990; Preisser et al., 1992). Thus, in mature tissue, the amount of sucrose accessible to neutral invertase may be much more than was previously thought. The increased sucrose concentration may also have implications for the possible role of SuSy. SuSy activity is known to be controlled by substrate availability *in vivo* (Avigad, 1982). Thus while SuSy is close to equilibrium in immature tissues with low cytosolic sucrose concentrations, in mature tissue, equilibrium should be displaced towards sucrose cleavage (Whittaker, 1997).

Early studies suggested that sucrose cleavage in the apoplastic space is a necessary part of sugar uptake. As a result of this it was proposed that cell-wall-

bound acid invertase played an important role in controlling photosynthate import (Hawker and Hatch, 1965). More recent work has, however, found that sucrose cleavage is not obligatory (Lingle, 1989; Thom and Maretzki, 1992). The contradiction has been blamed on the treatment of tissue slices prior to radiolabeling. In earlier studies tissues were washed in water for 1 hour before being radiolabeled. This pre-radiolabeling wash is proposed to have inactivated the sucrose transporter, thereby resulting in the exclusive uptake of hexoses. *In vivo* sugar uptake is suggested to occur as both sucrose and hexose sugars (Moore, 1995). Thus, the importance of cell-wall-bound acid invertase in controlling photosynthate import may have been over-estimated. It is now thought that the main function of cell-wall-bound acid invertase is to retrieve sugars 'leaked' to the apoplast (Moore, 1995). In addition to the possible roles of the invertases in regulation, sucrose synthesizers have also been implicated in the regulation of sucrose accumulation (Hatch, 1964). However, due to problems with the assaying techniques for both SPS and SuSy, improved techniques were required before their importance could be determined.

Work at the tissue slice level established the occurrence of a cyclic sucrose accumulation pathway in which sucrose storage is controlled by changes in the rates of sucrose synthesis and degradation.

2.2.4 Confirmation of the occurrence of the sugar accumulation cycle at the cell level

Sucrose accumulation was re-examined with the use of sugarcane suspension cells grown in batch culture. Results obtained from work using cell suspension cultures supports the occurrence of a sucrose accumulation cycle, as described in tissue slices. Wendler and co-workers (1990) found that even in sugarcane cells that were no longer carrying out net storage of sucrose, SPS activity was still high. In addition, in cells that were undergoing sucrose storage, the activities of the sucrose degraders, SuSy and neutral invertase, were still high. These unexpected high enzyme activities and the continuous incorporation of ^{14}C

fructose into sucrose, irrespective of whether sucrose was being stored or mobilised, confirmed the presence of a cycle in which sucrose is simultaneously synthesized and degraded (Wendler et al., 1990). Further, the net rate of sucrose synthesis was found to exceed the net rate of sucrose storage (Wendler et al., 1990), indicating a rapid cycle of sucrose synthesis and hydrolysis. It was hypothesized that this seemingly 'futile' cycle allows for rapid switches between net storage and mobilisation with only small changes in enzyme and metabolite levels.

The relationship between growth, SAI activity and sucrose accumulation in batch cultures of sugarcane cell suspensions was found to be similar to that described in tissue slices (Goldner et al., 1991). In cells grown in continuous culture, SAI activity was found to be low during low culture growth rates and high at high culture growth rates. Thus there is support for the regulatory role of vacuolar acid invertase at both the tissue slice and cell levels. However, work at the cell level did little to clarify the role of neutral invertase. Wendler and co-workers (1990) presented work showing that the decline in neutral invertase activity and SAI activity was similar during sucrose accumulation. Later work showed that under conditions of nutrient limitation, neutral invertase activity could not be correlated with growth or sucrose storage in cell suspensions (Veith and Komor, 1993). No work on the role of cell-wall-bound acid invertase using cell suspension cultures has been carried out.

The proposal that SPS plays an important role in mediating sucrose accumulation is strengthened by results obtained using cell suspension cultures with improved assaying techniques. These improved techniques enabled rapid separation of SPS activity from UDP hydrolysing activities (Dancer et al., 1990) and allowed for increased SPS stability (Hubbard et al., 1989). Pulse radiolabeling experiments indicated that sucrose synthesis is catalysed entirely by SPS (Wendler et al., 1990). When sugarcane cell suspensions were grown in batch culture, the depletion of nitrogen resulted in an increase of SPS activity

and the commencement of sucrose accumulation. SPS activity doubled during active sucrose storage and remained high once storage had ceased. SuSy activity was also found to be high during sucrose storage but pulse feeding experiments with [^{14}C] glucose and non-radiolabeled fructose indicate that SuSy does not play a substantial role in sucrose synthesis (Wendler et al., 1990; Goldner et al., 1991). The equilibrium constant for SuSy formation of sucrose was previously found to be only 5, much less than the value calculated value for SPS, 3250 (Goodwin and Mercer, 1983). The dominance of SPS activity was therefore not unexpected.

Work using cell suspension cultures confirmed the occurrence of a cyclic sucrose accumulation pathway. It also added support for the role of SAI- and confirmed the importance of SPS in regulating sucrose storage. However, cell suspension work shed little light on the possible roles of neutral invertase, SuSy and cell-wall-bound acid invertase in the sucrose accumulation process.

2.3 Developmental relationship between sucrose accumulation and carbon partitioning

Whittaker and Botha (1997) presented work on the temporal relationship between sucrose accumulation and carbon partitioning in developing sugarcane internodes. The main aim of the study was to characterize developmental changes in the partitioning of carbon into the different cellular organic fractions in internodal sugarcane tissues. Possible regulatory factors were also examined.

In the study, the rate of sucrose accumulation down the developing culm was calculated using the change in sucrose content during the period in which a new internode was produced. Results obtained indicate that the sucrose accumulation rate increases sharply between internodes 4 and 7. This increase was attributed to a re-direction of carbon entering the internode from biosynthesis

and respiration to sucrose storage. Thus, during maturation, incoming carbon is partitioned less into proteins, structural carbohydrates, amino acids and organic acids and more into sucrose. It was proposed that a reduction in sucrose cleavage or an increase in sucrose synthesis, or a combination of the two, was responsible for the re-direction of carbon. Thus, the rate of sucrose accumulation is controlled by a cycle of synthesis and degradation as described initially by Sacher and co-workers (1963a). The decreased allocation of carbon to respiration also coincided with a reduction in the flux of hexose monophosphates into the respiratory pathway. It was proposed that underlying regulatory changes in the pathways of cell wall polysaccharide synthesis, glycolysis and the OPP pathway may operate to downregulate the entry of hexose monophosphates into structural matter synthesis and respiration. However, the reduction in respiratory flux could not be attributed to the fine control of any of the investigated steps in the glycolytic pathway.

These findings are supported by work carried out using sugarcane cell suspension cultures grown in batch culture. Marked changes in the sucrose accumulation rate were also observed during the growth cycle of sugarcane cell-suspension cultures (Wendler et al., 1990; Goldner et al., 1991). Under nitrogen limiting conditions, increased carbon partitioning into sucrose occurs at the expense of both respiration and structural material (Veith and Komor, 1993). Decreased partitioning of carbon into the non-sucrose water soluble component has also been noted during sugar beet maturation (Giaquinta, 1979).

2.4 Solute distribution between the vacuole and cytoplasm in storage tissue

As previously indicated in section 2.2.3, the distribution of sucrose between the vacuole and cytoplasm in storage tissue can alter the accessibility of sucrose to certain enzymes involved in sucrose metabolism. This alteration in accessibility may have notable implications for sucrose accumulation in storage tissue.

Consequently, it is necessary to briefly discuss our current knowledge of sugar distribution between the vacuole and cytoplasm in sugarcane tissue.

Early work carried out by Sacher and co-workers (1963a) using radiolabeled sucrose and non-radiolabeled fructose provided evidence in support of the spatial separation of the metabolic (cytosol) and storage (vacuole) compartments. In plant storage tissue the vacuole can constitute up to 90% of the total cell volume; thus most of the stored compounds must be located in the vacuole. However, it is not clear whether the stored solutes are actively accumulated in the vacuole. Work done on various plant systems has revealed that there is no standard rule concerning accumulation. In barley mesophyll cells, the uptake of sucrose into isolated vacuoles is passive (Kaiser and Heber, 1984; Martinoia et al., 1987). In isolated vacuoles of red-beet tissue (Doll et al., 1979) and in tonoplast vesicles of sugar beet (Saftner et al., 1983) uptake was found to be active with sucrose accumulation occurring in the vacuole. The presence of a group translocator in the tonoplast of red- and sugar beet has not been ruled out but as yet no conclusive evidence of its existence has been obtained (Hawker et al., 1991). In contrast to red beet and sugar beet, no active sugar uptake by isolated vacuoles (Thom et al., 1982; Thom and Maretzki, 1985) or tonoplast vesicles (Maretzki and Thom, 1986) of sugarcane has been reported. In addition, Preisser and Komor (1988) presented work showing that sucrose accumulation does not occur via UDP-glucose-group translocation.

To determine whether sugarcane vacuoles had lost their sugar uptake capacity due to the osmotic shock method of isolation or whether they actually do not accumulate sugars, Preisser and co-workers (1992) conducted work on whole sugarcane cells. Sugarcane suspension cells at different stages of the cell cycle were treated with CuCl_2 . CuCl_2 treatment results in the permeabilisation of the plasma membrane while the tonoplast remains intact. This allows for the determination of the degree of compartmentation of sucrose in the sugarcane cells.

The results of the study showed that there is no active accumulation of sucrose inside the vacuoles of sugarcane suspension cells during the sucrose-storage phase. In the early growth phase more sucrose was found in the vacuole but the concentration in the cytosol and the vacuole were approximately equal. In the sucrose storage phase the concentration of sucrose in the vacuole and cytosol rose to the same extent with no accumulation being observed. At the end of the cell cycle the sucrose concentration in the cytosol was actually higher than that in the vacuole. Thus the results confirmed earlier results from work on isolated vacuoles. The interesting finding of the work on whole cells was that while sucrose accumulation did not occur, hexose sugar accumulation in the vacuole was significant. CuCl_2 treatment of cells in the pre-sucrose storage phase resulted very low hexose efflux, indicating that most of the hexoses are located in the vacuole. It is not clear whether the observed accumulation of hexoses is due to active uptake via a H^+ -sugar antiport (Thom and Komor, 1984) or whether it is due to the activity of acid invertase, which is known to be present in vacuoles (Wendler et al., 1990).

The study of sugar distribution between the vacuole and cytosol in storage tissue, although still in its formative years, indicates that the availability of the substrate to sucrose metabolizing enzymes may also need consideration before conclusions are drawn about the regulation of sucrose accumulation. Although active accumulation of sucrose in the vacuole does not seem to occur, the readiness of sucrose transfer across the tonoplast might influence the accessibility of re-synthesized sucrose to metabolic degradation. In turn this might influence the cycle of sucrose breakdown and re-synthesis, the cycle which is considered important in the regulation of sucrose accumulation (Moore, 1995). More intensive study will reveal the actual significance of sucrose distribution between the vacuole and cytosol.

2.5 Sucrose accumulation in other plants

As a result of the commercial importance and apparent simplicity of the sugarcane system, sucrose accumulation has been studied more intensively in sugarcane than in any other plant system. However, study of other plants, although not as definitive, has revealed that sucrose accumulation in sugarcane cannot be used as a universal standard. Aside from sugarcane, the other sucrose storing crop of commercial importance is sugarbeet. Consequently, the main focus of this part of the review will be on sugar beet, but sucrose accumulation in other plant systems will also be briefly reviewed.

2.5.1 Sucrose accumulation in Sugar beet

The root system of the sugar beet can be divided into a taproot and fibrous root component. The taproot, which ultimately forms the sugar beet, consists of an inner core region and a peripheral meristematic region. Sucrose accumulation occurs predominantly in the inner core. In immature root tissue, 8-30 days after emergence, sucrose content is very low. In sugar beet grown under normal non-limiting conditions, sucrose storage usually commenced between 30 and 60 days after emergence (Giaquinta, 1979). As in sugarcane, sucrose is the carbohydrate produced in the leaves, translocated through the phloem and accumulated in the storage tissue. The process of sucrose accumulation in the storage tissue of sugar beet is, however, notably different from that in sugarcane. As in sugarcane, workers studying sucrose accumulation in sugar beet have focused on the physiological roles of the enzymes that catalyze the last steps of sucrose biosynthesis and the enzymes responsible for sucrose cleavage.

The majority of early studies reported a small or zero activity of SPS in beet tissue (Pavlinova and Prasolova, 1970; Vieweg, 1974; Giaquinta, 1979). Consequently, the role of SPS in sucrose metabolism was considered less important than that of SPS in sugarcane. However, more recent studies indicate

that SPS may play a larger role in sucrose accumulation than was previously thought. Using improved assaying techniques which allowed for better isolation of SPS activity, Fieuw and Willenbrink (1987) showed that SPS is not only active at significant levels but is also positively correlated with beet sucrose content. It is postulated that SPS activity is responsible for the steady increase in sucrose content in the growing beet (Fieuw and Willenbrink, 1987). In addition, SPS activity was found to be higher in the core region of the sugar beet, a region that is known to contain the highest sucrose content. More work needs to be done to establish the exact relationship between sucrose content and SPS activity.

Appreciable activity levels of the other enzyme capable of sucrose synthesis, sucrose synthase, have been found in the growing sugar beet (Kursanov, 1974; Giaquinta, 1977; Giaquinta 1979; Fieuw and Willenbrink, 1987). As in sugarcane *in vivo* conditions are thought to favour sucrose hydrolysis rather than sucrose synthesis. SuSy activity was found to be very low or absent in the pre-sucrose storage phase of beet growth. Significant activity levels were only noted at 30-35 days after emergence (Giaquinta, 1977). SuSy activity was 60% greater in the storage tissue than in the peripheral tissues of the sugar beet (Silvius and Snyder, 1979a). Based on these findings SuSy is considered to play a pivotal role in sucrose accumulation in the sugar beet. By catalysing sucrose hydrolysis, SuSy is thought to provide precursors for cell wall polymers (UDP-Glc) and catabolism (fructose-6-phosphate) (Kursanov, 1974). It is proposed that the rise in SuSy activity during sucrose accumulation reflects a need for carbohydrates in cell wall synthesis. This cell wall synthesis brings about an increase in sink capacity, which results in greater sucrose accumulation (Kursanov and Pavlinova, 1974; Giaquinta, 1979).

From the work on SuSy and SPS it is apparent that both enzymes play important roles in the sucrose accumulation process, with SuSy contributing to an increase in sink capacity while SPS provides the steady increase in sucrose content (Fieuw and Willenbrink, 1987). In sugarcane the importance of SPS as a

determinant of sucrose accumulation is recognized, but to date results suggest that SuSy only plays a nominal role in sucrose accumulation. The work on sugar beets together with recent work on compartmentalisation in sugarcane by Welbaum and Meinzer (1990) and Preisser and co-workers (1992) (refer to section 2.2.3) suggest that it is necessary to take a new look at the role of SuSy in sugarcane.

In sugar beet, as in sugarcane, sucrose is hydrolysed by invertase activity. Giaquinta (1979) reported single peaks of invertase activity at pH 4.5 but activity was also detectable above pH 7. In 28 day old sugar beets invertase activity in storage tissues was present at pH 8 but not at pH 4.5 (Silvius and Snyder, 1979b). This evidence suggests that both acid and alkaline invertase activity occurs but the existence of two separate invertases in sugar beet has not been clearly established. Alkaline invertase activity is present in both the storage and peripheral regions of the sugar beet and in both immature and mature sugar beet. However, unlike sugarcane, no work on sugar beet has reported an increase in alkaline invertase activity concomitant with an increase in sucrose accumulation.

The relationship between acid invertase activity and sucrose accumulation in sugar beet is very similar to that described in sugarcane. In sugar beet acid invertase activity is high in immature fast growing beets but declines notably at the onset of sucrose storage (12-21 days post emergence) (Giaquinta, 1979). It appears that in both sugarcane and sugar beet a decrease in invertase activity is necessary before sucrose accumulation can occur. This incompatibility between acid invertase and sucrose accumulation appears to be a common feature of many plant systems. The phenomenon has also been noted in carrot, turnip and radish (Ricardo and ap Rees, 1970; Ricardo and Soria, 1974). It has also been shown that sugar beet genotypes which retain more sucrose in the taproot had the least amount of invertase activity, as is the case in sugarcane (refer to section 2.2.3). A similar system of hormonal control is also proposed to regulate

invertase activity in both sugarcane and sugar beet (Silvius and Snyder, 1979b). In carrot and beet roots synthesis is inhibited by both auxin and kinetin and stimulated by gibberellic acid (Palmer, 1966; Ricardo, 1976). In addition to the reduction in *de novo* synthesis, invertase activity has also been shown to be reduced due to the presence of specific invertase inhibitors (Kursanov et al., 1971). The need for invertase activity to be reduced before sucrose accumulation can occur indicates that invertase activity is pivotal in the regulation of photosynthate partitioning between growth and storage.

As previously mentioned, experiments conducted on both immature and mature sugarcane culm revealed significant randomisation of the ^{14}C radiolabel after accumulation of asymmetrically radiolabeled sucrose into these tissues (Hatch et al 1963; Hawker and Hatch, 1965). Based on these experiments it was concluded that sucrose is hydrolysed prior to uptake from the apoplast and re-synthesized in the cytoplasm of sugarcane storage tissue. And, even though more recent studies have shown that sucrose hydrolysis prior to uptake is not obligatory (Lingle 1989; Thom and Maretzki, 1992), sucrose hydrolysis in the apoplastic space is still considered to be significant in sugarcane. Initial attempts to establish whether or not apoplastic sucrose hydrolysis occurred at significant levels in sugar beet were not successful. Russian workers (Angelova et al., 1974; Dubinina, 1970; Engel and Kholodova, 1970) who did not employ asymmetrically radiolabeled sucrose to study uptake, reported conflicting results on the presence or absence of invertase activity within the apoplast that could result in sucrose hydrolysis.

In a set of experiments similar to those used on sugarcane, Giaquinta fed radiolabeled hexoses, asymmetrically radiolabeled ^{14}C (fructosyl)-sucrose and uniformly radiolabeled sucrose to slices of sugar beet. The sucrose synthesized from radiolabeled glucose and fructose had a glucose/fructose (G/F) ratio of 1.2 and 0.7 respectively, indicating substantial randomisation due to isomerisation between the hexoses. When uniformly radiolabeled sucrose was offered, the

predicted G/F value of 1 was obtained. When ^{14}C (fructosyl)-sucrose was offered a G/F ratio of 0.007 was obtained, indicating that very little randomisation had occurred. These results demonstrated that sucrose hydrolysis does not occur prior or subsequent to accumulation in the sugar beet storage root under short-term conditions. Sucrose accumulation without free space hydrolysis has also been shown to occur in wheat grain (Jenner, 1974), bean pod tissue (Sacher, 1966), tomato roots (Chin and Weston, 1975) and immature pea (Dick and ap Rees, 1975). Thus, sucrose hydrolysis prior to uptake or accumulation is not a universal feature of sink tissue metabolism.

2.5.2 Sugar accumulation in fleshy fruits

Although grouped as one in this review, there is no clear set of principles governing sugar accumulation in fruits. Aside from sucrose and hexose sugars, sorbitol storage is also known to occur in many fruits. The storage of sorbitol will not be discussed in this review. In most cases, with the exception of fruits such as oranges, grapefruit and grape, sucrose content increases with maturation. In apricot, peach, apple and pear sucrose concentration increases linearly up to normal harvest, whereas in plum (Hulme, 1971) and pineapple (Dull, 1971) a rapid increase occurs only during the last ripening phase. To discuss the details of sucrose metabolism in each fruit is beyond the scope of this review. Nevertheless, an attempt will be made to give an overview of sucrose metabolism in certain fruits.

In muskmelon, raffinose saccharides are the primary form in which photosynthate is translocated to the fruit. Upon entry into the fruit the raffinose saccharides are metabolized and provide substrate for SPS. In immature fruit sucrose synthesized by SPS is rapidly degraded by SAI to provide precursors for growth and respiration. As the fruit matures SAI activity drops to negligible levels, while SPS activity increases to as much as $32 \mu\text{mol h}^{-1} (\text{g fresh weight})^{-1}$ (Hubbard et al., 1989). It is proposed that the increase in sucrose synthesis by SPS and the

decrease in sucrose degradation by SAI results in the observed sucrose accumulation. Hubbard and co-workers (1990) demonstrated that the sucrose concentration did not increase unless SPS activity exceeded the sum of activities of the sucrose degrading enzymes. A similar situation appears to exist in banana and in mango. Both mango (Morga et al., 1979) and banana (Beaudry et al., 1989; Hubbard et al., 1990; Tucker and Grierson, 1987) sweeten in post-harvest conditions as a result of starch degradation and subsequent conversion to soluble sugars (Morga et al., 1979). As in muskmelon sucrose accumulation in mango and banana only occurred when SPS activity was in excess of SAI activity (Hubbard et al., 1991).

In strawberry SPS activity also increased while SAI activity decreased with fruit development (Poovaiah and Veluthambi, 1985; Hubbard et al., 1991). However, it has not been demonstrated that SPS activity has to be in excess of SAI activity before sucrose accumulation can occur. Strawberries have a substantially higher concentration of hexose sugars than sucrose throughout development, yet sucrose is the primary form of translocated carbohydrate throughout the plant (Fomey and Breen, 1985). Fomey and Breen (1986) concluded from studies of [^{14}C]-sugar uptake by strawberry that sucrose is hydrolysed and re-synthesized on uptake. Hubbard and co-workers (1991) also presented work that is suggestive of sucrose turnover in strawberry. These findings parallel those made concerning the uptake of sugars in sugarcane. More intensive study into sugar uptake in strawberry is required to confirm that a rapid cycle of sucrose degradation and re-synthesis, as found in sugarcane, also occurs in strawberry.

Although SPS appears to play a significant role in sucrose accumulation in many fruits, it is not universally important. Low SPS activities in the developing peach fruit suggest that SPS may not play a pivotal role in sucrose accumulation in peach. In work presented by Hubbard and co-workers (1991) the observed increase in SPS activity during fruit development was found to be much less than the observed increase in sucrose synthase activity (SuSy). The increase in

sucrose concentration in the peach fruit was associated with a 5-fold increase in SuSy activity and a less than 1-fold increase in SPS activity, while SAI activity levels were undetectable throughout the sampling period. Based on the increase in SuSy activity, and on the higher affinity for fructose than sucrose by peach SuSy (Moriguchi and Yamaki, 1988) it is proposed that in peach SuSy replaces SPS as the primary sucrose synthesizer (Moriguchi et al, 1990).

Sugar accumulation in grape differs notably from the previously discussed fruits in that glucose and fructose are the primary storage sugars and sucrose is only detected in trace amounts. Sucrose is translocated from the leaves to the berries where it is cleaved to glucose and fructose. Glucose and fructose accumulation, commences at the inception of berry ripening and continues throughout ripening (Davies and Robinson, 1996). Both sugars are accumulated in roughly equal amounts (Kliwer, 1965). As is to be expected acid invertase, the primary sucrose degrader, and not sucrose phosphate synthase, plays an important role in determining the relative levels of sucrose and hexose accumulation.

Hawker (1969) reported that acid invertase activity in Sultana grape berries is substantially higher than SPS activity during all stages of development. From work on Shiraz berries invertase activity was found to increase during the initial phase of berry growth, reaching a maximum at approximately 8 weeks after flowering and then remaining constant during ripening. SPS and SuSy activities were also found to increase during berry development but activities remained low in comparison to invertase activity (Hawker, 1969; Takayanagi and Yokotsuka, 1997). From studies on purified invertase by Nakanishi and co-workers (1991) it appears that the predominant invertase activity in grape berries is located in the vacuole. It is proposed that SPS facilitates the transport of sucrose across the tonoplast and into the vacuole where it is cleaved by soluble acid invertase (Takayanagi and Yokotsuka, 1997). In work similar to that carried out by Glasziou and Waldron (1964) on sugarcane, Davies and Robinson compared a low hexose sugar storing variety of grape with a high hexose sugar storing

variety. It was found that SAI activity was substantially higher in the high hexose sugar storing variety than in the low hexose sugar storing variety. Thus, as in sugarcane, SAI appears to play a pivotal role in determining the composition of stored sugars within the grape berry.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

Radiolabeling studies were carried out on field grown, mature (12 months old), non-flowering plants of the variety NCo 376 (Saccharum spp. hybrid cv. Nco376). Plants were radiolabeled in early autumn (April/May). All plants studied experienced similar environmental conditions for the duration of the study period.

3.1.2 Biochemicals

Radiolabeled Sodium bicarbonate, specific activity 55mCi mmol^{-1} , was supplied by ICN Radiochemicals Irvine, CA 92715. Soluene-350 tissue solubilizer, HIONIC FLUOR and Ultima Gold XR quench resistant scintillation cocktail were purchased from Packard. Dowex-Anion exchange resin (Dowex, AG 1-X8) and Dowex-Cation exchange resin (Dowex, AG 50W-X4) were obtained from Sigma while the Supelcosil LC-NH² carbohydrate column was purchased from Supelco Inc. The sucrose/D-glucose/D-fructose biochemical analysis kit was purchased from Boehringer Mannheim.

3.2 Methods

3.2.1 Radiolabeling protocol

One-hundred and twenty culms were radiolabeled. Culms of similar girth and total internode and leaf number were chosen. A sugarcane plant, or stool complex, consists of numerous culms connected by underground lateral branches (tillers). The stool complex can be widely scattered and is not always well delineated. Hartt and co-workers (1963) has shown that inter-

culm photosynthate transport within the stool complex occurs at significant levels. Thus, to minimize the possibility of a culm being 'double' radiolabeled by radiolabeled photosynthate produced in the fed leaf and by radiolabeled photosynthate imported from another radiolabeled culm, no two culms within 60 cm (base-base) of each other were labeled. All labeling was carried out between the hours of 08:00 to 12:00 under full sunlight.

Only leaf 6, as measured from the first unfurled leaf, was used for labeling in all experiments (figure 3.1). ^{14}C Sodium bicarbonate with a specific activity of 55 mCi mmol⁻¹ was used for all labeling. A leaf area of approximately 88 cm² was enclosed in a gas tight glass cuvette. The volume of the cuvette was 0.596 litres. A glass chamber containing 0.1ml labeled NaHCO₃ under vacuum, was positioned adjacent to the cuvette. To produce labeled carbon dioxide an equivalent amount of 0.1 mHCL was added to the sodium bicarbonate in the glass chamber. A total of 6.8 μmol of radiolabeled CO₂ was produced. The glass chamber and glass cuvette were then linked via plastic tubing. All the radiolabeled CO₂ was pushed into the glass cuvette by filling the chamber and tubing with acidic water. The glass cuvette contained 0.37 mCi or 13977 kBq of radiolabel. The enclosed leaf was allowed to photosynthesize in full sunlight for a period of 10 min, after which the cuvette was unsealed and the leaf removed. Time trial studies showed that at least 92% of the radiolabeled CO₂ was taken up within the first 10 min.

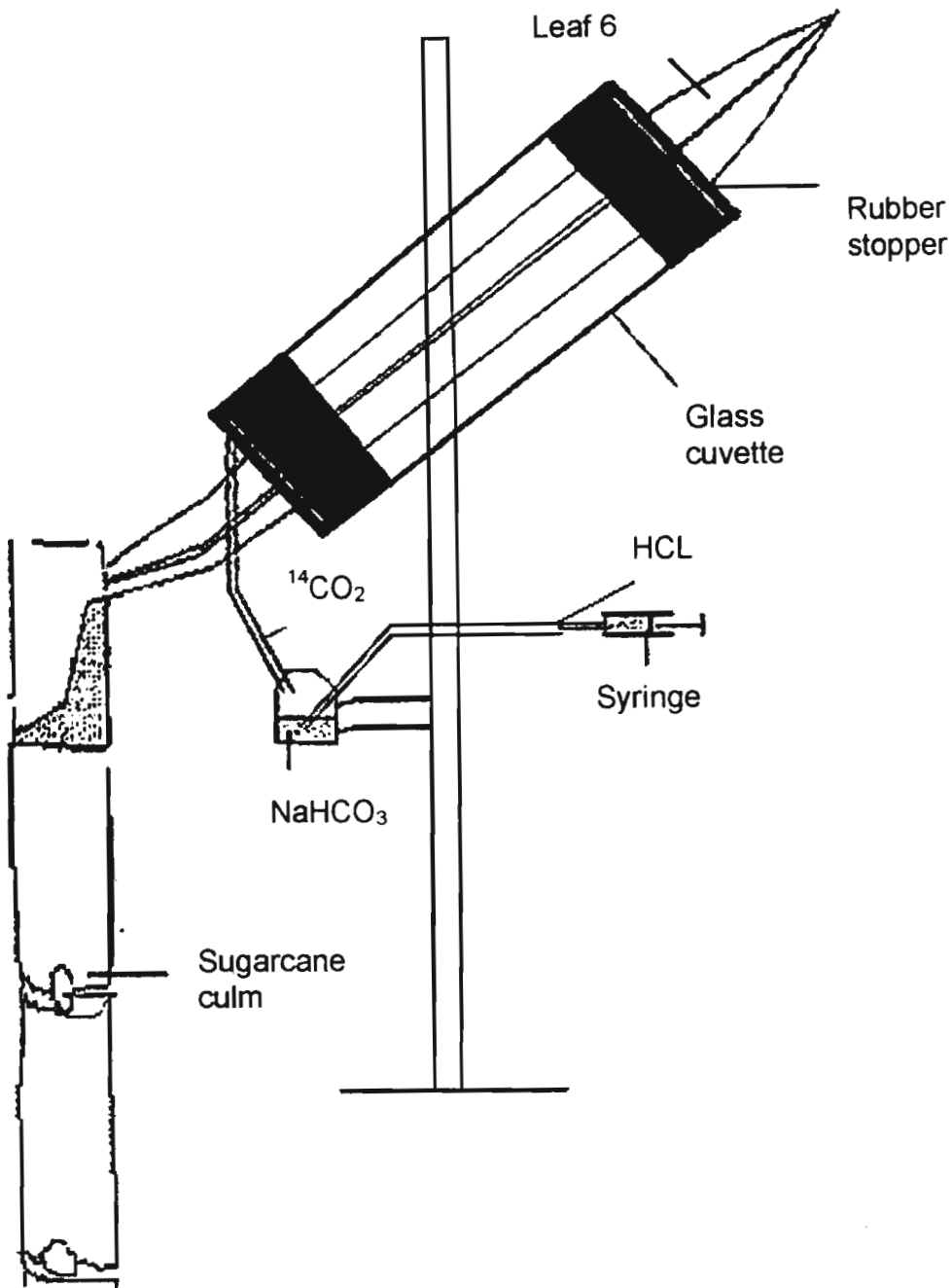


Figure 3.1 Illustration of the radiolabeling protocol described in section 3.2.1

3.2.2 Determination of fixation rate of leaf 6

Leaf 6 was radiolabeled as in section 3.2.1 for a radiolabeling period of 40 minutes. During the first 16 minutes, 1mL of air was removed from the sealed cuvette with the aid of a syringe at every 1 minute interval. Prior to the sample

removal, 1ml of air was injected into the cuvette to compensate for pressure changes. The carbon dioxide in each 1mL sample was removed by passing each sample through 5mL of a 12% KOH solution. One milliliter of the 12% KOH solution was removed and combined with 4mL scintillant in a 5mL scintillation vial. The amount of ^{14}C in each sample was determined using scintillation spectroscopy. Data obtained was used to determine the amount of radiolabeled carbon dioxide in the cuvette at each time interval. The rate of label loss from the glass cuvette during the 40 minute period was used to estimate a carbon fixation rate for leaf 6.

3.2.3 Determination of radiolabel in the fed part of leaf 6

The fed region of leaf 6 was solubilized using SOLUENE-350 (Packard), for a period of 24h. One millilitre of the solubilized material was removed and combined with 5 mL scintillant, HIONIC FLUOR. Radioactivity was detected and quantified by liquid scintillation counting using a Packard Tricarb 1900 TR counter.

3.2.4 Harvesting

The destructive nature of culm tissue analysis prevents any long-term study on the same culm tissue. To obtain a general picture of the changes occurring over the long-term, a population of radiolabeled culms, which could be 'sampled' at different time points, was created. A total of 120 culms were radiolabeled. Culms were harvested at four time points after labeling, namely, 6 hours, 24 hours, 7 days and 6 weeks. A total of thirty culms were harvested at each time point.

From each culm, three internodes were excised, namely internode 6 (the internode subtending the radiolabeled leaf 6) internode 3 and internode 9 (figure 3.2). Internodes were numbered according to the number of the leaf they subtended. Each internode was divided into a top and bottom half and each half

was further divided into core tissue, outer ring tissue and rind tissue. The rind tissue was discarded while sample tissue was removed from the core and outer ring tissue of both internode halves. These samples were then frozen in liquid nitrogen and homogenized using a mortar and pestle. The homogenized material was stored at -20°C for later use.

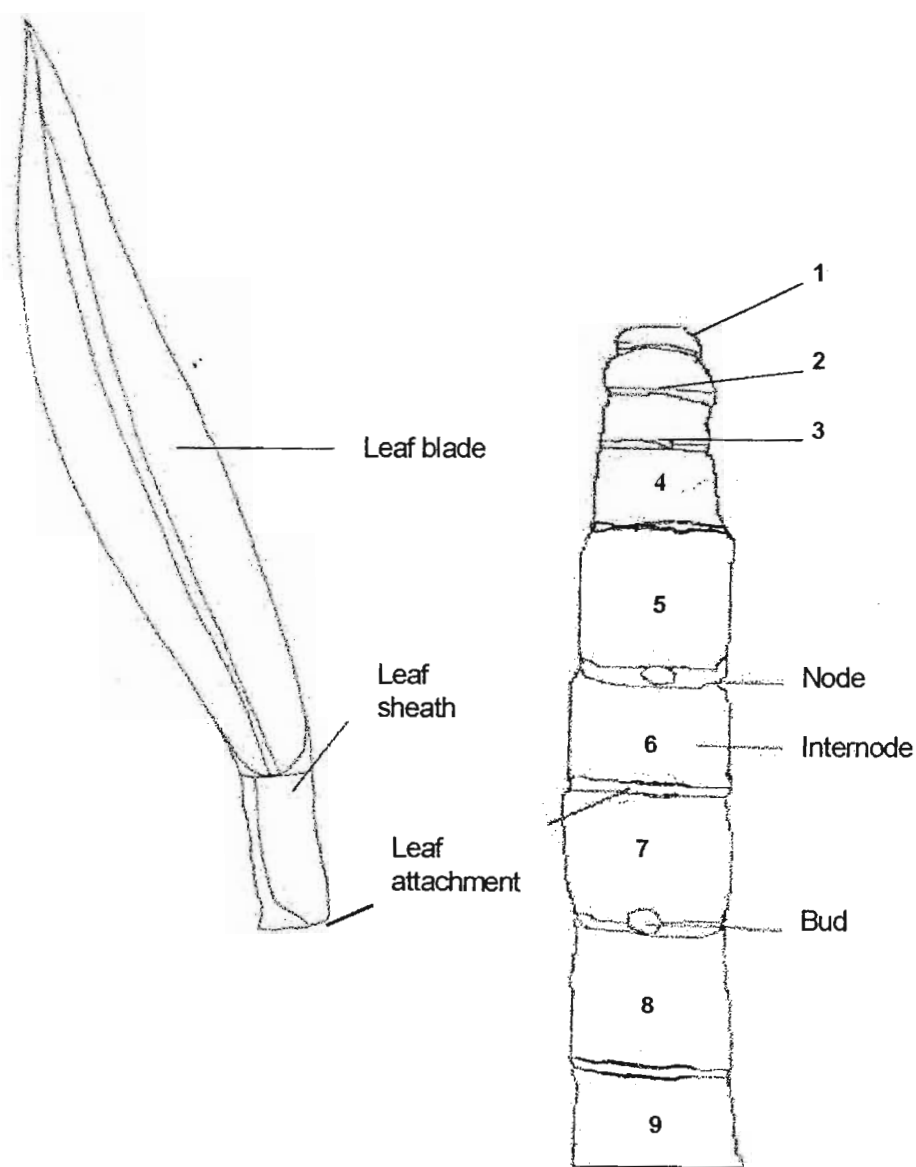


Figure 3.2 Sugarcane culm showing internodes 1-9. The internode subtending the first unfolded leaf is labeled as internode 1. Leaf 6 is attached to the base of internode 6. Tissue samples were removed from internodes 3, 6 and 9.

3.2.5 Determination of total radiolabel per internode

A gram of homogenized tissue was removed from storage (-20°C) and immersed in 5mL tissue solubilizer, SOLUENE-350 (Packard), for a period of 24h. One millilitre of the solubilized material was removed and combined with 5mL scintillant, HIONIC FLUOR. Radioactivity was detected and quantified by liquid scintillation counting using a Packard Tricarb 1900 TR counter.

Liquid scintillation counting is an analytical technique which is defined by the incorporation of the radiolabeled analyte (the solubilized material) into uniform distribution with a liquid chemical medium (the scintillant) capable of converting the kinetic energy of nuclear emissions into emitted photons. Based on these photon emissions the Packard Tricarb 1900 TR counter determines the number of nuclear disintegrations per minute (DPM) in each sample. The counter uses an external standard to correct for quenching in each sample.

The number of DPM per gram fresh weight was calculated for each sample. This value was then multiplied up to the total weight of each internode (rind tissue excluded) to obtain an estimate of the total labeled carbon in each internode.

3.2.6 Determination of radiolabeled internodal tissue components.

3.2.6.1 Tissue extraction

One gram of homogenized material from each internode was removed from cold storage (-20°C) and heated to 75°C in 40 mL 80% (v/v) Etoh for 40 minutes. After centrifugation at 6000 g for 10 min, the supernatant was removed and both the pellet and supernatant fractions retained. The pellet fraction was washed with two 5mL aliquots of 80% (v/v) Etoh. The washings and the supernatant were combined in a 100 mL glass beaker.

The washed pellet fraction was suspended in scintillant (HIONIC FLUOR) and the amount of radioactivity (^{14}C) determined by liquid scintillation spectroscopy.

3.2.6.2 Ion exchange separation of the radiolabeled water-soluble component

The combined washings and supernatant fraction was dried down at 50°C overnight. Each dried sample was re-suspended in 5 mL 80%(v/v) EtOH and loaded onto a tandem ion exchange column. The column consisted of a cation exchange column (Dowex, AG 50W-X4) over an anion exchange column (Dowex, AG 1-X8) loaded with H⁺ and COOH⁻ ions respectively. Each column was packed to a bed length of 4cm and washed to neutral pH with 80%(v/v) EtOH. The neutral sugar fraction was eluted from the tandem column with 80%(v/v) EtOH and 40 mL eluate collected. The columns were then separated and the organic acids and amino acids eluted from their respective columns. Organic acids were eluted from the anion exchange column with 10mL 4M formic acid. A 1mL aliquot of the eluate was transferred to a 5mL scintillation vial. 4mL scintillant (Ultima Gold) was added and the radioactivity determined by scintillation spectroscopy.

Amino acids were eluted from the cation exchange column with 10mL 4M ammonia.

3.2.6.3 Fractionation of the radiolabeled neutral water-soluble component

The neutral fraction was dried down at 50°C overnight and re-dissolved in 500µL 70 % (v/v) EtOH. Sugars within the neutral fraction concentrate (20µL injection volume) were resolved by H.P.L.C. on a Supelcosil LC-NH² carbohydrate column (Supelco inc., Bellefonte, PA USA) by isocratic elution with a mobile phase made up of 80% acetonitrile and 20 % H.P.L.C. grade H₂O at a flow rate of 1.5 mL min⁻¹. The mode of H.P.L.C. separation is described as liquid-solid adsorption. It involves the interaction between the adsorbent (usually silica) and the solute and solvent molecules in solution. The process can be considered as a competition between the solute and the solvent molecules for adsorption sites on the solid surface. Since different molecules are absorbed and displaced

differently, separation is effected (Harbourne, 1984). In the case of carbohydrates (sucrose, glucose and fructose), separation is based on the total number of hydroxyl groups, their distribution on the molecule and their linkage positions. Figure 3.3 shows a typical elution chromatograph for a sucrose, glucose and fructose sugar mixture. The retention time for fructose was approximately 6 minutes, for glucose 7 minutes and for sucrose 10 minutes.

The amount of ^{14}C in sucrose, glucose and fructose was quantified with a Packard Flo-one Beta in-line isotope detector. Ultima Flo scintillant was used at a ratio of 2:1. Extraction efficiencies for sucrose, glucose and fructose were $92\pm 7\%$.

3.2.7 Determination of radiolabeled components in the leaf sheath

The process in section 3.2.6 was repeated, using leaf sheath tissue to determine which tissue components of the leaf sheath were radiolabeled.

3.2.8 Determination of sucrose, glucose and fructose content

Sucrose, glucose and fructose content was assayed enzymatically using the Boehringer Mannheim sugar food analysis kit according to the manufactures instructions. The principle of the analysis was described by Bergmeyer and Bernt (1974)

3.2.9 Determination of insoluble matter content

A gram of internodal tissue was powdered in liquid nitrogen. Powdered samples were solubilized in purified H_2O overnight. Thereafter, the insoluble matter was thoroughly washed under partial vacuum with additional volumes of H_2O to remove residual water-soluble material. The washed insoluble matter was then oven-dried at 80°C for 12 h and weighed.

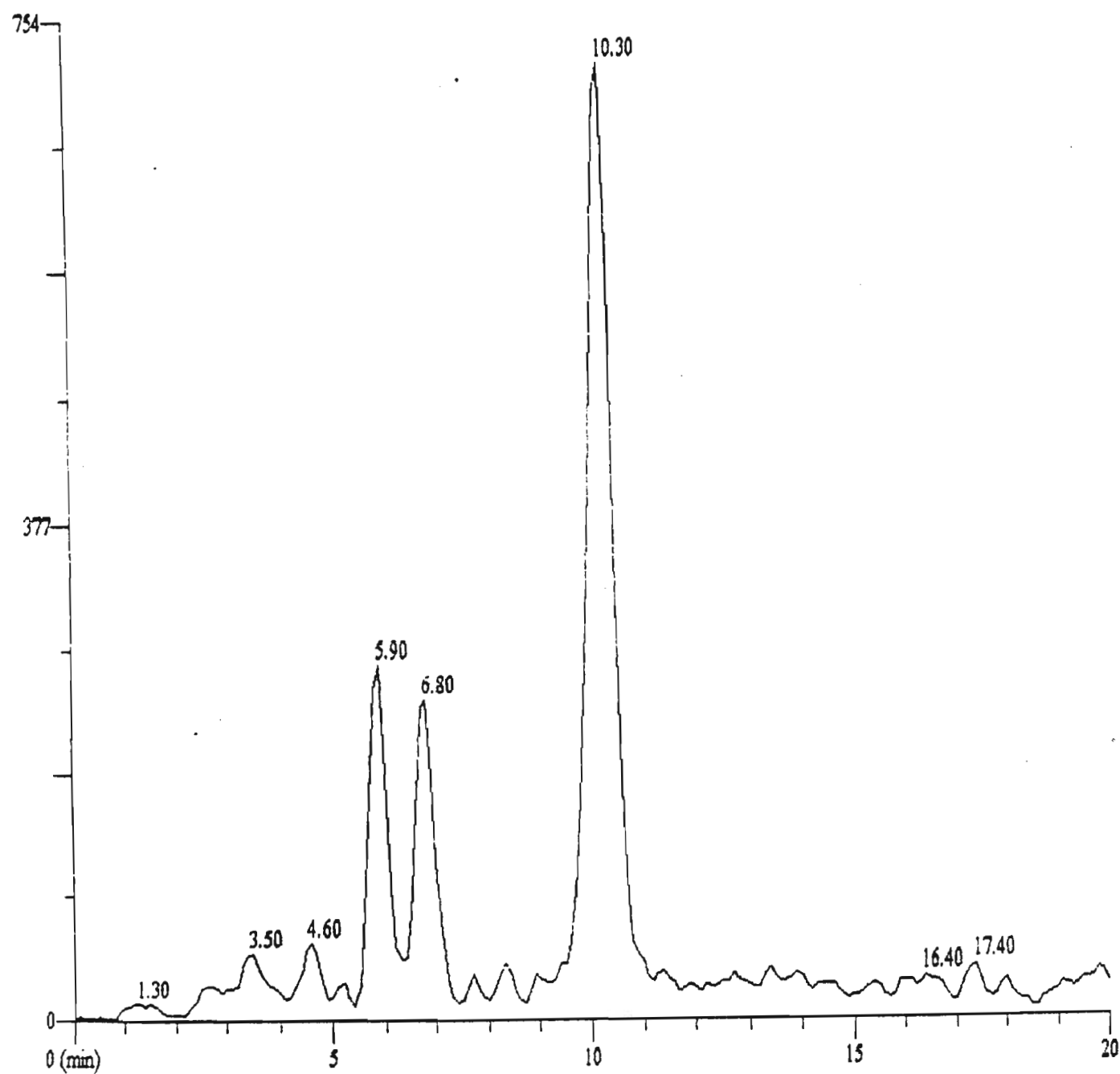


Figure 3.3 A typical radiograph showing peaks for glucose fructose and sucrose. Glucose is eluted first from the H.P.L.C. column at approximately 6 minutes (5.90) followed by fructose at approximately 7 minutes (6.80) and finally sucrose at approximately 10 minutes (10.30). Total amounts of radiolabel were calculated from the area enclosed by the positive and negative arms of each peak.

CHAPTER 4

RESULTS

4.1 Developmental status of internodes 3, 6 and 9

Although there are presently no universally accepted definitions for mature and immature sugarcane internodal tissues, it appears from a review of the available literature (Chapter 2) that a common naming protocol has been adopted. In general the term mature has been used to describe internodal tissue with a very high sucrose content ($\sim 160 \text{ mg suc g}^{-1} \text{ fw}$) and a low hexose sugar content ($\sim 1.5 \text{ mg glc\&fruc g}^{-1} \text{ fw}$), while the term immature has been used to describe internodal tissue with a very high hexose sugar content ($\sim 45 \text{ mg glc\&fruc g}^{-1} \text{ fw}$) and a low sucrose content ($\sim 10 \text{ mg suc g}^{-1} \text{ fw}$). In order to continue using this informal naming protocol, and thereby allow comparisons to be made between the present and previous studies, the sucrose and hexose sugar, together with the insoluble matter content of internodes 3, 6 and 9 was determined.

The work revealed that internode 9 has the highest sucrose and the lowest glucose and fructose content and insoluble matter content of all three internodes. A gram of fresh internodal tissue taken from internode 9 at 24 hours after radiolabeling was found to contain 85.5 mg insoluble matter, 157.3 mg sucrose and 4.3 mg hexose sugars (Table 4.1). In contrast, internode 3 has the lowest sucrose content and the highest glucose and fructose content (Table 4.1). A gram of fresh internodal tissue taken from internode 3 at 24 hours after radiolabeling was found to contain 111.2 mg insoluble matter, 14.5 mg sucrose and 42.88 mg hexose sugars (Table 4.1). The sucrose and hexose sugar content of internode 3 and internode 9 are respectively similar to the sugar contents of tissues described as immature and mature tissues in previous studies (Chapter 2). Internode 6 was also analysed as it was the internode subtending the fed leaf. As is to be expected internode 6 occupies an intermediary position between internode 3 and 6 with its sucrose content higher

than its hexose sugar content, but with the hexose sugar content still being notable at 15 mg glc&fruc fw g⁻¹.

Table 4.1 Insoluble matter, sucrose and hexose sugars content * and fresh weight of sugarcane internodal tissue at 24 hours and 6 weeks after radiolabeling with ¹⁴CO₂. * (sugar and insoluble matter content was assayed directly)

24hrs				
Internode no.	mg insol fw g ⁻¹	mg suc fw g ⁻¹	mg glc&fruc fw g ⁻¹	Internode fw (g)
3	111.25 ± 12.0	14.5 ± 1.3	42.88 ± 7.9	4.6 ± 0.8
6	107.3 ± 10.3	50.3 ± 3.5	15.2 ± 2.9	17 ± 5.2
9	85.56 ± 6.4	157.3 ± 7.8	4.3 ± 0.4	28.2 ± 5.3
6 weeks				
Internode no.	mg insol fw g ⁻¹	mg suc fw g ⁻¹	mg glc&fruc fw g ⁻¹	Internode fw (g)
3	100 ± 14.4	47.8 ± 2.9	20.01 ± 5.1	20 ± 4.8
6	92.2 ± 8.3	158 ± 7.9	3.2 ± 0.9	22.4 ± 3.3
9	90.51 ± 7.3	163.3 ± 5.3	0.8 ± 0.03	33.8 ± 6.2

Each value is the mean ± SD of thirty samples

The most notable change in the physiological status of internode 9 over the 6 week period was a decrease in hexose sugar content from 4.3 to 0.8 mg gl&fruc fw g⁻¹. Increases in both the insoluble matter content and sucrose content of internode 9 are noted, but these are relatively small (Table 4.1).

As in internode 9 insoluble matter content in internodes 3 and 6 also decreases by a small margin during the 6 week. However, unlike in internode 9, a 3 fold increase in the sucrose content of both internode 3 and 6 is observed from the 24 hour time point to the 6 week time point. In internode 6 this increase in sucrose content is accompanied by a 3 fold decrease in hexose sugar content (Table 4.1). A notable decrease in hexose sugar content is also occurs in internode 3 over the 6 week period, but the extent of the decrease is less with only a two fold decrease being observed (Table 4.1).

At the 6 week time point it was found that the actual internode number of the studied internodes had changed as a result of the growth of new internodes. The actual numbers of internode 3,6 and 9 at 6 weeks after radiolabelling were internodes 6,9 and 12 respectively. However, to avoid confusion internode numbers have been maintained as defined at the start of the experiment.

4.2 Leaf as carbon source

4.2.1 $^{14}\text{CO}_2$ uptake by leaf 6

Work on $^{14}\text{CO}_2$ uptake by leaf 6 was carried out to determine an adequate feeding period. It was decided that during the feeding period the amount of $^{14}\text{CO}_2$ taken up by leaf 6 should not be less than 90% of the total $^{14}\text{CO}_2$ introduced into the sealed glass cuvette. In figure 4.1 it can be seen that the depletion of $^{14}\text{CO}_2$ from the glass cuvette is rapid within the first 6 minutes of exposure but slows down appreciably thereafter. Eighty-six percent of the $^{14}\text{CO}_2$ was depleted from the cuvette within the first 6 minutes of exposure. The rate of radiolabel loss during the first minute after exposure was used to estimate a fixation rate of $48 \text{ mg CO}_2 \text{ dm}^{-2} \text{ s}^{-1}$ for leaf 6. Within 10 minutes 90% of the original $^{14}\text{CO}_2$ had been depleted. Thus, a feeding period of 10 minutes was sufficient. After 16 minutes the remaining 10% of the original $^{14}\text{CO}_2$ had been depleted and no radioactivity could be detected in the sealed cuvette.

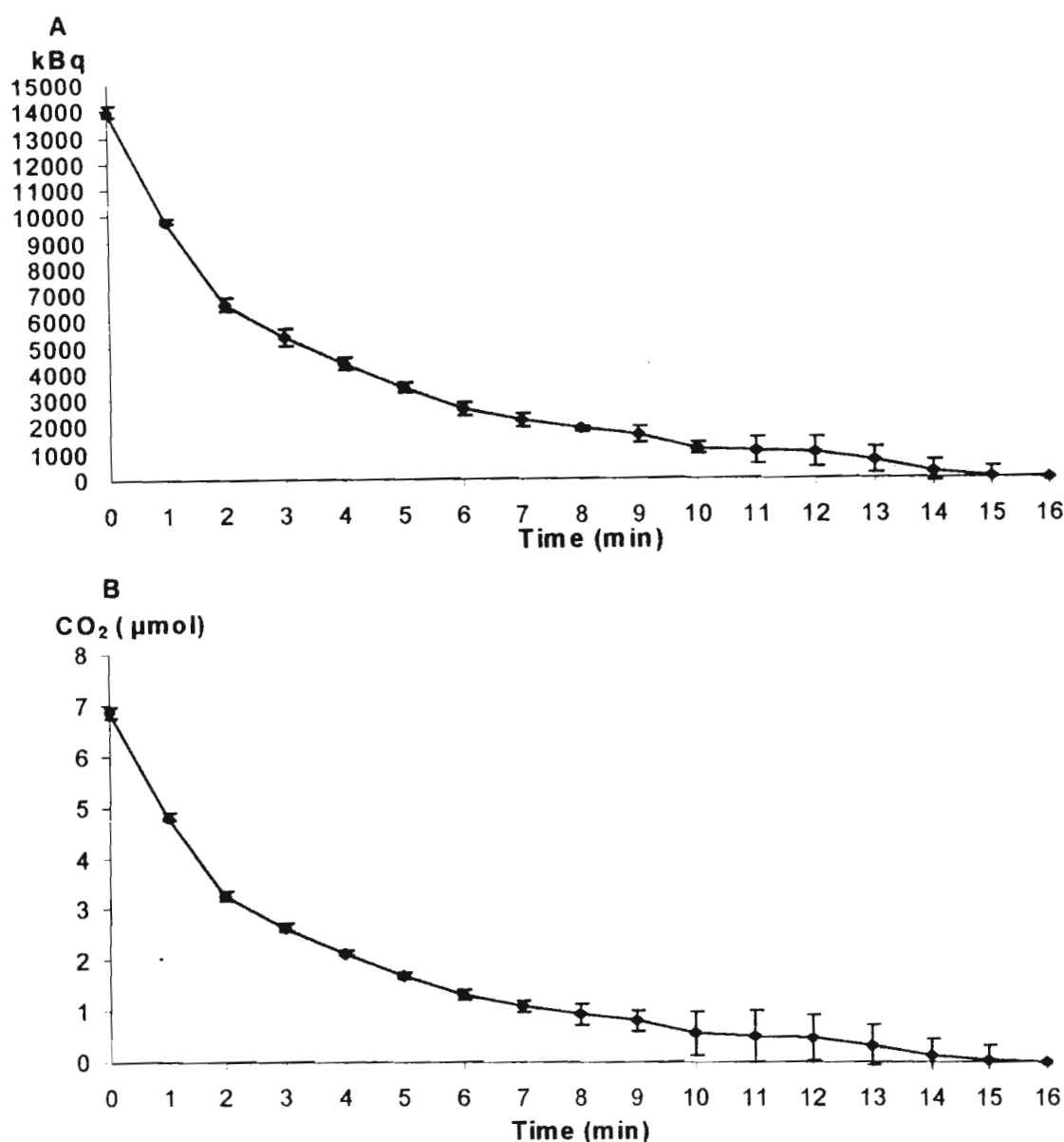


Figure 4.1 Depletion of radiolabel (A) and associated $^{14}\text{CO}_2$ (B) in a sealed glass cuvette containing a photosynthesizing sugarcane leaf. The mid-region of leaf 6 of a field growing sugarcane plant was enclosed in a glass cuvette with the aid of two split rubber bungs. $6.8 \mu\text{mol } ^{14}\text{CO}_2$ (total 13700 KBq) was injected into the cuvette. At 2 minute intervals 1 ml of air was removed from the atmosphere within the cuvette and passed through 4 ml of a 12% KOH solution. Prior to removing the air sample, 1 ml of air was injected into the cuvette to compensate for pressure changes. The amount of ^{14}C absorbed by the KOH solution was determined using liquid scintillation spectroscopy. This value was used to estimate the amount of $^{14}\text{CO}_2$ in the 1ml of extracted air. The amount of $^{14}\text{CO}_2$ in 1ml of air was then multiplied up to the total volume of the glass cuvette to estimate the total amount of $^{14}\text{CO}_2$ in the glass cuvette at each time interval. The experiment was carried out in triplicate. Each value is the mean \pm SD of three samples.

This implies that radiolabeled carbon dioxide release due to photorespiration or normal cellular respiration is negligible. This low level of photorespiration is to be expected as C_4 plants such as sugarcane, are known to have very low compensation points.

4.2.2 Dissemination of ^{14}C from leaf tissue after uptake

As the ultimate aim of feeding $^{14}CO_2$ to leaf 6 was to create a radiolabeled sucrose pool within the culm tissue, it had to be established that a sufficient amount of radiolabeled carbon was leaving the leaf in the form of radiolabeled sucrose.

Figure 4.2. shows the percentage radiolabel loss of from the fed part of leaf 6. The 30 and 60 minute lapse periods started directly after radiolabelling. The amount of radiolabel found in the fed part directly after radiolabeling, was used as the total to calculate each percentage. The radiolabel loss from leaf 6 is rapid within the first hour after radiolabeling with 85% of the original radiolabel leaving the fed part. Thereafter there is little change in the amount of radiolabel found in the fed part of the leaf 6. This indicates that 85% of the radiolabeled carbon leaves leaf 6 while 10 % is retained and utilized by the leaf. To establish in what form this radiolabeled carbon enters the culm tissue, sample tissue from the leaf sheath of each of the radiolabeled leaves removed at 30 and 60 minutes after radiolabeling, was analysed. Analysis revealed that on average 98% of the radiolabeled carbon in the leaf sheath sample tissue took the form of radiolabeled sucrose (Table 4.2). These results confirmed that a sufficient amount of radiolabeled carbon was leaving the fed leaf and entering the culm tissue in the form of radiolabeled sucrose.

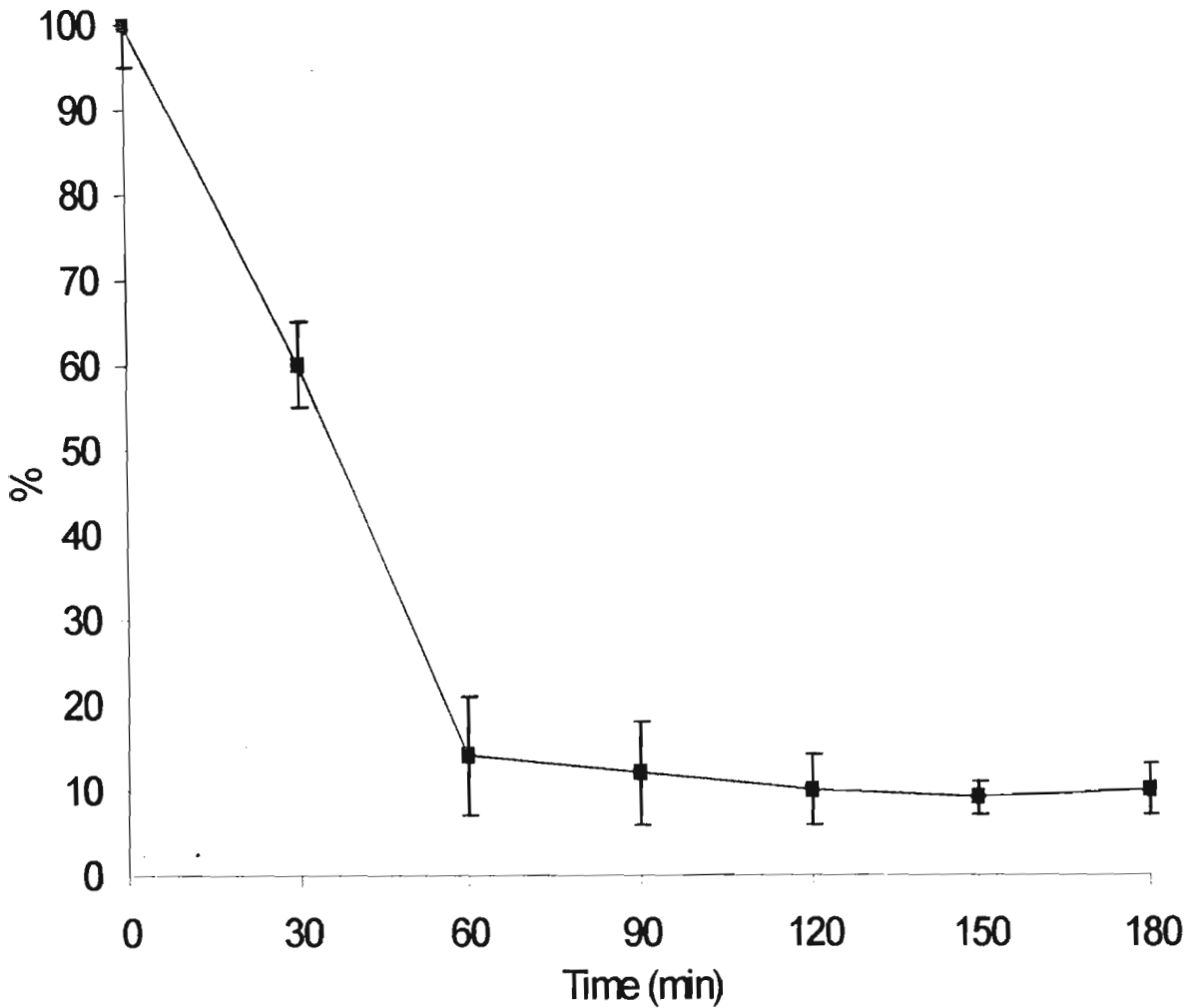


Figure 4.2 Translocation of radiolabel out of fed part of leaf 6. Twenty-one leaves were radiolabeled following the standard radiolabeling protocol. Directly after radiolabeling, the average amount of radiolabel in the fed part of three leaves was determined. This amount was used to represent the total radiolabel in the fed part of the leaf 6. At 30 minute intervals three leaves were detached and the average amount of radiolabel per fed region determined. These values were used to calculate the percentage radiolabel loss at each time point. Each value is the mean \pm SD of three samples.

Table 4.2 The percentage radiolabel in each of the cellular organic fractions analysed from the leaf sheath of radiolabeled leaves

% Radiolabel in each cellular constituent			
Sucrose	Hexose sugars	Insoluble matter	Ionic pool
98 ± 1.5	2 ± 0.5	-	-

Each value is the mean ± SD of 10 samples

4.3 ¹⁴C distribution in the culm

Having established that radiolabeled sucrose is being translocated into the culm, it was necessary to establish that radiolabel carbon was being delivered to all the studied internodes. Table 4.3 shows the average total amount of radiolabel (for all samples taken) in internodes 3, 6 and 9, individually and collectively, at 24 hours, 7 days and 6 weeks after radiolabeling. Radiolabel was found to be present in all three internodes at all three time points (Table 4.3). Thus, as carbon is translocated primarily as sucrose in the sugarcane culm, the presence of radiolabel in each internode confirmed that radiolabeled sucrose was being delivered to all three internodes.

Table 4.3 also shows that in internodes 6 and 9 the total amount of radiolabel in each internode remains fairly constant during the 6 week period. The total amount of radiolabel in internode 3 decreased by 20% between 24 hours and 7 days. During the subsequent 5 weeks a decrease of only 5% was observed. As a result of this radiolabel loss from internode 3, the total amount of radiolabel in all three internodes decreased by 3% during the 6 week period (Table 4.3).

To show the distribution of radiolabel between the three internodes more clearly, the amount of radiolabel in each internode is represented as percentage of the total radiolabel in all three internodes in figure 3.4. It is evident at the 24 hour time point that the distribution of radiolabel is not equal, with internode 9 containing the bulk of the radiolabel (77%), internode 6 containing much less (17%) and internode 3 containing the least (6%) (figure 4.3). This distribution pattern was found to change very little during the 6 week period (figure 4.3).

Table 4.3 Changes in the total radiolabel (¹⁴C) in internodes 3, 6 and 9 individually, and collectively during the 6 week period following radiolabel feeding

Internode no.	Total ¹⁴ C at each time point (kBq)		
	24hrs	7d	6 weeks
3	39 ± 4	34.3 ± 8	31.4 ± 6.3
6	81 ± 7.1	78 ± 5.5	77 ± 3.2
9	492 ± 15.2	488 ± 12.3	486 ± 16
3,6 and 9	612 ± 20	600.3± 13.7	594.4 ± 16.5

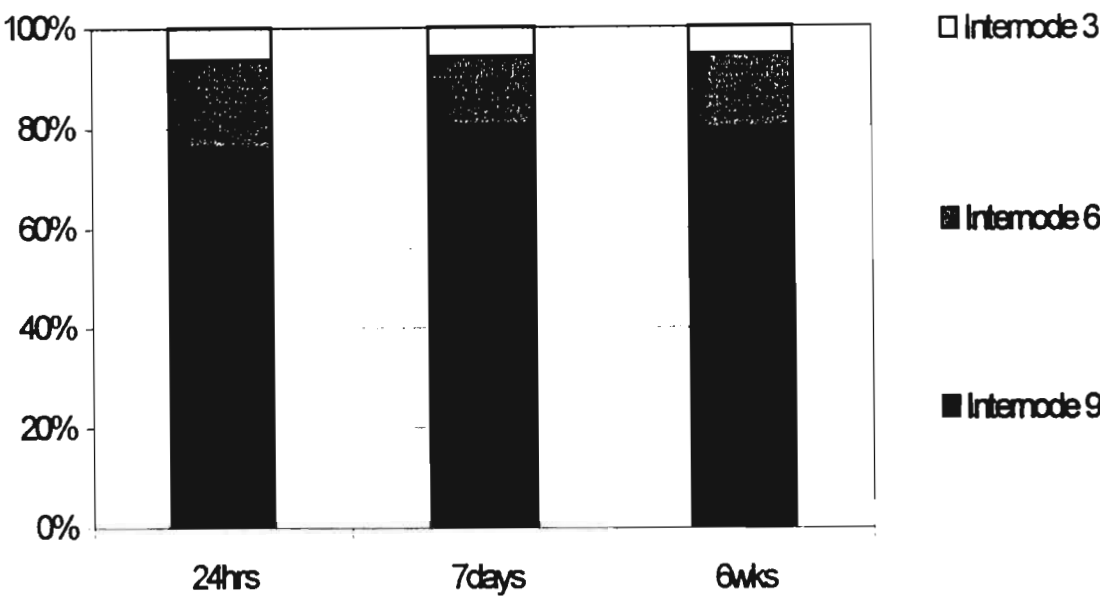


Figure 4.3 Radiolabeled carbon distribution between internodes 3,6 and 9. At each time point the average total amount of radiolabeled carbon in all three internodes was calculated. This average total was used to calculate the percentage distribution of radiolabel carbon in the three internodes.

4.4 Phloem unloading in sink tissue.

In section 4.2 it was established that radiolabeled carbon was fixed in leaf six and translocated to the culm mainly as radiolabeled sucrose. Based on previous work using tissue slices and cell suspension cultures (refer to chapter 2), this radiolabeled carbon could be taken up directly as sucrose, or it could be broken down in the storage cell apoplast to form hexoses prior to uptake. It is not evident from the available literature whether carbon uptake is divided equally between the two sugars or whether carbon uptake is predominantly in the form of one sugar. However, based on results obtained in the present study it would appear that *in vivo* carbon uptake is predominantly in the form of hexose sugar in immature tissue and elongating tissue, and is predominantly in the form of sucrose in mature tissue.

Table 4.4 shows the specific activities of the sucrose and hexose sugar pools in the three internodes at the 24 h time point. In the present study sugar specific activity is defined as the total radiolabeled carbon (in Bequerels (Bq) per μmol sugar. If radiolabeled carbon was entering the cell predominantly as sucrose then the specific activity of the hexose sugar pool would be lower than that of the sucrose pool, as the specific activity of the product, the hexose pool, can never exceed that of the source, the sucrose pool. However, this is found to be the case only in mature tissue(internode 9). In immature tissue (internode 3) and in internode 6 the hexose specific activity is higher than the sucrose specific activity. This suggests that radiolabeled hexoses and not radiolabeled sucrose is the main form in which radiolabeled carbon enters the cell in these two tissues.

Table 4.4 Specific activity of the sucrose and hexose sugar pools at the 24 h time point

24hrs		
Internode no.	Specific activity (Bq μmol^{-1})	
	Sucrose	Hexoses
3	6.6 \pm 1.2	6.9 \pm 3.1
6	8.1 \pm 2.2	11.04 \pm 3.3
9	7.5 \pm 2.1	6.9 \pm 1.8

-Each value is the mean \pm SD of thirty samples

4.5 Sucrose accumulation rate

The sucrose accumulation rate is defined as the change in sucrose content per unit time. Table 4.5 shows the sucrose accumulation rate for internodes 3, 6 and 9 for the six week period. During the six week period three new internodes developed from the apical shoot. Consequently, the internodes originally designated as internodes 3, 6 and 9 became internodes 6, 9 and 12 during the six week period. Thus, the given accumulation rates pertain to the period in which internode 3 develops into internode 6, internode 6 develops into internode 9 and internode 9 develops into internode 12.

The sucrose accumulation rate was found not to be constant throughout the developing culm. During early maturation, as internode 3 developed into internode 6, the accumulation rate was 0.054 mg suc g⁻¹ fw h⁻¹. As internode 6 developed into internode 9 the accumulation increased notably to 0.053 mg suc g⁻¹ fw h⁻¹. During late maturation, as internode 9 developed into internode 12, the sucrose accumulation rate decreased to 0.21 mg suc g⁻¹ fw h⁻¹ (Table 4.5).

Table 4.5 Sucrose accumulation rate in the developing culm

Developing internode	Sucrose accumulation rate (mg suc g ⁻¹ fw h ⁻¹)
Internode 3-6	0.054 ± 0.002
Internode 6-9	0.53 ± 0.032
Internode 9-12	0.21 ± 0.014

Each value is the mean ± SD of thirty samples

4.6 Carbon partitioning

4.6.1 Short-term partitioning

Having confirmed that radiolabeled sucrose was entering the sugarcane culm tissue (section 4.1), the partitioning of this radiolabeled carbon into cellular organic fractions of this tissue was characterized. Culm tissue from internodes 3, 6 and 9 was analysed to determine the amount of radiolabeled carbon in the sucrose pool, in the hexose sugar pool, in the insoluble matter pool and in the ionic pool. The ionic pool consists of anionic amino and cationic organic acids. In figure 4.4 the total amount of radiolabel in each of the cellular organic fractions is presented as a percentage of the total radiolabel in all of the cellular organic fractions. This presentation of the data enables direct comparison between internodes

As the sugarcane culm consists of tissues at different stages of development, it was necessary to characterise and compare carbon partitioning in a range of tissue types. These tissues included immature tissue, internode 3, intermediary tissue, internode 6 and mature tissue, internode 9. In all three tissues, the

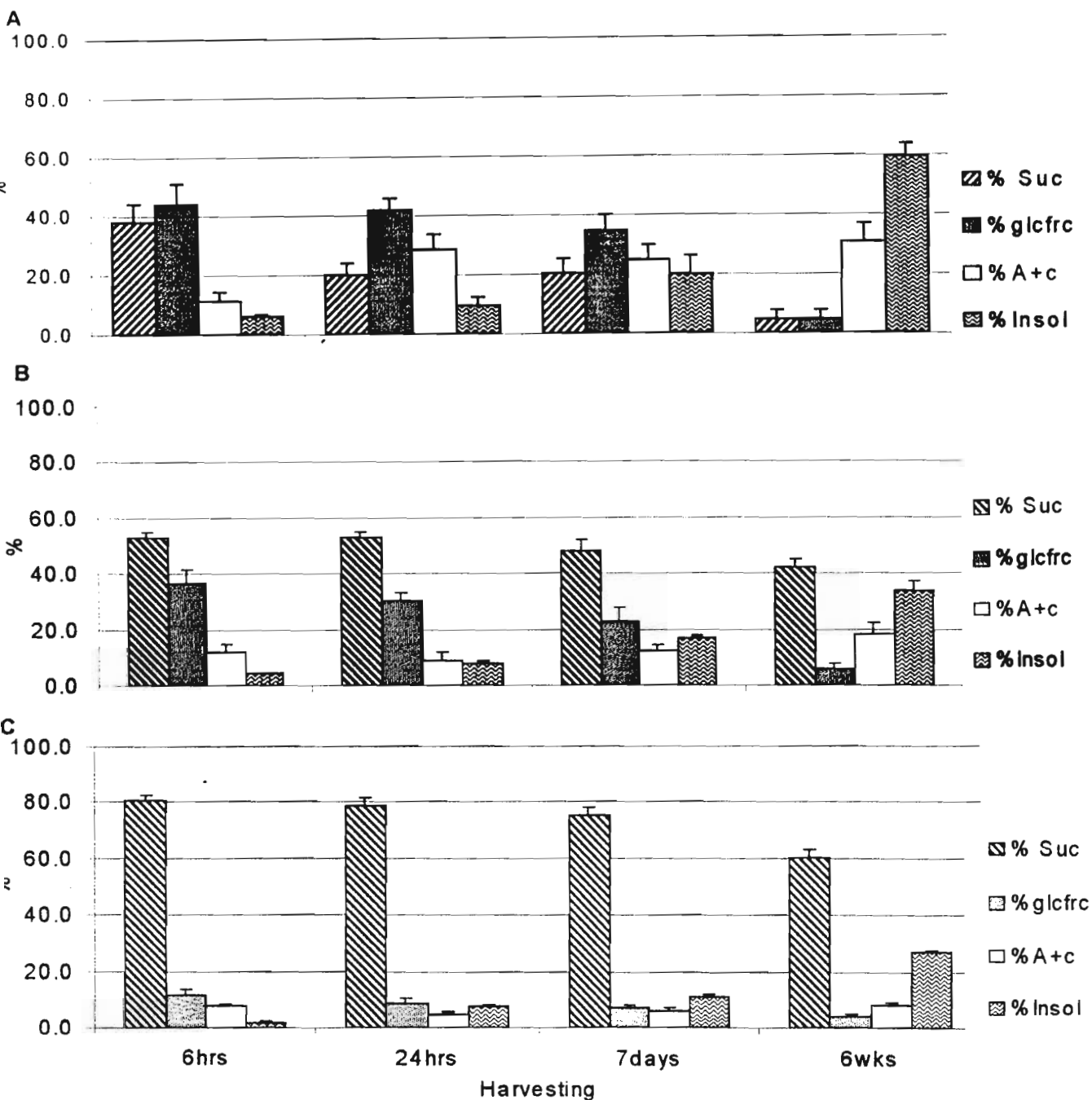


Figure 4.4 Percentage distribution of radiolabel in the cellular organic fractions of internode 3 (A), internode 6 (B) and internode 9 (C). A total of 120 culms were radiolabeled by feeding leaf 6 with $[^{14}\text{CO}_2]$. Thirty culms were harvested at 6 hours, 24hours, 7 days and 6 weeks after feeding. Internodes 3, 6 and 9 were excised from each culm and analysed to determine the percentage radiolabel in the sucrose (Suc) pool, the hexose pool (glcfr), the combined Anionic and Cationic pool (A+c) and the water insoluble matter pool (Insol). Each value is the average of thirty samples. Bars represent \pm SD

occurrence of radiolabeled sucrose turnover is confirmed by the presence of radiolabeled carbon in the non-sucrose cellular organic fractions of the tissue (figure 4.4). However, the partitioning of this carbon in each of these tissues is found to be notably different.

In internode 3 the percentage radiolabel in the sucrose pool constitutes only 39% of the total radiolabel at the 6h time point, the lowest for all three internodes (figure 4.4 (A)). At this time point radiolabel is found predominantly in the hexose sugars pool (44%) and the amounts of radiolabel in the cation and anion pool and the insoluble matter pool are the highest for all three internodes at this time point.

At the 24h time point the amount of radiolabel in the insoluble matter pool has increased almost two fold, while the amount of radiolabel in the cation & anion pool has doubled. Relative decreases in the percentage radiolabel in the hexose sugar and sucrose pools are observed. It appears that the extent of turnover of the radiolabeled sucrose pool is much greater in internode 3 than in either internode 6 or internode 9.

In internode 6 a significantly greater percentage of the total radiolabeled carbon was found in the sucrose pool (figure 4.4 (B)). At the 6h time point the amount of radiolabel in the sucrose sugar pool makes up 52% (as compared to 39% in internode 3) of the total radiolabel, while the radiolabeled hexose sugar only constitutes 38% (as compared to 44% in internode three) of the total radiolabel. The percentage radiolabel found in the insoluble matter pool also decreases slightly from 8% in internode 3 to 6% in internode 6. At the 24h time point radiolabel in the insoluble matter pool increases to 8% while radiolabel in the hexose sugar pool decreases to 30%. Radiolabel in the sucrose pool remains relatively constant from the 6h time point to the 24h time point.

For both the 6h and the 24h time point, radiolabeled carbon is found predominantly in the sucrose component in internode 9. At the 6h time point 80% of the radiolabel is in sucrose, 10.5% in hexose sugars and 7.5% in the anion and cation pool. Very little radiolabeled carbon was present in the insoluble matter component (<2%) (figure 4.4 (C)). At the 24h time point most of the radiolabel is still in sucrose but the percentage decreases to 77%. The hexose sugars component decreases to 8% and the anion and cation component decreases to 4% of the total radiolabel. The most significant difference between samples taken at 6h and 24h is in the percentage radiolabeled insoluble matter, with an eight-fold increase in the percentage insoluble matter being observed.

The differences in carbon partitioning between internodes 3, 6 and 9 suggest that internode maturation results in more of the incoming carbon being retained in the sucrose pool, and less carbon being allocated to respiration and other cellular organic fractions.

4.6.2 Long-term changes in carbon partitioning

Culm tissue from internodes 3, 6 and 9 was removed and analysed at a further two time points, 7 days and at 6 weeks after radiolabeling. The purpose of this analysis was to determine if there was long-term change in carbon partitioning in each of the internodes.

In internode 3 the rapid shift of radiolabeled carbon out of the sugar pool (hexoses and sucrose) observed between the 6 hour and 24 hour time points, continues until, at the 6 week time point, 60% of the radiolabel is in insoluble matter, 30% is in the cation and anion pool and only 10% remains in the hexose sugars and sucrose pools (figure 4.4 (A)). This indicates that both the hexose sugars pool and the sucrose pool are being turned over rapidly in internode 3.

As in internode 3, there is a notable increase in the percentage radiolabel in the insoluble matter pool during the 6 weeks (from 6% to 36%) in internode 6.

However, unlike internode 3, much more of the radiolabel is retained in the sucrose pool at the end of the 6 week period, with 42 % of the total radiolabel still found in the form of radiolabeled sucrose at the 6 week time point (figure 4.4 (B)). It appears that in internode 6 radiolabeled carbon transfer is mainly from the hexose sugar pool to the insoluble matter and ionic pools and not from both the hexose sugar and sucrose pool, as is the case in internode 3. The percentage radiolabel in the hexose sugar pool was found to decrease from 38% at 6h to 6% at the 6 week time point while the percentage radiolabel in the sucrose pool decreased from 54% to 40%. At the 6 week time point the percentage radiolabel in the insoluble matter and anion/cation (amino acid/organic acid and sugarphosphate) pools is considerably lower than that observed in internode 3. Thus, in internode 6 a large percentage of radiolabel is initially lost to the sucrose pool, but the radiolabeled sucrose pool that remained was not utilized greatly during the 6 week period. This initial high, followed by low utilisation of the radiolabeled sucrose could indicate that the radiolabeled sucrose is being maintained as storage sucrose and not being metabolised, or it could indicate that accessibility to the radiolabeled carbon is being reduced as a result of cold sucrose dilution. The latter is more likely as the sucrose pool size increased from 855 mg per internode to 3539.6 mg per internode in internode 6, a four fold increase (Table 4.6).

In internode 9, as in internode 3 and 6, there is a shift of radiolabel from the sucrose pool to the insoluble matter pool during the 6 week period (figure 4.4). However, in internode 9 the magnitude of the shift is much smaller. Consequently, in internode 9 the majority of the radiolabeled carbon is still in its original radiolabeled sucrose form at the 6 week time point. In comparison to internodes 6 and 3, the percentage radiolabeled carbon in the sucrose pool is 1.5 times that found in internode 6 and is almost 12 times that found in internode 3 at the 6 week time point. At the same time point, the percentage radiolabeled carbon in the insoluble matter pool is one-half of that found in internode 6 and just above one-third of that found in internode 3. The percentage radiolabeled

carbon in the anion and cation pool is also the lowest (10%) for all three internodes.

Table 4.6. Changes in specific activity and sugar pool size
from the 24h time point to the 6 week time point

24hrs				
Internode no.	Specific activity (Bq μmol^{-1})		Sugar pool size (mg per internode)	
	Sucrose	Hexoses	Sucrose	Hexoses
3	6.6 ± 1.2	6.9 ± 4.1	65.55 ± 11.5	197.24 ± 12.1
6	8.19 ± 2.2	11.04 ± 3.3	855.1 ± 28.73	258 ± 22.9
9	7.5 ± 4.1	6.9 ± 4.0	4444.8 ± 120	121.26 ± 12
6 week				
Internode no.	Specific activity (Bq μmol^{-1})		Sugar pool size (mg per internode)	
	Sucrose	Hexoses	Sucrose	Hexoses
3	0.01 ± 0.009	0.15 ± 0.002	940.4 ± 42.2	401.6 ± 19.6
6	1.18 ± 0.7	6.53 ± 3.6	3539.6 ± 74.7	208.32 ± 17.1
9	3.2 ± 0.96	28.47 ± 7	5519.54 ± 92.9	27.08 ± 10.3

-Each value is the mean \pm SD of thirty samples

From the long-term analysis of carbon partitioning it is clear that partitioning is not fixed in any of the studied internodes. The most significant changes in partitioning were observed in immature tissue, internode 3. In this tissue, the majority of the radiolabeled carbon, initially found in the sucrose pool, is transferred into the ionic and insoluble matter pools during the 6 week period. The ultimate result of this transfer is that after 6 weeks radiolabeled carbon in the

sucrose pool only constitutes 10% of the total radiolabeled carbon, while radiolabeled carbon in the ionic and insoluble matter pool constitutes 90% of the total radiolabeled carbon. In elongating tissue (internode 6) 42% of the total radiolabeled carbon is present in the sucrose pool at the 6 week time point, while in mature tissue, internode 9, as much as 60% of the total radiolabeled carbon remains in the sucrose pool. Thus, the extent of transfer of radiolabeled carbon from the sucrose pool to the ionic and insoluble matter pools decreases with internode maturation.

4.6.2.1 Changes in sugar specific activities

Further evidence indicating that there is long-term radiolabeled carbon loss from the sucrose pool is found in the changes in sugar specific activities from the 24 hour time point to the 6 week time point (Table 4.6). Due to the influx of 'cold' sucrose (non-radiolabeled sucrose) into each internode, one would expect the specific activity of the sucrose pool to decrease with time as the pool size became larger. This is shown to be true in Table 4.6, where specific activity decreases are observed in all internodes. However, as can be seen in Table 4.7, the specific activity values which this 'cold' sucrose dilution should have produced (the expected values), are greater than the observed specific activities. This difference between the expected specific activities and the observed specific activities can only be due to radiolabeled carbon being lost from the sugar pools. The expected values were estimated as follows:

Expected specific activity = Specific activity at 24 hrs X Dilution factor

Dilution factor = $1/(S_b / S_a)$

Where: S_a = sucrose pool size at 24 h

S_b = sucrose pool size at 6 weeks

In internode 3 the sucrose specific activity decreased from 6.6 Bq μmol^{-1} at 24h to 0.01 Bq μmol^{-1} at 6 weeks (Table 4.6). The average sucrose pool size increased from 65 mg at 24h to 940 mg at 6 weeks, a fifteen fold increase (Table 4.6). If dilution alone was responsible for the observed decrease in specific activity, the expected specific activity value for internode 3 is 0.44 Bq μmol^{-1} (Table 4.7). This value is much greater than the observed value of 0.01 Bq μmol^{-1} . Thus, the difference must be due to a loss of radiolabel from the sucrose pool.

In contrast to internode 3, the drop in sucrose specific activity from 8.19 Bq μmol^{-1} at 24h to 1.18 Bq μmol^{-1} in internode 6 (Table 4.6) must be mainly due to isotopic dilution as relatively little radiolabeled carbon is lost from the sucrose pool during the 6 weeks (figure 4.4). This is confirmed by the marginal difference between the observed and expected specific activities. The expected value of

Table 4.7 Comparison between observed specific activities and expected specific activities

6 weeks		
Internode no.	Sucrose specific activity Observed (Bq μmol^{-1})	Sucrose specific activity Expected (Bq μmol^{-1})
3	0.01 \pm 0.009	0.44 (0.066)
6	1.18 \pm 0.1	1.6 (0.26)
9	3.2 \pm 0.9	6.25 (0.83)

-Each value is the mean \pm SD of thirty samples
Values in parenthesis are dilution factors

1.6 Bq μmol^{-1} is very close to the observed value of 1.18 Bq μmol^{-1} (Table 4.7). Thus, only the 0.42 Bq μmol^{-1} difference between the expected specific activity

and the observed specific activity can be attributed to radiolabel loss from the sucrose pool.

In internode 9 sucrose specific activity decreased from 7.5 at 24h to 3.2 Bq μmol^{-1} at 6 weeks. If only isotopic dilution was responsible for the drop in specific activity, the expected specific activity value would be 6.25 Bq μmol^{-1} . Thus, both isotopic dilution and the loss of radiolabeled carbon must have contributed to the observed drop in specific activity.

CHAPTER 5

DISCUSSION

Prior to the present study, the majority of the detailed work on sink metabolism in sugarcane had been conducted using either tissue slices or cell suspension cultures. This raises questions as neither system has been conclusively proven to be representative of the whole plant system. By investigating sink metabolism in the whole sugarcane plant, the current study aimed to determine if conclusions drawn from the previous non-whole plant work hold true for the whole sugarcane plant system. The study concentrates on three aspects of sucrose/sink metabolism, namely the partitioning of carbon in sink tissues, the rate of sucrose accumulation in sink tissues and the occurrence of sucrose turnover in sink tissues. Although these sink related aspects can be regarded as the focal point of this discussion, results obtained from preliminary work on sucrose production in the source leaf, the distribution of sucrose within the culm and phloem unloading, also warrant discussion.

Leaf as a carbon source

The average photosynthetic rate for leaf 6 in full sunlight was estimated at $48 \text{ mg CO}_2 \text{ dm}^{-2} \text{ s}^{-1}$. This value is expected for a commercial sugarcane variety such as NCo 376 (*Saccharum* hybrid used in present study), as most commercial varieties average above $50 \text{ mg CO}_2 \text{ dm}^{-2} \text{ s}^{-1}$. The observed photosynthetic rate can be attributed to anatomical and physiological adaptations which allow the leaves of C_4 plants, such as sugarcane leaves, to fix carbon at very high rates (Hatch and Osmond, 1976; Karpilov, 1960; Kortschak et al., 1957, 1965).

The initial discovery of the C_4 photosynthetic pathway was in sugarcane (Hatch and Slack, 1966). From work on sugarcane and other C_4 species, especially maize, it appears that the enzymes and metabolites of the C_4 pathway and the enzymes and metabolites of the RPP pathway are separated spatially between

photosynthetic cells respectively (Hatch and Osmond, 1976; Furbank et al., 1985; Stitt and Heldt, 1985). Carbon dioxide is fixed in the KM cells by phosphoenolpyruvate (PEP) carboxylase, with the initial product, oxaloacetate, converted to the 4-carbon organic acids, malate and aspartate. Malate is then transferred to the BS cells where it is decarboxylated to produce CO₂ and reduced nicotinamide-adenine dinucleotide phosphate (NADPH). In the BS cells the CO₂ released from the malate is fixed by Rubisco of the RPP pathway to 3-PGA. The by-product of malate decarboxylation, pyruvate, is returned to the KM cells and converted to PEP in the KM cells (Hatch and Osmond, 1976; Furbank et al., 1985; Stitt and Heldt, 1985). Thus, the supplementary C₄ pathway and the compartmentation of photosynthetic enzymes serve to concentrate CO₂ in the vicinity of Rubisco.

The adaptive significance of the CO₂ concentrating mechanism is that it allows for very high photosynthetic rates by reducing the inhibitory effect of O₂ on photosynthesis, reducing photoassimilate losses due to photorespiration and by reducing water loss during high rates of carbon fixation. Photosynthetic rates for sugarcane have been shown to increase with increasing irradiance up to full sunlight (Moore and Maretzki, 1997). Maximum photosynthetic rates as high as 86.4 mg CO₂ dm⁻² s⁻¹ have been recorded in certain varieties of sugarcane (Irvine, 1967). However, the photosynthetic rate of most sugarcane varieties, like most C₄ plants, ranges between 34 and 49 mg CO₂ dm⁻² s⁻¹ (Zelitch, 1992; Grodzinski et al., 1998). In comparison, photosynthetic rates of C₃ plants only range between 12 and 30 mg CO₂ dm⁻² s⁻¹ (Zelitch, 1992; Grodzinski et al., 1998).

In most plants, including sugarcane, sucrose and starch are the principal end products of photosynthesis (Lunn et al., 1995; Moore and Maretzki, 1996). Carbon fixed during photosynthesis can be exported from the chloroplast and used for sucrose synthesis in the cytosol or it can be converted to starch and temporarily stored in the chloroplast. The synthesized sucrose can be utilized

for cellular respiration, temporarily stored in the vacuole or immediately exported to the rest of the plant. Carbohydrate stored either as starch or sucrose is usually utilized at night to maintain sucrose export to the rest of the plant. The co-ordination of primary partitioning with sucrose utilisation (sucrose demand) has been found to be centred on the relationship between triose-phosphate export from the chloroplast and Pi import into the chloroplast, and the signal metabolite fructose-2,6-bisphosphatase, (Hawker et al., 1991).

In C_3 plants triose-phosphate is exported from the chloroplast and converted to sucrose in the cytosol. The Pi released during sucrose synthesis reenters the chloroplast in exchange for more triose phosphate. The presence of triose phosphate in the cytosol inhibits the synthesis of the signal metabolite, fructose-2,6-bisphosphate (Stitt and Quick, 1989). As fructose-2,6-bisphosphatase inhibits sucrose synthesis, inhibition of its production results in greater sucrose synthesis. When sucrose synthesis exceeds sucrose withdrawal from the cytosol, the subsequent build up of sucrose inhibits sucrose phosphate synthesis and hydrolysis. This results in a buildup of fructose 6-P, an inhibitor of fructose-2,6 breakdown. The reduction in sucrose-P hydrolysis also results in less Pi re-entering the chloroplast. This in turn results in a reduction in the level of triose phosphate in the cytosol, thereby causing a reduction in the inhibition of fructose-2,6- P_2 synthesis. The resultant increased amount of fructose-2,6- P_2 inhibits sucrose synthesis (Stitt, 1990). The reduction in sucrose synthesis results in a buildup of triose phosphate within the chloroplast. This buildup of triose phosphate promotes starch synthesis by activating adenosinediphosphate glucose synthetase, the enzyme providing adenosinediphosphate glucose, the immediate precursor of starch synthesis (Preiss and Sivak, 1996).

As a result of the apparent intercellular compartmentation of sucrose and starch synthesis, the situation in C_4 plants is rather more complicated. In the most well studied C_4 species, maize, the synthesis of sucrose appears to occur largely in the Kranz-mesophyll cells while starch synthesis occurs mainly in the bundle

sheath cells (Leegood, 1985; Furbank et al., 1985). Thus, in C_4 plants, co-ordination between sucrose export and primary partitioning is not only at the intracellular level (chloroplast/cytosol) but must also be at the intercellular level. As in the chloroplast/cytosol situation in C_3 plants the basis of this additional co-ordination is considered to be the intercellular exchange of P_i and triose phosphate. However, the exact mechanisms of this additional co-ordination are still unclear.

In light of the previous findings on co-ordination, one would expect that in sugarcane, a system with a high rate of carbon fixation, there would either be a high level of carbohydrate storage or a high level of carbohydrate export. Results obtained in the present study indicate that relatively little of the fixed carbon is stored. The amount of radiolabel present in the fed area of leaf 6 directly after radiolabeling was found to be only 30% of the estimated amount of radiolabeled carbon fixed during the 10 minute radiolabeling period. Thus, during photosynthesis, only 30% of the fixed carbon is partitioned into the storage carbohydrate pool while the remaining 70% is partitioned into sucrose for immediate export from the leaf. High rates of sucrose export have also been noted previously in other C_4 plants such as maize, sorghum and pigweed (Grodzinski et al., 1998). In the study conducted by Grodzinski and co workers (1998) it was found that the average export rate, expressed as a percentage of the rate of photosynthesis, for all the C_4 plants studied was 73%. In the same study the average export rate for C_3 plants was found to be much lower at 48%. Thus, it appears that the maintenance of high photosynthetic rates in C_4 plants necessitates a high rate of carbon removal from the leaf.

The pathway taken by sucrose from the photosynthetic tissues to the phloem is still debatable. Phloem loading has been recently reviewed by van Bel (1993). Theoretically the movement of sucrose from source cells to the phloem can be via the symplasm or via the apoplast. In the case of a purely symplastic route, sucrose moves from the mesophyll cells to the sieve tube via the plasmodesmata

of the interconnecting cells. Since plasmodesmata are not known to be capable of selective active uptake, the accumulation of sucrose must occur primarily by the mesophyll cells to achieve the high concentrations in the sieve tube sap. Thus, in the symplastic case, only the mesophyll cells determine the quantity of sucrose to be loaded. In a purely apoplastic route, sucrose released from the mesophyll cells into the apoplast is taken up by plasmalemma-bound transport systems in the phloem cells. Consequently, in the apoplastic case, both the mesophyll cells and the phloem cells can influence the amount of sucrose loaded. Unfortunately, there is little information on the exact mechanisms of regulation of loading in response to changed photosynthetic rates or sink demand.

The exact form (sucrose or starch) in which the fixed carbon is temporarily stored in leaf 6 was not determined in the present study. However, regardless of the type of storage carbohydrate, results indicate that there is rapid remobilisation of the stored carbohydrate in the first 60 minutes after radiolabeling. As much as 86 % of the radiolabel found in the fed part of leaf 6 directly after radiolabeling, was translocated out of the leaf during this time period. In similar translocation studies conducted on sugarcane by Hartt and Kortschak (1967) and on maize by Hofstra and Nelson (1969), radiolabeled carbon was also found to be rapidly exported from the fed leaf. Sugarcane was found to translocate about 74% of the original radiolabel within the first 90 minutes while maize was found to translocate almost 80% within 2 hours after radiolabeling. Similar high rates of translocation were also found in two other C_4 species, namely sorghum and millet (Hofstra and Nelson, 1969). In the C_3 species investigated, the retention of radiolabeled carbon has been shown to be much higher. Sugarbeet translocates about 60% in 3 hours (Mortimer, 1965), soybean a maximum of 45% in 2 hours (Thrower, 1967), tobacco about 22% in 5.5 hours (Shiroya et al., 1961) and pine seedlings about 15% in 7 hours (Shiroya et al., 1961). Based on these previous studies and on the present study it would appear that high rates of photosynthesis and high rates of translocation go hand in hand in C_4 plants.

Further work is required to establish if the relationship between the high rates of photosynthesis and the high percentage of exported assimilates is causal or not.

Even though relatively high translocation rates have been noted in previous studies on C_4 plants, the amount of carbohydrate retained in leaf 6 is still exceptionally low. To explain this, one needs to consider the time of day during which radiolabeling occurred, and the possible effect of changes in leaf irradiance levels brought about by the radiolabeling method. A diurnal variation in both the rate of sucrose synthesis (Köhler et al., 1988) and the rate of sucrose translocation (Hartt and Kortschak, 1967) has been noted in sugarcane. At the start of the photoperiod leaf sucrose concentrations are usually low and the production of sucrose is favoured over starch production. At the end of the photoperiod, when sucrose concentrations are high, the rate of sucrose synthesis slows and a shift in partitioning toward storage carbohydrates can occur (Köhler et al., 1987). Translocation rates have been found to follow a similar pattern, being high in the morning and low in the afternoon. Thus, as all radiolabeling was carried out during mid-morning, the amount of retained carbohydrate is low as the majority of the fixed carbon is being partitioned into sucrose and being exported from the leaf. In addition it is proposed that alterations in the levels of leaf irradiance resulted in an extraordinary amount of stored carbohydrate being translocated out of the leaf. In most field grown C_4 plants the photosynthetic carbon fixation rate has been found to vary significantly during the photoperiod. This variation has been attributed to natural fluctuations in light intensity and to the fact that the photosynthetic rate in C_4 plants is light dependant over a very large range since it is hardly saturated. In contrast to fluctuations in photosynthetic rate, assimilate export has been found to be much less variable (Kalt-Torres et al., 1987). The uncoupling between the two processes has been attributed to the buffering role of stored carbohydrates (Kalt-Torres et al., 1987; Servaites, 1989). It is suggested that during periods of low irradiance, the stored carbohydrate pool is remobilized to counter changes in the amount of carbon being fixed. During radiolabeling in the present study, leaf 6 was positioned to

maximize the amount of light striking the enclosed leaf area. In most instances this resulted in the enclosed leaf being irradiated by full direct sunlight. Once leaf 6 was removed from the glass cuvette, it almost always returned to a position where the level of irradiance was much lower, either as a result in a change in orientation or as a result of shading. Thus, carbohydrate that might have normally been retained for night translocation is utilized to stabilize the export of assimilate to the rest of the plant.

Distribution of carbon in culm

Sucrose is considered to be the main form in which carbon is translocated in the phloem in most plants (Hawker, 1985) and in sugarcane (Hartt et al., 1963; Hatch and Glasziou, 1963). In the present study, leaf sheath analysis confirms that 98% of the carbon entering the sugarcane culm is in the form of sucrose. Münch (1927) proposed that the flow of solution through the sieve tubes of phloem tissue is driven by a pressure difference generated by loading of osmotically active carbohydrate within the source and unloading in the sink. With a few additions pertaining to the creation of axial phloem pressure by Lang (1983), Lang and Thorpe (1986) and Wolswinkel (1990), this pressure flow mechanism is widely accepted as the means by which long-distance phloem transport occurs.

In a simplified system with one non-limiting source and one sink, the sink tissue need only maintain a slight pressure difference to ensure that it attracts assimilate. However, in a normal plant system where there are numerous sinks and the source can be limiting, assimilate attraction is dependant on the tissues ability to maintain a competitive pressure difference. The term sink strength has been used to describe this competitive ability of sink tissue to attract assimilates (Wareing and Patrick, 1975; Wolswinkel, 1985; Farrar, 1993). In figure 4.3 the percentage distribution of radiolabeled carbon in all three of the studied internodes is shown. The majority of the radiolabeled carbon is found in the mature internode (internode 9), with relatively little being found in the intermediary tissue (internode 6) and in the immature tissue (internode 3). Thus,

it appears that mature tissue attracts the most assimilate. Based on this, one would be tempted to conclude that the mature internode has the greater sink strength. However, it is possible that the observed distribution is not indicative of sink strength.

By using carbon distribution to estimate sink strength, one is essentially basing sink strength on the flow of carbon into the sink tissue. In a simplified system with one source and two competing sinks, the flow of carbon into the sink tissue would be a true reflection of the sink's ability to attract assimilates. However, in a normal plant there are numerous sinks and sources. In the present study, the elongating tissue and immature tissue sinks may be attracting less of the assimilate produced in leaf 6, not because they are weaker sinks, but because sucrose supply from leaves above leaf 6 fulfill their requirements. Thus, it could be tentatively concluded that while mature tissue is not necessarily the strongest sink within the culm, the occurrence of most of the radiolabel in this tissue suggests that it does place the greatest demand on assimilate originating in leaf 6.

An alternative explanation for the observed distribution pattern centres on the actual anatomy of the vascular tissue within the culm. It has been found that leaf traces, continuations of leaf vasculature often extend basally to the next node or below, before uniting with the stem vasculature (Esau, 1965; Wardlaw, 1968). In sugarcane (Hartt et al., 1963) and in other plant species such as rye (Mayer and Porter, 1960) and soybean (Vernon and Aronoff, 1952), it has been found that photosynthate usually follows the pattern created by the interconnections between these leaf traces and the stem vasculature. Hartt and co-workers (1963) found that in sugarcane, photosynthate produced by middle ranked leaves (leaves 5-8) moved down as far as 9 nodes before finding its way into an upward moving system. In the same study it was found that the leaf traces of these middle ranked leaves usually lost their identity about 9 nodes below the associated leaf. In the present study the leaf sheath of leaf 6 joins the culm at the

base of internode 6. Therefore, assimilate following the leaf traces would pass through internode 7 first and continue for an undetermined number of nodes before entering an upward moving system. Thus, it is possible that the occurrence of most of the radiolabeled carbon in the tissues below leaf 6 is not due to these tissues placing a greater demand on the assimilate, but is due to these tissues gaining first access to the assimilate from the leaf traces.

Phloem unloading

It was initially proposed that sucrose moves from the sieve elements (the sieve tube-companion cell complex) through the free space and into the metabolic compartment of the parenchyma cells in the sugarcane culm (Sacher et al. 1963). The presence of invertase activity in the apoplast (Hatch and Sacher, 1963; Hatch and Glasziou, 1963; Hawker and Hatch, 1963) and the ability of parenchyma cells to take up sugars from the apoplast (Bieleski, 1960; Hatch and Glasziou, 1963) was seen as evidence that post-phloem transport in the culm was through the apoplast (Sacher et al., 1963a; Glasziou and Gaylor, 1972). However, this evidence does not exclude several alternative pathways. Sucrose could follow the symplastic path and pass directly from the sieve elements into the storage parenchyma via plasmodesmata (Oparka and Prior, 1988). The leakage of sucrose from the cell could then result in the need for invertase activity in the apoplast and a plasmamembrane that is able to retrieve sugars. Alternatively, sucrose may pass from the sieve elements to the 'endodermis-like' cells surrounding the phloem via plasmodesmata and then move into the apoplast and be taken up by the storage parenchyma cells (Hawker, 1985). The path would then be partly symplastic and partly apolastic. Another alternative is that both the apoplastic and purely symplastic paths are used simultaneously so that the sugars in the apoplast originate from direct phloem unloading and from leakage of storage cells.

With no definitive conclusions drawn on the chosen path, phloem unloading in the sugarcane culm may occur through either the symplasm, the apoplasm, or

both. However, based on the specific activity values of radiolabeled sugars in immature and elongating tissue of the present study, it appears that in these tissues at least, apoplastic unloading predominates. If radiolabeled carbon was entering the cell mainly as sucrose, the specific activity of the sucrose pool should be in excess of the hexose sugar pool. This assumption is based on the fact that the specific activity of the product, in this case the hexose sugars, can never exceed the specific activity of the source, in this case the radiolabeled sucrose. However, the specific activity of the hexose sugar pool was found to be greater than the specific activity of the sucrose pool (refer to section 4.4 Table 4.4) in immature and elongating tissues. This suggests that in immature and elongating tissue, radiolabeled hexose and not radiolabeled sucrose is the main source of radiolabeled carbon in the cell. This can only be explained if a greater proportion of the incoming sucrose is being hydrolysed prior to uptake. As sucrose cleavage is not known to occur within phloem tissue (Hartt et al., 1963) and invertase activity is associated with the apoplast of storage parenchyma tissue, from this it can be concluded that a greater proportion of the incoming sucrose must be unloaded apoplastically than symplastically. Although this argument does not occlude the possibility that symplastic unloading is occurring, it does suggest that in immature and elongating tissues the movement of sucrose from the phloem tissue to the storage parenchyma is predominantly via the apoplastic pathway.

In mature tissue the situation is reversed, with the specific activity of the sucrose pool being higher than that of the hexose sugar pool. Thus, sucrose must be the main form in which radiolabeled carbon is entering mature storage parenchyma tissue. By applying the reasoning used in the previous hexose sugar case, one would be tempted to conclude that the predominant pathway taken by sucrose in mature tissue must therefore be the symplastic pathway. The problem with applying the same reasoning is that, unlike previously where the hexoses must have originated from the apoplast, sucrose could have moved through the symplasm into the cell or it could have been taken up intact from the apoplast by

the plasmamembrane (Lingle, 1989; Thom and Maretzki, 1992). Thus, based only on the fact that sucrose is the main form in which carbon enters storage parenchyma tissue, it cannot be conclusively stated that the unloading pathway is mainly symplastic. However, in light of work carried out on the developmental changes in stem anatomy during internode maturation, it is probable that unloading in mature tissue is mainly symplastic.

It has been found that as culm tissue matures there is an increase in the level of lignification and suberisation of the cell walls of the vascular tissue and the storage parenchyma tissue (Clements, 1980; Jacobsen et al., 1991). This lignification and suberisation ultimately results in the vascular tissue becoming isolated from the storage parenchyma tissue due to the formation of thick walled, lignified and suberized schlerenchyma cells. Experiments with apoplastic tracer dyes (of similar molecular sizes to sucrose) revealed these thick walled cells were effective in confining the dyes to the vascular tissues in mature tissues (Jacobsen et al., 1991). Collectively this work suggests that apoplastic transfer from phloem in mature internodes is very improbable. Thus, there appears to be a developmental change in the pathway of phloem unloading from predominantly apoplastic in immature tissue to predominantly symplastic in mature tissues.

The flux of sucrose from the phloem to the storage parenchyma cells is governed by differences in solute chemical potentials (diffusion) or in pressure (bulk flow) (Patrick, 1997). In immature internodal tissue, where sucrose is rapidly hydrolysed and utilized for growth and respiration, it is likely that a favourable concentration difference exists for unloading by diffusion. In contrast, diffusion driven sucrose unloading is less likely to occur in high sucrose content mature tissue, where the difference in sucrose concentration between the phloem tissue and storage parenchyma tissue may not be sufficient to result in diffusion (Patrick, 1992). Rather, in mature tissue it is thought that symplasmic sucrose unloading occurs by bulk flow driven by a hydrostatic pressure gradient

established between the phloem sieve elements and the storage parenchyma (Murphy, 1989).

To promote bulk flow, storage parenchyma tissue cells would have to maintain low cell turgor despite high sucrose concentrations (Minchin and Thorpe, 1987; Patrick, 1991). This implies that the cells must have a turgor-sensing mechanism and the capacity to regulate turgor pressure. As cell turgor is determined by the difference between cell osmotic potential and apoplastic water potential (Patrick, 1991), it is proposed that this regulation involves controlled sucrose leakage into the apoplast and retrieval from the apoplast. This proposal is supported by the finding that cellular solutes in the apoplast are usually in the same proportion as the solutes in the symplast (Welbaum and Meinzer, 1990). The occurrence of high apoplastic concentrations of osmotically active solutes, primarily sucrose, has also been confirmed in numerous other studies (Hawker, 1965; Glasziou and Gaylor, 1972; Welbaum and Meinzer, 1990; Moore and Cosgrove, 1991). The partitioning of osmotica between different cellular compartments is also proposed to be important in the maintenance of low turgor during sucrose accumulation in sugar beet (Leigh and Tomos, 1983; Tomos et al., 1992). The main difference between sugarcane and sugarbeet is that while sugars make up the majority of the partitioned osmotica in sugarcane, in sugar beet the osmotica consists mainly of K^+ and other non-sugar solutes.

In order for the proposed turgor regulating mechanism to be effective the apoplasts of the phloem and the storage parenchyma tissue have to be physically separated. If the two were not separated the turgor of the storage parenchyma tissue could not be regulated independently of that of the phloem tissue and thus, a pressure gradient could not be established. This need for separation provides an explanation for the creation of the before mentioned lignified, suberized sclerenchyma cell barrier during internode development.

Sucrose accumulation rate in sink tissues

The maturity of each of the studied internodes was established by determining the sucrose content of the culm tissue. Sucrose content in internodes 3, 6 and 9 was determined at 24 hours after radiolabeling and again at 6 weeks after radiolabeling. An increase in sucrose content was observed from immature internode to the mature internode. Utilizing the data obtained from the sucrose content determinations, the sucrose accumulation rate ($d\text{Sucrose Concentration} / d\text{Time}$) for each of the internodes was calculated. It should be noted that at the six week sucrose content determination, the internodes originally labeled as internodes 3, 6 and 9 had become internodes 6, 9 and 12 due to the production of three new internodes at the shoot tip. Thus, the rate of sucrose accumulation for internode 3 represents the accumulation rate in internode 3 as it develops into internode 6. Similarly for internodes 6 (becomes internode 9) and 9 (becomes internode 12). There appears to be three phases of sucrose accumulation in the developing culm. Initially, the accumulation rate in rapidly growing tissue (internode 3 develops into internode 6) is relatively low. This is followed by a rapid increase in sucrose accumulation during internode elongation as internode 6 becomes internode 9. Finally, a slowing down in the rate of sucrose accumulation is observed during late maturation as internode 9 becomes internode 12.

Changes in the rate of sucrose accumulation in the developing culm have also been noted in work on tissue slices and on cell-suspension cultures. Whittaker and Botha (1997) found that there was a sharp increase in the rate of sucrose accumulation between sliced internodal tissue from internodes 4 and 7, while Wendler and co-workers (1990) noted that the sucrose accumulation rate changed during the growth cycle of cells in culture. Thus, from results obtained in the present study and from previous studies it can be concluded that the higher sucrose content in more mature internodes is not merely a result of a longer growth period, but is due to an increased sucrose accumulation rate. The change in sucrose accumulation rate could be a result of increased carbon

uptake or it could be due to a redirection of carbon away from respiration and growth and into sucrose storage. With the lack of conclusive studies on carbon uptake and related membrane transport, the redirection of incoming carbon towards sucrose storage serves as the best explanation for the changes in sucrose accumulation during culm development. This redirection of carbon to sucrose storage has been attributed to reduced cleavage, higher synthesis, or a combination of both. Thus, the rate of sucrose accumulation is controlled by cycles of synthesis and degradation.

Carbon partitioning in sink tissues

Short-term partitioning

Upon entering storage tissue, the radiolabeled carbon contained in the radiolabeled sucrose could either become part of the stored sucrose pool or be utilized for growth (cell wall synthesis) and respiration (production of CO₂ and the acidic and basic cellular components). In the present study carbon allocation to storage increased from internode 3 to internode 9. Conversely, carbon allocation to hexose sugar synthesis, insoluble matter synthesis and amino acid and organic acid biosynthesis decreases from internode 3 to internode 9. Thus, internode maturation down the developing stalk is associated with an increased allocation of carbon into the stored sucrose pool. This supports the proposal that the increased rate of sucrose accumulation noted in the previous section is due to a re-direction of carbon away from respiration and biosynthesis and towards sucrose storage.

At 6 hours after radiolabeling, radiolabel was found predominantly in the hexose sugar pool in internode 3. At the same time point only a very small percentage of the total radiolabel is present in the hexose sugar pool in internode 9. The decrease in percentage radiolabel recovered in the hexose sugar pool from internode 3 to internode 9 suggests that the observed increase in stored radiolabeled sucrose could be either a result of reduced sucrose cleavage and

higher sucrose synthesis, or a result of each factor acting individually. The machinery for sucrose breakdown and re-synthesis is present in both internode 3 and internode 9, with significant activity levels of both the primary sucrose degrader, SAI, and the primary sucrose synthesizer, SPS, known to occur in both immature and mature tissues (Hatch and Glasziou, 1963; Batta and Singh, 1986). It is proposed that changes in the activities of these enzymes play important roles in determining carbon partitioning down the developing sugarcane culm.

SAI activity levels have been found to be high in immature tissue, fast growing tissues and very low or absent in mature tissue (Hatch and Glasziou 1962). The developmental loss of soluble acid invertase activity from sucrose accumulating tissue has also been noted in carrot (Ricardo and ap Rees, 1970) in citrus fruit (Kato and Kubota, 1978) in sugar beet (Giaquinta, 1979) in muskmelon (Hubbard et al., 1989, 1991; Lingle and Dunlap, 1987; McCollum et al., 1988) in tomato (Yelle et al., 1988; Stommel, 1992) and in pear (Moriguchi et al., 1992). As soluble acid invertase is localized in the vacuole (storage compartment) and sucrose is sequestered in the vacuole, a reduction in invertase activity should result in more sucrose being stored in the vacuole due to the slower turnover of the sucrose pool. There is also evidence that suggests that SPS may play a role in determining partitioning in the developing culm. This evidence has originated mainly from studies on sugarcane suspension cells. In certain studies on whole sugarcane tissue, SPS activity has been found to increase with internode maturation, but data obtained from these studies are questionable (Moore and Maretzki, 1997). In studies on cell suspension cultures it has been demonstrated that the activity of SPS in sugarcane can be sufficient to account for the observed rate of sucrose accumulation (Wendler et al., 1990; Goldner et al., 1991). SPS activity was found to double during the phase when cells were actively storing sucrose and remain high once storage had ceased and when the sucrose was remobilized (Wendler et al., 1990). Increase in SPS activity associated with increases in sucrose concentration has also been noted in fruits such as peach,

strawberry, mango and kiwi (Hubbard et al., 1991). Thus, the high percentage of radiolabel recovered in non-sucrose components from internode three could be a result of high sucrose cleavage with very low levels of re-synthesis. Conversely, low levels of sucrose cleavage due to low SAI activity and higher levels of sucrose re-synthesis due to higher SPS activity could be responsible for the higher percentage of total radiolabeled carbon found in stored sucrose in internode 9.

A similar change in carbon partitioning during internode maturation has been observed in work carried out on tissue slices (Whittaker and Botha, 1997). As in the present study it was found that during maturation incoming carbon is partitioned less into the non-sucrose components and more into sucrose storage. Whittaker and Botha (1997) found that when tissue slices were fed with either radiolabeled glucose or radiolabeled sucrose, the percentage of radiolabeled carbon lost as CO₂ (catabolic respiration) was significantly higher in immature tissue than in mature tissue. Carbon allocation to amino acid, organic acid and lipid biosynthesis (anabolic respiration) was also found to be much higher in immature tissue than in mature tissue. Whittaker and Botha (1997) proposed that this decreased allocation of carbon to both catabolic and anabolic respiration is a result of a reduction in the flux of hexoses into the respiratory pathway. In the present study overall ¹⁴CO₂ loss and carbon flux from the hexose sugar pool could not be determined. However, the determination of percentage total radiolabel in the ionic (anion and cation) pool in internodes 3,6 and 9 confirms findings made by Whittaker and Botha (1997). At the 6 hour time point a two fold decrease in the amount of radiolabel in the ionic pool is observed from internode 3 to internode 9. Thus, it is confirmed at the whole plant level that internode maturation is associated with the redirection of carbon away from respiration and towards sucrose storage.

The previous findings are both supported and questioned by work carried out using sugarcane cell suspension cultures. As in tissue slice work marked

changes in the sucrose accumulation rate were also observed during the growth cycle of sugarcane cell-suspension cultures (Wendler et al., 1990; Goldner et al., 1991). In cells maintained under continuous culture, nitrogen starvation resulted in increased carbon partitioning into sucrose. This increased partitioning into sucrose was found to occur at the expense of both respiration and structural material (Veith and Komor, 1993). However, in cells grown in batch culture changes in sucrose storage are shown to occur without any change in respiration (Wendler et al., 1990). The reasons for this apparent contradiction are unclear. Decreased partitioning of carbon into the non-sucrose water soluble component has also been noted during sugar beet maturation (Giaquinta, 1979) and an inverse relationship between respiration and sucrose storage is shown to exist in the developing carrot root (Steingrover, 1981).

The significant difference between results obtained by Whittaker and Botha (1997) and those in the present work lies in the percentage distribution of radiolabel within the non-sucrose cellular component. In the previous study radiolabeled hexose sugar constituted less than 10% of the total radiolabel, amino acids/organic acids/sugar phosphates constituted 27% and water insolubles constituted 15% of the total radiolabel in internode three. In tissue from internode 7 the percentage radiolabel in the organic acid/amino acid/sugar phosphate and water insoluble components decreased notably to 12 and 5% respectively. No significant decrease in the percentage radiolabel in the hexose sugar component is observed from internode three to internode seven. In the present study the percentage radiolabel in the combined ionic (cation and anion) and water insoluble pool is less than 20% while radiolabel in the hexose sugar pool makes up as much as 42% of the total radiolabel in internode 3. Thus, in immature tissue of the present study, a much greater percentage of the total radiolabel is found in the hexose sugar pool. In internode 9 of the present study the percentage radiolabel in the hexose sugar pool (11%) is very similar to that found in mature tissue of the previous study (10%). The exact reason for the difference between the amounts of radiolabeled carbon in the hexose sugar of

the present study and that of the previous study is unclear. However, it is plausible that the enlarged radiolabeled hexose sugar pool is due to an increased *in vivo* intake of radiolabeled hexoses from the apoplast and a decreased level of sucrose re-synthesis within the cytoplasm of the storage tissue cells.

Initially Sacher and co-workers (1963a) concluded that sucrose was cleaved in the apoplast and the resultant hexose sugars taken up by a hexose transporter in the cell membrane. More recent work suggests that sucrose cleavage is not obligatory and that both a hexose and sucrose transporter operate *in vivo* (Lingle, 1989; Thom and Maretzki, 1992). However, it has not been determined if both transporters are equally active. From study of specific activities of both the sucrose and hexose sugar pool in the present study, it appears that at the whole plant level the hexose sugar transporter actually predominates in immature and elongating tissue. Thus, the larger pools of hexose could be indicative of a hexose uptake system.

The maintenance of a large percentage of the total radiolabel in the hexose sugar pool may also indicate that within immature (internode 3) and elongating (internode 6) storage parenchyma tissue, the re-synthesis of sucrose is relatively low and sucrose breakdown is relatively high. In immature internodal tissue sucrose cleavage could be accomplished by either neutral invertase in the cytosol or by soluble acid invertase (SAI) in the vacuole. The higher levels of SAI activity found in immature tissue suggest that most of this cleavage occurs in the vacuole (Sacher et al., 1963a; Glasziou and Gaylor, 1972) but neutral invertase mediated cleavage also occurs in the cytosol. Hexoses produced in the cytosol can be phosphorylated and either used in the SPS mediated re-synthesis of sucrose or they can be used in cell wall polysaccharide synthesis, amino- and organic biosynthesis and in the provision of energy (resulting in CO₂ production). However, due to the absence of SPS and respiratory enzymes within the vacuole, hexose sugars can only be used for sucrose re-synthesis and respiration once they have left the vacuole. It is therefore possible that the

proposed decrease in sucrose re-synthesis is a result of more of the hexose sugar pool being contained in the vacuole in immature tissue of whole plants.

Long-term partitioning

In addition to investigating short-term (24 hours after radiolabeling) sucrose turnover and carbon partitioning, long-term change in these two aspects of sucrose metabolism was also investigated. The uniqueness of the study prevents any discussion of findings in light of previous findings. The long-term analysis of carbon partitioning confirmed that in mature tissues a larger amount of sucrose is stored in comparison to immature tissue. The persistence of a high amount of radiolabel in the hexose sugar pool in internode 3 in the first week after radiolabeling also confirms that the level of cycling in immature tissue is higher than that in mature tissue. Thus, it would appear that the short-term study alone would have sufficed. However, the long-term study did reveal that there is a continuous transfer of radiolabel from the stored radiolabeled sucrose pool to the insoluble matter pool in all internodes at all stages of development. This could only occur if the stored sucrose pool is being continually turned over. The difference in expected specific activity and observed specific activity of the sucrose pool is taken as further evidence that sucrose turnover is occurring in both immature and mature tissues. Thus, the long-term study confirmed that the sucrose pool is being continually turned over in both immature and mature sugarcane internodal tissues.

The occurrence of sucrose turnover in sink tissues

In a detailed review on work on sucrose accumulation in the sugarcane culm, Glasziou and Gaylor (1972) presented a widely accepted model of the pathways for uptake and transformation of sucrose by internode tissues. In short it proposes the following path of sucrose accumulation (see figure 2.1, Chapter 2 for details). Sucrose unloaded in the apoplast is cleaved by acid invertase and the resultant hexoses are taken up by the plasmamembrane. The presence of

invertase activity in the apoplast (Hatch and Sacher, 1963; Hatch and Glasziou, 1963; Hawker and Hatch, 1965), the randomisation of asymmetrically radiolabeled sucrose during radial transfer from the phloem into the storage tissue (Sacher et al., 1963a; Hawker and Hatch, 1965) and the ability of the plasmamembrane to take up hexoses (Bieleski, 1960; Hatch and Glasziou, 1963) was all interpreted to mean that sucrose hydrolysis prior to uptake was obligatory. Within the metabolic compartment sucrose is resynthesized by sucrose phosphate synthase (SPS) and sucrose phosphatase (SPP) (Hatch and Sacher, 1963; Hatch and Glasziou, 1963; Hatch, 1964). The main evidence in support of sucrose resynthesis by SPS was the fact that when either ^{14}C fructose or ^{14}C glucose was fed to tissue slices, both the fructosyl and glucosyl moieties of the synthesized sucrose molecule were labeled at more or less the same frequency (Sacher et al., 1963a). The resynthesized sucrose is then transferred into the vacuole, the storage compartment. Based on the presence of invertase activity in the metabolic compartment (neutral invertase) and in the storage compartment (soluble acid invertase) it is proposed that sucrose hydrolysis occurs in both of these compartments. The model also suggests that there is movement of sugars from the vacuole to the metabolic compartment and from the metabolic compartment to the apoplast. Thus, according to the model, there is both a cycle of sucrose synthesis and degradation in the metabolic compartment and a cycle of sugar movement between all three compartments.

Although the compartmentation and cyclic nature of the sucrose accumulation pathway remains undisputed, more recent work has both substantiated certain parts of the model and cast doubt on certain parts of the model. At the time the model was proposed, the difficulties associated with measuring SPS activity impeded the search for direct evidence of SPS mediated sucrose synthesis. However, as a result of improved protocols for stabilizing SPS (Hubbard et al., 1989) it was confirmed that SPS activity was sufficient to account for observed rates of sucrose accumulation (Wendler et al., 1990; Goldner et al., 1991). Work on sugarcane cell suspension also established the cytosolic phosphorylated

sucrose synthesis pathway (Wendler et al., 1990; Goldner et al., 1991). As mentioned previously in the discussion on phloem unloading, a purely apoplastic transfer from the phloem in mature internodes is unlikely due to increased lignification and suberisation of the storage parenchyma tissue (Jacobsen et al. 1992; Welbaum et al, 1992). In addition, evidence of the uptake of intact sucrose (Lingle, 1989) and fluorosucrose (Thom and Maretzki, 1992), an analogue of sucrose that cannot be cleaved by invertase, suggests that sucrose cleavage in the apoplast prior to uptake is not obligatory. Criticism leveled at Glasziou and Gaylor's model and new findings regarding membrane transport, suggest that there is a need for a new synthesis and a new model. Nevertheless, it is likely that even in this new model the two salient features of the previous model, namely, the compartmentation of different parts of the sucrose accumulation process and sugar cycling within and between compartments, will still be prominent features of the new model.

The phenomenon of carbon cycling between sucrose and hexoses, as a result of simultaneous synthesis and degradation of sucrose was initially described only in immature storage tissue slices (Sacher et al., 1963a). More recently it has also been described in mature storage tissue slices (Batta and Singh, 1986; Whittaker and Botha, 1997). Whittaker and Botha (1997) found that feeding radiolabeled glucose to tissue slices resulted in significant amounts of radiolabel being found in the fructose moiety of sucrose in both immature and mature tissue slices, thereby indicating that sucrose hydrolysis and resynthesis is occurring. Additional evidence in support of a cycle of sucrose synthesis and hydrolysis has been obtained from studies on cell suspension cultures. In cell suspension cultures it was found that the rate of sucrose synthesis was usually much higher than the net rate of sucrose storage, indicating a fast cycle of sucrose synthesis and hydrolysis (Wendler et al., 1990). High enzyme activities in excess of the requirements for respiration and storage, and the continuous incorporation of ^{14}C into sucrose also lent support for the proposed cycle of sucrose hydrolysis and resynthesis (Wendler 1990; Veith and Komor, 1993).

Assuming that cycling is occurring in storage parenchyma at the whole plant level, one would expect to find evidence of constant sucrose turnover throughout the developing culm. Thus, it is surprising that in whole plant work carried out by Hartt and co-workers (1963) on sucrose translocation in the culm, sucrose turnover and thus sucrose cycling does not appear to occur in mature tissues. The results presented by Hartt and co-workers (1963) showed that after feeding leaves with $^{14}\text{CO}_2$, the resulting radiolabeled sucrose was only converted to radiolabeled hexoses and amino acids in the fed internode and internodes above it. In more mature internodes all the recovered radiolabel was in the form of sucrose, thereby suggesting that no sucrose turnover occurs in mature tissues. However, in the present study, radiolabel is recovered in the form of sucrose, hexoses, amino acids and insoluble matter from both immature and mature internodal tissue. These results confirm that sucrose turnover does occur in both immature and mature internodal tissue. This confirmation together with the known occurrence of significant activity levels of SAI, NAI and SPS and SuSy, the machinery of sucrose hydrolysis and resynthesis in mature culm tissue (Whittaker, 1997), suggests that sugar cycling is occurring in mature sugarcane tissue at the whole plant level. The reason for the discrepancy between the present work and the work carried out by Hartt and co-workers (1963) is unclear.

The cycling of sugars in sink tissue has been referred to as a 'futile cycle' (Dancer et al., 1990) because the simultaneous synthesis and degradation appears to involve energy 'wastage'. However there are numerous benefits in maintaining a sugar cycle within the storage parenchyma tissue. The cycle would allow for relatively large changes in net mobilisation and storage, and thus the level of sugar accumulation, to be effected by relatively small changes of enzymes and metabolites (Wendler et al., 1990). This type of fine control would allow sugarcane to respond quickly and efficiently to changes in environmental conditions. Another important consequence of cycling is that cycling results in the partitioning of osmotica among the three cellular compartments. With

reference to the previous section on 'Phloem unloading', this partitioning of osmotica is considered to be the mechanism by which storage parenchyma tissue maintains low turgor despite increases in sugar concentration (Moore and Cosgrove, 1991). By maintaining low turgor within the sink, translocation of sucrose to the sink by mass transfer is promoted (Minchin and Thorpe, 1987; Patrick, 1991) and the sink tissue is maintained as an active sink (Wolswinkel, 1985; Ho 1988; Patrick, 1992). Thus the benefits of maintaining a sugar cycle might outweigh the apparent energy wastage.

The occurrence of sucrose breakdown and resynthesis is believed to occur to varying degrees in various sink tissues (Quick and Schaffer, 1997). In studies based on the randomisation of asymmetrically radiolabeled sucrose breakdown and resynthesis has been shown to occur in sucrose accumulating tomato fruit (Dali et al., 1992). In the hexose accumulating grape a hydrolysis-resynthesis-rehydrolysis scheme has been proposed (Brown and Coombe, 1982). In the other commercially important sucrose accumulating crop, sugar beet, the lack of radiolabel randomisation of asymmetrically radiolabeled sucrose has been taken as evidence that cycling does not occur. However, the presence of SPS in the sugar beet taproot (Fieuw and Willenbrink, 1987) and evidence of hexose uptake into protoplasts and its subsequent incorporation into sucrose (Giaquinta, 1977; Fieuw and Willenbrink 1990; Ho et al., 1991) suggests that it is likely that sucrose turnover is occurring to some degree. Evidence suggestive of a rapid cycle of sucrose breakdown and re-synthesis has also been found in strawberry. Forney and Breen (1985) noted that the concentration of hexose sugars in strawberry is consistently higher than the sucrose concentration at all stages of development. In a later study Forney and Breen (1986) concluded from ^{14}C uptake studies that as in sugarcane, sucrose is hydrolysed and re-synthesized on uptake. Hubbard and co-workers (1991) also presented work that is suggestive of rapid sucrose turnover in strawberry. Although much more conclusive evidence needs to be obtained in relation to sugar cycling in storage tissues, it appears the sucrose

breakdown and resynthesis may be a part of the sugar accumulation process in most sugar storing tissues.

CONCLUSIONS

The occurrence of sucrose turnover in both immature and mature tissue is confirmed at the whole plant level. Internode development is found to coincide with a redirection of incoming carbon from anabolic and catabolic respiration to sucrose synthesis. The increased sucrose content in mature internodal tissues is attributed to an increased rate of sucrose accumulation and not to the longer growth period of mature internodes. As these conclusions have also been drawn from work conducted on tissue slices, it would appear that the tissue slice system is representative of the whole plant system. However, the difference observed between the percentage radiolabeled carbon in the hexose sugar pools of the present and previous tissue slice study indicates that more detailed work is still needed for conclusive verification. This work would have to concentrate on determining if the proposed differences in cell compartmentalisation and cell membrane sugar transport between internodal tissue slices and intact internodal tissue are real or apparent.

Aside from investigating sink related metabolism, the experimental design also allowed for a certain degree of source metabolism investigation. This investigation revealed that leaf 6 is an excellent source of carbon for the rest of the plant. Not only does it fix carbon at a relatively high rate, but the carbon that is fixed is exported rapidly and efficiently to the rest of the plant. This suggests that in sugarcane, as in C₄ plants in general, crop yield and productivity is not source limited. Rather the ultimate determinants of productivity are probably within the sink tissues.

The majority of the carbon produced in leaf 6 is sequestered in mature tissue. This distribution pattern could be a result of mature tissue placing the greatest demand on assimilate produced in leaf 6, or it could be a result of mature tissue gaining first access to the assimilate due to the anatomy of the culm vasculature.

More detailed work on the anatomy of the culm vasculature and the movement of sucrose is required to establish which of these explanations is most plausible.

Theoretically, sucrose unloading in the storage sink tissue could be via the apoplastic path or symplastic path, or it could via both paths. Placing the anomalous specific activity results in the context of previous findings on anatomical changes during internode maturation, it is proposed that the path changes from mainly apoplastic in immature tissue to mainly symplastic in mature tissues. As characteristics of the path of sucrose unloading could determine accumulation rates, the proposed developmental changes in path utilisation warrants more detailed study.

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