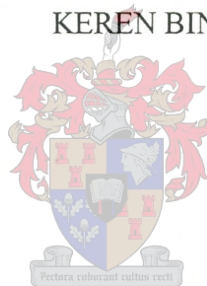


CARBON PARTITIONING IN SUGARCANE INTERNODAL TISSUE  
WITH SPECIAL REFERENCE TO THE INSOLUBLE FRACTION

by

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Thesis presented in fulfilment of the requirements for the degree of

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## **DECLARATION**

I, the undersigned, hereby declare that the work contained in the thesis is my own original work, and that I have not previously in its entirety or in part submitted it an any university for a degree.

26 May 2000

## ABSTRACT

The changes in carbon allocation to sucrose, hexoses, fibre, starch and respiration were investigated in developing internodes of sugarcane. Radiolabelling studies were conducted on internode 3, 6 and 9 tissue, representing three stages of increasing maturity. It was apparent that a high rate of cycling between triose-phosphate and hexose-phosphate occurred. A maximum of 50% of carbon entering triose-phosphates was returned to hexose-phosphate in internode 3 tissue, and this flux decreased with tissue maturity to 30%. Carbon partitioning into sucrose increased from 34% of total  $^{14}\text{C}$  uptake in internode 3, to 61% in internodes 6 and 9. In immature tissue, the protein and fibre components were the dominant competing sinks with sucrose for incoming carbon, to which 14 and 16% of carbon were allocated respectively. Increased carbon allocation to sucrose with tissue maturity, coincided with a decrease in partitioning to fibre. This indicated that previous studies had underestimated total carbon allocation to respiration, since the protein component was not considered. In contrast with earlier work, the respiratory pathway was the strongest competitor with sucrose for incoming carbon, even in mature tissue. Between internodes 3 and 6, carbon allocation to total respiration did not change significantly, but decreased 50% in mature tissue. Starch was a weak competitor with sucrose, for incoming carbon, to which a maximum of 2% of  $^{14}\text{C}$  was allocated in immature tissue. In cane harvested in early spring, radiolabelled maltose was recovered in internode 3 tissue of ripening cane, indicating that concomitant starch synthesis and degradation occurred. The redistribution of C-1 and C-6 in starch glucose was analysed following feeding of tissue with [1- $^{14}\text{C}$ ]- and [6- $^{14}\text{C}$ ]-glucose. Randomization of label in starch indicated that the pathway for carbon movement into sugarcane plastids for starch synthesis is primarily through the triose-phosphate translocator. Finally, this study indicated that radiolabelling of tissue discs is a suitable experimental system to determine carbon flux in sugarcane. During the 3 h labelling period the rate of  $^{14}\text{CO}_2$  release became linear, indicating that the system approached isotopic steady state between the external and internal glucose pool; and the respiratory processes involved in  $\text{CO}_2$  release.



## OPSOMMING

Die veranderinge in koolstoftoedeling na sukrose, heksoses, vesel, stysel en respirasie is in ontwikkelende internodes van suikerriet ondersoek. Die koolhidraatmetabolisme in internodes 3, 6 en 9, wat drie stadiums van toenemende rypheid verteenwoordig, is met behulp van  $^{14}\text{C}$  merkingstudies ondersoek. Dit is duidelik dat daar 'n hoë mate van koolstofsirkulering tussen die heksose- en triose-fosfaat poele voorkom. In internode 3 word tot 50% van die koolstof wat in triose-fosfate geïnkorporeer is, weer na heksose-fosfaat omgeskakel. Selfs in volwasse weefsel vind daar nog soveel as 30% koolstofsirkulering plaas tussen die twee poele plaas. Koolstoftoedeling vanaf glukose na sukrose het van 34% in internode 3, tot 61% in internodes 6 en 9 toegeneem. Proteïen en selwandkomponente was die belangrikste swelgpunte vir koolstof in onvolwasse weefsel gewees. Namate die weefsel meer volwasse word, word sukrose 'n belangriker swelgpunt. Die koolstoftoedeling aan sukrose is veral ten koste van toedeling aan die selwandkomponente. Die bevinding dat die proteïenpoel 'n sterk swelgpunt is dui aan dat vorige studies die belang van respiratoriese koolstofvloei onderskat het. In teenstelling met vorige aansprake is dit duidelik dat selfs in volwasse weefsel respirasie die grootste swelgpunt vir die inkomende organiese koolstof in die internode vorm. Koolstoftoedeling aan respirasie het nie noemenswaardig tussen internodes 3 en 6 verskil nie, maar het met 50% in volwasse weefsel afgeneem. Stysel is deurgaans 'n swak swelgpunt vir koolstof met die hoogste toedeling aan die poel (2%) in die jong weefsel (internode 3). Na toediening van [ $^{14}\text{C}$ ]-glukose is radioaktief gemerkte maltose gevind in suikerriet wat vroeg in die lente geoes is. Dit dui aan dat gelyktydige afbraak en sintese van stysel plaasgevind het. Die herverdeling van C-1 en C-6 in glukose afkomstig van stysel is na toediening van [ $^{14}\text{C}$ ]- en [ $^{14}\text{C}$ ]-glukose ontleed. Die ewekansige verspreiding van radioaktiwiteit tussen koolstof 1 en 6 van die glukose in stysel dui aan dat dit hoofsaaklik die triose-fosfaat translokeerder is wat in die plastied verantwoordelik is. Hierdie studie het ook aangetoon dat radioaktiewe merking van weefselsnitte 'n geskikte eksperimentele sisteem is om koolstoftoedeling in suikerriet te ondersoek. Die patroon van  $^{14}\text{CO}_2$  vrystelling dui daarop dat die weefsel na 'n 3 h inkuberingsperiode isotopiese ewewig tussen die eksterne en interne glukose poele en die respiratoriese prosesse begin bereik het.



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**LIST OF ABBREVIATIONS**

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
Bq	Bequerel
CHO	carbohydrate
DHAP	dihydroxyacetone-phosphate
EtOH	ethanol
FBPase	fructose-1,6-bisphosphatase (EC 3.1.3.11)
Fru-1,6-P <sub>2</sub>	D-fructose-1,6-bisphosphate
Fru-2,6-P <sub>2</sub>	D-fructose-2,6-bisphosphate
fructose-6-P	D-fructose-6-phosphate
f. wt.	fresh weight
Glucose-1-P	D-glucose-6-phosphate
Glucose-6-P	D-glucose-6-phosphate
GPT	glucose-6-phosphate/phosphate translocator
HPLC	high performance liquid chromatography
K <sub>m</sub>	concentration of substrate that produces half-maximal velocity
MES	(2 [N-morpholino] ethanesulfonic acid)
NADP	oxidised nicotinamide-adenine phosphate dinucleotide
NADPH	reduced nicotinamide-adenine phosphate dinucleotide
n.d.	not determined
OPP	oxidative pentose phosphate
PCR	polymerase chain reaction
PFK	6-phosphofructokinase (EC 2.7.1.11)
PFP	pyrophosphate-dependent phosphofructokinase (EC 2.7.1.90)
P <sub>i</sub>	inorganic phosphate
PP <sub>i</sub>	inorganic pyrophosphate
PK	pyruvate kinase (EC 2.7.1.40)
SD	standard deviation
SPS	sucrose phosphate synthase (EC 2.4.1.14)
SuSy	sucrose synthase (EC 2.4.1.13)
TCA cycle	tricarboxylic acid cycle
TPT	triose-phosphate translocator
UDP	uridine 5'-diphosphate
V <sub>max</sub>	maximal velocity of reaction at unlimiting substrate concentration



## CHAPTER 1

### GENERAL INTRODUCTION

Previous advances in sucrose output of the sugar industry have been through manipulation of the harvest index, i.e., the yield of sucrose per stem, and the quantity of cane per unit land (Moore and Maretzki, 1996). This has been primarily through the production of new cane varieties using traditional plant breeding techniques, or improved agricultural practices. The challenge currently facing the industry is to further increase productivity, although it is recognised that there is a reciprocal relationship between growth rate in plant tissue and sucrose recovery (Komor *et al.*, 1987). Furthermore, increased sucrose content attained using traditional plant breeding strategies has reached an apparent threshold. To break this limit, new plant improvement strategies are needed. Hence, the use of molecular technology to increase sucrose yield is becoming an increasing focus alongside traditional plant breeding programmes.

Current research strategies to improve sugarcane productivity have taken the viewpoint that the product yield of photosynthate is limited at a sink rather than at a source level (Gifford and Evans, 1981; Krapp *et al.*, 1993). At the sink level, therefore, an understanding of the regulation of sucrose import and storage becomes essential. Genetic engineering requires identification and manipulation of key pathways in metabolism, to increase carbon allocation to one or more sinks. Traditionally, key regulatory sites have been identified according to criteria defined by the theory of metabolic control analysis (ap Rees and Hill, 1994). Enzymes which possess regulatory properties, catalyse irreversible reactions, and show reciprocal changes in flux and substrate concentration fall into this category (Stitt and Sonnewald, 1995).

In sugarcane, the study of the regulation of heterotrophic sucrose metabolism has largely followed this approach, and provided valuable insight into the pathways of sucrose synthesis and degradation. However, it is becoming increasingly apparent that this approach is limited, in that quantitative changes in the balance of enzyme activities,



rather than qualitative differences in enzymes are now thought to influence the processes governing sucrose accumulation (Moore and Maretzki, 1996). Furthermore, the regulatory capacity of enzymes; i.e. the correlation of enzyme activity to actual metabolic flux within the system, has not been extensively considered (Stitt and Sonnewald, 1995).

In the light of the above, a more system-oriented approach is necessary for the study of metabolic flux. For the purposes of this study, carbon partitioning is defined as allocation of carbon to pathways or sinks within the cell, and does not include extra-cellular metabolism. In sugarcane, the direct sampling of tissue discs from culm tissue is potentially the most effective system to study carbon partitioning of [ $^{14}\text{C}$ ]-substrates *in vivo*. Currently, the effectiveness of the technique is limited by a lack of knowledge as to whether the system meets criteria for metabolic flux analysis. A key factor in the calculation of metabolic flux using [ $^{14}\text{C}$ ]-substrates is the establishment of isotopic steady state in the experimental system (ap Rees and Hill, 1994). Given this, flux estimates can be determined based on the specific activity of the feeding pool of radiolabel, either external or endogenous to the tissue under study.

The hypotheses of the current study are based on the concept that metabolic competition for carbon occurs within plant cells. The hexose-phosphates form a dynamic pool, which can be partitioned between various pathways, one of which is sucrose synthesis (Kruger, 1997). It must be considered that carbon partitioning to pathways not directly involved in sucrose synthesis may significantly impinge on the amount of carbon allocated to sucrose. Thus, increased allocation to sucrose is potentially accompanied by a redirection of carbon from other sinks. Previous studies of [ $\text{U-}^{14}\text{C}$ ]-glucose and [ $\text{U-}^{14}\text{C}$ ]-sucrose partitioning in sugarcane tissues at increasing stages of maturity, showed that the primary competitive pathways for carbon in sugarcane are respiration, and a water-insoluble component, assumed to consist primarily of fibre (Botha *et al.*, 1996; Whittaker and Botha, 1997). The contribution of protein and starch to this fraction were not considered.



Hexose-phosphates in heterotrophic tissues are synthesized either from the direct phosphorylation of hexose sugars formed from starch or sucrose breakdown; or from resynthesis from triose-phosphates via gluconeogenesis. A futile cycle between sucrose and hexoses has been identified in sugarcane tissues at all stages of development (Sacher *et al.*, 1963; Whittaker and Botha, 1997; Vorster and Botha, 1999), and has been proposed to regulate net sucrose storage or degradation through fine changes in the enzyme properties and metabolites of the cycle. Thus, increased sucrose storage with tissue maturity in sugarcane could theoretically involve a decrease in sucrose degradation.

In other plant species, a further cycle between triose-phosphate and hexose-phosphate occurs, mediated primarily by PFP (Dancer *et al.*, 1990; Hatzfeld *et al.*, 1990; Hajirezaei *et al.*, 1994). It has been proposed to operate in conjunction with the sucrose-hexose cycle to balance the supply of sucrose, and the demand for carbon in respiration and biosynthesis (Moore and Maretzki, 1996). In sugarcane, this cycle was reported to be absent (Sacher *et al.*, 1963). However, more recent evidence has shown a significant activity of PFP in sugarcane (Lingle and Smith, 1991; Botha *et al.*, 1996; Whittaker and Botha, 1999). The estimated mass-action ratio of this reaction indicates that it operates at an apparent equilibrium (Whittaker and Botha, 1997). Thus, PFP in sugarcane could theoretically operate in both the forward and reverse direction. In the light of this, the potential exists for the recycling of carbon from the triose-phosphate pool, to hexose-phosphate, in sugarcane.

A further aspect which has not been addressed in sugarcane research is the contribution of the plastid as a carbon sink in sink tissue. Starch is a primary sink of plastid metabolism in many heterotrophic tissues, and the route of carbon entry to the plastid for starch synthesis has been found to be chiefly as hexose-phosphate in these tissues (Kruger, 1997). In sugarcane, the point at which this organelle competes for carbon with cytosolic pathways will depend on the route of carbon entry, either as hexose-phosphate or triose-phosphate.

The goals of this study were four-fold. 1. To establish whether the study of carbon partitioning of [ $^{14}\text{C}$ ]-substrates by tissue discs of sugarcane meets the criteria for metabolic flux analysis (Chapter 3). 2. Developmental changes in carbon partitioning to the components of the insoluble fraction, namely: protein, starch and fibre, were investigated. The significance of fibre synthesis and respiration were assessed as competitive sinks with sucrose-storage (Chapter 4). The contribution of the starch pool to metabolism, and its turnover was determined (Chapter 5). 3. To establish whether triose-phosphate is recycled to the hexose pool in sugarcane, and to determine the changes in the cycle associated with sucrose accumulation (Chapter 4). 4. The route of carbon entry to the plastid for starch synthesis; either as triose-phosphate or hexose-phosphate, was investigated (Chapter 5).



## CHAPTER 2

### LITERATURE OVERVIEW

This chapter is primarily aimed at introducing the concept of metabolic competition for carbon in plant cells, such that the hexose-phosphates form a dynamic pool, which can be partitioned between key pathways. Firstly, the concept of futile cycling from the hexose-phosphate pool is introduced, with speculation as to the role of cycling in sucrose metabolism. Secondly, in sugarcane, biochemical pathways such as fibre synthesis and respiration may significantly impinge upon sucrose metabolism, such that decreased partitioning to these pathways is a primary cause for sucrose accumulation with maturation (Glasziou and Gayler, 1972; Lingle and Smith, 1991; Whittaker and Botha, 1997). Finally, the potential of the plastid as a carbon sink in sugarcane has not previously been studied. In sugarcane, the point at which this organelle competes for carbon with cytosolic pathways will depend on the route of carbon entry, either as hexose-phosphate or triose-phosphate. The enzymes and metabolic intermediates of the biochemical pathways discussed are shown in Figure 2.1.

#### 2.1 Hexose-phosphate metabolism

The original hypothesis for the source of carbon in plant cells was derived from the classical metabolism of hexose-phosphate by animal cells, and likewise assumed to be hexose-phosphate (ap Rees, 1988). However, it is now generally accepted that in plant cells, sucrose is the primary form in which photoassimilate arrives at sink cells, where it is subsequently cleaved to hexose and hexose-phosphates (ap Rees, 1988). Sucrose can be degraded to hexoses by acid invertase in the apoplastic space or vacuole, or by neutral invertase in the cytosol (Hatch *et al.*, 1963; Gayler and Glasziou, 1972; Hawker, 1985). Furthermore, SuSy can operate to cleave sucrose in the cytosol, yielding UDP-glucose and fructose (Wendler *et al.*, 1990).

The primary pathway for sucrose import and cleavage in sugarcane cells has been a source of debate, although it is currently accepted that hexose uptake is a key route for carbon import to the cell (Moore, 1995 and references therein). Free hexose, or hexose



formed following cleavage of sucrose are phosphorylated via the hexokinases, to generate a pool of hexose-phosphate (Copeland, 1990). This pool, along with that of UDP-glucose formed during sucrose cleavage via SuSy, or conversion from glucose-1-P via UDP-glucose pyrophosphorylase (Edwards and ap Rees, 1986), form a central pool upon which various metabolic pathways converge. It has been proposed that plant metabolism in storage organs, such as sugarcane, can be viewed as a competition between central pathways for these metabolic intermediates (Blakely, 1997).

The hexose-phosphate and UDP-glucose pools in plants do not only originate from translocated sucrose, but depending on the nature of the sink tissue, can arise from degradation of pre-existing carbohydrates or through gluconeogenesis (ap Rees, 1988). The hexose-phosphate pool is therefore dynamic, and the characterization of the pathways influencing its turnover is an important starting-point for biochemical study.

### **2.1.1 The regulatory properties of “futile cycles”**

In the field of carbohydrate metabolism of plants, research has been dominated by studies of individual enzymes; their properties and regulation (Quick and Schaffer, 1996). In most cases, a series of enzymes involved either in synthesis or degradation will be tightly regulated, preventing both pathways from being active at the same time. However, where such antagonistic reactions are functioning simultaneously, a “futile cycle” is set up (Dancer *et al.*, 1990). A classic futile cycle is characterised as a metabolic conversion, the net balance of which is made up by hydrolysis of ATP into ADP and Pi (Hers and Hue, 1983). When antagonistic reactions operate at the same rate there is no net flux of metabolites, but an apparently futile loss of energy as heat. It would appear to be an energetically wasteful system, rapidly utilizing ATP in the cell (Hers and Hue, 1983).

Futile cycling can be avoided if the antagonistic reactions operating in the pathway are controlled by an on/off mechanism (Hers and Hue, 1983). In other words, the system functions to prevent activation of one of the enzymes, when the other is functional. However, a futile cycle is set up in cases where the  $K_m$  of the antagonistic enzymes exceeds the usual availability of substrate (Hers and Hue, 1983), preventing feedback



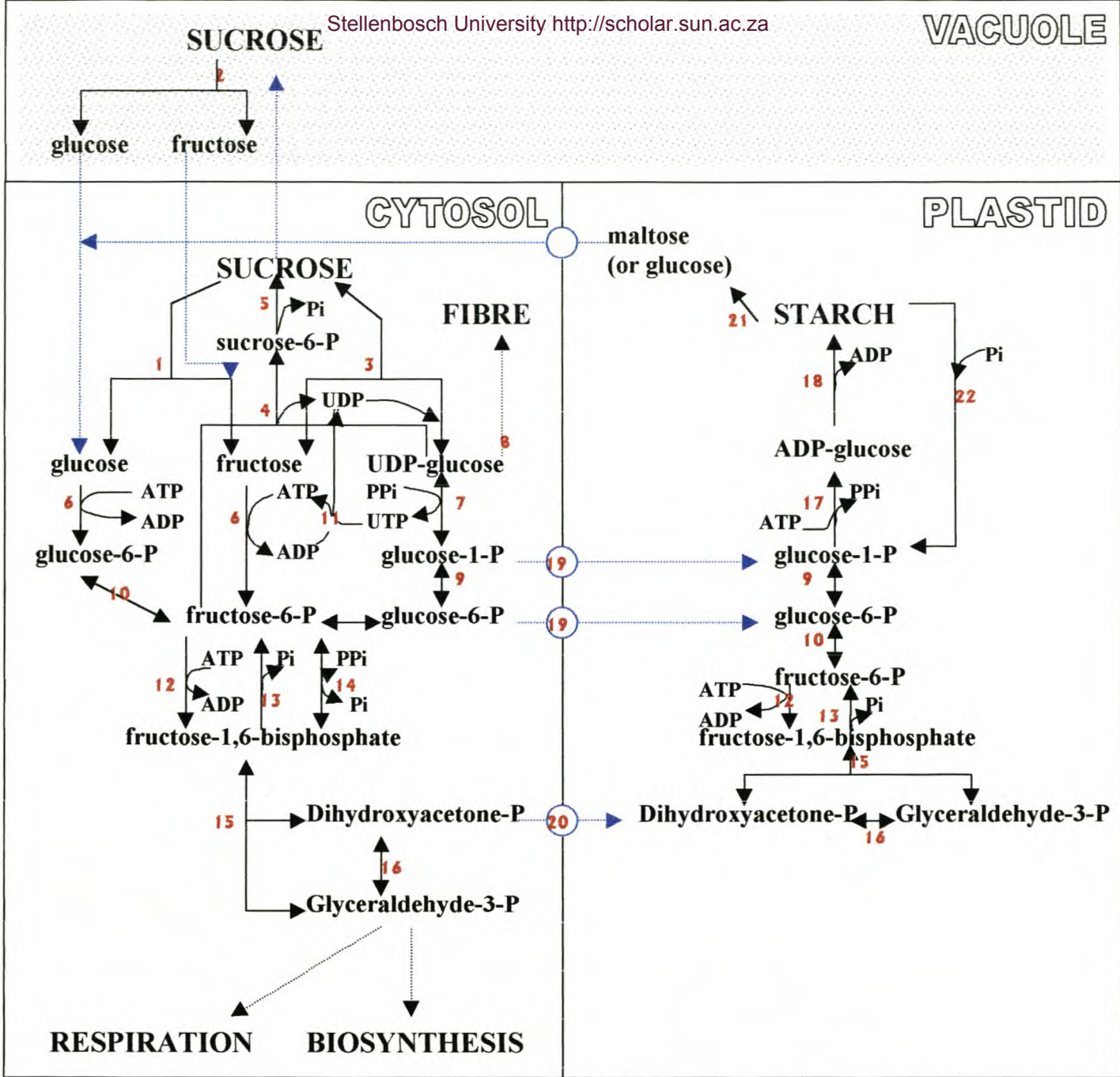
inhibition of the enzymes by substrate. Hence, their activity is essentially controlled by substrate concentration. This allows for significant changes in the net fluxes of the cycle to be finely regulated by only small alterations in the activity of enzymes or metabolite levels within the cycle (Dancer *et al.*, 1990; Wendler *et al.*, 1990). Various cycles have been reported in association with the hexose-phosphate pool in plants, and it has been proposed that they may interact in the regulation of net flux toward storage sinks and respiration (Hatzfeld and Stitt, 1990; Dancer *et al.*, 1990). For the purposes of this study, futile cycling is confined to the characterization of carbon turnover via simultaneously-operating antagonistic reactions. No attempt has been made to integrate the downstream or upstream effects of the cycles on other pathways.

### **2.1.2 Sucrose cycling in sugarcane**

The presence of a cycle of sucrose synthesis and degradation to hexoses was first characterised in immature sugarcane internodal tissues through radiolabelling studies and enzyme measurements on tissue slices (Glasziou, 1961; Sacher *et al.*, 1963). Following uptake of radiolabelled glucose by tissue discs of immature sugarcane internodes in a 4 h pulse, degradation of labelled sucrose to glucose and fructose occurred over a 24 h chase in cold glucose (Glasziou, 1961). This was the first indication that hydrolysis of sucrose occurred following synthesis in sugarcane, and it was proposed that this was catalysed by a vacuolar acid invertase. This result was recently confirmed for tissue discs from young internodes in a short-term radiolabelling experiment, of a 30 minute pulse in 1:1 labelled glucose and unlabelled fructose, followed by a 6 h chase in unlabelled hexoses (Komor *et al.*, 1996). The decay of sucrose was found to be  $4 \mu\text{mol.g}^{-1}.\text{h}^{-1}$ .

The potential for sucrose resynthesis from the hexose moieties derived from sucrose degradation was confirmed in a study by Sacher *et al.* (1963). Here, tissue discs were fed radiolabelled glucose for 10 h, and then enclosed in a humid atmosphere to simulate a closed system. It was found that radiolabel in sucrose decreased to a stable level, and radiolabelled hexoses increased to a constant value. The proposal was made that all the [ $^{14}\text{C}$ ]-labelled hexoses derived from sucrose passed back to the metabolic compartment for resynthesis to sucrose. This assumption was supported by the observation that only





- |                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <ul style="list-style-type: none"> <li>1. neutral invertase</li> <li>2. acid invertase</li> <li>3. sucrose synthase</li> <li>4. sucrose-phosphate synthase</li> <li>5. sucrose phosphatase</li> <li>6. hexokinase</li> <li>7. UDP-glucose pyrophosphorylase</li> <li>8. UDP-glucose dehydrogenase</li> <li>9. phosphoglucomutase</li> <li>10. phosphoglucomutase</li> <li>11. nucleoside diphosphate kinase</li> <li>12. 6-phosphofructokinase</li> </ul> | <ul style="list-style-type: none"> <li>13. fructose-1,6-bisphosphatase</li> <li>14. pyrophosphate-dependent phosphofructokinase</li> <li>15. aldolase</li> <li>16. triose-phosphate isomerase</li> <li>17. ADP-glucose pyrophosphorylase</li> <li>18. starch synthase</li> <li>19. glucose-phosphate translocator</li> <li>20. triose-phosphate translocator</li> <li>21. α- or β-amylase</li> <li>22. starch phosphorylase</li> </ul> <p>                 —▶ enzyme-mediated reaction<br/>                 .....▶ entry of compound to biochemical pathway<br/>                 .....▶ transfer of compound between compartments             </p> |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

**Figure 1.1** Pathways of carbohydrate synthesis and degradation



10% of the total radioactive compounds soluble in ethanol was lost in the time course of the experiment. Since the initial work in the 1960s and 70s, this model of sucrose cycling was not further developed. More recently, a cycle of sucrose turnover has been reported in other plant species, namely: pea roots (Hargreaves and ap Rees, 1988a), *Chenopodium rubrum* cell suspension cultures (Dancer *et al.*, 1990), *Ricinius communis* L. seedlings (Geigenburger and Stitt, 1991) and in climacteric banana (Hill and ap Rees, 1994). In sugarcane, further evidence for the cycle was obtained from pulse feeding experiments with [U-<sup>14</sup>C]-fructose and unlabelled glucose on cell suspension cultures (Wendler *et al.*, 1990). This led to the labelling of free glucose, indicating the degradation of sucrose via invertase. Vorster and Botha (1999) confirmed this result in tissue discs of sugarcane, where metabolism of labelled fructose led to the appearance of label in the glucose pool after a 5.5 h incubation. Furthermore, this study also compared sucrose cycling between young and mature tissues *in vivo*, indicating significant invertase-mediated hydrolysis of sucrose at all stages of tissue development. The theoretical turnover time for the sucrose pool by combined neutral and acid invertase activity was calculated to be 0.14 days in internode 3, ranging up to 1.77 days in internode 10. This confirmed earlier work using pulse-chase experiments, which indicated that degradation of synthesised [<sup>14</sup>C]-sucrose was slower in mature internodes (Glasziou, 1961; Komor *et al.*, 1996)

A possible sucrose-degrading enzyme not included in the original Glasziou and Gayler (1972) model is SuSy. In sugarcane culm tissue, this enzyme was found to be associated with the vascular strands of immature (Hatch *et al.*, 1963) and mature (Hawker and Hatch, 1965) internodes. Little activity was found in the storage parenchyma cells (Hatch *et al.*, 1963). This enzyme is currently thought to function primarily in sucrose degradation in plant cells (Hawker, 1985). Pulse feeding of cell suspension cultures (Wendler *et al.*, 1990) and tissue discs (Whittaker and Botha, 1997; Botha and Black, 2000) with [U-<sup>14</sup>C]-glucose revealed that the fructosyl moiety of sucrose was derived from the hexose-phosphate pool, ruling out SuSy as making a major contribution to sucrose synthesis. Furthermore, 3-fold higher SPS activity, over that of SuSy was reported in mature internodes of sugarcane (Botha and Black, 2000).



However, in cell suspension cultures of sugarcane, SuSy activity was high during the culture period, including the phase of sucrose storage, and was implicated in sucrose breakdown (Wendler *et al.*, 1990). Both the activity of SuSy and the cytosolic enzyme, neutral invertase, were high relative to that of acid invertase (Wendler *et al.*, 1990). Thus, the contribution of the cytosolic enzymes SuSy and neutral invertase to sucrose degradation cannot be ignored.

Currently, sucrose synthesis in sugarcane is thought to be primarily mediated via the enzyme SPS (Moore and Maretzki, 1996). Early studies indicated that the precursor for sucrose in sugarcane was sucrose-phosphate (Hatch, 1964), although the assay of SPS was achieved only more recently (Hubbard *et al.*, 1989; Dancer *et al.*, 1990). The activity of SPS is sufficient to catalyse the observed rate of sucrose synthesis in sugarcane cell suspension cultures (Wendler *et al.*, 1990; Goldner *et al.*, 1990). The work of Wendler *et al.* (1990) also showed a decrease in SPS sensitivity to inhibition by Pi, and an increase in the concentration of the activating metabolite glucose-6-P, at the onset of sucrose storage. Furthermore, SPS levels were correlated with high sucrose content in cell suspension cultures (Wendler *et al.* 1990). In culm tissue, SPS activity has been found to increase with tissue maturity, showing a significant positive correlation with sucrose content (Botha and Black, 2000).

It has been proposed that the sucrose cycle in sugarcane functions as a futile cycle, with SPS mediating sucrose synthesis, and degradation being catalysed by three possible pathways: acid invertase, neutral invertase and SuSy (Wendler *et al.*, 1990). The work of Wendler *et al.* (1990) indicates that there is rapid turnover via these antagonistic pathways, with the difference between their rates allowing for net sucrose storage to occur. Small shifts in the metabolite pools and enzyme activities of the pathways were proposed to result in larger changes in net sucrose synthesis or degradation (Dancer, 1990; Komor, 1994). On a cell or tissue level, there would be resultant net storage or mobilization. Thus, sucrose storage in sugarcane could theoretically reflect a reduction in sucrose degradation, as well as an increase in its synthesis.



On a whole plant level the ability to turn over sucrose between compartments would allow for the rapid removal of photoassimilates from the source, to prevent sink inhibition of source activity (Krapp *et al.*, 1993), and in the long term allow for the remobilization of stored sucrose in periods of rapid growth, e.g. axillary bud formation from the stem (Bull and Glasziou, 1963).

### 2.1.3 Cycling between hexose-phosphates and triose-phosphates

The entry of hexose-phosphate to the glycolytic pathway leads to the formation of Fru 1,6-P<sub>2</sub>, either by the action of PFK in the cytosol and plastid, or PFP in the cytosol (Dennis *et al.*, 1997). This molecule is in turn degraded by aldolase to two three-carbon molecules: dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Dennis *et al.*, 1997). These triose-phosphate molecules are readily interconverted through the reversible action of triose-phosphate isomerase (Dennis *et al.*, 1997). Consequently, carbons 1, 2 and 3 of the original glucose molecule are rendered equivalent to carbons 4, 5 and 6. At this stage, triose-phosphates can enter glycolysis, through oxidation of glyceraldehyde-3-phosphate; or can be recycled via the gluconeogenic pathway. The incorporation of randomized 3-carbon molecules to hexose-phosphate, effectively causes a 50% redistribution of C-1 of the original glucose to position C-6 in the newly-synthesised 6-carbon molecule. The reverse occurs for C-6 of the original glucose. This phenomenon has led to the development of techniques which employ the labelling of tissues with glucose, specifically labelled at position C-1 or C-6. The extent of redistribution of C-1 and C-6, or *vice versa*, in the hexose moieties of sucrose gives an estimate of the contribution of triose-phosphate recycling to the hexose-phosphate pool (Hatzfeld and Stitt, 1990).

Early work by Sacher *et al.* (1963) indicated that triose-phosphate was not returned to the hexose-phosphate pool for synthesis to sucrose in sugarcane. In this study, sucrose produced after feeding tissue discs with [1-<sup>14</sup>C]-glucose was hydrolysed, and the extent of randomization of label in the glucosyl and fructosyl moieties determined as an estimate of triose-phosphate recycling to hexose-phosphate in the cytosol. Here, 95% of



radioactivity was recovered in mesaxoldehyde osazone, a derivative of carbons 1-3 of the hexoses of sucrose .

However, more recent work by Hatzfeld and Stitt (1990) revealed a triose-phosphate cycle in heterotrophic cell suspension cultures of *Chenopodium rubrum*, tissue discs of potato tuber; and maize kernels. Here, the extent of label randomization in the glucosyl and fructosyl moieties of sucrose was determined after feeding either [6-<sup>14</sup>C]-glucose or [1-<sup>14</sup>C]-glucose. There was significant randomization found, between 30%-40% in *Chenopodium*, 20%-26% in potato, and 8-12% in maize, indicative of a considerable rate of recycling of triose-phosphate to hexose-phosphate in the cytosol. In *Chenopodium rubrum*, the estimated flux from triose-phosphates was 0.15  $\mu\text{mol}$  hexose resynthesised per gram fresh weight per minute, and was 1.2 times higher than the net glycolytic flux.

In concurrent work by Hatzfeld *et al.* (1990) on *Chenopodium rubrum*, PFP was found to catalyse this rapid recycling of triose-phosphates. PFP activity was observed to triple during glucose uptake and assimilation in the cell-suspension cultures, whereas no change was observed for PFK. The activity of FBPase was 20 to 50 times lower than that of PFP, apparently ruling out a significant contribution of the this reaction in triose-phosphate recycling. However, no correlation could be made between Fru-2,6-P<sub>2</sub> concentration in the tissue, and partitioning to sucrose or starch. The presence of a PFP-PFK-mediated cycle was proposed, with its primary purpose being the maintenance of high levels of inorganic pyrophosphate (PPi) and triose-phosphate, necessary for the remobilisation of sucrose via SuSy (ap Rees *et al.*, 1985; Huber and Azakawa, 1986). This cycle was proposed to interact with the sucrose cycle, based on the observation that an increase in PFP activity and PPi corresponded to an increased rate of sucrose degradation after adding glucose to *Chenopodium* cells (Hatzfeld *et al.*, 1990). Dancer *et al.* (1990) later proposed that although these cycles may interact, they would not be stoichiometrically coupled due to the involvement of PPi in many other cytosolic reactions. Therefore, it was suggested that the PFP-mediated step served as a balancing mechanism between PPi-generating and PPi-consuming processes.



Triose-phosphate recycling has been reported for other plant species, and has been found to be between 11-14% in seeds of faba bean (Viola *et al.*, 1991), 60% in maize root tips (Dieuaide-Noubhani *et al.*, 1995), and between 18-45% in carrot (Krook *et al.*, 1998). Hill and ap Rees (1994) studied various aspects of metabolism during the mass conversion of starch to sucrose in banana ripening. In radiolabelling experiments similar to those previously described, the estimated return of carbon to hexose-phosphates from triose-phosphates was  $0.14 \mu\text{mol hexose.g}^{-1} \text{ f.wt.min}^{-1}$ . Unlike for *Chenopodium rubrum* (Hatzfeld *et al.*, 1990), this value was less than the estimated flux of glycolysis, at  $0.5\text{-}1.6 \mu\text{mol hexose.g}^{-1} \text{ f.wt.min}^{-1}$ , and lower than the OPP pathway, which was  $0.48 \mu\text{mol hexose.g}^{-1} \text{ f.wt.min}^{-1}$ . Increased exogenous glucose concentration (to 200 mM) led to a 5-fold reduction in the extent of triose-phosphate recycling from that in the control sample, as measured through feeding with  $[\text{U-}^{14}\text{C}]$ -glycerol (Hill and ap Rees, 1995). Earlier work on banana had shown that the steady-state concentration of Fru<sub>2,6</sub>P<sub>2</sub> decreases to one-half of its preclimacteric value during the onset of rapid gluconeogenesis associated with ripening (Beaudry *et al.* 1989). During the later stages of ripening, carbon flux increases relative to gluconeogenic carbon flux, accompanied by an increase in Fru<sub>2,6</sub>P<sub>2</sub> (Beaudry *et al.* 1989). This may indicate the activation of PFP, and the relative inactivation of fructose-1,6 biphosphatase (FBPase) during banana ripening.

The participation of PFP in triose-phosphate cycling was conclusively demonstrated by Hajirezaei *et al.* (1994). In this study, downregulation of PFP activity by more than 90% in transgenic potato tubers led to a decrease in the rate of triose-phosphate recycling. The extent of randomization in the glucosyl and fructosyl moieties of sucrose derived from  $[\text{1}^{14}\text{C}]$ -glucose decreased from 13.8 and 15.7% respectively in wild-type tubers, to 3.6 and 3.9% in the antisense transformant. No phenotypic change was observed in transgenics, but feeding experiments with  $[\text{U}^{14}\text{C}]$ -glucose led to a 15-fold increase in carbon-partitioning towards sucrose, and it was proposed that the reduction in starch synthesis was primarily a result of resynthesis (cycling) of sucrose in transgenic tubers.



In sugarcane, PFP activity has been reported in the sugarcane culm (Lingle and Smith, 1991; Botha *et al.*, 1996; Whittaker and Botha, 1999). Lingle and Smith (1991) reported that no developmental changes in PFP activity were observed down the sugarcane culm. However, in another study PFP activity was inversely correlated with sucrose content between different sugarcane varieties (Whittaker and Botha, 1999), indicating that the enzyme may have a regulatory role in sugarcane metabolism. A study of the mass-action ratio of the PFP reaction indicated that it is in apparent equilibrium in all internodes of the culm (Whittaker and Botha, 1997). Thus, the reaction could theoretically operate in both the forward (glycolytic) and reverse (gluconeogenic) direction. The Fru-2,6-P<sub>2</sub> concentration in all sugarcane tissues is sufficient to fully activate PFP *in vivo* (Lingle and Smith, 1991; Whittaker and Botha, 1997; Whittaker and Botha, 1999), and to significantly inhibit cytosolic FBPase when compared to the levels required for inhibition in other plant species (Kruger and Beevers, 1984). The potential for the return of carbon to hexose-phosphates from the triose-phosphate pool therefore exists in sugarcane, possibly via the enzyme PFP.

#### **2.1.4 The simultaneous synthesis and hydrolysis of starch in sink tissues**

Plant starches fall into two categories, depending upon their stability and location within the system; i.e. source versus sink (Boyer, 1996). Storage starches are found in sink tissues, in large quantities, and can be present over long periods of time, and in different stages of development (Boyer, 1996). The metabolism of storage starches is primarily unidirectional; i.e. during sink development synthesis predominates, and during sink mobilisation, degradation occurs (Boyer, 1996). On the other hand, the metabolism of transitory starch is dynamic. Synthesis and degradation occur simultaneously, resulting in alternating periods of net storage and mobilisation (Stitt and Hildt, 1981).

In sugarcane, the developmental and temporal metabolism of starch has not been addressed. In sink tissues of other plants, concomitant synthesis and degradation of starch has been reported, but the role of this cycle in metabolism is not yet known. In ripening banana tissue, metabolism of [U-<sup>14</sup>C]-glucose resulted in a small, but significant, incorporation of label to starch, whilst a 15-fold higher concomitant flux was from starch



hydrolysis (Hill and ap Rees, 1994). From calculations of ATP availability in the system, it was concluded that starch breakdown in banana is phosphorolytic, i.e. requiring the action of a phosphorylase (Steup, 1989). A later study by Hill and ap Rees (1995) showed that high levels of exogenous glucose (100 mM and 200 mM) increased the rate of starch synthesis, and inhibited its degradation.

Dancer *et al.* (1990), also reported that starch breakdown occurred during its net accumulation by cell suspension cultures of *Chenopodium rubrum*. It was proposed that small changes in the rates of synthesis or degradation in the sucrose-hexose cycle, and a possible starch-glucose cycle observed might regulate net flux to sucrose or starch. Furthermore, transient storage of starch has been described in carrot, where the removal of photoassimilate supply led to a rapid decrease in the amount of stored starch (Sturm *et al.*, 1999). This rapid turnover in the starch pool was suggested to regulate the diurnal flux of stored starch into sucrose in carrot.

Hargreaves and ap Rees (1988a) determined the extent of starch and sucrose turnover during a pulse labelling experiment with [U-<sup>14</sup>C]-glucose and chase with unlabelled glucose. Here, the sucrose pool was found to have a rapid turnover, losing 90% of incorporated label during the chase. On the other hand, turnover in the starch pool was slower, and it lost only 25% of its radioactivity in the chase. The conclusion of this work was that sucrose was a more readily available metabolic substrate than starch in pea roots.

## **2.2 Carbon partitioning to insolubles and respiration**

In sink tissues of higher plants, the principle pathways that consume hexose-phosphates are structural and storage polysaccharides, and respiration (ap Rees, 1988). However, in sugarcane, the primary sink for incoming carbon is sucrose. Accumulation of sucrose increases down the sugarcane culm with tissue maturity (Glasziou and Gayler, 1972). Because of this, studies of carbon partitioning have largely focussed on the soluble sugars, namely: fluxes between sucrose, fructose and glucose (Glasziou, 1961; Sacher *et al.*, 1963; Batta and Singh, 1986; Lingle, 1989).



Recently, carbon partitioning studies were extended to include other cell constituents (Whittaker and Botha, 1997), and it was found that sucrose accumulation in culm tissue coincided with a decreased partitioning of carbon derived from [U-<sup>14</sup>C]-glucose and [U-<sup>14</sup>C]-sucrose to water-insoluble matter, amino acids, phosphorylated intermediates and respiration. It was proposed that the sucrose accumulation might reflect a redirection of available carbon from other metabolic sinks, toward sucrose synthesis. This was an additional hypothesis to the sucrose cycle: i.e. that sucrose accumulation primarily reflects a decrease in sucrose degradation, resulting in increased storage.

### 2.2.1 The insoluble component

In the study of Whittaker and Botha (1997), the assumption was made that <sup>14</sup>C recovered in the water-insoluble fraction was primarily cell wall material. Partitioning to the insoluble fraction was at a maximum in internode 3, at 36% of total metabolised [U-<sup>14</sup>C]-glucose. This value decreased with tissue maturity, to 13% in internode 7. From these data, the cell wall fraction was proposed to contribute significantly as a competing sink with sucrose in sugarcane, since labelling of sucrose increased from 31% to 66% between internodes 3 and 7 respectively. Fibre synthesis competes with sucrose for carbon at two levels: withdrawal of available UDP-glucose, and through direct consumption of sucrose. UDP-glucose is the primary precursor of cellulose synthesis, and also very likely the compound from which nucleoside diphospho-sugars used in non-cellulose structural polysaccharides are derived (ap Rees, 1988). Evidence has accumulated that UDP-glucose enters cellulose synthesis primarily through cleavage of sucrose via SuSy (Amor *et al.*, 1995; Delmer *et al.*, 1995). Labelling experiments with [<sup>14</sup>C]-sucrose led to a significant labelling of structural polysaccharides in pea roots (Dick and ap Rees, 1976) and cotton seeds (Pillonel and Meier, 1985). Furthermore, feeding of pea roots with [<sup>14</sup>C]-sucrose led to a 6-fold higher incorporation of <sup>14</sup>C to fibre than with either [<sup>14</sup>C]-glucose or [<sup>14</sup>C]-fructose (Hargreaves and ap Rees, 1988b).

In sugarcane, total fibre content is at a maximum of 50% on a dry mass basis in immature sugarcane internodes, decreasing to 34% in mature tissue (Botha *et al.*, 1996). On the other hand, the contribution of protein and starch is small by



comparison, at 2% (Botha *et al.*, 1996) and 0.1% (Wood, 1962) of dry mass respectively. It would, therefore, be expected that their contribution to the insoluble fraction in radiolabelling experiments is low.

However, in sugarbeet tubers labelled with [U-<sup>14</sup>C]-glucose, further fractionation of the insoluble component revealed a significant protein fraction (Giaquinta, 1979). Allocation to protein was highest in immature sugarbeet tissue, consuming 40% of total <sup>14</sup>C uptake. This amount decreased significantly in mature tissue, to 5%, whereas sucrose production consumed approximately 80% (Giaquinta, 1979). After sucrose, the greatest proportion of radioactivity was incorporated into protein, followed by starch, structural material and lipids. Therefore, protein synthesis could potentially contribute to total metabolism in a high-sucrose storing system, such as sugarcane.

Furthermore, cell suspension cultures of sugarcane grown under nitrogen limitation accumulate sucrose as amino acid availability decreases (Wendler *et al.*, 1990; Veith and Komor, 1993). It was later proposed that the amino acid pool, or products thereof may be linked to the regulation of carbon partitioning between sucrose synthesis and degradation (Komor, 1994). In the light of this, the potential contribution of protein to the insoluble fraction in sugarcane needs to be revisited.

### **2.2.2 The contribution of respiration to sugarcane metabolism**

The respiratory pathway not only supplies the end-products of ATP and reductant for use in metabolism, but has as its primary purpose the function of providing intermediates for biosynthetic pathways (Dennis *et al.*, 1997). Intermediates of the TCA cycle are either completely oxidised for the provision of energy, or partially oxidised for amino acid or organic acid biosynthesis (Plaxton, 1996). In sugarcane culm tissue, the study by Whittaker and Botha (1997) indicated that partitioning of carbon via the respiratory pathway is dominated by the biosynthetic component. The partitioning of carbon to catabolic respiration was found to be between 24 and 33% of total respiration, with the remainder entering anabolic pathways. The anabolic component was estimated from the incorporation of <sup>14</sup>C into CO<sub>2</sub>, along with organic acid and amino acid biosynthesis, but



did not include the protein fraction. Therefore, total anabolic metabolism may have been underestimated in this case. Further details of the study revealed that incorporation of  $^{14}\text{C}$  to respiration as a proportion of total  $[\text{U-}^{14}\text{C}]$ -glucose uptake decreased with increasing partitioning to sucrose. In internodes 2, 3 and 7, respiration constituted 27, 19 and 15% of total  $^{14}\text{C}$  incorporation respectively. This implicated the respiratory pathway as a major competitor for carbon that could potentially be allocated to sucrose. However, analysis of the metabolite ratios of the PK, PFK, PFP and FBPase reactions indicated that fine metabolic regulation of glycolysis did not change with tissue maturity.

Studies with cell suspension cultures of sugarcane indicated that catabolic respiration rate, as measured by  $\text{O}_2$  uptake, exceeded sucrose accumulation rates by 2-fold (Wendler *et al.*, 1990). The accumulation of sucrose induced by nitrogen-limiting conditions accompanied a reduction in respiration, to less than half that estimated under unlimiting growth conditions (Veith and Komor, 1993). However, no evidence indicated that sucrose storage was driven by a restriction in respiration, in that maximal  $\text{O}_2$  uptake occurred at the onset of sucrose storage, along with maximal activity of glycolytic enzymes and the content of glycolytic intermediates (Wendler *et al.*, 1990).

### **2.3. Compartmentation in heterotrophic tissues: the plastid**

Plastids in heterotrophic tissues have various functions, including metabolic steps in the pathways of, glycolysis, the OPP (Dennis, 1997), nitrogen reduction and in synthesis of amino acids, nucleic acids, fatty acids, pyrimidines, terpenes and tetrapyrrole (Mullet, 1997). Starch synthesis is exclusively located in plastids, and these are specialised to form amyloplasts in starch-storing tissues (Preiss, 1982). At an ultrastructural and physiological level, sugarcane is markedly different as a storage tissue from starch-storing sink tissues. In sucrose-storers, such as sugarcane and sugarbeet the vacuole becomes larger as the tissue matures and functions primarily in sucrose storage, acquiring over 90% of the cell volume (Ehwald *et al.*, 1980). Starch-storers, on the other hand, have a large central vacuole, and generally contain many amyloplasts. (Zamski, 1996). However, cell ultrastructure is altered in sugarcane cell suspension cultures, which display 40% lower vacuolisation than in culm tissue (Komor, 1994). Furthermore, starch content was found



to predominate under unlimited growth conditions, and scanning electron microscopy revealed the presence of starch-storing amyloplasts (Veith and Komor, 1993). Thus, plastid metabolism can potentially influence sugarcane metabolism at specific developmental stages, or growth conditions. To date, however, it has not been characterized in sugarcane.

Because plants are sessile, there is a need for a high degree of homeostatic flexibility, and this is found at a cellular level in the duplication of metabolic pathways and enzymes; as well as division of metabolic labour through compartmentation (Dennis *et al.*, 1997). Two cytosolic pathways have complementary pathways in the plastid, namely: glycolysis (Miernyk and Dennis, 1982; MacDonald and ap Rees, 1983a; Journet and Douce, 1985; Echeverria *et al.*, 1988) and the OPP pathway (Dennis and Miernyk, 1982; Bowsher *et al.*, 1989), although some reports have claimed that the OPP pathway is exclusive to plastids (Dieuaide-Noubhani *et al.*, 1995; Schnarrenberger *et al.*, 1995; Krook *et al.*, 1998).

Given the report of the significant contribution of respiration to metabolism in sugarcane culm tissue, feeding into smaller sinks of amino acid and lipid biosynthesis (Whittaker and Botha, 1997), it would be expected that plastid metabolism contributes to this pathway. The estimation of the OPP pathway contribution to metabolism through CO<sub>2</sub> release patterns has not been possible due to interference of CO<sub>2</sub> from pentan synthesis in sugarcane (Whittaker, 1997). Presently, the harsh shearing techniques necessary for the homogenisation of sugarcane tissue prevent the isolation of intact plastids by density gradient centrifugation (MacDonald and ap Rees, 1983a; Echeverria *et al.*, 1985). Thus, other techniques are necessary for the study of plastid metabolism *in vivo*. Since starch and lipid are end-products of plastid-based processes, these components could theoretically indicate the significance of this compartment as a carbon sink. Partitioning of carbon to lipid in sugarcane is low, at a maximum of 2% of total <sup>14</sup>C uptake from [U-<sup>14</sup>C]-glucose in mature tissue (Whittaker and Botha, 1997). However, carbon partitioning of [U-<sup>14</sup>C]-glucose to starch has not yet been characterized in sugarcane. Should this pool be a potential competitor for carbon with sucrose, it is



necessary to determine the point at which the plastid competes for intermediates of cytosolic pathways. Thus the route of carbon entry to the plastid as hexose-phosphate or possibly, triose-phosphate is a key question in sugarcane metabolism.

### **2.3.1 Route of carbon uptake by amyloplasts for starch synthesis**

Communication between the cytosol and plastid takes place via metabolite translocators in the plastid envelope (Flügge and Heldt, 1991). The route of carbon entry into the amyloplast for starch synthesis was initially the source of much debate. The original hypothesis that triose-phosphates were the primary precursors of starch synthesis in the amyloplast was backed by the finding that all the enzymes necessary for the gluconeogenic conversion of triose to starch were present in isolated amyloplasts of a number of tissues, namely: maize endosperm (Echeverria *et al.*, 1988), cauliflower buds (Journet and Douce, 1985), soybean suspension cultures (MacDonald and ap Rees, 1983a) and potato tubers (Mohabir and John, 1988). However, these results were brought into question on the basis that PFP contamination of the extracts could have occurred, giving an apparent FBPase activity (Entwhistle and ap Rees, 1988). Later research further indicated that plastidic FBPase is absent or low in starch-storing tissues (Entwhistle and ap Rees, 1988, Entwhistle and ap Rees, 1990; Neuhaus *et al.*, 1993b). However, significant plastidic FBPase was demonstrated in barley leaf etioplasts (Neuhaus *et al.*, 1993b), which were also shown to preferentially incorporate DHAP as a precursor for starch synthesis (Batz *et al.*, 1992). Since etioplasts are plastids grown under dark conditions, which differentiate into chloroplasts upon exposure to light (Newcomb, 1997), it is apparent that they retain a functional chloroplastic TPT.

A further research area undertaken was the investigation of specific precursors to the plastid for starch metabolism. MacDonald and ap Rees (1983b) demonstrated that the labelling of starch in suspension cultures of soybean was appreciable from fed [U-<sup>14</sup>C]-glycerol over that of [U-<sup>14</sup>C]-sucrose, as evidence for the conversion of triose-phosphates to starch. This was initially challenged by Keeling *et al.* (1988), who fed developing wheat grains [<sup>13</sup>C]-glucose, specifically labelled at position C-1 or C-6. They demonstrated that a low level of randomization of carbon from position C-1 to C-6 in



starch glucose occurred, indicating the preferential incorporation of carbon into starch via hexoses as opposed to triose-phosphate. Subsequently, the body of evidence favouring this hypothesis has accumulated from similar experiments using specifically labelled [ $^{13}\text{C}$ ]-glucose or [ $^{14}\text{C}$ ]-glucose, for various plant tissues: potato (Hatzfeld and Stitt, 1990; Viola *et al.*, 1991), faba bean (Viola *et al.*, 1991), *Chenopodium rubrum* (Hatzfeld and Stitt, 1990), maize endosperm (Hatzfeld and Stitt, 1990), carrot (Krook *et al.*, 1998) and maize root tips (Dieuaide-Noubhani *et al.*, 1995). Furthermore, Hill and ap Rees (1995) in their work on ripening bananas showed that starch was not formed following labelling with [U- $^{14}\text{C}$ ]-glycerol, although [U- $^{14}\text{C}$ ]-glucose led to 5% of total radioactivity appearing in starch.

In isolated amyloplasts, it was initially demonstrated that triose-phosphate could be transported by the amyloplast envelope of non-photosynthetic tissues (Liedvogel and Kleinig, 1980; Emes and Traska, 1987; Alban *et al.*, 1988). Yet again, subsequent studies revealed that although triose can be transported by the amyloplast membrane, it does not appear to be a precursor for starch synthesis. Here, the preferential metabolite transported and assimilated by amyloplasts was found to be glucose-6-P in pea cotyledons (Hill and Smith, 1991), pea roots (Borchert *et al.*, 1989) and cauliflower bud amyloplasts (Neuhaus *et al.*, 1993a). In some instances the transported hexose was reported to be glucose-1-P in wheat endosperm (Tyson and ap Rees, 1988), or ADP-glucose in sycamore suspension cultures (Pozueta-Romera *et al.*, 1991; Okita, 1992).

Recently, molecular techniques have provided further evidence for glucose-phosphate uptake by the plastid. Kammerer *et al.* (1998) isolated a plastidic glucose-6-P/Pi translocator (GPT) from heterotrophic maize endosperm tissue. The TPT gene was found predominantly localised in photosynthetic tissues, whereas the GPT was primarily expressed in heterotrophic tissues. The mode of action was shown to be a 1:1 counter-exchange of glucose-6-P with Pi and triose-phosphate. Other authors have also indicated that counter-exchange of triose-phosphate with hexose-phosphate, and the necessity of triose-phosphate and ATP in promoting starch synthesis in isolated amyloplasts (Batz *et*



*al.*, 1994; Flügge, 1995; Hill and Smith, 1991; Neuhaus *et al.*, 1993a, b; Tetlow *et al.*, 1996; Tyson and ap Rees, 1988).

However, in tomato fruits and potato tubers, the expression of a TPTgene has been reported (Schünemann *et al.*, 1996), in addition to the GTP gene (Shünemann and Borchert, 1994). TPT cDNA from potato tubers and tomato fruits showed high homology with the sequence of potato leaf cDNA (Schünemann *et al.*, 1996). Therefore, the possibility for the movement of triose-phosphates into amyloplasts of sink tissues does exist.

## CHAPTER 3

### TISSUE DISCS AS AN EXPERIMENTAL SYSTEM FOR METABOLIC FLUX ANALYSIS IN THE SUGARCANE CULM

(Submitted to the South African Journal of Botany)

#### 3.1 ABSTRACT

This study investigated the suitability of tissue discs as an experimental system to determine carbon flux in sugarcane. The number of cells per tissue disc derived from young and older internodes differed largely. However, protein content per cell was constant on a disc basis and is therefore most probably the best unit to accurately express metabolic flux. The rate of glucose uptake during the 3 h incubation period was linear, and in young internodes up to 25% of the supplied glucose was taken up. This indicates that the active uptake mechanism is probably saturated even at 4 mM glucose. No significant changes in the glucose, fructose and sucrose pools of tissue discs occurred during the labelling period, which suggests that the uptake of sugars from the medium was adequate to balance the demand by the tissue. This also indicates that no major disturbances in the *in vivo* metabolism occurred. During the 3 h labelling period the rate of  $^{14}\text{CO}_2$  release became linear, indicating that the system was approaching isotopic steady state between the external and internal glucose pool; and the respiratory processes involved in  $\text{CO}_2$  release. Despite this observation, the specific activity of the internal glucose pool was much lower than that of the external glucose pool. This is probably as a result of the cellular compartmentation of glucose, with most of it present in a cellular compartment making a small contribution in the provision of substrate for respiration.



### 3.2 INTRODUCTION

The ripening of sugarcane is associated with an increase in sucrose concentration in mature stalk tissue (Glasziou and Gayler, 1972). This phenomenon has been extensively studied in the past. Most of these studies have concentrated on aspects such as sugar levels and some of the enzymes involved in primarily sucrose synthesis and breakdown. Despite these numerous investigations, the biochemical basis for sucrose accumulation in sugarcane is still poorly understood (Moore, 1995 and references therein). Further progress in understanding the processes which are involved and controlling sucrose accumulation in the sugarcane culm will be dependent on more insight into metabolic flux in the tissue.

It is extremely difficult to investigate any aspect of metabolism in the internodal tissue of the sugarcane culm. This is due to the hard rind, fibrous nature of the tissue and size of the culm (more than two meters long and several centimetres thick). Since the whole culm constitutes a storage organ, it cannot be sampled for analysis without sacrificing the entire plant (Moore, 1995). As an experimental system it therefore presents several limitations, and studies on intact material have been limited. In order to overcome these problems, two different approaches have been followed, namely: tissue discs of internodal tissue, (Bialeski, 1962; Glasziou, 1961; Sacher *et al.*, 1963; Hawker, 1965; Hawker and Hatch, 1965; Bowen, 1972; Bowen and Hunter, 1972; Glasziou and Gayler, 1972) and cell suspension cultures derived from callus material (Maretzki and Thom, 1972; Komor *et al.*, 1981; Wendler *et al.*, 1990; Goldner *et al.*, 1991). Both these systems allow more detailed analysis, such as short-term radiolabelling experiments.

However, both approaches have been criticised in the past. Some limitations previously pointed out in the tissue disc system include: wound reactions caused by tissue slicing, long diffusion pathways of labelled sugars in tissue discs, and the inability to repeatedly and precisely associate a morphological stage of internode development with sucrose levels (Moore and Maretzki, 1996). However, sugarcane cell suspension cultures show atypical sucrose metabolism, in which 1-6% of total carbon is allocated to sucrose (Veith



and Komor, 1993) compared with 66% in tissue discs of mature sugarcane tissue (Whittaker and Botha, 1997). Furthermore, vacuolization is 40% lower in cell suspension cultures than in culm tissue (Komor, 1994), potentially altering the correlation of enzyme activity with sucrose metabolism from that found *in vivo* (Ebrahim *et al.*, 1999).

Therefore, despite some of the limitations associated with the tissue disc system, it would appear that it best approximates the intact sugarcane culm. Careful selection of osmotic conditions of the incubation medium (Lingle, 1989) and washing after wounding (Lingle, 1989; Whittaker and Botha, 1996) significantly contribute to eliminating some of the potential problems with tissue discs. Short-term labelling experiments on tissue-discs, (Botha *et al.*, 1996; Whittaker and Botha, 1997; Vorster and Botha, 1999) can also help to reduce possible disruption of metabolism caused by depletion of substrates after long incubation periods.

A prerequisite for the quantification of fluxes in metabolic pathways *in vivo* using labelling experiments, and mathematical modelling is a system that approximates an internal steady state (Salon and Raymond, 1988). A requirement for experimental steady-state conditions is the equilibration of internal metabolic pools with the substrate in the external medium. The time necessary to reach steady state depends on the ratio of metabolic fluxes to the pool sizes of intermediates, such that the greater this ratio the more rapidly steady state is reached (Salon and Raymond, 1988). Therefore, it is possible that in a short-term uptake experiment, uptake might be so slow that isotopic steady state may only be reached after long incubation periods. None of these parameters have previously been analysed in the sugarcane tissue disc system.

Comparison of data from previous studies is difficult, because fresh weight, a whole tissue disc basis (Glasziou, 1960; Bielecki, 1962; Sacher *et al.*, 1963; Bowen, 1972; Bowen and Hunter, 1972; Gayler and Glasziou, 1972) or protein (Whittaker and Botha, 1996; Vorster and Botha, 1999) have been used as a basis to normalise data. Since the number of cells per internode reach a maximum early in internode development, with the



later expansion being as a result of cell enlargement (Komor, 1994; Botha *et al.*, 1996) it is evident that cell number per tissue disc will considerably differ.

Here we report that the protein content per cell for tissue discs derived from different internodes is constant. Therefore, it is proposed that the preferable unit to express metabolism is tissue protein content. In addition, it is shown that internal sugar pools remain constant during a 3 h labelling period, and that the cytosolic metabolic compartment probably approached isotopic steady state. It is also evident that at least two separate pools of hexoses are present in the tissue.

### 3.3 MATERIALS AND METHODS

#### 3.3.1 Biochemicals

All coupling enzymes, cofactors and substrates used for sugar determinations were purchased from Sigma-Aldrich S.A. (Pty) Ltd. (P.O. Box 1460, Brackenfell, 7561, South Africa) or Roche Biochemicals (9 Will Scarlet Road, Randburg, 2125, South Africa). The [U-<sup>14</sup>C]-glucose was from Amersham International (P.O. Box 23298, Claremont, 7735, South Africa). Sep-pak 1ml (100mg) Alumina A and TC-18 cartridges were purchased from Waters Corporation (34 Maple Street, Milford, Massachusetts, 01757, USA). All other solvents and biochemicals were of analytical grade.

#### 3.3.2 Tissue selection and preparation

Mature, non-flowering sugarcane plants of variety N19 were randomly sampled between 09:00 h. and 10:00 h. from Welgevallen experimental farm in Stellenbosch, South Africa. The natural break-point of the sugarcane stalk was defined as internode 3, and internodes below this point were numbered sequentially. Internodes 3 and 4 represent immature tissue, internodes 5-7 actively accumulate sucrose, and 8-10 represent mature tissues which have accumulated high levels of sucrose. Internodes were selected for analysis, excised and longitudinal cores were sectioned from the tissue mid-way between the core and periphery of the internode, using a cork borer 6 mm in diameter. One mm slices were sectioned using a hand microtome, from internodes at different stages of maturity. For the



determination of cell number per tissue disc, transverse sections were cut from discs, stained with 0.05% (m/v) toluidine blue, and viewed under a light microscope. Repeated counts were made for cell number across a 1x1 mm section, and total cell number then estimated for a calculated disc volume. Protein in the tissue was assayed according to the method of Bradford (1976), using gamma-globulin as a standard.

### 3.3.3 [ $^{14}\text{C}$ ]-radiolabelling experiments

In metabolic studies, sectioned tissue discs were immediately placed in 50 ml buffer containing 25 mM K-Mes (pH 5.7), 250 mM mannitol and 1 mM  $\text{CaCl}_2$  (Lingle, 1989; Whittaker and Botha, 1997; Vorster and Botha, 1999) and washed 15 min. Excess buffer was removed from tissue discs, 20 of which were then transferred to 1.5 ml buffer containing 25 mM K-Mes (pH 5.7) and 250 mM mannitol in 250 ml Erlenmeyer flasks (Lingle, 1989). For radiolabelling experiments, 5 mM glucose, 5 mM fructose and [ $\text{U-}^{14}\text{C}$ ]-glucose were included in the incubation buffer, to give a final specific activity of 23 Bq  $\text{nmol}^{-1}$ . For experiments with unlabelled sugars, 5 mM glucose and fructose alone were added to the incubation medium. Discs were vacuum-infiltrated for approximately 5 seconds and the flasks sealed with rubber stoppers. Samples were incubated for 3 h on a rotary shaker at 115 rpm.  $^{14}\text{CO}_2$  released over the incubation period was collected in 500  $\mu\text{l}$  12% (m/v) KOH contained in a central well. An aliquot of the incubation buffer was diluted 1:4 with Ultima Flo<sup>TM</sup> M scintillation cocktail, and counted for 5 min in a Beckman LS 1801 scintillation counter to determine total  $^{14}\text{C}$  uptake. After incubation, discs were washed three times (2 min) in 15 ml ice-cold 1% (m/v)  $\text{CaCl}_2$ . The first two washes removed 98% of unincorporated label.

### 3.3.4 Extraction of the EtOH-soluble component

Discs were transferred to 20 ml 80% (v/v) EtOH in sealed 50 ml centrifuge tubes, and incubated in an 80°C water-bath overnight. The extracts were centrifuged at 12 000 g in a Sorvall SLA-600TC rotor for 15 min at 25°C. The EtOH-soluble supernatant was removed and reduced completely in a vacuum centrifuge at 25°C. Extracts were resuspended in HPLC-grade  $\text{H}_2\text{O}$ . The average recovery of label was 84%, 97% and 95% for internodes 3, 6 and 9 respectively.



### 3.3.5 HPLC separation of [<sup>14</sup>C]-sugars

For the determination of [<sup>14</sup>C]-labelled sugars by HPLC, samples were passed through Alumina A and TC-18 cartridges in tandem. Sugars were fractionated by HPLC (Shimadzu SCL-10AVP system). Samples labelled with [U-<sup>14</sup>C]-glucose were separated using a Supelco™ LC-NH<sub>2</sub> column, over 20-25 min with 80% (v/v) acetonitrile at 1.2 ml min<sup>-1</sup>. <sup>14</sup>C in glucose, fructose and sucrose was determined by liquid scintillation spectroscopy (Radiomatic A-500 inline radio-chromatography detector). Peak elution times were obtained using standards of [U-<sup>14</sup>C]-glucose, [U-<sup>14</sup>C]-fructose and [U-<sup>14</sup>C]-sucrose. The peak areas of the sugars fractionated by HPLC were integrated using a Shimadzu Class-VP software package.

### 3.3.6 Enzymatic assay of sucrose, glucose and fructose

Unlabelled sucrose, glucose and fructose were assayed according to the procedure of Bergmeyer and Bernt (1974). Aliquots of the samples were pipetted into the wells of a microplate. The production of NADPH was measured at 340 nm after the sequential conversion of glucose with 0.6 U hexokinase and 0.3 U glucose-6-P dehydrogenase. This reaction was carried out for 15 min at 25°C with 0.5 mM NADP and 1.8 mM ATP in triethanolamine buffer (pH 7.6). Glucose from sucrose was assayed following hydrolysis with 47 U ml<sup>-1</sup> invertase in citrate buffer (pH 4.6) for 10 min at 37°C. Fructose was converted to glucose with 5.6 U ml<sup>-1</sup> phosphoglucose isomerase. The recovery of standard samples of sucrose, glucose and fructose was 108 ± 4%, 105 ± 0.4% and 100 ± 2% respectively.

### 3.4 RESULTS

#### 3.4.1 Number of cells, fresh weight and protein content of discs

direct count of cells in tissue-disc sections was carried out, and expressed as  $10^6$  cells per unit fresh weight. This value decreased markedly as the tissue matured (Table 3.1) which is consistent with previous observations that growth in the internodes during internodal maturation is primarily due to cell enlargement. On average there was an 8-cell per mm layer in discs derived from the youngest internode and this decreased to approximately 5 per mm in the older internodal discs. Protein content per unit fresh weight also decreased with tissue maturity (Table 3.1). However, values of protein content expressed on a cell basis, showed no significant differences as estimated by a t-test ( $P = 0.05$ ) (Table 3.1). This most probably implies that cell enlargement is due to an increase in the vacuolar volume.

**Table 3.1** Cell number and protein content in tissue discs (1 disc =  $28 \text{ mm}^3$ ; each value is the mean  $\pm$  SD of three separate samples)

Internode number	$10^6$ cells disc <sup>-1</sup>	$10^6$ cells g <sup>-1</sup> fresh wt	mg protein g <sup>-1</sup> fresh wt	mg protein $10^6$ cells <sup>-1</sup>
3	$0.07 \pm 0.01$	$2.30 \pm 0.21$	$2.05 \pm 0.26$	$0.90 \pm 0.19$
5	$0.02 \pm 0.004$	$0.52 \pm 0.07$	$0.41 \pm 0.03$	$0.79 \pm 0.06$
8	$0.02 \pm 0.002$	$0.52 \pm 0.09$	$0.50 \pm 0.06$	$1.00 \pm 0.27$

#### 3.4.2 Maintenance of internal sucrose, glucose and fructose pools

Concentrations of sucrose, glucose and fructose in tissue discs were determined prior to labelling, and at the end of a 3 h incubation in buffer containing 5 mM glucose and fructose. A paired t-test of the sugar concentrations before and after incubation revealed that no significant change occurred in both immature and mature internodes ( $n = 5$ ,  $P = 0.05$ ). A paired t-test indicated that no significant change in the specific activity of the labelling medium occurred in the labelling period ( $n = 3$ ,  $P = 0.05$ ). Following feeding with  $[\text{U-}^{14}\text{C}]$ -glucose, no labelled sucrose or fructose were returned to the medium. Firstly, this indicates that synthesis and/or breakdown of sucrose did not occur in the



medium as a result of the release of cell constituents from cell breakage. Secondly, it demonstrates that leakage of sucrose and fructose from the cells is occurring at a slow rate.

### 3.4.3 Uptake of sugar and metabolism of labelled glucose

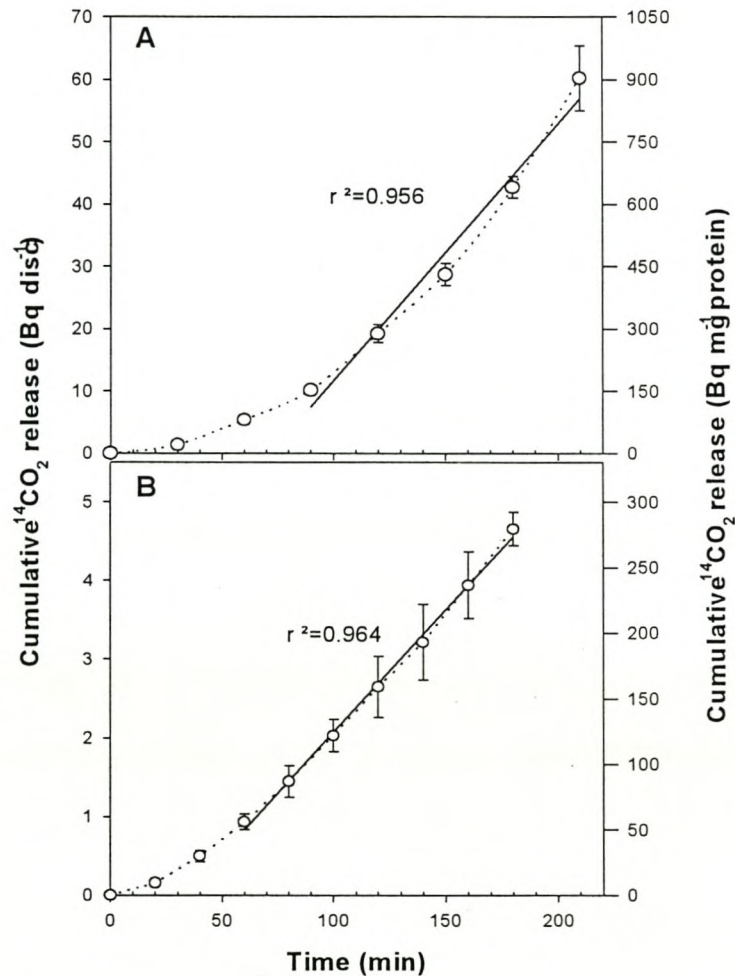
The uptake of glucose by the tissue discs (on a fresh mass or tissue disc basis) is much higher in the young than older internodes (Table 3.2). The tissue discs from the young internodes took up 24% of the supplied glucose and the internode 9 discs, only 10% (Table 3.2). Thus in internode 3, total uptake by the tissue was highest, giving a final external glucose concentration of 4 mM. However, expression of uptake on a protein basis showed that the highest uptake occurred in cells of internode 6 (Table 3.2). It is this region of the culm that also exhibits the highest rate of sucrose accumulation (Whittaker and Botha, 1997). The uptake of isotope during the labelling period was linear for all the internodal tissue (result not shown). This clearly shows that even in internode 3, with external glucose at 4 mM, the glucose transport-system is saturated, i.e. it is functioning at  $V_{max}$ .

**Table 3.2** Total uptake of glucose after 3h incubation in 23 Bq.nmol<sup>-1</sup> glucose (1 disc = 38 mm<sup>3</sup>; each value is the mean  $\pm$  SD of three separate samples)

Internode number	Uptake of glucose			
	% uptake of total isotope	nmol 20 discs <sup>-1</sup> min <sup>-1</sup>	nmol g <sup>-1</sup> fresh wt min <sup>-1</sup>	nmol mg <sup>-1</sup> protein min <sup>-1</sup>
3	23.85 $\pm$ 2.26	9.63 $\pm$ 1.02	14.42 $\pm$ 1.09	8.77 $\pm$ 0.64
6	16.77 $\pm$ 2.15	6.47 $\pm$ 0.80	9.13 $\pm$ 1.32	12.12 $\pm$ 2.23
9	10.51 $\pm$ 0.41	3.89 $\pm$ 0.14	5.22 $\pm$ 0.17	7.29 $\pm$ 0.95

The pattern of CO<sub>2</sub> release initially increased exponentially, but apparently became linear after 90 min in internode 4 tissue, and after 60 min in internode 9 (Figure 3.1. A, B). This was confirmed by a linear regression analysis on the rate after 90 and 60 minutes in the respective tissues. For the internode 4 tissue  $R^2 = 0.956$  ( $P < 0.001$ ,  $n = 21$ ), and for internode 9 tissue  $R^2 = 0.964$  ( $P = 0.001$ ,  $n = 15$ ) was found. The rate of <sup>14</sup>CO<sub>2</sub> release on a disc basis was 10 times higher in young internodal tissue than in mature tissue

(internode 9) (Figure 3.1 A,B). However, when expressed on a protein basis this difference was only 3-fold. The very rapid attainment of a linear CO<sub>2</sub> release pattern indicates that diffusion of the glucose, even in the discs derived from the young internodes (a layer of 8 cells) was not hindered by long diffusion distances.



**Figure 3.1:** Cumulative release of <sup>14</sup>CO<sub>2</sub> from tissue discs fed [U-<sup>14</sup>C]-glucose. A. internode 4 tissue, B. internode 9 tissue (n = 3). Solid line indicates the results of a linear regression analysis on the data after 90 minutes (A), and after 60 minutes (B), of CO<sub>2</sub> release (each value is the mean ± SD of three separate samples).



The specific activity of endogenous glucose varied between internodes, and was significantly lower than the external glucose specific activity (Table 3.3). The specific activity of sucrose was 18-fold higher than that of glucose in internode 3 tissue (Table 3.3). In internode 9 the reverse was apparent. The sucrose content of the tissue increased to 45-fold that found in internode 3, resulting in a sucrose specific activity 3 times lower than that of glucose (Table 3.3).

**Table 3.3.** Concentration, and specific activity of endogenous pools after 3 h labelling with [U-<sup>14</sup>C]-glucose (each value is the mean  $\pm$  SD of three separate samples)

Internode number	Sugar concentration ( $\mu\text{mol mg}^{-1}$ protein)			Specific Activity Bq nmol <sup>-1</sup>	
	Endogenous glucose	Endogenous sucrose	Exogenous glucose	Endogenous glucose	Endogenous sucrose
3	7.51 $\pm$ 3.49	2.10 $\pm$ 0.73	23.05 $\pm$ 1.36	0.07 $\pm$ 0.02	1.25 $\pm$ 0.16
9	5.34 $\pm$ 2.39	90.18 $\pm$ 16.60	23.05 $\pm$ 1.36	0.17 $\pm$ 0.08	0.05 $\pm$ 0.01

### 3.5 DISCUSSION

#### 3.5.1 Units for the measurement of metabolism

The correlation of protein content and cell number in internode discs indicates that protein content can be used to more accurately represent cellular metabolism than fresh mass. In the past, various measures have been used for the expression of metabolic data in sugarcane, namely: dry mass (Veith and Komor, 1993; Botha *et al.*, 1996; Whittaker and Botha, 1997; Lingle, 1999), fresh mass (Glasziou, 1961; Sacher *et al.*, 1963; Batta and Singh, 1986; Wendler *et al.*, 1990; Goldner *et al.*, 1991), protein content (Whittaker and Botha, 1997; Vorster and Botha, 1999) or on a whole-internode basis (Botha *et al.*, 1996; Lingle, 1999), which have produced conflicting results.



This discrepancy is apparent in the representation of glucose uptake and  $^{14}\text{C}$  release on a disc basis (fresh mass) or per mg protein. A study by Glasziou (1961) showed that glucose uptake on a dry weight basis decreased with tissue maturity. The current data show a similar trend on a fresh weight basis. However, the differing pattern produced by representing glucose uptake on a protein basis indicates that maximal glucose uptake occurs during elongation, rather than in immature tissue. This is in agreement with the finding that maximal sucrose accumulation takes place at this stage (Whittaker and Botha, 1997). Similarly, the expression of  $^{14}\text{CO}_2$  release on a tissue disc (fresh mass) basis would give a large overestimation of the reduction in respiratory carbon flow that occurs during internodal maturation.

### **3.5.2 Establishment and maintenance of steady state conditions**

Under steady state conditions, all reactions in the pathway proceed at the same rate, and all metabolite contents and enzyme activities are invariant over the period of study (ap Rees and Hill, 1994). In a tissue like sugarcane where a large portion of the sugars are probably in the vacuole it is virtually impossible to reach isotopic steady state in a reasonable short period. The release of  $^{14}\text{CO}_2$  initially accelerated, as would be expected, since the intermediate pools in the glycolytic pathway sequentially accumulate label. As these pools approach isotopic equilibrium with the feeding pool of radiolabelled glucose, the rate of  $^{14}\text{CO}_2$  release becomes constant. There are three important conclusions to be made from this data. Firstly, it is evident that diffusion of the external sugar to the cell surface for uptake, even in the young internodes is not limiting, as it would be impossible to reach a linear pattern of  $\text{CO}_2$  release so rapidly. Secondly, isotope (sugar) availability did not become limiting, as uptake remains constant during the labelling period. Thirdly, continuous dilution of the high specific activity sugar pools in the cytosol from the low specific activity storage pools in the vacuole probably occurs at a very low rate.

The  $\text{CO}_2$  release is an indication of glycolysis, the OPP pathway or pentan synthesis (ap Rees and Hill, 1994) and we therefore assume that all these reaction are approaching steady state with the cytosolic glucose pool during the 3 h labelling period. However, it is conceivable that one or more of these routes are occurring at a very slow rate and as a result their contribution to total  $\text{CO}_2$  is too small to measure accurately. Previous work has



indicated that significant labelling of the organic acid and amino acid pools occur (Whittaker and Botha, 1996) so we assume that at least isotopic steady state between the cytosolic glucose and the glycolytic intermediates has been reached. It should therefore be possible to use the external specific activity of glucose in the medium to determine carbon flux in the tissue discs. However, this will not allow for the calculation of fluxes in sub-cellular compartments, since it is possible that both cytosolic and plastidic glycolysis (ap Rees and Hill, 1994) can occur in the sugarcane culm.

The fact that the specific activity of the external glucose is much higher than that of the internal glucose pools, despite the apparent isotopic steady state at least in part indicate sub-cellular pools of glucose. The data would be consistent with most of the glucose, especially in the young internodes being in the vacuole which can be up to 90% of the cell volume (Komor, 1994). The labelling patterns observed in glucose and sucrose is also consistent with the findings by Preisser *et al.* (1992), who showed that glucose and fructose accumulated exclusively in the vacuole in the early stages of sugarcane cell suspension culture. Toward the end of the culture period, i.e. maturity, equal distribution of glucose and fructose was found between the cytosol and the vacuole. Sucrose was reported not to be actively stored in the vacuole, but represented an equilibration of sucrose between the cytosol and the vacuole, such that regulation of the cytosolic sucrose pool might influence sucrose storage in the vacuole. From this, it is evident that the use of the internal specific activity of glucose should be avoided in the calculation of metabolic flux in sugarcane.

### 3.6 CONCLUSIONS

It is evident that radiolabelling of tissue discs is an effective way to assess sugarcane metabolism *in vivo*. There are no significant changes in the sugar pools of the tissue discs, when incubated in isosmotic buffer containing 23 Bq.nmol<sup>-1</sup> glucose and 5 mM fructose. Furthermore, no efflux of radiolabelled products was observed in the buffer following labelling, indicating integrity of the symplast. It is proposed that the disruption of tissue during sectioning, previously used as a critique of the method, does not obviously alter metabolism.

In addition, the estimation of metabolic flux on a protein basis, removes the error incurred by changes in cell size with tissue maturity. For calculations of metabolic flux, the exogenous specific activity of the labelling substrate can be used. The reason for this is two-fold. Firstly, glucose, fructose and sucrose appear to be compartmented *in vivo*, which would cause an underestimation of the specific activity of a cytosolic glucose pool for metabolic flux analysis. Secondly,  $^{14}\text{CO}_2$ -release data indicate that the cytosolic intermediates of respiration approach isotopic equilibrium during the labelling period. In turn, this means that the cytosolic glucose pool is close to isotopic equilibrium with the exogenous glucose pool.



## CHAPTER 4

### CARBON ALLOCATION TO THE INSOLUBLE FRACTION, RESPIRATION AND TRIOSE-PHOSPHATE CYCLING IN SUGARCANE

(Submitted to *Physiologia Plantarum*)

#### 4.1 ABSTRACT

The changes in carbon allocation to sucrose, hexoses, fibre and respiration were investigated in developing internodes of sugarcane. Radiolabelling studies were done on internode 3, 6 and 9 tissue, representing three stages of increasing maturity. Carbon partitioning into sucrose increased from 34% of total  $^{14}\text{C}$  uptake in internode 3, to 61% in internodes 6 and 9. In immature tissue, the protein and fibre components were the dominant competing sinks with sucrose for incoming carbon, to which 14 and 16% of carbon was allocated. Increased carbon allocation to sucrose with tissue maturity coincided with a decrease in partitioning to fibre. Between internodes 3 and 6, carbon allocation to total respiration did not change significantly, but decreased 2-fold in mature tissue (internode 9). Triose-phosphate recycling activity was found in all internodes. A maximum of 50% of carbon entering triose-phosphates was returned to hexose in internode 3 tissue, and this flux decreased with tissue maturity to 30%.

#### 4.2 INTRODUCTION

In developing sugarcane, a sharp gradient in sucrose content is evident in the top 10 internodes of the culm (Moore, 1995; Moore and Maretzki, 1996; Botha *et al.*, 1996; Whittaker and Botha, 1997). The biochemical basis for the transition of internodes from low sucrose levels at an immature stage, to the mature state of high sucrose content is not yet well understood (Moore and Maretzki, 1996). Previous studies of carbon partitioning of sucrose and hexose in sugarcane focussed primarily on the sugar pool, and have revealed that a cycle of rapid sucrose synthesis and degradation exists in sugarcane



(Sacher *et al.*, 1963; Batta and Singh, 1986; Wendler *et al.*, 1990; Komor *et al.*, 1996). It has been proposed that this cycle operates to facilitate the metabolic transition between net storage and synthesis of sucrose. In effect, rapid cycling allows only small changes in the enzymes and metabolites of the pathway to bring about significant changes in the rate of synthesis or degradation of sucrose (Dancer *et al.*, 1990).

However, sucrose cycling does not operate in isolation in the regulation of sucrose metabolism. Theoretically, allocation of available photosynthate to metabolic sinks other than sucrose may limit carbon allocation to sucrose storage. Recent studies on carbon allocation in sugarcane have included metabolic pathways not directly associated with sucrose synthesis or degradation, in order to assess their importance as competitive sinks for carbon (Veith and Komor, 1993; Botha *et al.*, 1996; Whittaker and Botha, 1997). Two such sinks have been identified, namely: the respiratory pathway, as defined by carbon allocation to CO<sub>2</sub>, organic acids and amino acids, and an insoluble component, assumed to be primarily fibre (Botha *et al.*, 1996; Whittaker and Botha, 1997). Total allocation to these pathways, expressed as a proportion of total carbon uptake, decreased with tissue maturation, accompanied by a concomitant rise in partitioning to sucrose (Whittaker and Botha, 1997). Furthermore, sucrose accumulation occurring in sugarcane cell suspension cultures under N-limiting conditions, was found to be at the expense of respiration and structural carbohydrate (Veith and Komor, 1993).

There are, however, two potential problems arising from these reports. Firstly, the insoluble component might consist of more than just structural carbohydrate, and as such could have led to an underestimation of carbon flow into other pathways. Although protein constitutes only 2% on a dry mass basis in immature sugarcane tissue, decreasing to 0.5% in mature internodes (Botha *et al.*, 1996), it might still represent an important sink for carbon. In sugarbeet, further fractionation of this insoluble component revealed a significant protein fraction (Giaquinta, 1979). Allocation of carbon from [U-<sup>14</sup>C]-glucose to protein was highest in immature sugarbeet tissue, consuming 40% of total <sup>14</sup>C uptake. This amount decreased significantly in mature tissue, to 5%, whereas allocation to sucrose was approximately 80% (Giaquinta, 1979).



A second problem is that the return of carbon from triose-phosphate to hexose-phosphate was assumed to be negligible in previous studies, based on a previous report by Sacher *et al.* (1963). This may potentially underestimate carbon flow toward the trioses. Significant triose-phosphate recycling has been reported in a number of plant species, namely: *Chenopodium rubrum*, maize, potato (Hatzfeld and Stitt, 1990), faba bean (Viola *et al.*, 1991) and banana (Hill and ap Rees, 1994). In ripening banana, this recycling activity was estimated to be between 8% and 28% of glycolytic flux (Hill and ap Rees, 1994). A higher return of triose was found in *Chenopodium rubrum* cell suspension cultures, estimated at 158% of flux into glycolysis (Hatzfeld and Stitt, 1990).

Here we report that as much as 50% of the carbon partitioned to respiration can be allocated to the protein fraction. In addition, a very high cycling of carbon between the triose-phosphate and hexose-phosphate pools are evident in sugarcane, returning up to 50% of carbon entering the triose-phosphate pool.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Sample preparation and processing

Samples were prepared and processed according to the procedure described in sections 3.3.1 to 3.3.6 of Chapter 3.

### 4.3.2 Ion-exchange fractionation of the EtOH soluble component

Dowex cation- $H^+$  and anion-formate exchange resins were prepared through immersion in 4M ammonia (70% v/v EtOH) and 4M formate (70% v/v EtOH) respectively. The resin was then washed with distilled water until neutral. Columns were packed with 1 ml resin, and EtOH-extracts of the samples were fractionated according to Dickson (1979). Aliquots of the acid, basic and neutral fractions were diluted 1:4 with Ultima Flo™ M scintillation cocktail. Samples were counted 5 min in a Beckman LS 1801 scintillation counter. Column efficiency with standards of  $[U-^{14}C]$ -protein hydrolysate and  $[U-^{14}C]$ -malate were 98% and 86% respectively.



### 4.3.3 Fractionation of the EtOH-insoluble component

The EtOH-insoluble component was fractionated to protein according to Dickson (1979). Pellets were homogenized in a mortar and pestle at room temperature, transferred to 2 ml Eppendorf tubes, and dried down in a vacuum centrifuge to remove any remaining EtOH. Control samples were resuspended in 1.5 ml H<sub>2</sub>O to estimate [<sup>14</sup>C]-sucrose background. Experimental samples were resuspended in 1.5 ml 12U ml<sup>-1</sup> pronase, in 0.05 M Tris-HCl (pH 7.4). Samples were incubated for 18 h at 37°C in an oven, with gentle shaking. The release of radioactivity into solution by pronase reached a maximum by 18 h. After incubation, all samples were centrifuged at 12 800 g for 30 min. Aliquots were removed and counted as protein.

Sample pellets were washed sequentially in 1.5 ml H<sub>2</sub>O until low counts were recorded in the supernatant. The samples were then dried in a vacuum centrifuge, and resuspended in 1.5 ml H<sub>2</sub>O, and an aliquot counted as representative of background radioactivity. Samples were then autoclaved for 1 h (Beutler, 1984), cooled, and incubated at 55-60°C for 3 h with 1.5 U amyloglucosidase and 3 mM acetate (pH 4.8). Maximum glucose release from samples was obtained after a 2 h incubation. After incubation, the samples were spun down at 12 800 g for 15 min, and the supernatant counted as starch. A 94% recovery of radioactivity in sugars after samples were passed through Alumina A cartridges confirmed the presence of starch glucose.

Samples were sequentially washed in 1.5 ml H<sub>2</sub>O. The supernatants were removed and discarded, and 1 ml Soluene<sup>®</sup>-350 (Packard) was added to the pellet and vortexed. The same procedure was performed on control pellets prior to fractionation for the determination of total counts in insolubles. Samples were left at room temperature for 24 h, and then counted. Recovery of the insoluble fractions was 79 ± 3% of total counts in the insoluble component.

### 4.3.4 Determination of <sup>14</sup>C in position C-6 of glucose

Sucrose in extracts from samples labelled with [1-<sup>14</sup>C]- and [6-<sup>14</sup>C]-glucose was hydrolysed in 0.3 M citric acid for 5 h in a 60°C water bath and prepared for HPLC



according to section 3.3.5 of Chapter 3. Glucose and fructose moieties of sucrose were separated using a Supelco™ LC-Pb column, 25 min at 0.6 ml min<sup>-1</sup> HPLC-grade H<sub>2</sub>O, at 80°C. Sugars were collected following peak detection with a Shimadzu RID-10A refractive index detector. In unhydrolysed sucrose samples, glucose was at a maximum 4% of total label in all tissues.

Radioactivity as <sup>14</sup>C in C-6 of glucose was determined according to the protocol of Dunn *et al.* (1956) Glucose fractions were reduced in a vacuum centrifuge and resuspended in 300 µl 10 mg ml<sup>-1</sup> glucose. To this, 600 µl sodium phosphate buffer (0.5 M, pH 7.5) was added, followed by 360 µl 0.5 M sodium periodate. The solution was mixed well, and left to stand for 1 h. Then 150 µl dimedone (80 mg ml<sup>-1</sup> 100% v/v EtOH) was added, mixed, and left to stand overnight for the precipitation of C-6 of glucose as formaldemethone. Samples were then centrifuged 12 800 g for 30 min, and the supernatant removed by pipetting and retained. The pellet was washed in 1 ml H<sub>2</sub>O, and again centrifuged. The supernatant was combined with the first and counted with the first as representative of carbons 1-5 of glucose. The pellet was counted as representative of C-6 of glucose. Standards were included with each experiment, using 0.5 µCi ml<sup>-1</sup> [1-<sup>14</sup>C] or [6-<sup>14</sup>C]-glucose, or a 1:1 combination of both types. Recovery of C-1 of glucose was 99 ± 0.3 %, and C-6 was 92 ± 0.2% (n = 3). For a 1:1 combination, recovery in C-1 was 44 ± 0.8% and in C-6, 56 ± 0.8%.

## 4.4 RESULTS

### 4.4.1 Calculation of metabolic flux in developing internodes of sugarcane

Preliminary investigations indicated that the protein content of tissue discs could be correlated with cell number (Bindon and Botha, submitted, and Chapter 3). Glucose uptake was determined from the total recovery of <sup>14</sup>C in metabolic components and expressed on a protein basis to normalize the data to represent cellular metabolism (Table 4.1). Maximal glucose uptake occurred in internode 6, at 49.31 ± 14.69 kBq mg<sup>-1</sup> protein (Table 4.1). From the values for glucose uptake, flux of glucose into the system was estimated as nmol mg<sup>-1</sup> protein min<sup>-1</sup>, using the external specific activity of glucose



(23 Bq nmol<sup>-1</sup>) in the labelling medium (Figure 4.1). This decision was based on the finding that the system approached isotopic steady state between the external and internal glucose pool, and the respiratory processes involved in CO<sub>2</sub> release (Bindon and Botha, submitted, and Chapter 3). The maximal flux of glucose into the system in internode 6 was  $11.91 \pm 3.54$  nmol mg<sup>-1</sup> protein min<sup>-1</sup> (Figure 4.1B). Carbon partitioning into various tissue components was calculated as a proportion of total glucose uptake into the tissue, and from this, estimates of fluxes into metabolic sinks were derived (Figure 4.1).

#### 4.4.2 Sucrose synthesis and degradation

In internode 3, return of label was found in fructose, which very likely represents the mobilisation of fructose from [<sup>14</sup>C]-sucrose via invertase and/or SuSy. Sucrose synthesis was estimated from the combined value of synthesis and degradation. Based on the premise that the cytosolic sucrose pool was at equilibrium with the feeding glucose pool, the estimated degradation rate was  $0.29 \pm 0.09$  nmol mg<sup>-1</sup> protein min<sup>-1</sup> (Figure 4.1A). This represents a minimum, because the return of labelled fructose into cytosolic metabolism is not considered. A maximal degradation rate was also calculated assuming that the entire endogenous pool of sucrose participated in metabolism (i.e. no compartmentation), from the specific activity of sucrose. This gave a flux value of  $5.40 \pm 2.09$  nmol mg<sup>-1</sup> protein min<sup>-1</sup>. Therefore, estimates of total sucrose synthesis in internode 3 varied from a minimum of  $2.65 \pm 0.51$  nmol mg<sup>-1</sup> protein min<sup>-1</sup> and a maximum of  $8.00 \pm 2.05$  nmol mg<sup>-1</sup> protein min<sup>-1</sup>. The return of label in fructose was not observed within the labelling period in internodes 6 or 9. Flux into sucrose was calculated as a proportion of total uptake in these samples. In internode 6, sucrose flux was estimated at  $7.41 \pm 2.97$  nmol mg<sup>-1</sup> protein min<sup>-1</sup> (Figure 4.1A, B). In internode 9, this flux was lower than in internode 6, at  $4.43 \pm 1.89$  nmol mg<sup>-1</sup> protein min<sup>-1</sup> (Figure 4.1C). In 9-month old cane [<sup>14</sup>C]-fructose was recovered in internodes of all ages following a 3 h feeding with [U-<sup>14</sup>C]-glucose. [<sup>14</sup>C]-Fructose recovered was 9.11, 3.74 and 0.93 % of radiolabel taken up into sucrose, in internodes 3, 6 and 9 respectively (mean of 3 replicates, result not shown).



**Table 4.1:** Incorporation and percentage distribution (in parenthesis) of  $^{14}\text{C}$  in internodal tissue slices supplied 3 h with  $[\text{U-}^{14}\text{C}]$ -glucose (each value is the mean  $\pm$  SD of three separate samples).

Internode number	Total uptake	Component (kBq mg <sup>-1</sup> protein)								
		protein	starch	fibre	sucrose	glucose	fructose	organic acids	amino acids	CO <sub>2</sub>
3	30.65 $\pm$ 6.15	4.19 $\pm$ 1.35 (13.5%)	0.71 $\pm$ 0.16 (2.3%)	4.79 $\pm$ 0.10 (16.0%)	10.57 $\pm$ 2.09 (34.5%)	1.78 $\pm$ 0.37 (6.1%)	1.21 $\pm$ 0.36 (4.1%)	2.71 $\pm$ 0.29 (9.1%)	0.98 $\pm$ 0.12 (3.3%)	0.34 $\pm$ 0.26 (1.0%)
6	49.31 $\pm$ 14.67	3.36 $\pm$ 0.31 (7.2%)	0.24 $\pm$ 0.03 (0.5%)	1.26 $\pm$ 0.20 (2.8%)	30.69 $\pm$ 12.28 (60.1%)	1.64 $\pm$ 0.56 (4.0%)	-	4.59 $\pm$ 0.92 (9.5%)	1.87 $\pm$ 0.26 (6.0%)	0.14 $\pm$ 0.08 (0.3%)
9	29.46 $\pm$ 10.29	0.48 $\pm$ 0.18 (1.4%)	0.14 $\pm$ 0.05 (0.5%)	0.37 $\pm$ 0.06 (1.3%)	18.33 $\pm$ 7.82 (61.0%)	2.96 $\pm$ 0.41 (10.6%)	-	2.67 $\pm$ 0.67 (9.3%)	1.72 $\pm$ 0.38 (6.0%)	0.05 $\pm$ 0.01 (0.2%)

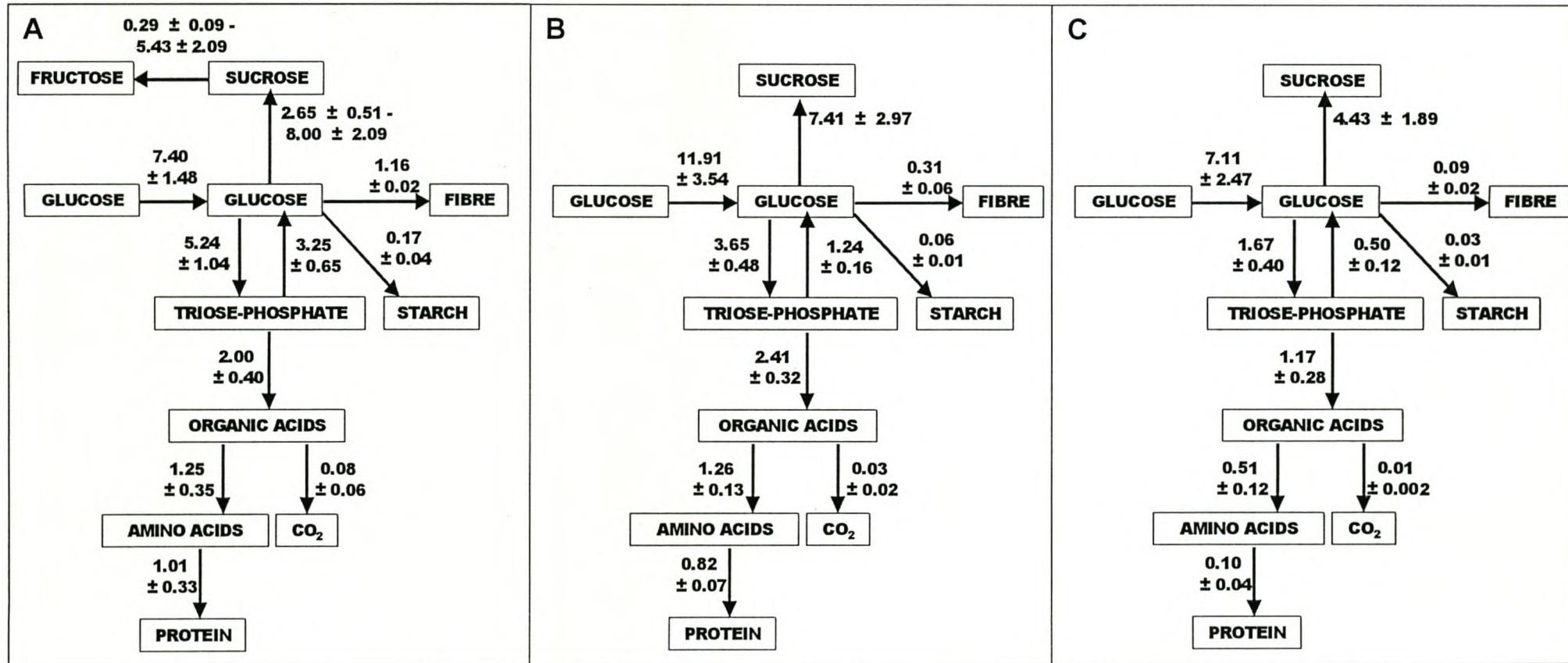


Figure 4.1 Metabolic profiles of internodal development in A. internode 3; B. internode 6; C. internode 9 (Flux values in nmol glucose mg<sup>-1</sup> protein min<sup>-1</sup>; each value is the mean ± SD of three separate samples).



#### 4.4.3 Carbon flux into glycolysis, fibre and starch

The insoluble component of the extraction procedure was sub-fractionated to fibre, starch and protein. Of the total  $^{14}\text{C}$  taken up in internode 3, 16% was allocated to fibre (Table 4.1). Flux into fibre was two-fold less than into sucrose in immature tissue (Figure 4.1A), and decreased as the tissue matured (Figure 4.1B, C). Flux into starch also decreased with tissue maturity: two-fold at each stage of development (Figure 4.1). However, the contribution of the starch pool to total metabolism was always low, at a maximum of 2% in young tissue (Table 4.1).

Flux into respiration was represented by the combined uptake into protein, organic acids, amino acids and  $\text{CO}_2$ . Flux into this pathway was similar between internodes 3 and 6 (Figure 4.1A,B), but was halved in mature tissue (Figure 4.1C). The extent of  $^{14}\text{C}$  allocated to protein as a proportion of total respiration decreased with tissue maturity, from 50% in internode 3, to 33% and 8% in internodes 6 and 9 respectively (Figure 4.1). The relative contribution of organic and amino acids to respiration increased with internode development, whereas  $^{14}\text{CO}_2$  release was less than 4% in all tissues and decreased with tissue maturity (Figure 4.1).

#### 4.4.4 Triose-phosphate recycling activity

The extent of triose-phosphate recycling was estimated from the amount of label redistribution between C-1 and C-6 of the glucose moieties of sucrose, following feeding of tissues with either  $[1-^{14}\text{C}]$ -glucose or  $[6-^{14}\text{C}]$ -glucose. The premise of this method is that sucrose is formed from a hexose-phosphate pool, derived from both unrandomised glucose, and randomised molecules which have been recycled from triose-phosphates. Redistribution of label occurred in all internodal tissues (Table 4.2), and the extent of redistribution was comparable when feeding with either  $[1-^{14}\text{C}]$ -glucose or  $[6-^{14}\text{C}]$ -glucose for the respective internodes.

In internode 3, redistribution following feeding with  $[1-^{14}\text{C}]$ -glucose was  $24.62 \pm 4.71\%$  (Table 4.2). For every randomized triose molecule reincorporated to glucose, a second, unrandomized molecule is also recycled. To calculate recycling of triose, the label-



redistribution data were doubled, indicating a 50% recycling of triose-phosphate to hexose in immature tissue. In the more mature internodes, 6 and 9, the extent of redistribution approximated 15% (Table 4.2), corresponding to a 30% return of triose-phosphate to the hexose-phosphate pool. The flux of carbon from the triose-phosphate/hexose-phosphate cycle was estimated from the net glycolytic flux and decreased with internode maturity (Figure 4.1).

**Table 4.2** Label redistribution in C-1 and C-6 of the glucose moieties of sucrose from either [1-<sup>14</sup>C]-glucose or [6-<sup>14</sup>C]-glucose fed to internodes 3, 6 and 9 of N19 (n = 3; \* = 2 replicates).

Internode number	Specifically-labelled glucose	% Redistribution of label	
		C-1	C-6
3	C-1	75.36 ± 4.71	24.64 ± 4.71
	C-6	30.74 (*)	69.25 (*)
6	C-1	81.32 ± 5.12	18.68 ± 5.12
	C-6	14.92 ± 2.67	84.75 ± 2.67
9	C-1	84.76 ± 0.59	15.24 ± 0.59
	C-6	14.33 ± 1.05	85.68 ± 1.05

## 4.5 DISCUSSION

### 4.5.1 Glucose uptake and sucrose accumulation

The current data for N19 cane indicate that the highest rate of glucose uptake and sucrose accumulation are in internode 6. Previous studies have shown maximal sucrose accumulation to occur in this region of the culm (Hartt, 1963; Whittaker and Botha, 1997). From the available literature, it is not certain whether this higher rate is due to a higher uptake of carbon, or a redirection of incoming carbon toward sucrose storage (Moore, 1995; Whittaker and Botha, 1997). The current data suggest that higher uptake of glucose in this tissue may in part account for the increased rate of sucrose accumulation. However, increased synthesis, and/or reduced cleavage of sucrose could also contribute to the observed increase in sucrose storage in internode 6 (Whittaker and Botha, 1997). Two flux rates into sucrose were determined for internode 3, based on a maximum and minimum calculated rate of sucrose degradation. The maximum rate of sucrose synthesis



calculated for internode 3 approximated that found in internode 6, indicating that the potential for a high rate of sucrose synthesis exists in young tissue. Therefore, increased sucrose accumulation in internode 6 could also be as a result of decreased degradation of sucrose. The absence of radiolabelled fructose following feeding with glucose does not negate the presence of a sucrose cycle, since return of label in fructose has been observed when longer labelling times were used, showing that cycling occurs mature tissues (Whittaker and Botha, 1997). Moreover, [U-<sup>14</sup>C]-glucose feeding of immature (9-month old) cane led to the appearance of label in fructose in internodes 3, 6 and 9, indicating that cycling occurred. A high rate of sucrose cycling throughout the sugarcane culm may therefore be dependent on the age of the cane. However, a reduced level of sucrose cycling may occur with internode development, since return of label in fructose, as a percentage of total label recovered as [<sup>14</sup>C]-sucrose declined as the tissue matured. Longer turnover times for the sugar pool have been reported in more mature tissues in sugarcane (Glasziou, 1961; Komor *et al.*, 1996; Vorster and Botha, 1999). Thus, sucrose accumulation could also result from a decrease in sucrose degradation in more mature tissues, leading to a higher net storage.

#### **4.5.2 Carbon partitioning to respiration and fibre**

A primary goal of this work was to fractionate the insoluble component of the extraction procedure, and to re-assess its contribution to total metabolism. The insoluble fraction was previously assumed to be fibre (Whittaker and Botha, 1997), and it is apparent from the [U-<sup>14</sup>C]-glucose partitioning data, that this component is further made up of protein and starch, as well as fibre. Previous work by Whittaker and Botha (1997) estimated partitioning to the insoluble pool to consume between 29 and 36% of incoming [U-<sup>14</sup>C]-glucose in immature tissue. From this, fibre was assumed to be a significant sink for incoming carbon, with increased allocation to sucrose potentially being as a result of a redirection of carbon away from fibre synthesis, toward sucrose.

The current study indicates that incorporation to fibre is less than previously supposed. However, it is still a significant drain on carbon which could potentially be allocated to sucrose, consuming 16% of incoming carbon in immature tissue. Moreover, it is the only



significant sink which undergoes marked change with the transition from immature tissue, to sucrose accumulation in internode 6. Therefore, it is possible that the observed increase in sucrose storage in this internode involves redirection of incoming carbon from fibre synthesis.

In view of the fact that up to 50% of the insoluble pool consists of protein, the estimated partitioning into respiration of Whittaker and Botha (1997) represents an underestimation. Therefore, drainage from the hexose phosphate pool via the glycolytic pathway is more significant than previously supposed. The onset of sucrose accumulation in internode 6 was not accompanied by a restriction in flux to respiration, as has been previously claimed (Veith and Komor, 1993; Whittaker and Botha, 1997). Furthermore, the increase in flux to sucrose in internode 6 was not immediately coupled to reduced flux into amino acids and protein. Other reports have shown that sucrose accumulation was accompanied by a decrease in amino acid and/or protein biosynthesis (Wendler *et al.*, 1990; Thom *et al.*, 1981; Whittaker and Botha, 1997). Furthermore, the amino acid level has been proposed to have a regulatory role in sucrose accumulation in sugarcane (Veith and Komor, 1993). The reduced respiratory flux observed in mature, internode 9, tissue was primarily due to a reduction in allocation to protein, but flux into amino acids was similar to that observed in internode 6. Therefore, although mature tissue is characterised by reduced carbon partitioning into protein, the current data do not suggest that it can directly be correlated to the regulation of sucrose accumulation. Internodes 3 and 6 are still undergoing development, and are therefore characterised by high growth rates (Moore, 1995; Lingle, 1999). It would therefore be expected that they should display a higher rate of protein synthesis than more mature internodes, which are fully elongated.

#### **4.5.3 Flux between triose-phosphate and hexose-phosphate: the case for PFP**

The presence of a triose-phosphate recycling activity in sugarcane contradicts the negative result of Sacher *et al.* (1963), who found 95% of the original label was retained in position C-1 of the hexoses of sucrose after a 4 h pulse of sugarcane discs in [1-<sup>14</sup>C]-glucose. A cycle between triose-phosphate and hexose-phosphate has been reported for various plant species (Hatzfeld and Stitt, 1990; Hatzfeld *et al.*, 1990; Viola *et*



*al.*, 1991; Hill and ap Rees, 1994; Dieuaide-Noubhani *et al.*, 1995; Krook *et al.*, 1998). In *Chenopodium rubrum* cell suspension cultures (Hatzfeld *et al.*, 1990), and climacteric banana (Hill and ap Rees, 1994) flux from Fru-1,6-P<sub>2</sub> to fructose-6-P was attributed primarily to PFP. These results were based on the finding that FBPase activity was absent or limiting in these tissues (Hatzfeld and Stitt, 1990; Ball *et al.*, 1991). Further, germinating cucumber seeds undergo rapid gluconeogenesis, and PFP activity is three-fold higher than cytosolic FBPase (Botha and Botha, 1993).

In sugarcane, the concentration of Fru-2,6-P<sub>2</sub> in sugarcane tissue is sufficient to significantly inhibit FBPase activity when compared to the levels required for inhibition in other plant species (Kruger and Beevers, 1984), and to fully activate PFP (Whittaker and Botha, 1997). Therefore, it is possible that PFP is the primary enzyme catalyzing gluconeogenic flux from triose-phosphate. The calculated mass-action ratio for PFP in sugarcane indicates that the reaction is at equilibrium *in vivo* (Whittaker and Botha, 1997). Thus, the enzyme can theoretically catalyze flux in the glycolytic or gluconeogenic direction. Furthermore, the maximum catalytic activity of PFP measured in internode 7 of N19 is in excess of the fluxes estimated for this pathway in the current study, at  $32.8 \pm 3.5$  nmol glucose mg<sup>-1</sup> protein min<sup>-1</sup> (Whittaker and Botha, 1999). Thus, PFP could contribute significantly to the observed triose-phosphate recycling. The levels of Fru-2,6-P<sub>2</sub> drop 4-fold between internodes 3 and 9 (Whittaker and Botha, 1997). Although the concentration of this metabolite was sufficient to fully activate PFP (Whittaker and Botha, 1997) it is known that Fru-2,6-P<sub>2</sub> can bind allosterically to enzymes in some instances (Stitt, 1987; Nielson and Wischmann, 1995), rendering a portion unavailable for metabolic regulation. Therefore, a decreasing Fru-2,6-P<sub>2</sub> concentration in mature tissue may downregulate PFP, leading to lower levels of PFP-mediated triose-phosphate recycling in mature tissue.

In sugarcane, PFP activity has been negatively correlated to sucrose content between different varieties of sugarcane (Whittaker and Botha, 1999). Total respiration, on the other hand, was positively related to PFP, indicating that the enzyme plays a role in the regulation of respiratory flux (Whittaker and Botha, 1999). Therefore, it would be

expected that PFP regulates the balance of the supply of sucrose and the demand for carbon in respiration and biosynthesis in sugarcane. Using transgenic potato with a reduced PFP activity to 1-3% that of wild type, Hajirezaei *et al.* (1994) found a 4-fold reduction in triose-phosphate recycling. Although no phenotypic change was observed in transgenics, feeding experiments with [U-<sup>14</sup>C]-glucose in growing transgenic tubers, showed a 13-fold increase in carbon partitioning toward sucrose at 25°C, compared with that observed in wild-type tubers. In the light of this, the current data indicating reduced triose-phosphate recycling activity in mature sugarcane internodes, may be related to the increase in sucrose accumulation in these tissues.

#### 4.6 CONCLUSIONS

It is apparent that the study of sucrose metabolism in sugarcane can no longer focus primarily on carbon partitioning within the sugar pool. Within the system, there are two primary pathways which compete with sucrose for incoming carbon, namely: fibre synthesis and respiration. In immature tissue, these are significant metabolic sinks, and impact on the amount of carbon which is allocated to sucrose. Furthermore, previous estimates of respiratory flux neglected the presence of a significant protein component, thereby underestimating flux via this pathway. A further pathway which was previously overlooked in studies of sugarcane metabolism is a cycle between triose-phosphate and hexose-phosphate. This decreases in activity concomitant with sucrose accumulation, and could potentially regulate sucrose metabolism.



## CHAPTER 5

### STARCH METABOLISM IN SUGARCANE

#### 5.1 ABSTRACT

Starch content in sugarcane internodal tissue increased with tissue maturity in both the high-sucrose variety N19 and in low-sucrose variety US6656-15. Starch content as a proportion of total storage carbohydrate decreased with tissue maturity in N19, and increased in US6656-15. Partitioning of carbon to starch as a proportion of uptake into storage carbohydrate was highest in immature tissue of N19 (10%). This value decreased in mature tissue, to 0.4%. In [U-<sup>14</sup>C]-glucose radiolabelling studies, carbon partitioning to starch was found to decrease with tissue maturity. A two-fold higher flux into starch was observed in immature tissue of N19 cane harvested in early spring than that harvested in late summer. In the cane harvested in early spring, radiolabelled maltose was recovered in internode 3 tissue of ripening cane, indicating that the higher flux into starch coincided with starch degradation. The redistribution of C-1 and C-6 in starch glucose was analysed following feeding of tissue with [1-<sup>14</sup>C]- and [6-<sup>14</sup>C]-glucose. Randomization of label in starch indicated that the pathway for carbon movement into sugarcane plastids for starch synthesis is primarily through the triose-phosphate translocator.

## 5.2 INTRODUCTION

Starch metabolism in sugarcane has received little attention in carbon partitioning studies, since the levels attained in culm tissue are low compared with sucrose. Starch constitutes on average 0.01% of cane fresh mass, whereas sucrose can reach up to 20% on a fresh mass basis (Hawker, 1985). However, starch levels in the culm have been found to vary, depending on developmental stage, growth season and variety (Godshall *et al.*, 1996). Within the developing culm, the contribution of starch content to total storage carbohydrate has been found to attain up to 12% in young tissue, and decrease with tissue maturity, to 3% in mature tissue (Batta and Singh, 1986). Starch content in the sugarcane culm also varies seasonally, increasing initially at the onset of ripening, and decreasing into the growth season (Chen, 1968; Batta and Singh, 1986). Furthermore, those cane varieties with a high proportion of wild-cane parentage, e.g. NCo310, attain high starch levels (Wood, 1962; Chen, 1968). Evidence from sugarcane cell suspension cultures indicate that starch can attain levels 5-fold those of sucrose under unlimited growth conditions (Veith and Komor, 1993). In the light of these factors, carbon partitioning between sucrose and starch needs to be addressed in sugarcane.

A further aspect of starch metabolism that has not been considered in sugarcane is the potential return of carbon to the hexose-phosphate from starch during its degradation. In cell suspension cultures of *Chenopodium rubrum* (Dancer *et al.*, 1990) and cores of banana, (Hill and ap Rees, 1994) concomitant synthesis and breakdown of starch has been reported. Furthermore, the route of carbon entry to the amyloplast for starch synthesis, either as triose-phosphate or hexose-phosphate is not known in sugarcane. This has been extensively studied in starch-storing sink tissues, and has been addressed in four possible ways, namely: assay of the enzyme activities of starch-storing tissues or amyloplasts, (Echeverria *et al.*, 1988; Journet and Douce, 1985; MacDonald and ap Rees, 1983; Entwistle and ap Rees, 1990) uptake affinities of precursors of starch synthesis by amyloplasts, (Emes and Traska, 1987; Tyson and ap Rees, 1988; Hill and Smith, 1991; Neuhaus *et al.*, 1993b) expression of cDNA transcripts of amyloplast membrane translocators in heterotrophic tissues, (Kammerer *et al.*, 1998; Shünemann and Borchert,



1994) and finally studies of C-1:C-6 randomization in the glucose moieties of starch using [ $^{13}\text{C}$ ]- or [ $^{14}\text{C}$ ]- specifically-labelled glucose (Keeling *et al.*, 1988; Hatzfeld and Stitt, 1990; Viola *et al.*, 1991; Dieuaide-Noubhani *et al.*, 1995; Krook *et al.*, 1998)

Keeling *et al.* (1988) fed developing wheat grains [ $^{13}\text{C}$ ]-glucose, specifically labelled at position C-1 or C-6 and demonstrated a low level of randomisation of carbon from position C-1 to C-6 in starch glucose occurred. This indicated the preferential incorporation of carbon into starch via hexoses as opposed to triose-phosphate. Subsequently, the body of evidence favouring this hypothesis has accumulated from similar experiments using specifically labelled [ $^{13}\text{C}$ ]-glucose or [ $^{14}\text{C}$ ]-glucose, for various plant tissues: potato (Hatzfeld and Stitt, 1990; Viola *et al.*, 1991), faba bean (Viola *et al.*, 1991), *Chenopodium rubrum* (Hatzfeld and Stitt, 1990), maize endosperm (Hatzfeld and Stitt, 1990), carrot (Krook *et al.*, 1998) and maize root tips (Dieuaide-Noubhani *et al.*, 1995). The potential for starch to directly compete with sucrose as a sink for carbon will depend on the rate of hexose-phosphate uptake by the plastid.

A goal of this investigation was to investigate starch and sucrose content within the developing culm, between a high sucrose-storing variety (N19) and a low sucrose-storing variety (US6656-15) with a high level of wild-cane (*Saccharum spontaneum*) parentage. Furthermore, analysis of the partitioning between sucrose and starch in culm tissue was undertaken, to establish the changes in the starch pool associated with development in the culm. From this, the contribution of starch as a competing pool with sucrose could be established. Finally, this study aimed to ascertain the route of carbon entry into starch through observation of the extent of randomization of [ $1\text{-}^{14}\text{C}$ ]-glucose and [ $6\text{-}^{14}\text{C}$ ]-glucose incorporated into starch glucose residues.

## 5.3 MATERIALS AND METHODS

### 5.3.1 Sample preparation and processing

Mature, non-flowering sugarcane plants of the high-sucrose variety N19 were obtained from Elsenburg experimental farm in Stellenbosch, South Africa, and samples of the low-sucrose variety US6656-15 were grown under greenhouse conditions. Samples were prepared and processed according to sections 3.3.1 to 3.3.4, and 3.3.6 of Chapter 3. In section 3.3.3, samples were also labelled with [1-<sup>14</sup>C]-glucose and [6-<sup>14</sup>C]-glucose at a specific activity of 23 Bq nmol<sup>-1</sup>.

### 5.3.2 HPLC fractionation of labelled sugars

EtOH-soluble extracts were prepared and processed according to section 3.3.5 of Chapter 3. Radioactivity appearing in maltose was quantified.

### 5.3.2 Assay of starch

Starch was extracted from the insoluble component of unlabelled tissue according to the procedure outlined in section 4.3.3 of Chapter 4. Glucose released from starch was assayed according to section 3.3.6 of Chapter 3. The addition of 1 mg of soluble starch to tissue samples prior to extraction in EtOH gave a 90% recovery of starch glucose.

### 5.3.3 Determination of <sup>14</sup>C in position C-6 of glucose

Starch was hydrolysed from the insoluble component of [1-<sup>14</sup>C]- and [6-<sup>14</sup>C]-glucose-labelled extracts according to section 4.3.3 of Chapter 4. Glucose from starch was collected following HPLC separation according to section 4.3.4 of Chapter 4. Sucrose from the EtOH-soluble extracts of [1-<sup>14</sup>C]- and [6-<sup>14</sup>C]-glucose-labelled samples was also processed according to section 4.3.4 of Chapter 4.



## 5.4 RESULTS

### 5.4.1 Comparison of seasonal, varietal and developmental levels of starch

In N19 and US6656-15 cane harvested in late summer (March) and in N19 cane harvested in early spring (September), starch content was found to increase as the tissue matured (Tables 5.1 and 5.2). Starch was found in internode 3 of immature cane harvested in winter (July), and was not detected in older internodes (Table 5.1). Sucrose content within the culm of N19 samples increased as the tissue matured (Table 5.1), and was similar between seasons (result not shown). The level of starch in mature tissue (internode 10) of US6656-15 (Table 5.2) was significantly higher than that found in equivalent tissue of N19 (internode 9) (Table 5.1). Sucrose content increased with tissue maturity in both varieties, although the maximum sucrose level attained in equivalent tissue of N19 (Table 5.1) was 4-fold those of US6656-15 (Table 5.2). As a percentage of total storage carbohydrate (starch + sucrose), starch content was highest in internode 3, at 9.8%, decreasing to 0.4% in internode 9 (Table 5.1). The reverse was apparent in US6656-15 tissue, where starch increased as a proportion of total stored carbohydrate with tissue maturity, reaching 8.00% in internode 10. The level of glucose was similar between the N19 and US6656-15 varieties (Tables 5.1 and 5.2), however, in N19 tissue, glucose levels decreased 3-fold in internode 11 (result not shown).

**Table 5.1:** Starch and sugar concentrations in the developing culm of N19 sugarcane harvested in winter (July) early spring (September) and late summer (March)  
((1) = July (2) = September; (3) = March; each value is the mean  $\pm$  SD of three separate samples)

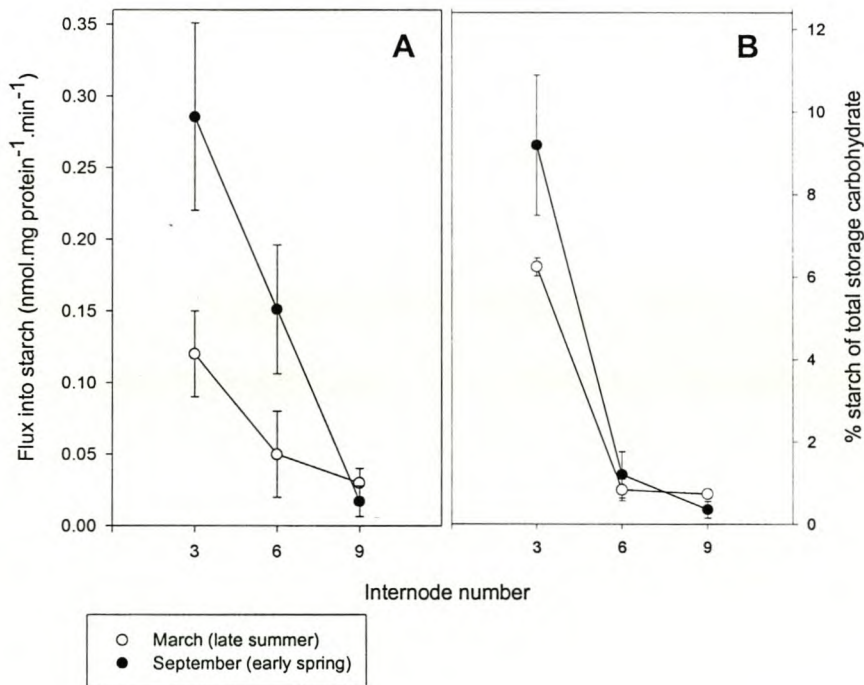
Internode number	Component (mg mg <sup>-1</sup> protein)					%starch/ storage CHO
	starch <sup>(1)</sup>	starch <sup>(2)</sup>	starch <sup>(3)</sup>	sucrose <sup>(2)</sup>	glucose <sup>(2)</sup>	
3	0.10 $\pm$ 0.01	0.25 $\pm$ 0.08	0.10 $\pm$ 0.09	2.42 $\pm$ 0.76	2.47 $\pm$ 1.95	9.84
6	-	0.13 $\pm$ 0.06	0.13 $\pm$ 0.09	27.80 $\pm$ 8.29	18.38 $\pm$ 0.52	0.53
9	-	0.78 $\pm$ 0.09	0.50 $\pm$ 0.17	100.00 $\pm$ 12.03	2.17 $\pm$ 1.18	0.43

**Table 5.2:** Starch and sugar concentrations in the developing culm of US6656-15 sugarcane harvested in late summer (March); (each value is the mean  $\pm$  SD of three separate samples).

Internode number	Component (mg mg <sup>-1</sup> protein)			%starch/ storage CHO
	starch	sucrose	glucose	
4	0.04 $\pm$ 0.02	2.13 $\pm$ 0.75	3.39 $\pm$ 0.76	2.16
7	0.92 $\pm$ 0.60	20.57 $\pm$ 2.91	14.71 $\pm$ 0.87	4.18
10	1.85 $\pm$ 0.45	22.19 $\pm$ 5.28	6.90 $\pm$ 2.30	8.00

### 5.4.2 Developmental pattern in carbon partitioning to starch

Conversion of [U-<sup>14</sup>C]-glucose into starch was highest in immature tissue (internode 3), and decreased as the tissue matured (Figure 5.1A). The flux of glucose into starch was estimated from the external specific activity of glucose in the incubation medium, based on preliminary data indicating equilibration of the cytosolic glucose pool with the isotope fed to the system. In cane harvested in September, the estimated flux into starch was higher in immature and elongating (internode 6) tissues, than in cane sampled in March (Figure 5.1A). In internode 9, flux into starch was similar in cane in both seasons. Starch synthesis as a percentage of flux into sucrose was low at all stages of development, having a maximum of 6-10% in immature tissue (Figure 5.1B).



**Figure 5.1:** A. Glucose flux into starch in developing internodes of N19 sugarcane, at stages of ripening and maturity. B. Flux into starch as a percentage of flux into total storage carbohydrate (each value is the mean  $\pm$  SD of three separate samples)



### 5.4.3 Starch cycling in immature internodal tissue

Following feeding of internode 3 tissue of cane harvested in September with [U-<sup>14</sup>C]-glucose, labelled maltose was recovered in the soluble fraction (Table 5.3), indicating amyolytic breakdown of starch. To estimate the rate of starch breakdown, a number of factors need consideration. Firstly, the whole starch pool in the tissue may not be participating in metabolism, i.e. starch is stored in reserves. Thus, a minimum estimate of calculation of flux to maltose was made assuming the specific activity of starch approached that of glucose in the labelling medium, (Table 5.3). A maximum flux (Table 5.3) was determined assuming complete mobilization of starch in the tissue, using the specific activity of total endogenous starch, of  $0.94 \pm 0.10$  Bq nmol<sup>-1</sup>. Neither estimate of starch degradation accounted for flux out of maltose, or possible phosphorolytic starch breakdown.

**Table 5.3** Carbon entry to starch, and return in maltose in internode 3 (each value is the mean  $\pm$  SD of three separate samples; n.d. not determined)

	Component	
	Starch	Maltose
Total uptake (kBq mg <sup>-1</sup> protein)	0.90 $\pm$ 0.21	0.25 $\pm$ 0.08
Flux (nmol mg <sup>-1</sup> protein min <sup>-1</sup> )		
Estimated minimum	0.36 $\pm$ 0.09	0.08 $\pm$ 0.03
Estimated maximum	n.d.	1.50 $\pm$ 0.65

### 5.4.4 Route of carbon entry to the plastid in sugarcane

Glucose moieties were isolated from starch and sucrose labelled with either [1-<sup>14</sup>C]-glucose or [6-<sup>14</sup>C]-glucose, and the redistribution between carbons 1 and 6 assessed. The redistribution pattern between C-1 and C-6 of glucose from sucrose was 13.76% following labelling with [1-<sup>14</sup>C]-glucose, and 15.34% from [6-<sup>14</sup>C]-glucose (Table 5.4). Following labelling with [6-<sup>14</sup>C]-glucose, the randomization of carbons 1 and 6 in glucose isolated from starch was found to be complete, giving an approximately 50% redistribution of label (Table 5.4). Since for every one randomized triose molecule another unrandomized one is returned, this corresponds to 100% randomization. The

pattern of label redistribution was only 37% from C-1 to C-6 following feeding with [1-<sup>14</sup>C]glucose. This value was significantly different from that obtained with [6-<sup>14</sup>C]-glucose as indicated by a student t-test ( $P = 0.05$ ). Recoveries of label in starch glucose did not differ following feeding with either [6-<sup>14</sup>C]-glucose or [1-<sup>14</sup>C]-glucose (result not shown).

**Table 5.4:** Comparison of label randomization in C-1 and C-6 of the glucose moieties of sucrose and starch in internode 7 tissue fed [1-<sup>14</sup>C]-glucose or [6-<sup>14</sup>C]-glucose (each value is the mean  $\pm$  SD of three separate samples).

Component	Specifically- Labelled glucose	% Redistribution of label in glucose moiety	
		C-1	C-6
starch	[1- <sup>14</sup> C]-glucose	62.78 ( $\pm$ 7.00)	37.21 ( $\pm$ 7.00)
	[6- <sup>14</sup> C]-glucose	50.82 ( $\pm$ 4.21)	49.18 ( $\pm$ 4.21)
sucrose	[1- <sup>14</sup> C]-glucose	86.24 ( $\pm$ 2.05)	13.76 ( $\pm$ 2.05)
	[6- <sup>14</sup> C]-glucose	15.34 ( $\pm$ 2.84)	84.66 ( $\pm$ 2.84)

## 5.5 DISCUSSION

### 5.5.1 Developmental changes in starch metabolism in the culm

The apparent increase in starch content with tissue maturity in the culm differs with previous findings that starch content decreases as the cane internodes matured (Wood, 1962; Batta and Singh, 1986). However, earlier studies were aimed at determining whole-cane starch levels, and sections of cane spanning a number of internodes were used to assess the changes in starch and sugar content in the culm (Wood, 1962; Batta and Singh, 1986). It is known that nodes of sugarcane contain 4-fold more starch than is found in internodal sections (Wood, 1962), and that any factor increasing internode number in sugarcane increases total recovered starch in the culm (Clarke, 1996). Therefore, since there are many, shorter developing internodes in the uppermost sections of cane, it would be expected that node number, and total starch would be highest in this region.



On the other hand, the uptake of  $^{14}\text{C}$  into starch decreased as the tissue matures, which suggests a decrease in carbon allocation to starch with tissue maturity. However, the simultaneous synthesis and degradation of starch in immature tissue may indicate that starch is cycled (Hill and ap Rees, 1994). Starch degradation in September cane was accompanied by a 2-fold higher rate of flux into starch than that observed in immature tissue of cane harvested later in the growing season. Cycling of starch may therefore be characterized by high rates of starch synthesis and degradation. The starch content in this tissue was lower than in more mature internodes, where starch degradation to maltose was not observed. Since cycling of starch would allow for a constant turnover of the starch pool, and utilization of degradation products, it would prevent the formation of long-term storage reserves of starch. In more mature tissue, starch degradation to maltose was not observed. Although uptake into starch was lower than in internode 3, this may reflect the long-term synthesis and storage of starch in reserves, leading to a build-up in the starch content of the tissue as it matured (Preiss and Sivak, 1996).

### **5.5.2 Carbon partitioning between starch and sucrose**

The seasonal increase in starch content from samples harvested in winter (July), to those collected in spring (September) and summer (March) reflects a similar trend to that observed by Batta and Singh (1986) and Chen (1968). In those studies, starch content was found to increase 6-fold between early stages of growth, and the onset of ripening. Later in the growth season, a 1.5-fold decrease in starch content occurred. When starch content was expressed as a proportion of total stored carbohydrate, contrasting trends were observed for N19 and US6656-15 cane. Since the levels of sucrose attained in mature tissue of US6656-15 were considerably lower than in N19, with higher starch levels, the contribution of starch to total stored carbohydrate was higher in mature internodes of this variety. Starch may therefore become an increasingly significant competitor with sucrose for incoming carbon allocated to storage carbohydrate in US6656-15.

In N19 the sharp increase in sucrose content along the culm led to a decrease in the contribution of starch to total stored carbohydrate with tissue maturity. Radiolabelling experiments with  $[\text{U-}^{14}\text{C}]$ -glucose confirmed this trend, and uptake to starch as a



proportion of total storage carbohydrate metabolism decreased markedly with tissue maturity. This was due primarily to increased partitioning to sucrose, and may reflect a decrease in carbon allocation to starch synthesis concomitant with sucrose accumulation. Since the contribution of  $^{14}\text{C}$  uptake to starch to total carbohydrate reached a maximum of 10% in immature tissue, starch may contribute significantly as a competitor for incoming carbon allocated to storage carbohydrate.

### 5.5.3 Route of carbon uptake to the plastid for starch synthesis

Due to the complete randomization of the hexose moieties of starch following feeding with  $[6-^{14}\text{C}]$ -glucose, it can be inferred that there was triose-phosphate movement into the plastid, with subsequent resynthesis of hexoses and ultimately, starch. Previous reports of randomization patterns in the moieties of starch and sucrose have given similar results (Keeling *et al.*, 1988; Hatzfeld and Stitt, 1990) indicating that equilibrium exists between the cytosolic and plastidic hexose pools. The approximately 3-fold lower degree of randomization in the glucose moieties of sucrose compared with those of glucose, indicates that the hexose pool feeding starch metabolism is not directly derived from the cytosolic glucose-phosphate pool. The higher level of randomization in starch glucose would necessitate a significant contribution from triose-phosphate taken up by the plastid. However, a significant difference in the randomization pattern was observed with  $[1-^{14}\text{C}]$ -glucose, compared with the value obtained using  $[6-^{14}\text{C}]$ -glucose. It is proposed that examination of larger sample sizes are required to minimise this discrepancy.

Resynthesis of hexoses from triose-phosphates would require the presence of a plastidic FBPase to convert Fru-1,6-P<sub>2</sub> to fructose-6-P. However, this enzyme has been demonstrated to have a low activity (Entwhistle and ap Rees, 1990) or to be absent (Neuhaus *et al.*, 1993a) in starch-storing sink tissues. In sugarcane, the activity of this enzyme has not been studied. One study indicated a significant plastidic FBPase activity in barley leaf etioplasts (Neuhaus *et al.*, 1993a), which were also shown to preferentially incorporate DHAP as a precursor for starch synthesis (Batz *et al.*, 1992). Since etioplasts are plastids grown under dark conditions, which differentiate into chloroplasts upon exposure to light (Newcomb, 1997), it is apparent that they retain a functional



chloroplastic TPT. Nothing is yet known about the ultrastructure of sugarcane plastids in culm tissue. However, the presence of a triose-phosphate translocator has been reported for heterotrophic tissues (Schünemann *et al.*, 1996), which would allow for the incorporation of triose-phosphate to the plastid.

## 5.6 CONCLUSIONS

Although starch forms a small component of whole-sugarcane metabolism, it makes a more significant contribution when viewed in terms of total carbon allocated to storage. It is apparent from ratios of sucrose and starch content, and carbon-partitioning patterns in radiolabelling experiments, that starch can contribute up to 10% of total storage carbohydrate in immature tissue. Thus, starch may compete with sucrose as a sink for incoming carbon in developing internodes. Tissue maturation may then be accompanied by a redirection of carbon away from allocation to starch, toward sucrose synthesis. The presence of a cycle of starch synthesis and degradation may account for the observed higher flux into starch in immature internodes, although total starch content is lowest in this tissue. The data obtained indicate entry of triose-phosphate to the plastid for starch synthesis is likely in sugarcane. This result contradicts the current consensus on carbon uptake by heterotrophic plastids, and will therefore need to be verified by the presence of plastidic FBPase activity.

## CHAPTER 6

### CONCLUSIONS

A current trend in sugarcane-improvement research is toward the use of molecular technology to increase sucrose yield at a sink level. In view of this fact, the goals of the current study were two-fold. Firstly, in the production and assessment of genetically modified plants, a system is necessary whereby the *in vivo* metabolism of wild-type and transgenic plants can be compared. Currently the most effective technique to directly assess sugarcane metabolism is through the radioactive feeding of tissue discs, although this approach has been extensively criticised. Thus, an aim of this study was to optimise the use of tissue discs as a method of flux analysis in sugarcane. Secondly, production of genetically engineered sugarcane initially requires the identification of primary pathways involved in the regulation of sucrose accumulation. Therefore, a primary goal of this work was to characterise developmental aspects of sucrose metabolism which have been neglected in previous studies, namely: the contribution of components of a water-insoluble fraction previously identified by Whittaker and Botha (1997); and the presence of a cycle between triose-phosphate and hexose-phosphate.

An important contribution in the optimisation of the tissue-disc technique for metabolic analysis was the finding that the experimental system approached isotopic steady-state. During the experimental period, cumulative  $^{14}\text{CO}_2$  release became linear, indicating that the respiratory pathways involved in  $\text{CO}_2$  release, and in turn the cytosolic glucose pool, were at isotopic equilibrium with the external feeding pool of glucose. This has significant implications for the study of metabolic flux analysis in this tissue, in that [ $^{14}\text{C}$ ]-glucose uptake and partitioning data can be interpreted to flux estimates, using the specific activity of the labelling medium. Previous estimates of carbon flux have been made using the internal specific activity of glucose (Whittaker and Botha, 1997). However, the current study indicated that glucose may be compartmented in the cell, rendering a significant proportion unavailable for metabolism. Therefore, use of the internal specific activity of glucose for flux calculation is likely to yield overestimates, and it is suggested that it should be avoided in future analyses.



Based on the premise that glucose flux in the tissue can be estimated from total  $^{14}\text{C}$ -uptake during the experimental period, flux from glucose was estimated for internodes of different maturity. These values were expressed on a protein basis, due to the finding that the protein content of the tissue approximated cell number. Thus, flux values between tissues could be normalized to represent cellular metabolism. Comparison of data from previous studies has been complicated by the normalization of data to fresh mass, dry mass, protein content or a whole tissue-disc basis. It was found that maximal glucose uptake and flux into sucrose occurred in internode 6 tissue. This is an important finding, because it is not yet known whether sucrose accumulation is as a result of increased uptake at the sink, or due to increased synthesis only. The current data suggest that increased flux into sucrose is at least in part due to higher uptake by the tissue.

A further aspect that requires consideration is that knowledge of the compartmentation of sucrose, glucose and fructose is necessary to calculate the turnover of these pools in the sucrose-hexose cycle. In the current study, cycling of carbon between sucrose and hexose sucrose was found in internode 3. When the rate of sucrose degradation was estimated assuming turnover of the entire sucrose pool in the tissue, the corresponding rate of synthesis was similar to that found in internode 6. This result suggests that the increase in sucrose accumulation in internode 6 could largely be as a result of a decrease in degradation, rather than an increase in the rate of sucrose synthesis. If a certain portion of the sucrose does not participate in metabolism, i.e. it is stored, it will significantly alter the estimate that is obtained. Therefore, a priority in future studies will be to determine the extent of, and the localisation, of stored sucrose and hexose, in sugarcane.

A highlight in the current study was the analysis of the insoluble component, which was previously found to make a significant contribution to metabolism by Whittaker and Botha (1997), and assumed to be primarily fibre. Further fractionation of the insoluble component revealed three carbon sinks: starch, protein and cell wall material (fibre). Starch was the least significant of these components, to which a maximum of 2% of total metabolism was allocated in immature tissue. However, when starch was



considered as a competitive sink with sucrose in terms of carbon allocated to storage carbohydrate, it contributed 10% in immature tissue. The recovery of labelled maltose in young tissue following feeding with [U-<sup>14</sup>C]-glucose indicated that concomitant synthesis and degradation of starch occurred. In the light of this, if starch is cycled, the actual flux into starch synthesis may be greater than is indicated by partitioning data. Therefore, starch may be an important competitive sink for carbon in immature tissue. Furthermore, in the manipulation of metabolism for the increase of sucrose yield, it may be preferential to opt for manipulation of a storage sink, rather than pathways which may interfere with the developmental or structural properties of the plant; e.g. glycolysis, biosynthesis or fibre synthesis. The finding that carbon enters the plastid for starch synthesis primarily as triose-phosphate in sugarcane was unexpected, since it is currently accepted that this occurs via the hexose-phosphates in sink tissues (Kruger, 1997). A further point of study will be to verify the capacity of the plastid to covert triose to hexose, via a plastidic FBPase.

The discovery that protein constitutes up to 45% of the insoluble component in immature tissue, was unexpected. This means that fibre is a less significant sink for carbon than previously supposed, consuming 16% of incoming carbon in immature tissue as opposed to the 36% found by Whittaker and Botha (1997). The contribution of protein biosynthesis to respiratory metabolism has not been considered in other studies, and was found to be at a maximum of 50% in immature tissue. Thus, total respiration was shown to be a primary competitive sink for incoming carbon in sugarcane. Flux via this pathway did not decrease with the onset of sucrose storage, between internodes 3 and 6. This differs from the result of Whittaker and Botha (1997), who found that total respiration decreased with tissue maturity. Although respiratory flux was halved in internode 9 tissue, flux to sucrose was also significantly reduced from that observed in internode 6. Thus respiration remained a dominant competitive sink for carbon even in mature tissue. Other studies have proposed that a decline in amino acid synthesis is involved in the regulation of sucrose storage (Veith and Komor, 1993). The current data indicate that carbon allocation to amino acid and protein biosynthesis did not decrease with the onset of sucrose storage in internode 6.



Although mature tissue had reduced allocation to protein from that observed in internode 6, this probably indicates the cessation of growth following complete internode expansion. Thus, the data suggests that this pool does not directly regulate carbon partitioning to sucrose.

A further discovery was the presence of a cycle between triose-phosphate and hexose-phosphate. This is a previously unexplored aspect of sugarcane metabolism (Sacher *et al.*, 1963). From studies of other tissues, this cycle has been proposed to regulate changes in the balance and supply of sucrose, and the demand for carbon in respiration and biosynthesis (Moore and Maretzki, 1996). The rate of cycling was high, returning up to 50% of carbon entering the triose pool in immature tissue. The sharp decrease in the extent of triose-phosphate cycling with tissue maturity and the onset of sucrose accumulation may indicate that it plays a regulatory role in sugarcane metabolism. Other studies have indicated that PFP is the primary enzyme mediating this cycle (Hatzfeld *et al.*, 1990; Hill and ap Rees, 1994). The potential for this enzyme to mediate triose-phosphate cycling in sugarcane is substantiated by evidence from Whittaker and Botha (1997), showing that PFP operates at equilibrium. Therefore, it could potentially operate in either the reverse or forward direction. Genetic manipulation causing downregulation of potato PFP decreased triose-phosphate cycling, without marked phenotypic change (Hajirezai *et al.*, 1994).

It is proposed that the genetic manipulation of PFP levels may significantly affect carbon partitioning between respiration and sucrose accumulation in sugarcane. The downregulation of PFP may cause a reduced level of triose-phosphate cycling, which according to the trend observed as the tissue matured, may in turn increase the amount of carbon partitioned to sucrose, and decrease flux to respiration. Since the potential exists for carbon uptake to the plastid via triose-phosphate, this may also decrease carbon partitioning to starch. In the South African sugarcane biotechnology programme, genetic manipulation of PFP levels is currently underway. The next step in this research will be the analysis of carbon partitioning and triose-phosphate cycling in transgenics, in order to assess the validity of current speculation as to the role of this enzyme.



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