Metabolic cycles in primary metabolism of cell suspensions of *Daucus carota* L. analysed by ^{13}C-NMR

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Stellingen

1. Bij onderzoek aan de oxidatieve pentose-fosfaat-route in planten wordt in het algemeen te weinig rekening gehouden met het feit dat deze gelocaliseerd is in zowel het cytosol als in de plastiden.
   (dit proefschrift)

2. De triose-hexose-fosfaat-cyclus in celsuspensies van de peen is altijd actief, onafhankelijk van de groeifase, omdat deze cyclus alleen gereguleerd wordt door de beschikbaarheid van koolstof-intermediairen.
   (dit proefschrift)

3. Bij de verklaring van fysiologische verschillen in de reacties op glucose en fructose wordt ten onrechte geen rekening gehouden met de ongelijke 'compartimentatie' van glucokinase en fructokinase activiteit in het cytosol.
   (dit proefschrift)

4. Zodra een behandeling van plantencellen leidt tot een sterk verhoogde ademhaling, zal hierdoor de gemeten activiteit van futiele cycli sterk onderschat worden.
   (dit proefschrift)

5. Aangezien de respiratie van celsupensies bij een relatieve zuurstofspanning van 20% niet maximaal is, zal de ademhaling in intact, niet-fotosynthetiserend plantenweefsel beperkt worden door de zuurstofspanning *in vivo*.

6. Indien futiele cycli een belangrijke rol spelen in de flexibiliteit van planten ten aanzien van hun milieu, zullen planten die een lage activiteit van deze cycli vertonen, slecht geadapteerd zijn aan het wisselende Hollandse weer en beter gedijen in een klimaat met constante zomers.

7. De regulatie van futiele cycli in plantencellen vormt een aangrijpingspunt voor veredelaars van glas-tuinbouwproducten: de energie die planten in deze cycli steken zou beter gebruikt kunnen worden voor een verhoging van de opbrengst, omdat aanpassing van de koolstofhuishouding via deze cycli overbodig is in kassen.

8. De aanpassing van zijn college-dictaten inzake de 'behandeling van homoseksualiteit' maakt, gezien het standpunt van de kerk, van bisschop Eijk een wolf in schaapskleren.

9. Het fokprogramma om de Korenwolf te redden, in plaats van het behouden van het leefgebied waarin deze dieren teruggeplaatst zouden kunnen worden, geeft aan dat mensen vaak hun geweten sussen met kortzichtige redeneringen.

10. Tijdens de zomermaanden voorkomt een NEE-NEE sticker op de brievenbus de toevloed van folders en huis-aan-huis-bladen nauwelijks, waaruit blijkt dat de vakantie-bezorger ook niet leest.

11. Het toiletgebruik wordt in veel openbare gebouwen onnodig verseksualiseerd door het instellen van aparte dames- en heren-toiletten.

12. Een reclame-aanduiding als "zuurkool vers uit het vat" verhult de werkelijke aard van een voedingmiddel.

Stellingen, behorend bij het proefschrift 'Metabolic cycles in primary metabolism of cell suspensions of *Daucus carota* L. analysed by ^{13}C-NMR', door Janhendrik Krook.

Wageningen, 7 december 1999.

Metabolic cycles in primary metabolism of cell suspensions of *Daucus carota* L. analysed by ^{13}C-NMR

Janhendrik Krook

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Dr. C.M. Karssen
in het openbaar te verdedigen
op dinsdag 7 december 1999
des namiddags te half twee in de Aula

ISN 972104

This research was financially supported by the Foundation for Life Science (SLW), which is subsidized by the Netherlands Organization of Scientific Research (NWO). The work was performed at the Wageningen University, Laboratories of Plant Physiology and Molecular Physics.

CIP-data Koninklijke Bibliotheek Den Haag

Krook, Janhendrik

Metabolic cycles in primary metabolism of cell suspensions of *Daucus carota* L. analysed by ^{13}C-NMR / Janhendrik Krook [S.I. : s.n.]

Thesis Wageningen - With ref. - With summary in Dutch.

ISBN 90-58080-137-0

Subject Headings: metabolic cycles / respiration / sugar utilization / *Daucus carota* L. / ^{13}C-NMR

Voorwoord

Aan het eind van mijn studie in Groningen, kreeg ik de mogelijkheid een stage te doen bij de vakgroep Moleculaire Fysica van de -toen nog- Landbouwuniversiteit Wageningen. Daaruit vloeide in 1992 een aanstelling voort als OIO bij de afdeling Plantenfysiologie: ik ging onderzoek doen aan de suikerhuishouding van plantencellen met behulp van 'NMR'. Veel mensen hebben hun steentje bijgedragen; anderen hebben zich 7 jaren afgevraagd wat ik in vredesnaam deed in Wageningen. Welnu, het wetenschappelijk eindverslag lees je in de volgende hoofdstukken, aan de gebeurtenissen die vooraf gingen -en met name de mensen die mij erbij hebben geholpen- wijd ik hier een paar woorden.

De betreffende groepen Moleculaire Fysica en Plantenfysiologie bedank ik voor mijn stationering op hun grondgebied. Bij de vakgroep Moleculaire Fysica stond Adri de Jager altijd op de achtergrond om technische problemen direct uit de wereld te helpen, waardoor de schaarse meettijd efficiënt benut kon worden. Cor Dijkema heeft mij -met veel geduld- geleerd om de NMR-machine nauwkeurig af te stellen met behulp van de UNIX-software. De laatste jaren, de schrijffase, heeft Cor als copromotor de NMR-kant van de artikelen en hoofdstukken voor mij bewaakt.

Bij de werkeenheid Moleculaire Biologie verdient Marijke Hartog alle eer. Zij heeft mijn onderzoek steeds voorzien van 'embryogene' celsuspensies. Marijke, jouw A10 en A+ lijnen zijn in dit proefschrift verwerkt!

Bij de werkgroep Plantenfysiologie, mijn vaste stekkie met laboratoriumruimte en kantoor, heb ik de meeste tijd doorgebracht. Ik heb daar de hele periode met fijne collega's mogen werken. Naast het echte werk was vooral de koffiehoek door de jaren heen een grote bron van inspiratie. Een aantal mensen verdient een extra bedankje, omdat zij mij vaak problemen hebben ontnomen. Wytske en Trees, bedankt voor het eindeloos verwerken van post, bestellingen en andere dingen. Jan en Ruth, als technische tovenaars hebben jullie vaak al 's ochtends om half negen mijn meetopstellingen gefixt! Op het lab zelf heb ik door de jaren heen heel wat collega's meegemaakt, die onontbeerlijk waren om de talloze problemen en proefopzetten te bespreken! Vooral Diaan, Marc & Mark hebben hun geestelijke ondersteuning gegeven. Mark, je was als kamergenoot ook onvergetelijk! Patrick en Wessel waren als "computer-assistants" altijd bereid om mijn gevecht met de diverse

computers in goede banen te leiden.

Vrij snel na mijn aantreden mocht ik een student begeleiden; samen met hem heb ik de eerste resultaten behaald, die het begin van de suiker-proeven in de andere hoofdstukken vormden. André, de resultaten in Hoofdstuk 5 zijn voor een deel van jou afkomstig!

De maandelijkse besprekingen met mijn copromotor Dick Vreugdenhil en promotor Linus van der Plas, zetten alles altijd weer op een rijtje, waarna een eindeloze reeks nieuwe proeven vanzelf volgde. Het was dan ook een hele omslag om los te komen uit dit ritme en om het lab in te ruilen voor de tekstverwerker. Jongens, -ook voor jullie- het zit er op! Ik heb de efficiënte besprekingen van artikelen erg gewaardeerd, deze leidden altijd tot een betere volgende versie!

In de afgelopen jaren is ook het leven buiten de universiteit heel belangrijk geweest. Naast alle dingen die ik heb meegemaakt met vrienden en kennissen, gaven ook alle mensen van het COC veel inspiratie en inzicht in het leven! De verschillende werkgroepen waar ik mee heb samengewerkt, en de laatste 1½ jaar het bestuur, hebben in mijn hart een warm plekje veroverd. Hans, het volgende boekje is vrijwel volledig op jouw ouwe rammelbak getypt!

Mijn familie wekte steeds de indruk er in te geloven dat ik dit boekje zou voltooien. Ik ben dan ook blij dat ik jullie kan laten zien waar ik mij de afgelopen jaren mee heb bezig gehouden. Mocht dit boekje niet tot duidelijkheid leiden, luister dan op de promotie zelf vooral naar mijn verhaaltje vooraf. Mam, alle zorgen en stimulering heb ik erg gewaardeerd: het is af!

Janhendrik

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Abbreviations

ADPG ADP-glucose

DW dry weight

FBPase fructose-1,6-bisphosphatase

FW fresh weight

K_e effector concentration at half maximum activity

K_m substrate concentration at half maximum activity

NMR nuclear magnetic resonance

NTP nucleotide triphosphate

OPPP oxidative pentose phosphate pathway

PEMs proembryogenic masses

PFP PP_i-dependent fructose-6-phosphate phosphotransferase

PFK ATP-dependent fructose-6-phosphate phosphotransferase

PP_i (inorganic) pyrophosphate

SPS sucrose phosphate synthase

SUSY sucrose synthase

UDPG UDP-glucose

UGPase UDP-glucose pyrophosphorylase

VACs large, vacuolated cells

Chapter 1

General introduction

Introduction

In plants photosynthesis is responsible for the primary production of carbohydrates. These carbohydrates are used as building blocks for biosynthesis and for respiratory purposes yielding ATP and reducing equivalents (NAD(P)H). ATP is used to supply the activation energy of biosynthetic reactions while in the reduction of nitrate and sulphate reducing equivalents are consumed. Carbon metabolism in green plants is complicated since they consist of a large number of different tissues and cell types, and sugars are synthesized and consumed simultaneously. However, since heterotrophic cell suspensions do not possess a photosynthetic apparatus, these cells will only take up and convert sugar. The amount of sugar supplied in the experiment is known, and suspension cultures generally form a relatively homogenous population of cells; representative samples can be taken easily and the partitioning of carbon between biomass production, storage carbohydrates and respiration can be determined. Therefore, heterotrophic cell suspensions are widely used as model-systems in research on carbohydrate and energy metabolism.

Embryogenic cell suspensions

Cells suspensions of *Daucus carota* often are not completely homogeneous since they are embryogenic. It was discovered in 1958 that suspension-cultured cells of *Daucus*, resuspended at low density in the absence of the auxin 2,4-D, form somatic embryos at high frequency (Steward 1958). These cell suspensions consist of different cell types, identified as clusters of small cytoplasm-rich cells, called proembryogenic masses ("PEMs"), large vacuolated cells ("VACs") and an intermediate state, the isodiametric cells (Fig. 1). Since cytoplasm-rich PEMs have a higher density than VACs, the different cell types can be separated by percoll density centrifugation (Ulmer and Flad 1979). PEMs show a high cell division activity and may differentiate into somatic embryos, while VACs show a low rate of cell division and low differentiating capacity. However, PEMs might develop from VACs and isodiametric cells, or *vice versa*, within *Daucus carota* suspension cultures (Steward 1958; Toonen *et al.* 1994).

Physiologically, the various cell types, i.e. PEMs and VACs, might differ significantly with respect to sugar and starch accumulation: PEMs store higher levels of sucrose and starch than

VACs (Wurtele *et al.* 1988). Dijkema *et al.* (1988) showed different ratios of sucrose/hexoses in the various cell types.

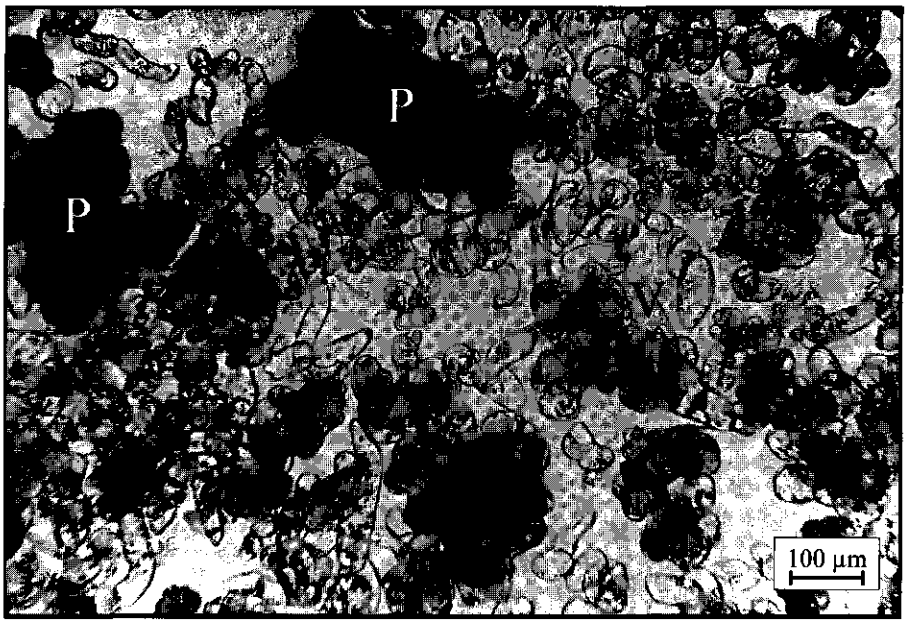

Figure 1 Photograph of cells of a 7 days old embryogenic batch culture of *Daucus carota* showing different cell types. P = proembryogenic mass, V = large vacuolated cell and I = isodiametric cell. Bar = 100 μm.

In addition, a cell line containing a high percentage of PEMs showed low label exchange from [1-^{13}C]-glucose to [6-^{13}C]-sucrose and hexoses, while a cell line with less PEMs showed a higher percentage of label exchange (Dijkema *et al.* 1990). Since this exchange is supposed to occur at the level of triose phosphates after which resynthesis of hexose (phosphates) occurs, the glycolytic and gluconeogenetic fluxes were suggested to be different in the various cell types. Furthermore, a relation between the embryogenic potential of PEMs and the level of glucose was proposed (Verma and Dougall 1977; Dijkema *et al.* 1988; Tremblay and Tremblay 1991).

Sugar uptake

In general, carbon dioxide fixation in plants takes place by photosynthesis in "source" tissues, after which specific transport sugars are translocated to the non-photosynthetic "sink" tissues. Although plants from some genera transport stachyose, raffinose or verbascose, e.g. in the Leguminosae family (Frias *et al.* 1999) or sorbitol as in the Rosaceae family (Berüter *et al.* 1997), most higher plants translocate sucrose; this also applies to carrot (Hole and Dearman 1994). Next to transport sugar, sucrose is generally known as a storage sugar in for instance red beet (Getz 1991) and carrot roots (Hole and Dearman 1994). In intact plants sucrose is transported through the phloem. Phloem loading is known to occur in the source apoplastically by means of sucrose carriers (Hole and Dearman 1994; Schulz *et al.* 1998) as in Fabaceae and Scrophulariaceae or symplastically via plasmodesmata in for instance Lamiaceae and Saxifragaceae (van Bel *et al.* 1994). In sink tissue, phloem unloading again might take place symplastically or apoplastically; in the latter case carrier-mediated sucrose uptake might take place in the sink parenchyma cells (Shakya and Sturm 1998). Also, hydrolysis of sucrose might take place in the apoplast, followed by concomitant uptake of the resulting hexoses.

Heterotrophically grown cell suspensions take up their sugars from the apoplast. Cell suspensions generally possess high activities of cell-wall-bound invertase (Kanabus *et al.* 1986; Callebaut *et al.* 1987); therefore it is most likely that they take up hexoses when sucrose is fed in the medium. Hexose carriers are reported for different plant species and are known to take up both glucose and fructose (Felker *et al.* 1991; Tubbe and Buckhout 1992). Different homologous hexose transporter proteins were reported for *Ricinus communis* seedlings showing different expression patterns in sink and source tissues (Weig *et al.* 1994). Furthermore, expression of a leaf sucrose transporter was found to be diurnally modified in *Solanum tuberosum* (Kuhn *et al.* 1997). Next to active uptake via carriers (Botha and Kennedy 1998), diffusion-like uptake is reported at high sugar concentrations (Stanzel *et al.* 1988b; Botha and Kennedy 1998). These sugars are thought to pass the lipid bilayer through spaces between lipid and transmembrane protein molecules. It is supposed, that this leakage is much faster through the plasmamembrane than through the tonoplast, since the first contains much more proteins (Aked and Hall 1993).

In the research described in this thesis, batch-grown cell suspensions of *Daucus carota* L. were used to study the uptake of hexoses, and subsequent conversion into sucrose and starch. By using specifically labelled [1-^{13}C]- and [6-^{13}C]-glucose and fructose in nuclear magnetic resonance (NMR) experiments it is possible to follow these molecules through the different pathways in primary metabolism, e.g. glycolysis, gluconeogenesis and the oxidative pentose phosphate pathway (OPPP).

Enzymes involved in hexose phosphorylation.

Once taken up by the cell, glucose and fructose might be transferred to the vacuole (Preisser *et al.* 1992) or phosphorylated in the cytosol at the expense of ATP. Hexose phosphorylation is known to take place by specific fructokinases and unspecific glucokinases, the latter phosphorylating glucose preferentially, but, also showing affinity towards fructose and mannose (Doehlert 1989). Therefore, the latter enzymes are often called "hexokinases" (Schnarrenberger 1990; Renz and Stitt 1993).

Fructokinases are known to be regulated by substrate levels and product inhibition (Renz and Stitt 1993). Furthermore, fructokinase might use UTP in addition to ATP (Yamashita and Ashihara 1988; Bayesdorfer *et al.* 1989; Schnarrenberger 1990). Hexokinases, on the other hand, are predominantly active with ATP and their activity with UTP is only low. Furthermore, hexokinases are not subject to product inhibition (Schnarrenberger 1990).

Sucrose and starch accumulation

Sucrose, synthesized in the cytosol may be stored either in the cytosol, in the vacuole or both (Preisser and Komor 1991; Preisser *et al.* 1992). A second, non-osmotic storage carbohydrate in plants is starch. Starch is synthesized inside the plastids after uptake of hexose phosphates from the cytosol (Hill and Smith 1991; Neuhaus *et al.* 1993; Ross and Murphy 1993). The conversion of hexose-phosphate into ADPG might also partially occur in the cytosol as found for cereals (Villand and Kleczkowski 1993).

Starch consists of mainly α(1,4)-glucan with various amounts of side chains coupled via α(1,6)-glucose. Starch may reach high levels in typical storage organs like potato tubers, seeds of faba bean (Viola *et al.* 1991) or cereals (Keeling *et al.* 1988; Batz *et al.* 1992). The

equilibrium between sucrose and starch synthesizing capacity is dependent on the species; *Daucus carota* roots and cell suspensions synthesize about equal amounts of sucrose and starch (Keller *et al.* 1988; Ross and Murphy 1993).

Cycling of carbohydrates and the use of parallel enzyme systems

Besides storage of sugars in sink tissues and degradation in respiratory metabolism, a continuous cycling of carbon within cells is a well known phenomenon in plants. During this cycling, metabolic pathways are active while their products are not used instantaneously (Plaxton 1996). Instead, as long as the end-products are not needed they are cycled back, resulting in a simultaneous synthesis and degradation of metabolites. Since these metabolic conversions do not result in net production of (carbon) metabolites which are converted by other pathways, they are called 'futile cycles'. Futile cycles are reported within the primary carbon metabolism between triose and hexose phosphates (Keeling *et al.* 1988; Hatzfeld and Stitt 1990; Viola *et al.* 1991; Kosegarten *et al.* 1995), between hexoses and sucrose (Dancer *et al.* 1990; Wendler *et al.* 1990), between hexoses and pentoses using the OPPP and the gluconeogenetic pathway from triose phosphates to hexose phosphates (Wagner *et al.* 1987; Emes and Fowler 1983; Hartwell *et al.* 1996; Redingbaugh and Campbell 1998) and to a lesser extent between hexoses and starch (Stitt and Heldt 1981; Hargreaves and ap Rees 1988).

Furthermore, plants possess different enzymes catalyzing the same reactions with different regulatory mechanisms. These 'enzyme-couples' are often connected with the futile carbon cycles and may therefore play a role in the physiological basis of these cycles in plants. For example, ATP-dependent fructose-6-phosphate phosphotransferase (PFK) and PP_i-dependent fructose-6-phosphate phosphotransferase (PFP) both convert fructose-6-phosphate into fructose-1,6-bisphosphate (Tobias *et al.* 1992). However, PFP catalyzes an reversible reaction while the PFK-catalyzed conversion is not reversible. Also, for the reverse reaction of fructose-1,6-bisphosphate into fructose-6-phosphate a irreversible enzyme exists, i.e. fructose-1,6-bisphosphatase (FBPase). This enzyme plays a role in photosynthetic tissues (Stitt *et al.* 1987); in non-green tissues it might be absent and PFP might replace its function (Entwistle and ap Rees 1990).

Although futile cycles may have appreciable costs in terms of energy consumption, advantages are also suggested, e.g. by Wendler *et al.* (1990), Hatzfeld and Stitt (1990) and Plaxton (1996). Futile cycles as well as the presence of parallel enzyme systems catalyzing the same reaction might make plants flexible in dealing with changing environmental conditions which occur very often in field situations. In this way futile cycles enable plants to restore the equilibrium between metabolites, if suddenly the flow through one or more pathways changes (Black *et al.* 1987). Possibly the coupling of a reversible and an irreversible reaction, using PP_i and ATP, respectively, in these cycles plays a role in maintaining the desired levels of ATP and PP_i (Dancer and ap Rees 1989).

Cycling through the OPPP

The OPPP converts hexose-6-phosphate into pentose-5-phosphate, producing 2 NADPH for each CO_2 which is released. The resulting pentose-5-phosphates may be cycled back to triose phosphates and hexose-phosphates, which might enter the OPPP again. In this way, glucose-6-phosphate might be completely oxidized, yielding only CO_2 and NADPH, the latter feeding biosynthetic and reduction-reactions (Emes and Fowler 1983; Redingbaugh and Campbell 1998). Alternatively, the OPPP is able to interconvert pentose, erythrose and heptulose sugars, which are used in the biosynthesis of nucleic acids, aromatic amino acids or secondary metabolites (Wagner *et al.* 1987; Hagendoorn *et al.* 1991; Hartwell *et al.* 1996).

Cycling of hexoses through sucrose

Sucrose is known to be synthesized by sucrose phosphate synthase (SPS) in the cytosolic compartment (Goldner *et al.* 1991; Zhu *et al.* 1997). Degradation might occur in the cytosol by sucrose synthase (SUSY) or invertase (Dancer *et al.* 1990; Wendler *et al.* 1990) resulting in cytosolic sucrose cycles. After synthesis sucrose is also transported into the vacuole where hydrolysis might take place by acid invertase. When the resulting hexoses are transferred back to the cytosol, they can again be used as precursors for sucrose synthesis, leading to a mixed "vacuolar/cytosolic" sucrose cycle (Goldner *et al.* 1991, Lee and Sturm 1996).
In the cytosol, hexose (phosphates) might immediately enter the sucrose cycle again, while in the vacuole hexoses are "protected" from hexose phosphorylating enzymes as long as they

are not transferred from the vacuole to the cytosol.

Cycling between triose and hexose phosphates

Young, meristematic tissue often possesses high hexose and sucrose importing (sink) properties as well as high sucrose hydrolysing activity by SUSY and high levels of PFP (Spilatro and Anderson 1988; Dancer and ap Rees 1989; Ashihara and Sato 1993). PFP is connected with triose-hexose phosphate cycling as was shown in antisense-PFP plants of *Solanum tuberosum* which had a much lower label exchange than the untransformed plants (Hajirezaei *et al.* 1994). It is still not clear whether PFP is working mainly in the glycolytic direction (Botha *et al.* 1992), the gluconeogenetic direction (Hatzfeld and Stitt 1990), or both (Hajirezaei *et al.* 1994). Tobias *et al.* (1992) and Sung *et al.* (1988) suggested that PFK may act as a maintenance enzyme supplying substrates for respiration, while PFP is an adaptive enzyme supplying intermediates for biosynthesis and cycling of sucrose.

Measuring of metabolic fluxes

In order to determine the dynamics of metabolic cycles in plants the flux of metabolites through the different pathways should be known. ^{13}C-labelled hexoses were supplied to the cells to study the conversion rate of hexoses by the various metabolic pathways by means of nuclear magnetic resonance (NMR). Next to the level of labelled sugar, also the total level of sugar must be known to calculate the changes in labelling percentage in time.

HPLC is a sensitive technique for measuring total sugar concentrations as low as 10 μM. On the other hand, ^{13}C-NMR is a less-sensitive method which requires concentrations in the mM-range. Since natural abundance ^{13}C is only 1.1%, sensitivity can be increased almost 100-fold by using labelled substrates.

The resulting pattern of labelling percentages provides information about the flux and turnover rate of carbohydrates through the various pathways and cycles.

^{13}C-Nuclear Magnetic Resonance (NMR)

NMR is a technique which has been developed already fifty years ago, but is used as a tool to study plant metabolism from the beginning of the seventies (Farrar and Becker 1971).

Various nuclei can be measured by means of NMR, e.g. ^{13}C, ^{14}N, ^{15}N, ^{23}Na and ^{31}P (Farrar and Becker 1971; Leibritz 1996; Schneider 1997). In biological research ^{1}H, ^{31}P and ^{13}C are most commonly used. ^{1}H is predominantly used for probing water (movement) to gain information about compartmentation or transport of water. ^{31}P is used for measuring phosphorylated cell components like sugar phosphates and nucleotide phosphates. Furthermore, its pH-dependence makes it very useful for determining the pH in compartmentation studies (Loughman *et al.* 1989; Fox and Ratcliffe 1990; Quiquampoix *et al.* 1993). Although ^{13}C is a less sensitive nucleus compared to ^{1}H and ^{31}P, ^{13}C-NMR can be used to measure glucose, fructose and sucrose in plant cells. Since the natural abundance of ^{13}C is only 1.1%, it is possible to specifically label the sugar molecules at a certain position (Breitmaier and Voelter 1987; Dijkema *et al.* 1990). Commercially 99.9% [n-^{13}C]-labelled sugars are available for nearly every carbon position in the glucose or fructose molecules. We used high resolution 1D Nuclear Magnetic Resonance (NMR) to measure the "amplitude" of different "resonance frequencies", to determine the amount of labelled carbon atoms within glucose, fructose and sucrose after feeding 99.9 % [1-^{13}C]- and [6-^{13}C]-labelled glucose and fructose, in extracts of cell suspensions and in cell suspensions *in vivo*. A major advantage of *in vivo* NMR-measurements is that they can be done non-destructively and are applicable to living plants, tissues or cell suspensions. Therefore, it is possible to follow the fate of a labelled substrate by gathering successive data points from the same sample.

Figure 2 gives examples of natural abundance reference spectra of 50 mM glucose (A), fructose (B) and sucrose (C); each carbon atom has its own resonance frequency (expressed in part per million relative to the basic frequency of a standard of tetra methyl silane). Secondly, molecules may appear in different conformations: i.e. an α- or ß- ring of 6 atoms (pyranose) or of 5 atoms (furanose). Thirdly, each atom has its own "response factor" which may differ significantly between the nuclei of sucrose, fructose and glucose depending on the molecular localisation (and therefore relaxation behaviour). The response factors of known concentrations of ^{13}C shown in Fig. 2 are used to calculate the concentration of label determined in the experiments. Carbons attached to -OH groups resonate in the range of 60-105 ppm (Figs 2, 3), and carboxylic carbons (attached to -OOH groups) in the range from 120-170 ppm (Fig. 4, Breitmaier and Voelter 1987).

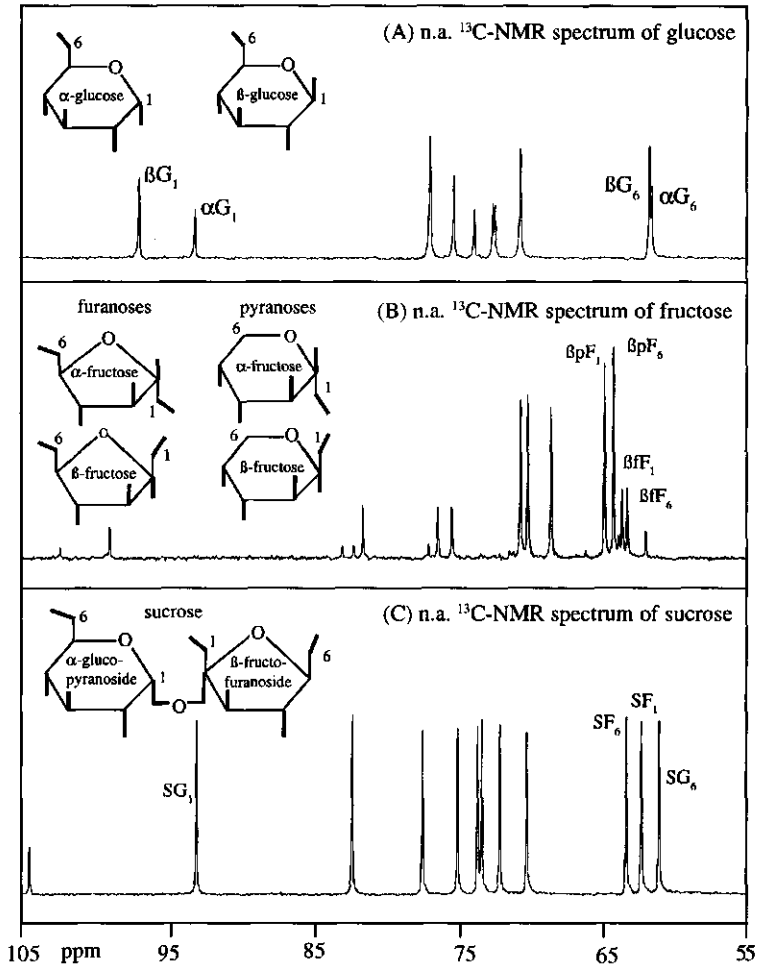

Figure 2 Natural abundance (n.a.) spectra of 50 mM solutions of glucose (A), fructose (B) and sucrose (C) in Gamborg's B5 medium. Glucose (G) shows 12 - partially overlapping- peaks corresponding to carbons 1-6 in α and ß configuration. Fructose (F) occurs mainly in the pyranose (p) and less in the furanose (f) form, both in the ß configuration. Minor peaks are from α-configurations. Sucrose shows 12 peaks of the ß-furanose of the fructosyl- (SF) and α-pyranose of the glucosyl-unit (SG). Molecules: schematic drawings of carbon-skeletons of sugars; only the C-1 and C-6 carbons are indicated, bold bars represent the -OH groups.

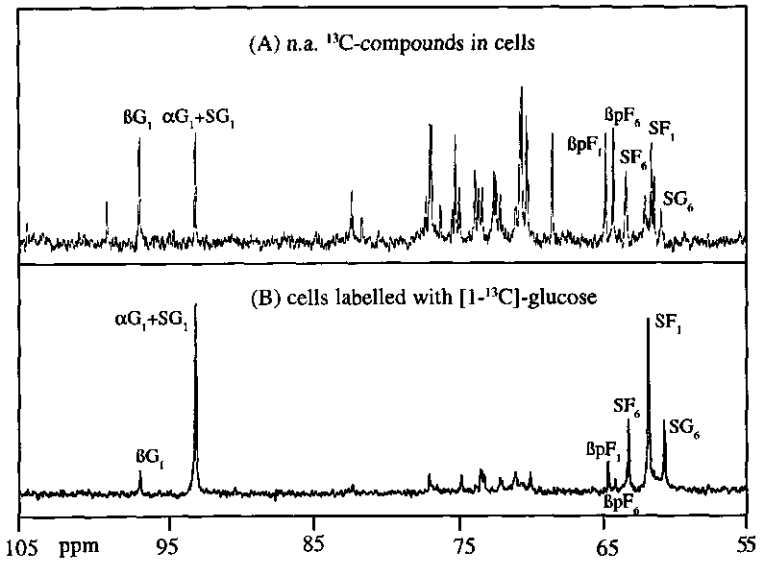

Figure 3 Representation of a natural abundance (n.a.) *in vivo* spectrum of 7 days old batch-cultured *Daucus carota* cells (A) and a spectrum of 14 days old *Daucus carota* cells after labelling with [1-^{13}C]-glucose in an airlift-system for 5 h (B). Symbols are as in Fig. 1. Spectra in A and B are accumulated scans of 1-h measurements, resulting in a higher signal/noise ratio for labelled carbon resonances in B.

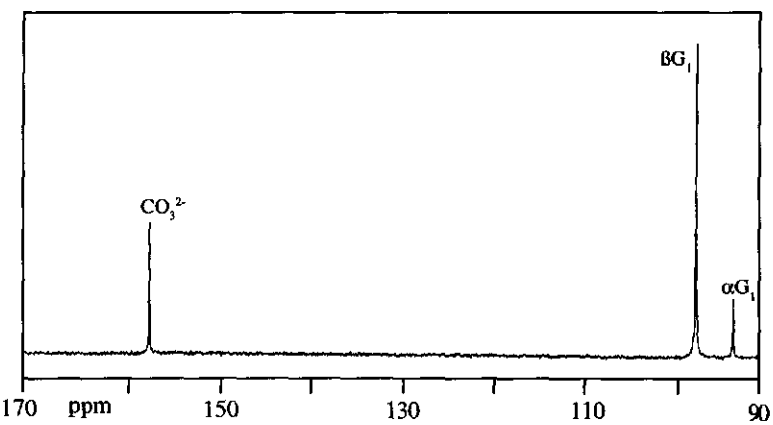

Figure 4 Example of the $^{13}CO_3^{2-}$ resonance at 164.5 ppm in a 10% KOH solution with 10 mM [1-^{13}C]-glucose (resonating at 96.8 and 93.0 ppm) as internal reference.

Figure 3A shows an example of a natural abundance ^{13}C-NMR spectrum of 7 days old *Daucus carota* cells. Figure 3B gives an example of 14 days old *Daucus carota* cells labelled with [1-^{13}C]-glucose for 5 h. Next to [1-^{13}C]-glucose (G_1) also [1-^{13}C]-fructose (F_1), sucrose-glucosyl C-1 (SG_1) and fructosyl C-1 (SF_1) are observed. Furthermore, C-6 carbons are observed in fructose (F_6) and sucrose (SG_6 and SF_6).

Next to the sugars also CO_2 produced by the cells was determined. Figure 4 shows an example of the CO_3^{2-} resonance at 164.5 ppm and a reference of 10 mM [1-^{13}C]-glucose resonating at 96.8 and 93.0 ppm. The differences in ratio of ß and α-glucose C-1 carbons in Fig. 2A and Fig. 4 are caused by differences in ionic composition of the solution (Gamborg's B5 medium *versus* 10% KOH), since ions influence the equilibrium between the α- and ß-conformation and relaxation behaviour of the spin labels.

The observed exchange of label from C-1 and C-6 carbons is supposed to occur at the level of triose phosphates, and thereby gives information about the glycolytic and gluconeogenetic flux; the difference in production of $^{13}CO_2$ from [1-^{13}C]- and [6-^{13}C]-glucose gives information about OPPP activity and the conversion from [1-^{13}C]-glucose into sucrose and fructose gives information about the flux through the sucrose cycles. From the time-course of the subsequent appearance of label in glucose, sucrose and fructose conclusions can be drawn about the cellular localisation of the various sugar pools.

High Performance Liquid Chromatography (HPLC)

Total sugar levels were measured to calculate labelling percentages during the various experiments. Glucose, fructose and sucrose can be determined by a sugar-specific HPLC-system of "DIONEX" to which a carbopac PA-1 column is connected which specifically binds -O^- groups of carbohydrates at high pH (Tetteroo *et al.* 1995). Glucose, fructose and sucrose were eluted with 100 mM NaOH solution (Fig. 5A) and sugar-phosphates with a Na-acetate gradient (Fig. 5B). The most abundant sugar phosphates in *Daucus carota* cells were glucose-6-phosphate, fructose-6-phosphate and glucose-1-phosphate.

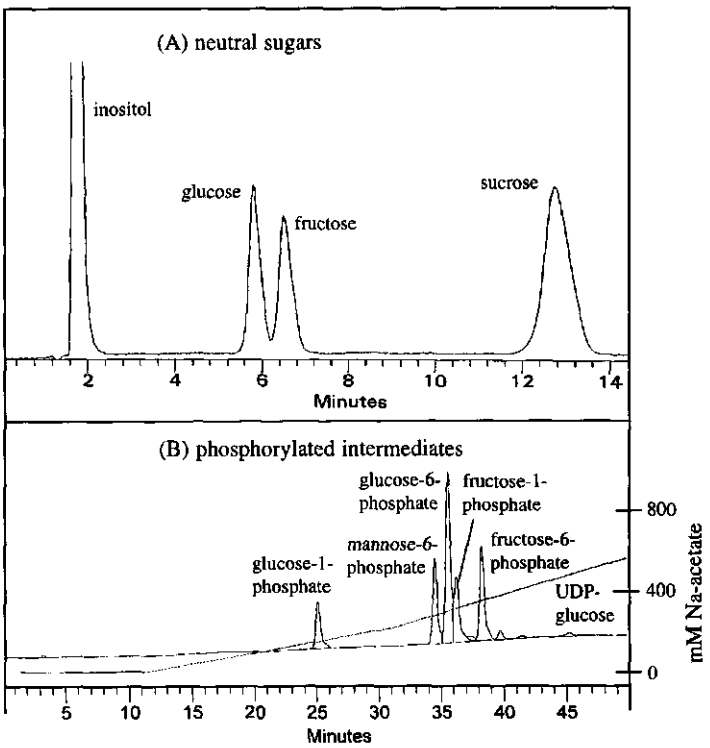

Figure 5 Standard HPLC chromatogram of neutral sugars separated with 100 mM NaOH (A) and of phosphorylated sugars separated with a Na-acetate gradient (50-650 mM, right axis) in 20 mM NaOH on a DIONEX HPLC-system equipped with a carbopac PA-1 column at room temperature and 4 °C, respectively.

Scope of the thesis

This thesis analyses the primary metabolism in *Daucus carota* cell suspensions, in order to understand more about the complex relationship between the different pathways, and the way they are regulated. Sugar uptake and conversion, including cycling of metabolites through different pathways were analyzed in logarithmic and stationary phase cells from batch cultures.

In Chapter 2, special attention is paid to the question whether differences exist between the use of glucose and fructose by plant cells. Both molecules are formed by hydrolysis of sucrose and form the main carbon-source of the cells. The molecules resemble each other: the overall chemical formula is the same (Figs 2A,B). In addition, the phosphorylation products of glucose and fructose, glucose-6-phosphate and fructose-6-phosphate are known to be rapidly interconverted by the enzyme phosphoglucoisomerase. However, the stereo-chemical composition of glucose and fructose is different (Figs 2A,B) which might influence the stereo-specific uptake (Kanabus *et al.* 1988; Botha and Kennedy 1998) and phosphorylation (Doehlert 1989; Steward and Copeland 1993) and the regulation of gene expression by sugar sensing (Jang and Sheen 1994; Smeekens and Rook 1997). Sucrose cycling might be influenced by the difference in the inhibition of the degrading enzymes (Sebková *et al.* 1995; Lee and Sturm 1996). A model is proposed in which glucose and fructose are taken up with different efficiencies by a hexose carrier in the plasmamembrane and are phosphorylated with different efficiencies by soluble fructokinases and mitochondrial-associated hexokinases.

The aim of the next two chapters (3 and 4) was to elucidate the extent to which carbon is cycled during different stages of batch cultured *Daucus carota* cells. Chapter 3 describes label exchange from C-1 to C-6 carbons and the localisation of the OPPP in cytosol and plastids during long-term labelling of batch cultures grown for 14 days. Chapter 4 analyzes short-term labelling for 8 h of cells from different growth stages of batch-cultured cells. It was found that label exchange between C-1 and C-6 carbons, the extent of cycling of sucrose and the amount of carbon cycling through the OPPP were dependent upon the growth stage and respiration rate.

Chapter 5 describes the effect of PFP and the oxygen concentration on cycling of carbon

between hexose phosphates and triose phosphates and sucrose cycling in two carrot cell lines differing in the composition of cell types, i.e. PEMs and VACs.

Finally, integrated models for the different carbon cycles described in the experimental chapters of this thesis are proposed for logarithmically growing cells (importing sugar and synthesizing storage sugars and starch) and stationary phase cells (mobilizing stored carbohydrates). The role of futile cycling of carbohydrates is discussed as a feature of plant metabolism necessary to overcome the changes in environmental conditions and stress factors to which plants are exposed.

Chapter 2

Sucrose and starch metabolism in carrot (*Daucus carota* L.) cell suspensions analyzed by ^{13}C-labelling: Indications for a cytosol and a plastid-localised oxidative pentose phosphate pathway

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published in Journal of Experimental Botany (1998) 49: 1917-1924.

Abstract

Cells were grown in batch culture on a mixture of 50 mM glucose and fructose as carbon source; either the glucose or the fructose was [1-^{13}C]-labelled. In order to investigate the uptake and conversion of glucose and fructose during long-term labelling experiments in cell suspensions of *Daucus carota* L., samples were taken every two days during a two weeks culture period and sucrose and starch were assayed by means of HPLC and ^{13}C-Nuclear Magnetic Resonance. The fructose moieties of sucrose had a lower labelling percentage than the glucose moieties. Oxidative pentose phosphate pathway activity in the cytosol is suggested to be responsible for this loss of label of especially C-1 carbons. A combination of oxidative pentose phosphate pathway activity, a relatively high activity of the pathway to sucrose synthesis and a slow equilibration between glucose-6-phosphate and fructose-6-phosphate could explain these results. Starch contained glucose units with a much lower labelling percentage than glucose moieties of sucrose: it was concluded that a second, plastid-localised, oxidative pentose phosphate pathway was responsible for removal of C-1 carbons of the glucosyl units used for synthesis of starch. Redistribution of label from [1-^{13}C]-hexoses to [6-^{13}C]-hexoses also occurred: 18-45% of the label was found at the C-6 carbons. This is a consequence of cycling between hexose phosphates and triose phosphates in the cytosol catalysed by PFP. The results indicate that independent (oxidative pentose phosphate pathway mediated) sugar converting cycles exist in the cytosol and the plastid.

Keywords: *Daucus carota* L. (cell suspensions), carbon-13 nuclear magnetic resonance (^{13}C-NMR), carbohydrate cycling, oxidative pentose phosphate pathway, plastid

Introduction

Heterotrophic plant cells growing in suspension cultures are dependent on import of carbohydrates from the medium. Sucrose which is usually used as the carbon source is very rapidly hydrolysed extracellularly by a cell-wall-bound invertase after inoculating the cultures and the resulting hexoses are taken up separately (Kanabus *et al.* 1986; Spilatro and Anderson 1988; Wickremesinhe and Arteca 1994). Although glucose and fructose are interconverted after phosphorylation, glucose is preferentially used compared to fructose in several plant cell suspensions as has been demonstrated for *Catharanthus roseus* (Sagishima *et al.* 1989), *Glycine max* (Spilatro and Anderson 1988), *Daucus carota* (Kanabus *et al.* 1986; Dijkema *et al.* 1988, 1990) and *Glycerrhiza glabra* (Arias-Castro *et al.* 1993). This points to either a difference in the uptake of the two hexoses or in their phosphorylation efficiency, i.e. in glucokinase or fructokinase activity as was described by Renz and Stitt (1993) and Schaffer and Petreikov (1997). With the high initial sugar concentrations used in cell suspension cultures (50 mM) diffusion rather than carrier mediated uptake is the main route of uptake (Stanzel *et al.* 1988), implying that a difference in uptake is unlikely to occur.

After phosphorylation, glucose and fructose are used either for respiration, for growth or for synthesis of storage materials: sucrose and starch. When [1-^{13}C]-labelled hexoses are supplied to plant cells, this label will be found also primarily at the C-1 positions of the cellular hexoses and the resulting hexose phosphates. A redistribution of label from C-1 to C-6 carbons has also been described, leading to C-6 labelled hexose phosphates that can be used for the synthesis of sucrose in the cytosol or for the synthesis of starch in the plastids. Hatzfeld and Stitt (1990) found for heterotrophic *Chenopodium rubrum* cell suspensions fed with [1-^{14}C]-glucose 15-20% of the ^{14}C-label at the C-6 carbons in sucrose. They concluded that intensive cycling of metabolites between hexose phosphates and triose phosphates occurs *in vivo* in the cytosol, resulting in the label redistribution from C-1 to C-6 carbons. A relationship was found between this cycling and the activity of the reversible PP_i-dependent fructose-6-phosphate phosphotransferase (PFP): it probably plays a key role in the gluconeogenetic reaction from fructose-1,6-bisphosphate to fructose-6-phosphate in the

cytosol which is necessary for the production of C-6 labelled hexoses (Hatzfeld *et al.* 1990). In *Chenopodium rubrum* cells the redistribution of label from the C-1 to the C-6 carbons was about the same in sucrose and starch; therefore they concluded that hexose phosphates which are used for starch synthesis are not subjected to triose phosphate cycling in the plastids of that species. However, they did report a lower redistribution of label in starch when [6-^{14}C]-glucose was used as a substrate instead of [1-^{14}C]-glucose. Similar results obtained with ^{13}C-labelled glucose in *Triticum aestivum* endosperm (Keeling *et al.* 1988), led to the conclusion that probably a significant part of the oxidative pentose phosphate pathway may be located inside the plastid. Dieuaide-Noubhani *et al.* (1995) found redistribution of label from [2-^{13}C]-glucose to [1-^{13}C]-hexose units in starch but not to [1-^{13}C]-moieties of sucrose, thus concluding that the oxidative pentose phosphate pathway was nearly exclusively located in plastids of maize root tips. Viola *et al.* (1991), however, found for *Solanum tuberosum* tubers and *Vicia faba* seedlings nearly no label redistribution from [2-^{13}C]-glucose to [1-^{13}C]-hexose moieties in sucrose and [1-^{13}C]-glucose units of starch, thereby concluding that the oxidative pentose phosphate pathway was not operating extensively in these tissues. Obviously the occurrence and detection of oxidative pentose phosphate pathway activity is dependent on the tissue examined and the labelling conditions. In all cases label was only applied during short-time intervals ranging from 2 to 5 hours.

Biochemical evidence for the occurrence of the oxidative pentose phosphate pathway in plastids is also reported. Emes and Fowler (1983) and Thom *et al.* (1998) found enzymes of the oxidative pentose phosphate pathway (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, transaldolase, transketolase) in isolated amyloplasts of *Pisum sativum* roots and *Capsicum annuum* fruits, respectively. Schnarrenberger *et al.* (1995) found all enzymes of the oxidative pentose phosphate pathway to be present in the chloroplasts of *Spinacia oleracea* leaves. However, they found an incomplete set of enzymes of the oxidative pentose phosphate pathway in the cytosol.

Next to the findings of oxidative pentose phosphate pathway activity during starch synthesis, evidence for a plastid localized oxidative pentose phosphate pathway was reported in relation to glutamate synthesis (Bowsher *et al.* 1992), fatty acid synthesis (Kang and Rawsthorne 1996) and NO_2^- reduction (Emes and Fowler 1983; Borchert *et al.* 1993; Hartwell *et al.*

1996).

To further investigate the relevance of triose phosphate cycling and cycling of hexoses through the oxidative pentose phosphate pathway in sucrose and starch metabolism, ^{13}C-nuclear magnetic resonance (NMR) was used to continuously measure the uptake and conversion of glucose and fructose to sucrose and starch during long-term labelling experiments ranging from 2 to 14 days. To follow the fate of glucose and fructose independently, parallel experiments were carried out to measure the conversion of [1-^{13}C]-glucose in the presence of unlabelled fructose as well as the conversion of [1-^{13}C]-fructose in the presence of unlabelled glucose. The incorporation of these specifically labelled carbon atoms into sucrose and starch and the redistribution of label from the C-1 to the C-6 carbons can be followed within the same experiments (Keeling *et al.* 1988; Viola *et al.* 1991).

In this report we deduce the existence of carbohydrate cycling in plants from the observed label distribution in sucrose and starch. Cycling in the cytosol between hexose-phosphates and triose phosphates mediated by PFP and cycling through the oxidative pentose phosphate pathway in both cytosol and plastids is suggested.

Materials and Methods

Cell suspensions

After being initiated from hypocotyl-derived callus of *Daucus carota* L. cv. Flakkese (Zaadunie, Enkhuizen, The Netherlands) "Line 10" cell suspensions were kindly provided by Sacco C. de Vries and Marijke Hartog (de Vries *et al.* 1988). Cells were subcultured every 14 days by diluting 2 ml of packed cells in 50 ml Gamborg's B5 medium (Gamborg *et al.* 1968) supplemented with 2.3 μM 2,4-D, 50 mM glucose and 50 mM fructose.

Two parallel types of experiments were performed: one in which glucose was 99.9% [1-^{13}C]-labelled in combination with unlabelled fructose and one in which fructose was 99.9% [1-^{13}C]-labelled and glucose was unlabelled. ^{13}C-labelled compounds were purchased from Isotec Inc. (Miamisburg, Ohio, USA). Samples were taken every two days. Cells were filtered over a Büchner funnel and washed 2 times with Gamborg's B_5 medium without sugar, after which

they were frozen in liquid nitrogen and stored at -80 °C until freeze-drying in a Modulyo 4k (Edwards, Crawley, Sussex, England).

Sugar and starch determinations

Soluble sugars were extracted by boiling 20 mg freeze-dried material in 1.5 ml 80% methanol for 15 minutes at 76 °C. Methanol was evaporated in a Speedvac (Savant Instruments Inc. Farmingdale, NY, USA) and the samples were dissolved in 2.25 ml ultra pure water (Millipore Intertech, Bedford, USA). For NMR spectroscopy, 50 μl 2.5 M Na-acetate at pH 5.9 was added as an internal reference and 200 μl D_2O was added for field lock. The remaining water-insoluble pellet was washed two times in 80% methanol to remove all soluble sugars. Hereafter starch was solubilized in 80% dimethylsulfoxide/1.6 N HCl at 60 °C for 60 minutes in a shaking waterbath. The acid hydrolysate was neutralized with NaOH and buffered with 100 mM citrate/200 mM phosphate to pH 4.6 in a final volume of 10 ml and further degraded by 20 mg (1400 U) *Aspergillus* amyloglucosidase (Fluka, Buchs, Zwitzerland) for 30 minutes at 55 °C. Dimethylsulfoxide was evaporated at 80 °C and the remaining glucose units were freeze-dried. The samples were diluted as described for sugar extracts.

Soluble sugars and glucose units derived from hydrolysed starch were measured with a Dionex HPLC system (Dionex Corporation, Sunnyvale, CA, USA) using a Carbopac PA-1 (guard)column coupled to a pulsed amperometric detector. Isocratic elution was performed with 100 mM NaOH for 15 minutes to separate glucose, fructose and sucrose. Peak areas were quantified using standard sugar solutions.

^{13}C-NMR

^{13}C-labelled sugars were analysed using a Brucker AMX-300 spectrometer (Brucker, Germany) equipped with a 10 mm internal diameter ^{13}C probe. The Waltz sequence was used for two-level proton decoupling. For each spectrum 7200 FID's were collected in 8k data points using a 60° pulse and a pulse repetition time of 0.5 s. A line broadening of 3 Hz was used and zero-filling to 16k data points was applied prior to fourier transformation. The C-2 resonance of acetate at 24.0 ppm was used as an internal reference for quantification.

Peak areas at 96.8 ppm (ß-glucose C-1), 93.0 ppm (sucrose-glucosyl C-1), 63.3 ppm (sucrose-fructosyl C-6), 62.0 ppm (sucrose-fructosyl C-1), 61.5 and 61.4 ppm (ß- and α-glucose C-6) and 60.9 ppm (sucrose-glucosyl C-6) were integrated. Spectra of standard solutions containing 50 mM of the various compounds recorded under similar experimental conditions were used for a proper quantification of the C-1 and C-6 carbons. The amounts of labelled C-1 and C-6 carbons were added and divided by the total concentration of sugar in order to calculate the labelling percentage. Label redistribution between C-1 and C-6 carbons was expressed as percentage labelled C-6 carbons of the sum of labelled C-1 and C-6 carbons.

Enzyme determinations

PP_i-dependent fructose-6-phosphate phosphotransferase (PFP, EC 2.7.1.90) and ATP-dependent fructose-6-phosphate phosphotransferase (PFK, EC 2.7.1.11) were assayed in freshly made extracts from freeze dried material. Ten to twenty mg samples were extracted in 1.2 ml buffer containing 50 mM HEPES at pH 7.5, 5 mM dithiotreitol, 5 mM Mg-acetate and 1 mM EDTA at 4°C. Low molecular weight components were removed on a Biogel P6 column (BioRad, Veenendaal, The Netherlands) (modified after Appeldoorn *et al.* 1997). Enzyme assays were performed under optimal conditions in a final volume of 1.2 ml containing 100 mM Tris/ acetic acid at pH 8.0, 0.15 mM NADH, 5.2 mM fructose-6-phosphate, 0.8 U aldolase, 0.8 U glycerol phosphate dehydrogenase and 0.8 U triose phosphate isomerase. In the PFK assay an additional 0.5 mM $MgCl_2$ was added. For assaying PFP 4.3 μM fructose-2,6-bisphosphate and 0.5 mM Mg-acetate were added. Reactions were started by the addition of 2.5 mM ATP (in the case of PFK) or 1.0 mM PP_i (in the case of PFP) (modified after Hatzfeld *et al.* 1990). NADH conversion was measured using a double beam spectrophotometer operating at 340 nm (Shimadzu, Kyoto, Japan).

Respiration measurements

Oxygen uptake was determined by transferring 2.5 ml of cell suspension directly from the batch culture into an oxygen electrode (Rank Bros., Bottisham, Cambridge, UK). Oxygen uptake was followed for about 10 minutes at 25°C while stirring the suspension. The amount

of hexoses respired per flask (called the cumulative respiration) was calculated from respiration data, by integration of the respiration rate divided by six, as a function of the amount of dry weight.

Results

Sugar uptake, cell growth and sugar content

Figure 1A shows the disappearance of glucose and fructose from the medium, during growth of the carrot cells, measured by HPLC. The [1-^{13}C]-labelled sugars were also measured by NMR, yielding similar results (*data not shown*). The disappearance of fructose from the medium was clearly delayed compared to that of glucose. During the first 6 days about 300 mg of glucose was taken up by the cells, but fructose uptake was only 100 mg (25%, Fig. 1A) and only 6.5 mg was present as free fructose (*data not shown*) in this period. From day 6 on the uptake rate of fructose was comparable to that of glucose (approximately 85 and 75 mg.flask^{-1}.d^{-1} respectively).

Sugar degradation via respiration (Fig. 1B) matched the production of dry matter (Fig. 1C): at the end of the culture about 50% of the initially added hexoses was converted to CO_2. Figure 1C shows that maximum dry weight was reached at day 10-12 (about 425 mg flask^{-1}). Total sucrose and starch levels increased in parallel and were maximal at day 12: about 16 mg flask^{-1} for sucrose and about 18 mg flask^{-1} for starch. After day 12 both levels were decreasing due to depletion of medium sugars and continuing respiration.

Labelling of the cells

Labelling percentages were calculated by adding the amount of labelled C-1 and C-6 carbons divided by the total amount of (labelled and unlabelled) sugars. Labelling percentages of the fructose moieties in sucrose were between 89-95% of that of the glucose moieties (Fig. 2). Labelling percentages for sucrose were generally higher than for starch (Fig. 2). Cells growing on a mixture of [1-^{13}C]-labelled glucose and unlabelled fructose showed a more or less constant maximum labelling percentage from day 2 until day 8 for sucrose (60%) and

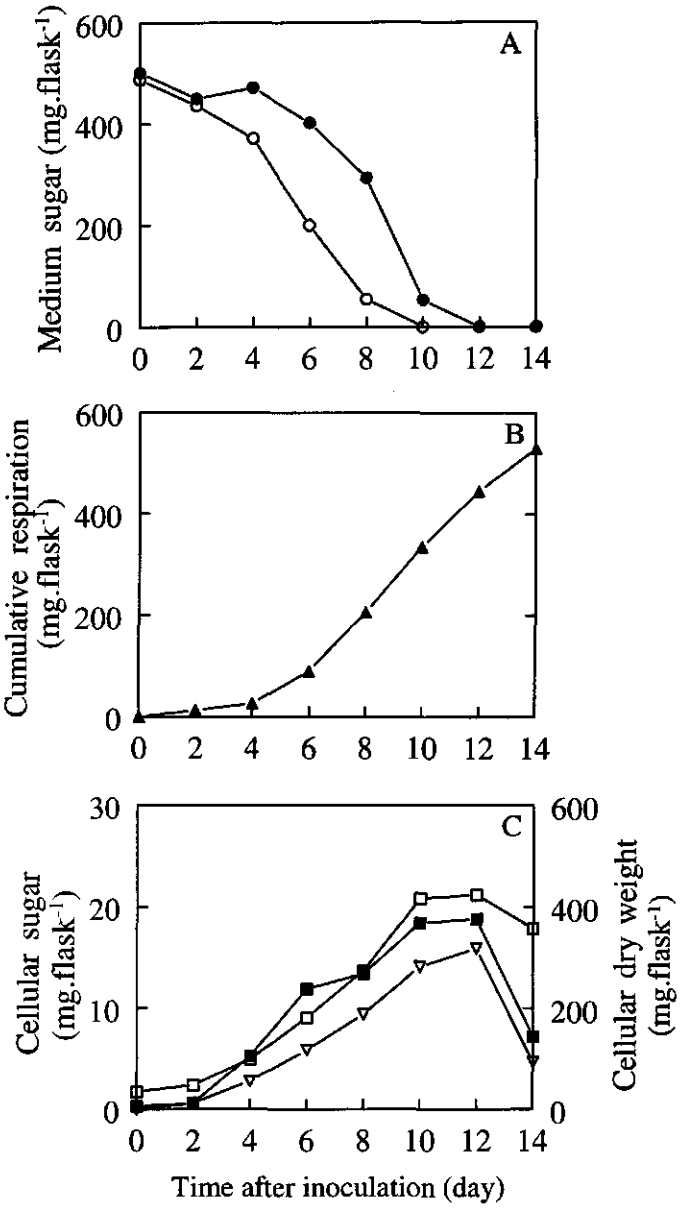

Figure 1 Decrease of total medium glucose (○) and fructose (●) (A), cumulative consumption of sugar in respiration, calculated as mg monosaccharides (▲) (B), and increase of cellular sucrose (▽), starch (■) and dry weight (□) (C) during the course of growth of *Daucus carota* cells in batch culture expressed in mg.flask^{-1}. Values are the means of two experiments.

from day 4 to day 8 for starch (22%, Fig. 2A). From day 8 the labelled glucose in the medium was almost exhausted and unlabelled fructose was taken up only (Fig. 1A); as a consequence labelling percentages decreased to about 18% for both sucrose and starch (Fig. 2A).

When cells were grown on a mixture of unlabelled glucose and [1-^{13}C]-labelled fructose, labelling of cellular sugars and starch started during the first days of growth, although labelling did not exceed 12% for starch and 30% for sucrose during the first 8 days of culture (Fig. 2B). Labelling percentage increased after day 8, when unlabelled glucose had disappeared from the medium and the remaining ^{13}C-labelled fructose was taken up by the cells. Maximum labelling of sucrose was 75% and was higher than that of starch which was maximal 25%.

Label redistribution from C-1 to C-6

Next to C-1 labelled hexoses, also C-6 labelled carbons were detected in the NMR spectra. Label redistribution was expressed as percentage labelled C-6 carbons of total labelled (C-1 and C-6) carbons (Fig. 3). Label redistribution within the glucosyl units of starch was always higher than in the glucose moieties of sucrose; they differed significantly as calculated with a paired Student t-test probability ($p \leq 0.007$) for both experimental data sets (i.e. labelling with [1-^{13}C]-glucose or [1-^{13}C]-fructose). However, label redistribution in the fructose moieties was significantly higher than in the glucose moieties of sucrose (Student t-test probability $p \leq 0.055$ for both data sets). Label redistribution increased during the culture period when cells were grown on [1-^{13}C]-labelled glucose. This increase was larger for sucrose (both glucose and fructose moieties) than for starch (Fig. 3A). If cells were labelled with [1-^{13}C]-fructose, label redistribution was more or less constant between day 4 and day 10 for both sucrose and starch: fructose moieties of sucrose were labelled for about 25%, glucose moieties for about 20% and glucosyl units of starch for about 30% (Fig. 3B).

PFP and PFK activity in relation to label redistribution

Because label redistribution from C-1 to C-6 carbons is thought to occur at the level of triose phosphates (Hatzfeld and Stitt 1990) and because PP_i-dependent fructose-6-phosphate

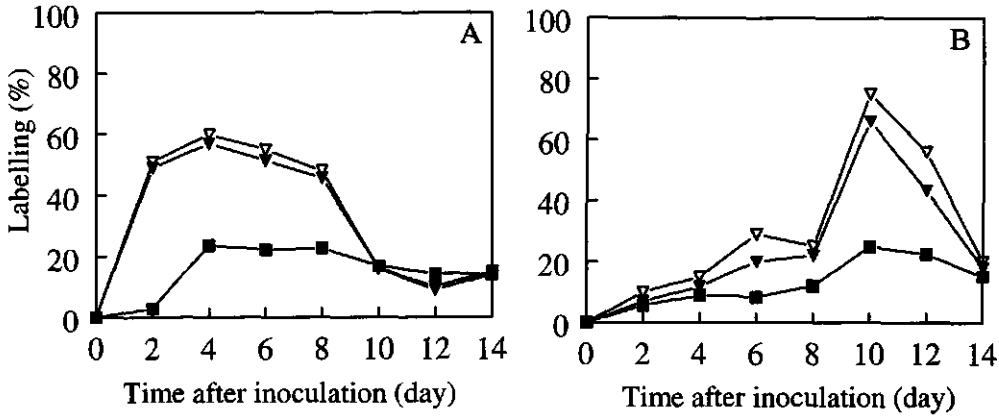

Figure 2 ^{13}C-Labelled glucose (▽) and fructose moieties (▼) of sucrose and ^{13}C-labelled glucosyl units of starch (■) calculated as labelling percentage during the course of growth of *Daucus carota* cells in batch culture grown on 99.9% labelled [1-^{13}C]-glucose and unlabelled fructose (A) or grown on unlabelled glucose and 99.9% labelled-[1-^{13}C]-fructose (B).

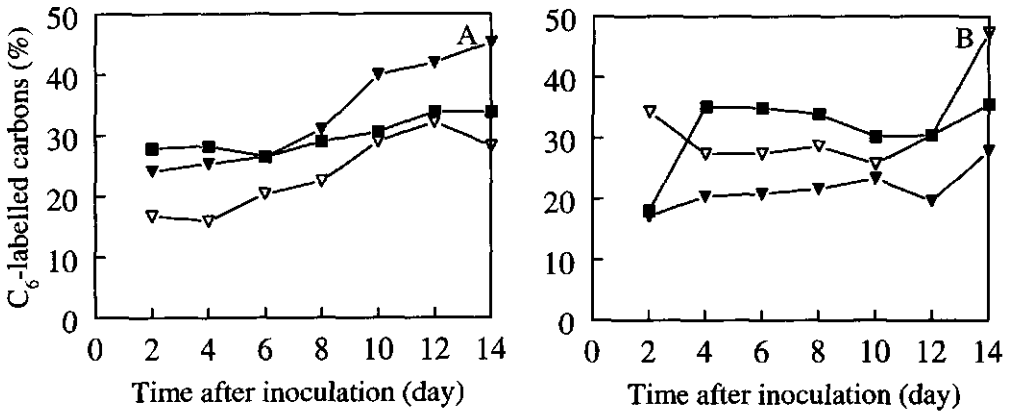

Figure 3 Redistribution of the incorporated label from the C-1 to the C-6 carbons in glucose (▽) and fructose moieties (▼) of sucrose and in the glucosyl units of starch (■) during the course of growth of *Daucus carota* cells in batch culture grown on 99.9% labelled [1-^{13}C]-glucose and unlabelled fructose (A) or 99.9% labelled [1-^{13}C]-fructose and unlabelled glucose (B). Values are expressed as percentages of labelled C-6 carbons of total labelled carbons.

phosphotransferase (PFP) in the cytosol may account for the gluconeogenetic reaction responsible for this conversion (Hatzfeld *et al.* 1990), its activity was determined. Optimised PFP assays showed maximum activity in the logarithmic growth phase (Fig. 4). Its activity was of the same order of magnitude as that of ATP-dependent fructose-6-phosphate phosphotransferase (PFK), indicating that its activity is high enough to support the observed redistribution of label from C-1 to C-6 carbons.

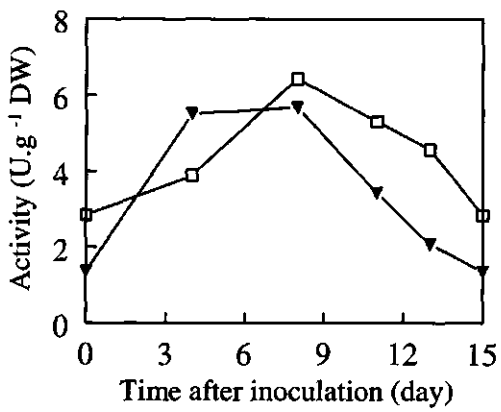

Figure 4 Activity of PP_i-dependent fructose-6-phosphate phosphotransferase (PFP, ▼) and ATP-dependent fructose-6-phosphate phosphotransferase (PFK, □) expressed in $U.g^{-1}$ DW during the course of growth of *Daucus carota* cells in batch culture. One Unit represents the amount of enzyme which liberates 1 μmol $product.min^{-1}$.

Discussion

Sugar uptake, cell growth and sugar content

The preferential use of glucose by *Daucus carota* cell suspensions is consistent with reports about other plant cell suspensions. In the first 6 days of the culture about 100 mg of fructose and 300 mg of glucose was taken up from the medium (Fig. 1A). This 100 mg of fructose was far more than the cellular level of fructose present at that time: only 6.5 mg was

detected as free fructose (*data not shown*). Thus, fructose was already being metabolized, although to a much lesser extent than glucose. This result is consistent with the observations of Fig. 2B: cells grown on unlabelled glucose and ^{13}C-labelled fructose showed some conversion of the ^{13}C-labelled fructose resulting in ^{13}C-labelled sucrose in the first 6 days.

Labelling percentage of soluble sugars and starch

About 50% of the hexoses was used in respiration (Fig. 1B). From the incorporated hexoses only 5-10% of the ^{13}C-label was present in monosaccharides, disaccharides and storage starch (Figs 1C, 2A,B). The remainder was used for synthesis of NMR invisible products other than starch, especially polymers like cell walls, lipids, proteins and polysaccharides.

Because the labelling percentage of sucrose never exceeded 60% in cells grown on [1-^{13}C]-glucose (Fig. 2A) and 75% in cells grown on [1-^{13}C]-fructose (Fig. 2B) it was concluded that dilution of label could occur by a combination of uptake and conversion of unlabelled sugar, by unlabelled sugars which were already present in the cells and by metabolic pathways like the oxidative pentose phosphate pathway removing specific the C-1 carbons of hexoses.

The total labelling percentage (C-1 and C-6 carbons) of the fructose moieties was slightly lower than that of the glucose moieties. This indicates that the labelling percentage of the fructose-6-phosphate pool (yielding the fructose moiety of sucrose) is lower than of the glucose-6-phosphate pool (responsible for the glucose moiety), independently whether [1-^{13}C]-glucose or [1-^{13}C]-fructose was used by the cells. This may be explained by oxidative pentose phosphate pathway activity in the cytosol: C-1 carbons are split off resulting in fructose-6-phosphate with a lower labelling percentage. This would only lead to the observed differences in labelling percentage if the interconversion of fructose-6-phosphate and glucose-6-phosphate is relatively slow compared to the flux of metabolites through the oxidative pentose phosphate pathway and the pathway responsible for sucrose synthesis (Fig. 5).

Hexose phosphates are also transported from the cytosol to the plastids, where starch is synthesized via ADP-glucose (Keeling *et al.* 1988; Hatzfeld and Stitt 1990; Viola *et al.* 1991; Hill and Smith 1991; Neuhaus *et al.* 1993; Viola 1996). Thorbjørnsen *et al.* (1996) reported ADP-glucose pyrophosphorylase to be present in the cytosol of *Hordeum vulgare* endosperm. They found one gene to encode for two different transcripts. One isoform is present in the

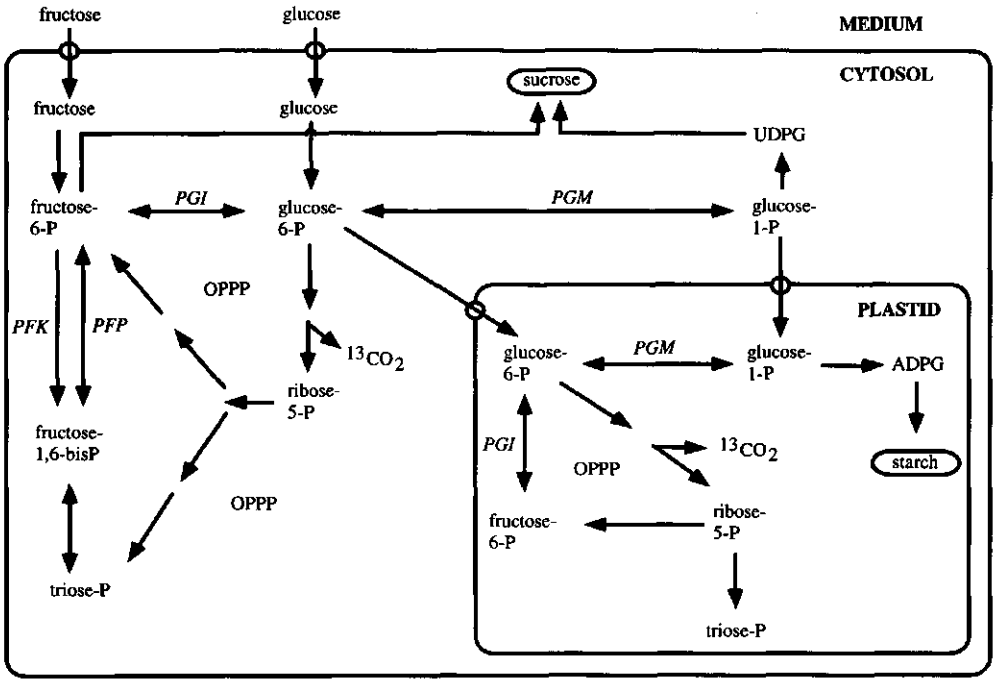

Figure 5 The proposed metabolic pathways in *Daucus carota* cells responsible for the observed label redistribution from C-1 to C-6 carbons in sucrose and starch. P = phosphate group, *PFK* = ATP-dependent fructose-6-phosphate phosphotransferase, *PFP* = PP_i-dependent fructose-6-phosphate phosphotransferase, *PGM* = phosphoglucomutase, *PGI* = phosphoglucoisomerase, PPP = oxidative pentose phosphate pathway, UDPG = UDP-glucose, ADPG = ADP-glucose.

plastid and one in the cytosol (Thorbjørnsen *et al.* 1998). In our experiments a difference in the total labelling percentage between sucrose and starch was found, indicating that the oxidative pentose phosphate pathway removed C-1 label from hexose phosphates in the plastids before ADP-glucose was synthesised.

Sucrose labelling reached a maximum of 75% (Fig. 2B); this labelling percentage quickly decreased as soon as labelled sugar in the medium was exhausted, indicating the fast turnover of sucrose in the cytosolic and vacuolar compartments as described by Wendler *et al.* (1990) and Dancer *et al.* (1990). Starch labelling never exceeded 25% and was less than the labelling percentage of sucrose for most of the growth period (Figs 2A,B). This can be

explained by the different label redistribution between C-1 and C-6 carbons.

Label redistribution from C-1 to C-6 carbons

Label redistribution from C-1 to C-6 carbons in hexoses was demonstrated in plants by Keeling *et al.* (1988), Hatzfeld and Stitt (1990) and Viola *et al.* (1991). PFP (only present in the cytosol) is supposed to catalyse the essential reaction from fructose-1,6-bisphosphate to fructose-6-phosphate after label redistribution from C-1 to C-6 carbons at the level of triose phosphates (Hatzfeld *et al.* 1990). Because label redistribution from C-1 to C-6 carbons in sucrose was high (18-45%), PFP activity (responsible for the glyconeogenetic reaction) is expected to be in the same order of magnitude as PFK activity (responsible for the glycolytic reaction, Fig. 5). Optimised PFP and PFK assays did indeed show this (Fig. 4). Fructose moieties of sucrose always had a higher label redistribution from C-1 to C-6 carbons than glucose moieties (Figs 3A,B). This difference in labelling between glucose and fructose moieties of sucrose was also published before (Keeling *et al.* 1988; Hatzfeld and Stitt 1990; Viola *et al.* 1991). Probably cycling through the oxidative pentose phosphate pathway in the cytosol produced fructose-6-phosphate that has lost its label at the C-1 position. Indeed, the NMR spectra showed that the difference in labelling was caused by a lower labelling of C-1 carbons of the fructose moieties (*data not shown*).

If the same pool of hexose phosphates, containing label at the C-1 and C-6 carbons, is used for sucrose synthesis and starch synthesis, label partitioning over C-1 and C-6 carbons is expected to be the same in sucrose and starch. This was indeed found for *Chenopodium rubrum* labelled with [1-^{14}C]-glucose (Hatzfeld and Stitt 1990), for *Triticum aestivum* (Keeling *et al.* 1988) and for *Solanum tuberosum* and *Vicia faba* (Viola *et al.* 1991) labelled with [1-^{13}C]-glucose. These authors all proposed that no extra round of triose phosphate cycling occurs in the plastids, and concluded that imported hexose phosphates are directly built into starch. However, Fig. 3 showed differences in label redistribution between the glucosyl moieties in sucrose and the glucose units in starch, indicating that cycling does occur in plastids of *Daucus* cells. The lower starch labelling was found to be caused mainly by a lower labelling of the C-1 carbons (*data not shown*). The increased label redistribution from C-1 to C-6 carbons observed in the glucosyl units of starch in combination with the lower

labelling percentage could be explained by cycling of hexose phosphates through the oxidative pentose phosphate pathway in the plastids. This was also found for maize root tips (Dieuaide-Noubhani *et al.* 1995). These workers showed that experiments with [2-^{13}C]--glucose did result in synthesis of C-1 labelled starch, and nearly no C-1 labelled sucrose, indicating that the oxidative pentose phosphate pathway was exclusively located in the plastids in this tissue.

Hatzfeld and Stitt (1990), Keeling *et al.* (1988) and Viola *et al.* (1991) all performed labelling experiments with a pulse labelling at short-term intervals varying from 2-5 h. They probably would not observe extensive cycling because steady-state conditions exhibiting oxidative pentose phosphate pathway activity in the plastids would not be reached during their treatments. Our experiments were performed by growing cell suspensions at a long-term interval for 2-14 d on medium containing 50% labelled hexoses. Steady-state conditions exhibiting extensive cycling during the 2 d intervals thus would be easily observed.

Conclusion

From literature it appears that the occurrence and detection of oxidative pentose phosphate pathway activity depends strongly on the species and tissue examined and the experimental set up. Our long-term labelling experiments of 2 weeks described here give a better insight into the available metabolic routes in plant cells than the short term labelling experiments published before.

From the observed label redistribution from C-1 to C-6 carbons in sucrose we conclude that in *Daucus* cells different types of cycling of hexoses phosphates occur. Firstly, cycling from hexose phosphates to triose phosphates and *vice versa* was probably catalysed by PFP in the cytosol. Secondly, the lower labelling percentage and the higher label redistribution from C-1 to C-6 carbons in the fructose moieties as compared to glucose moieties in sucrose can be explained by the presence of oxidative pentose phosphate pathway activity in combination with a relatively slow equilibration of glucose-6-phosphate and fructose-6-phosphate in the cytosol. Finally, the lower labelling percentage of starch and the higher label redistribution between C-1 and C-6 carbons in starch could be explained by the presence of a second, plastid-localised oxidative pentose phosphate pathway.

Acknowledgements

This project was financially supported by the Foundation for Life Science (SLW), which is subsidized by the Netherlands Organisation for Scientific Research (NWO). The authors are indebted to Sacco C. de Vries and Marijke Hartog of the Laboratory of Molecular Biology, Wageningen University for supplying embryogenic cell suspensions of *Daucus carota*.

Chapter 3

Uptake and phosphorylation of glucose and fructose in carrot (*Daucus carota* L.) cell suspensions are differently regulated

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Abstract

Cell suspensions of *Daucus carota* L. were grown in batch culture on 50 mM sucrose, 100 mM glucose or 100 mM fructose. Sucrose was rapidly converted extra-cellularly into equimolar amounts of glucose and fructose, and glucose was then taken up preferentially. The impaired uptake of fructose could only partially be explained by the lower affinity of the hexose carrier in the plasmamembrane, fructose and glucose showing K_m values of 650 and 80 μM, respectively. However, cells grown on fructose as the sole carbon source showed higher sugar uptake and conversion compared to glucose-grown cells. *In vitro* determination of hexose phosphorylating activities showed two distinct fractions: a soluble fraction containing mainly fructokinase activity (EC 2.7.1.4), and a membrane-bound, mitochondrial fraction showing similar amounts of glucose and fructose phosphorylating activity ("hexokinase", EC 2.7.1.1). Soluble fructokinase activity was thought to be connected with a "compartment" giving rise to a pool of UDPG resulting in structural cell components; as a result fructose-grown cells showed more production of biomass and a higher sucrose level compared to glucose-grown cells. About 95% of the hexokinase-activity was bound to mitochondria. It is suggested that the membrane-bound phosphorylating enzyme(s) are present in a respiratory "compartment" making glucose a better substrate for respiration compared to fructose. The membrane-bound enzyme(s) phosphorylated fructose less efficiently compared to glucose (with an at least ten-fold higher K_m value), which may result in a low initial respiration rate and less production of NTPs in fructose-grown cells. This might be the cause of the observed transient obstruction of the conversion of glucose-1-phosphate into UDP-glucose leading to glucose-1-phosphate accumulation. Furthermore, fructose-grown cells accumulated glucose up to 10-fold higher compared to glucose-grown cells, suggesting that glucose produced by the process of sucrose-cycling was inaccessible to the membrane-bound hexokinase. Although it is usually assumed that the cytosol contains one well-mixed pool of glycolytic intermediates, these results indicate that at least two functional pools might exist. A distinct physical location of the fructokinase and hexokinase might result in separated pools of glycolytic intermediates if the turnover rate of both pools is fast

compared to the diffusion between them.

Keywords: *Daucus carota* L. (cell suspensions), hexoses, (soluble) fructokinase, (membrane-bound) hexokinase, respiration, uptake

Introduction

Although glucose and fructose have similar molecular structures and are highly interconvertible in glycolysis, many plant cells respond differently to fructose and glucose with respect to uptake, conversion, growth and respiration. Different efficiencies for glucose and fructose with respect to growth were shown by Zwayyed *et al.* (1991), Gertlowski and Petersen (1993), Wickremesinhe and Arteca (1994) and Kanabus *et al.*. (1986).

Inhibition of fructose uptake by glucose is reported for Glycine max (de Klerk-Kiebert *et al.* 1983), *Daucus carota* (Kanabus. *et al.* 1986; Dijkema *et al.* 1988, 1990) and *Phaseolus vulgaris* cell suspensions (Botha and O'Kennedy 1998). At concentrations up to 2 mM a transmembrane-carrier is responsible for uptake of hexoses (Stanzel *et al.* 1988a; Sauer *et al.* 1990; Rausch *et al.* 1991; Verstappen *et al.* 1991). This carrier shows a higher affinity for glucose than for fructose; as a result glucose outcompetes fructose at these low concentrations (Botha and O'Kennedy 1998). At concentrations higher than 2 mM (facilitated) diffusion through the plasma membrane will take place next to this carrier-mediated uptake of hexoses (Stanzel *et al.* 1988b; Aked and Hall 1993; Botha and O'Kennedy 1998).

Inside the cells glycolytic breakdown of glucose and fructose starts with phosphorylation, leading directly (for fructose) or indirectly (via glucose-6-phosphate for glucose) to fructose-6-phosphate; after isomerization of the hexose phosphates conversion is generally assumed to be identical for both sugars. The phosphorylation of fructose to fructose-6-phosphate by fructokinase is regulated by substrate and product inhibition and nucleotide

specificity. In most plants fructokinases phosphorylate only fructose (Yamashita and Ashihara 1988; Doehlert 1989) and show optimum activity at fructose concentrations of 0.5 mM; at concentrations of 50 mM fructose they are inhibited for about 60% as is shown for developing *Zea mays* kernels (Doehlert 1989) and *Lycopersicon esculentum* fruit (Martinez-Barajas and Randall 1996). In the species mentioned above ATP is the preferred nucleotide triphosphate (NTP). Fructokinases from *Acer pseudoplatanus* cells (Huber and Akazawa 1986), *Catharanthus roseus* cells (Yamashita and Ashihara (1988) and *Spinacia oleracea* leaves (Schnarrenberger 1990) use UTP as the preferred phosphate donor.

Kanayama *et al.* (1998) found 2 isoforms of fructokinase in *Lycopersicon esculentum* fruit. One isoform which showed inhibition by high levels of fructose was related to starch synthesis in pericarp tissue. A second isoform did not show this inhibition and was found at constant levels in all tissues and was proposed to be a maintenance enzyme. So, depending on the type of tissue and species the presence of different NTPs and the concentration of fructose might influence fructose phosphorylation.

In contrast to fructokinases, glucokinases are known to be less sugar-specific and are able to phosphorylate other hexoses like fructose and mannose (Doehlert 1989; Schnarrenberger 1990; Renz and Stitt 1993; Steward and Copeland 1993). Therefore, they are more commonly entitled as "hexokinases". These hexokinases are also known to play a role in "sugar sensing" by transmitting signals for a broad range of hexoses concerning e.g. repression of photosynthetic genes in *Zea mays* seedlings (Jang and Sheen 1994) and α-amylase genes in *Oryza sativa* (Umemura *et al.* 1998). In addition, these hexokinases are known to be partially bound to mitochondrial membranes (Renz *et al.* 1993; Steward and Copeland 1993; Martinez-Barajas and Randall 1998) and chloroplast membranes (Singh *et al.* 1993). Hexokinases are known to be more active with ATP than with other NTPs (Yamashita and Ashihara 1988; Xu *et al.* 1989; Nakamura *et al.* 1991). Recently, hexokinases which are sensitive to inhibition by ADP are described for *Zea mays* (Martinez-Barajas and Randall 1998; Galina *et al.* 1999).

Here we investigated whether the difference in glucose and fructose utilization by *Daucus carota* cells as found by Kanabus *et al.* (1986) and Dijkema *et al.* (1988; 1990) is caused

by a lower uptake rate or by a lower phosphorylation rate of fructose. Therefore, cells were grown in batch culture on glucose ("glucose-grown cells"), fructose ("fructose-grown cells") or sucrose ("sucrose-grown cells"). The observed differences in conversion rate of glucose and fructose, in cell growth and in sugar content were explained by the properties of the uptake system and distribution of fructokinase and glucokinase activities within the cells.

Materials and Methods

Cell suspensions

After being initiated from hypocotyl-derived callus of *Daucus carota* L. cv. Flakkese (Zaadunie, Enkhuizen, The Netherlands) "Line 10" cell suspensions were kindly provided by Sacco C. de Vries and Marijke Hartog (de Vries *et al.* 1988). Cells were subcultured every 12 days by diluting 2 ml of packed cells in 50 ml autoclaved Gamborg's B5 medium (Gamborg *et al.* 1968) supplemented with 2.3 μM 2,4-D and 100 mM filter-sterilized glucose or fructose or 50 mM sucrose. Samples were taken every two days. Cells were filtered over a Büchner funnel and washed 2 times with Gamborg's B5 medium without sugar. Fresh weight was determined after which the samples were frozen in liquid nitrogen and stored at -80 °C. After freeze-drying in a Modulyo 4k (Edwards, Crawley, Sussex, UK) dry weight was measured.

Sugar determinations: neutral sugars and phosphorylated intermediates

Soluble sugars were extracted by boiling freeze-dried material in 80% methanol for 15 minutes at 76 °C. Methanol was evaporated in a Speedvac (Savant Instruments Inc. Farmingdale, NY, USA) and the samples were dissolved in ultra pure water (Millipore Intertech, Bedford, USA). Soluble sugars were measured with a Dionex HPLC system (Dionex Corporation, Sunnyvale, CA, USA) using a Carbopac PA-1 column with similar guard column and pulsed amperometric detection (Tetteroo *et al.* 1995). Isocratic elution was performed with 100 mM NaOH for 15 minutes to separate glucose, fructose and

sucrose. Hexose phosphates were measured with the same system with the column cooled to 4 °C. To separate glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate, elution was performed with 20 mM NaOH and a Na-acetate gradient: 50 mM from 0 to 10 minutes, a linear gradient from 50 mM to 200 mM from 10 to 30 minutes and a linear gradient from 200 mM to 650 mM from 30 to 65 minutes (de Bruijn *et al.* 1999). Peak areas were quantified using standard sugar and standard sugar phosphate solutions measured under identical conditions.

^{14}C-hexose uptake

[U-^{14}C]-glucose (specific activity 317 GBq/mmol) and [U-^{14}C]-fructose (specific activity 612 GBq/mmol) were purchased from Amersham Life Science (Buckinghamshire, UK). Cells of 6 days old sucrose-grown cultures were washed 2 times with Gamborg's B5 medium without sugar. After recovery of the cells for 2 hours at 25 °C, erlenmeyer flasks of 25 ml were incubated with 2.5 ml washed cells. At time zero 0.05 to 45 mM glucose or fructose supplemented with 716 to 1199 Bq ^{14}C-labelled glucose or fructose was added. Inhibition of fructose uptake by glucose was tested at low and high concentrations by adding 0-2 mM unlabelled glucose to 2 mM labelled fructose and 0-18 mM unlabelled glucose to 18 mM labelled fructose, respectively. After 15 minutes uptake was stopped by filtering the cells over a Büchner funnel and washing quickly 2 times with Gamborg's B5 medium without sugar. Cells were dried on filter paper and radioactivity was determined using a liquid scintillation counter (Beckman LS6000TA, Beckman Instr. Inc., Fullerton, CA, USA).

Glucokinase and fructokinase activity

Fructokinase (EC 2.7.1.4) and glucokinase (EC 2.7.1.1) activities were assayed in freshly made extracts from freeze-dried material of 3 days old sucrose-grown cells. Twenty-five mg samples were ground in a mortar with a pestle and extracted in 1.2 ml buffer containing 50 mM HEPES at pH 7.5, 5 mM DTT, 5 mM Mg-acetate and 1 mM EDTA at 4°C. Extracts containing soluble and membrane-bound enzymes were separated in an eppendorf centrifuge at full speed for 5 minutes. Low molecular weight components in

the supernatant were removed on a Biogel P6 column (BioRad, Veenendaal, The Netherlands) (modified after Appeldoorn *et al.* 1997). Pellets containing insoluble cell components were washed with buffer and resuspended in 1.0 ml buffer and assayed separately for insoluble, membrane-bound glucokinase and fructokinase activity. Standard enzyme assays were performed in a final volume of 1.2 ml containing 100 mM Tris at pH 8.0, 3 mM ATP, 0.22 mM NAD^+ and 0.8 U glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*). For fructokinase, an additional 0.8 U phosphoglucoisomerase was added. Reactions were started by the addition of fructose or glucose. NAD^+ reduction was measured using a double beam spectrophotometer operating at 340 nm (Shimadzu, Kyoto, Japan). For insoluble enzymes, assay volumes were centrifuged in an Eppendorf centrifuge for 20 s at full speed after which the supernatant was measured immediately at 340 nm. Glucose and fructose saturation curves for glucokinase and fructokinase were carried out at 3 mM ATP. NTP saturation curves were determined for ATP and UTP at 50 mM glucose or fructose.

Results

Sugar uptake and cell growth

Sugars disappeared from the medium in 6-8 days, depending on the type of sugar (Fig. 1A). When cells were grown on 100 mM fructose sugar disappearance from the medium was low during the first 2 days and sugar had completely disappeared from the medium at day 6. When cells were grown on 100 mM glucose the lag phase was extended from 2 to 4 days and medium depletion occurred after 8 days. Sucrose was completely hydrolysed within 2 days and thereafter glucose and fructose were taken up separately (Fig. 1A, dashed lines). From the resulting 50 mM glucose and 50 mM fructose, glucose was used preferentially: 50% of the glucose and 15% of the fructose had disappeared at day 4.

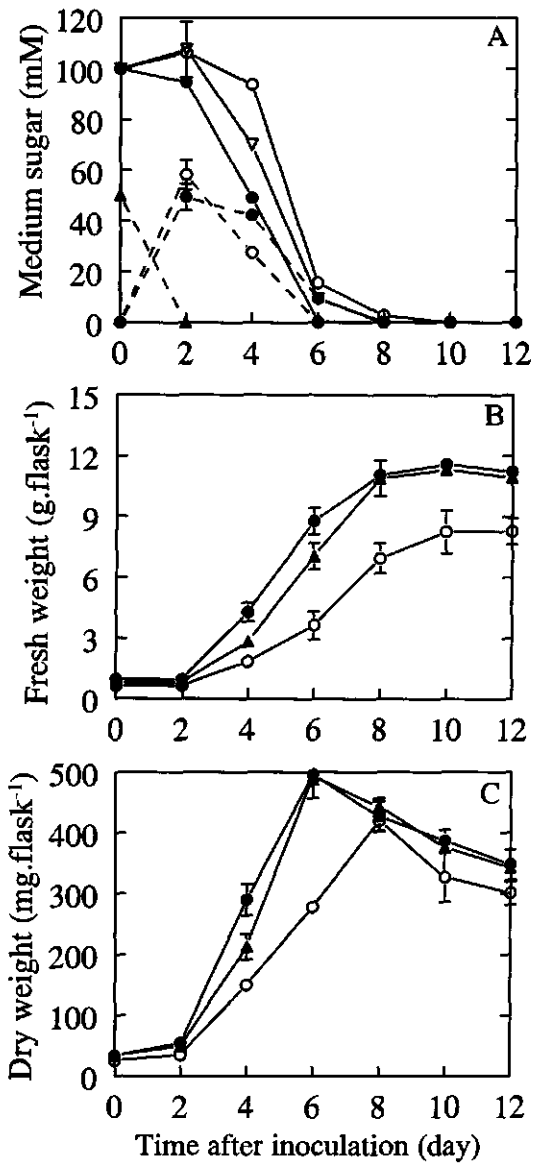

Figure 1 Medium sugars during the course of growth of *Daucus carota* cells in batch culture. Disappearance of sugar in fructose-grown cells (●) and glucose-grown cells (○). Symbols connected with dashed lines show disappearance of sucrose (▲) and changes in glucose (○) and fructose (●) and the sum of both (▽) in the medium of sucrose-grown cells (A). Fresh weight in $g.flask^{-1}$ (B) and dry weight in $mg.flask^{-1}$ (C) for glucose-grown cells (○), fructose-grown cells (●) and sucrose-grown cells (▲). Values are the means of 3 replicates ± S.D. If not shown, S.D. is smaller than symbol.

From day 4 on the rates of the disappearance of glucose and fructose were about equal; after 6 days the glucose and after 8 days also the fructose had completely been taken up from the medium by sucrose-grown cells.

Fresh weight of the cells increased from day 2 to day 10 (Fig. 1B): sucrose-grown and fructose-grown cells reached the same fresh weight: about 11.5 g.flask^{-1}; for sucrose-grown cells, however, this fresh weight increase lagged somewhat behind. Fresh weight production of glucose-grown cells was slower and ceased at about 75% of that of fructose- and sucrose-grown cells at 8.5 g.flask^{-1}.

Maximum dry weight productions (Fig. 1C) of fructose-grown and sucrose-grown cells were similar: about 500 mg.flask^{-1}, while maximum dry weight production of glucose-grown cells was reached 2 days later and was about 85% of that of fructose- and sucrose-grown cells (425 mg.flask^{-1}).

Uptake of ^{14}C-hexoses

Uptake of glucose and fructose was measured using ^{14}C-labelled hexoses. Both glucose and fructose showed a biphasic uptake curve; in the range of 0 to 2 mM uptake characteristics fitted active uptake via a carrier, showing saturation (Michaelis Menten) kinetics with a K_m value of about 80 μM for glucose and 650 μM for fructose. For both sugars a V_{max} of about 0.35 μmol.(g dry weight)$^{-1}$ min^{-1} was determined (deduced from Lineweaver-Burk plots, inserts of Figs 2A,B).

In the range of 5 to 45 mM uptake was diffusion-like as was concluded from the linear relationship between the rate of uptake and concentration. Uptake of fructose was not significantly different from that of glucose (Fig. 2, closed symbols). Uptake of fructose at low concentrations (2mM) in the presence of 2 mM glucose was about 20% of the uptake in the absence of glucose (Fig. 3A), while at high concentrations (18 mM) in the presence of 18 mM glucose fructose uptake was about 50% of the uptake without added glucose (Fig. 3B). Glucose uptake was inhibited for only 15% at concentrations of 2 mM of both glucose and fructose (*data not shown*).

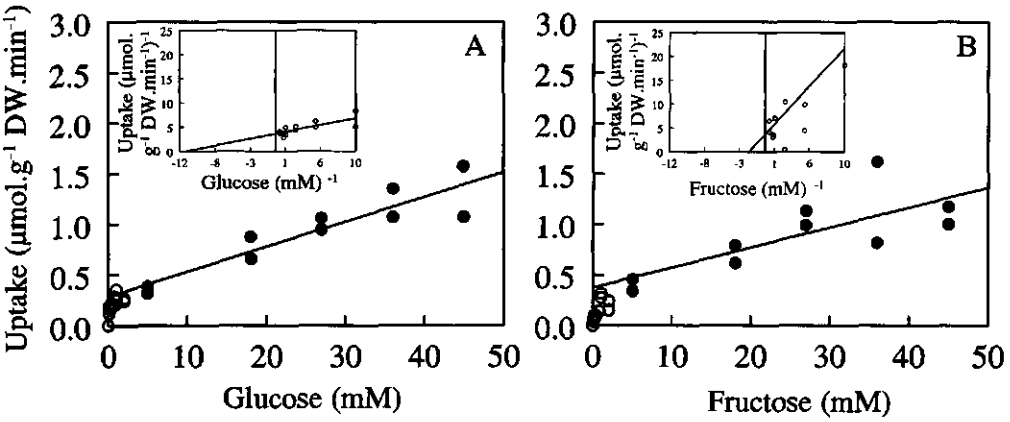

Figure 2 Uptake of ^{14}C-glucose (A) and ^{14}C-fructose (B) by 6 days old sucrose-grown *Daucus carota* cells expressed as µmol.g^{-1} DW.min^{-1}. Uptake at concentrations from 0.1 to 2.0 mM (○) are also shown in Lineweaver-Burk plots in the inserts. Uptake at concentrations from 5.0 to 45 mM (●) is linearly fitted. Pooled data of two experiments are shown from which K_m values of 80 µM and 650 µM were calculated for glucose and fructose uptake in the range of 0.1-2.0 mM, respectively.

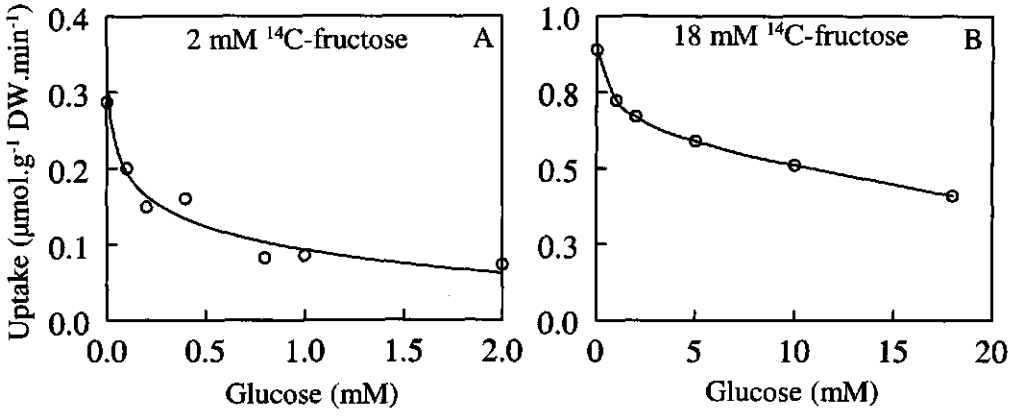

Figure 3 Inhibition of fructose uptake by glucose during incubation of 6 days old sucrose-grown *Daucus carota* cells. Representative data of uptake experiments of 2 mM (A) and 18 mM (B) ^{14}C-fructose at varying glucose levels expressed as µmol.g^{-1} DW.min^{-1}.

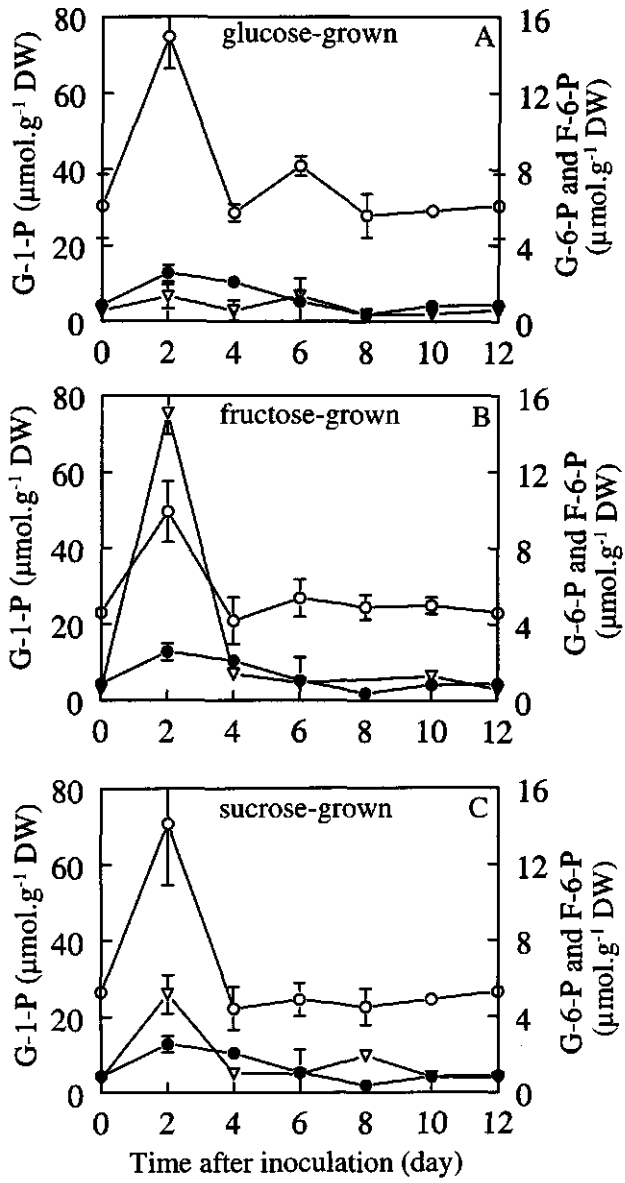

Figure 4 Levels of glucose-6-phosphate (G-6-P, ○), fructose-6-phosphate (F-6-P, ●) at the right y-axis and glucose-1-phosphate (G-1-P, ▽) at the left y-axis expressed as µmol.g^{-1} DW during the course of growth of batch-cultured *Daucus carota* cells grown on 100 mM glucose (A), 100 mM fructose (B) or 50 mM sucrose (C). Values are the means of 3 replicates ± S.D.

Cellular content of sugars and sugar phosphates

Glucose-6-phosphate, fructose-6-phosphate and glucose-1-phosphate were the most prominent phosphorylated intermediates. In all three cultures glucose-6-phosphate peaked at day 2. Fructose-6-phosphate also peaked at day 2, but decreased only gradually towards day 8 (Fig. 4). The most striking difference was the high level of glucose-1-phosphate at day 2 in fructose-grown cells, being 10 times higher than in glucose-grown cells and 3 times higher than in sucrose-grown cells (Fig. 4). From day 4 on, the levels of sugar phosphates were rather comparable for all three culture conditions, roughly 5, 1 and 7 μmol.(g dry weight)$^{-1}$ for glucose-6-phosphate, fructose-6-phosphate and glucose-1-phosphate, respectively.

Glucose-grown cells contained low levels of glucose (50 μmol.(g dry weight)$^{-1}$) and fructose (20 μmol.(g dry weight)$^{-1}$, Fig. 5A). On the contrary, fructose-grown cells contained high levels of glucose (up to 650 μmol.(g dry weight^{-1})) and low levels of fructose (40 μmol.(g dry weight)$^{-1}$, Fig. 5B). Sucrose-grown cells also showed low fructose levels (40 μmol.(g dry weight)$^{-1}$), while glucose was much lower (about 150 μmol.(g dry weight)$^{-1}$) than in fructose-grown cells (Fig. 5C). Sucrose increased to about 400 μmol.(g dry weight)$^{-1}$ in glucose and fructose-grown cells and to about 600 μmol.(g dry weight)$^{-1}$ in sucrose-grown cells. The maximal level of hexose units was highest in fructose and in sucrose-grown cells: up to 1200 μmol hexose units.(g dry weight)$^{-1}$ (Figs 5B,C). In glucose-grown cells maximal levels did not exceed 550 μmol hexose units.(g dry weight)$^{-1}$ (Fig. 5A). The maximal level of hexose units expressed per fresh weight was about 75 μmol for fructose and sucrose-grown cells and about 40 μmol for glucose grown cells (*data not shown*).

Soluble and membrane-bound fructokinase and glucokinase activity: kinetic properties

The sugar saturation curve of soluble fructokinase showed an optimum with maximum activity at 0.5 mM fructose; at 50 mM fructose fructokinase was inhibited for about 60% compared to this optimum. Soluble glucokinase showed maximal activity at 50 mM glucose. At this concentration activity was about 20% of that of fructokinase, at a concentration of 0.5 mM the relative activity of glucokinase was much lower (Fig. 6A).

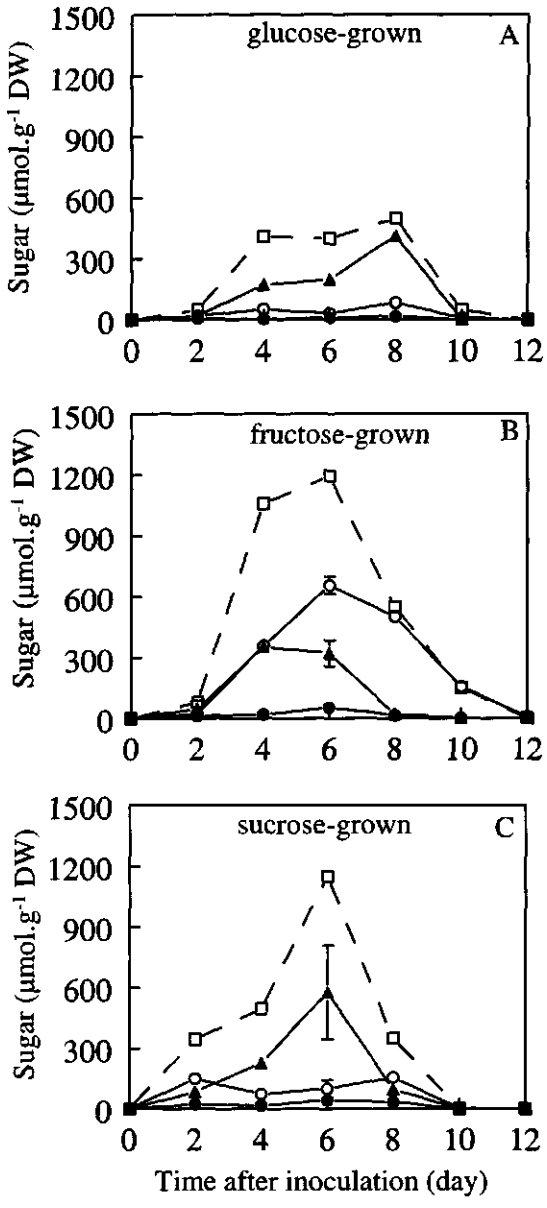

Figure 5 Levels of glucose (○), fructose (●), sucrose (▲) and total hexose units (□ , dashed lines) expressed as µmol.g^{-1} DW during the course of growth of batch cultured *Daucus carota* cells grown on 100 mM glucose (A), 100 mM fructose (B) or 50 mM sucrose (C). Values are the means of 3 replicates ± S.D.

The effect of different concentrations of ATP and UTP on soluble fructokinase and glucokinase was assayed at 50 mM fructose and glucose. Fructokinase showed a slightly higher activity with UTP as a phosphate donor compared to ATP although the affinity towards ATP was higher.

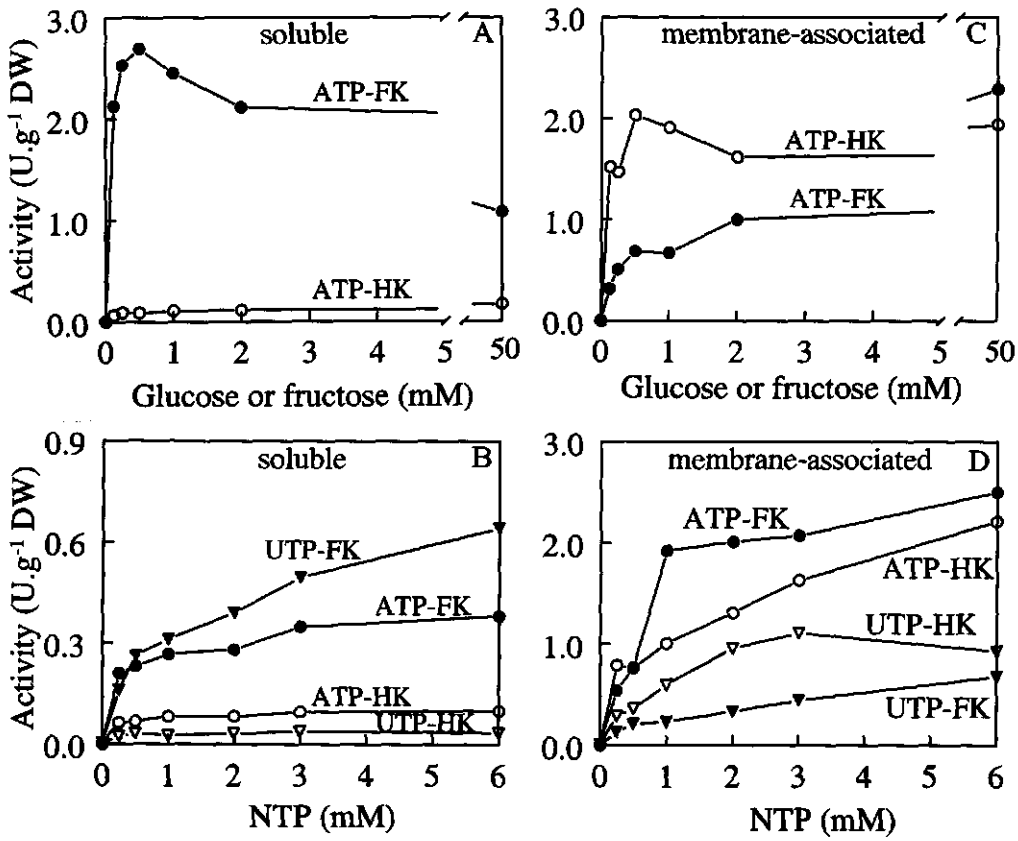

Figure 6 Kinetics of hexokinase and fructokinase activities in the soluble fraction (A,B) and the membrane-bound fraction (C,D) expressed as $U.g^{-1}$ DW. Sugar-saturation curves of fructokinase (FK, ●) and hexokinase (HK, ○) measured at 3 mM ATP (A,C). ATP (○, ●) and UTP (▽, ▼) saturation curves of fructokinase (●, ▼) and hexokinase (○, ▽) measured at 50 mM hexose (B, D). Extracts were prepared from 3 days old batch-cultured cells of *Daucus carota* grown on sucrose. One Unit represents the amount of enzyme which converts 1 µmol hexose.min^{-1}. Different batches were used for the experiments resulting in different absolute values in A, B.

Glucokinase activity was highest with ATP and maximal UTP dependent activity was about 35% of that of ATP activity (Fig. 6B).

In the membrane-bound fraction, activity of glucokinase was much higher than in the soluble fraction (Figs 6B,D). Glucose showed higher affinity towards the membrane-bound fraction with a K_m value at least 10 times lower than for fructose (Fig. 6C). Both membrane-bound glucose and fructose phosphorylating reactions showed a higher affinity for ATP than for UTP: maximal UTP-dependent activities were 70% and 25% for glucokinase and fructokinase, respectively (Fig. 6D).

Discussion

Sugar uptake and cell growth

Lineweaver-Burk plots of the uptake data of Figs 2A,B from 0.05 to 2 mM hexose resulted in a V_{max} of about 0.35 μmol.(g dry weight)$^{-1}$ for both glucose and fructose. At 2 mM total uptake was only slightly higher; it was calculated that the diffusion-like component accounted for only 12% of the total uptake and it was concluded that at low concentrations of hexoses uptake was controlled mainly by carrier-mediated transport. Corresponding K_m values for glucose and fructose in Daucus cells were about 80 μM and 650 μM, respectively (Figs 2A,B), which is in agreement with reports on transmembrane hexose carriers by Sauer *et al.* (1990), Rausch *et al.* (1991) and Verstappen *et al.* (1991). At concentrations higher than 2 mM diffusion occurred next to this carrier-mediated uptake. Uptake of glucose and fructose at concentrations of 50 mM was due to diffusion for about 70-80% (Figs 2A,B). The diffusion-mediated component of glucose and fructose uptake appeared to be about the same (Figs 2A,B). Most of the active uptake of fructose could be abolished by excess glucose (Fig. 3A), while the diffusion-mediated component of uptake of fructose was only slightly suppressed by glucose (Fig. 3B). Fructose uptake from an equimolar mixture of 50 mM of both sugars, therefore, is expected to be about 70% of that of glucose. However, fructose uptake was only 30% of that of glucose up to day 4 (Fig. 1A). Botha and O'Kennedy (1998) concluded for *Phaseolus vulgaris* cell

suspensions that differences in uptake, due to different affinities of the hexose carrier towards glucose and fructose could entirely explain the lower conversion rate of fructose; for *Daucus carota* cell suspensions, other factors seem to play an additional role.

Different hexose and sucrose transporters are known, which are often tissue-specific (Weig *et al.* 1994). Burkle *et al.* 1998 reported a developmental-specific sucrose carrier expressed only in mature (source) leaves of *Nicotiana tabacum*. However, it is assumed that cell suspensions represent one type of tissue with respect to sugar uptake. *Daucus carota* suspension cultures grown on glucose or sucrose for 6 months showed the same higher biomass production when transferred to fructose-medium, indicating that probably the same set of membrane carriers and phosphorylating enzymes was present or rapidly induced after inoculation, irrespective of the carbon source on which they were grown previously.

Our data suggest that in *Daucus carota* cells the conversion of fructose was impaired. However, fructose conversion was not impaired in cells growing exclusively on fructose (Figs 1A,B,C) suggesting that competition between glucose and fructose for the phosphorylating enzymes might occur.

Soluble and membrane-bound fructokinase and glucokinase: kinetic properties

Daucus cells showed two distinct fractions of hexose phosphorylating enzymes: a soluble fraction and a membrane-bound fraction. The soluble fraction contained mainly fructokinase and showed a higher activity with UTP as phosphate donor at concentrations above 0.5 mM NTP. Soluble glucokinase activity was most active with ATP but showed also some activity with UTP (Fig. 6B). It is concluded that the soluble fructokinase and glucokinase might use UTP in addition to ATP *in vivo*.

Up to 90-95% of the glucokinase activity was found in the membrane-bound fraction (Figs 6A,C). It is concluded that glucose is mainly phosphorylated by the membrane-bound enzyme(s). Steward and Copeland (1993) showed that 40% of the glucokinase activity was associated with mitochondria in *Persea americana* and Galina *et al.* (1995) found 45% of the glucokinase activity to be bound to mitochondria in *Zea mays* roots. These enzymes showed highest affinity towards glucose, although fructose might also be

phosphorylated and the enzyme is often referred to as "hexokinase" (Doehlert 1989; Schnarrenberger 1990; Renz and Stitt 1993, Figs 6C,D). Steward and Copeland (1993) found only one hexokinase present in *Persea americana*, although Renz and Stitt (1993) showed 3 hexokinases to be present in developing *Solanum tuberosum* tubers.

The K_m value of the membrane-bound hexokinase for glucose was more than 10 times lower than for fructose (Fig. 6C). Therefore, this hexokinase might use glucose preferentially to fructose in vivo. In our view, cells growing on a mixture of glucose and fructose will phosphorylate fructose mainly in the soluble fraction (Figs 6A,B) and glucose will be mainly phosphorylated by the membrane-bound hexokinase (Figs 6C,D, 7). ADP might inhibit glucose phosphorylation by mitochondrial hexokinase as was shown in *Zea mays* by Martinez-Barajas (1998) and Galina *et al.* (1999). The ATP/ADP ratio might be relatively low at the start of the culture priod; as a consequence glucose phosphorylation may be inhibited and glucose and sucrose-grown cells might show a longer lag-phase compared to fructose-grown cells.

Bender *et al.* (1987) found that *Daucus carota* root explants incorporated ^{14}C-glucose preferentially into malate and citrate while ^{14}C-fructose was incorporated more in sucrose. Sagishima *et al.* (1989) found more $^{14}CO_2$ produced by *Catharanthus roseus* cells fed with [U-^{14}C]-glucose, while [U-^{14}C]-fructose was incorporated more in sucrose. Preliminary results also showed more $^{13}CO_2$ produced by [1-^{13}C]-glucose than by [1-^{13}C]-fructose in *Daucus carota* cells (*results not shown*). Sagishima *et al.* (1989) suggested that fructose was incorporated in sucrose by sucrose synthase activity. However, experiments with cell suspensions labelled with fructose always showed a similar labelling percentage of the glucose and fructose moieties of sucrose, excluding sucrose synthase as a major sucrose synthesizing enzyme (Wendler *et al.* 1990; Kosegarten *et al.* 1995; Krook *et al.* 1998). We suggest that glucose phosphorylation may well be coupled to mitochondrial respiratory activity and that fructose is a better substrate for the synthesis of structural cell components necessary for growth and accumulation of sugar and starch.

It is suggested, therefore, that different "compartments" exist in the same cell: fructose will be phosphorylated by the soluble fructokinase creating a pool of glycolytic intermediates in a "compartment" resulting in structural components, while glucose

creates a pool of glycolytic intermediates surrounding the mitochondria in a "compartment" providing substrates for respiration (Fig. 7).

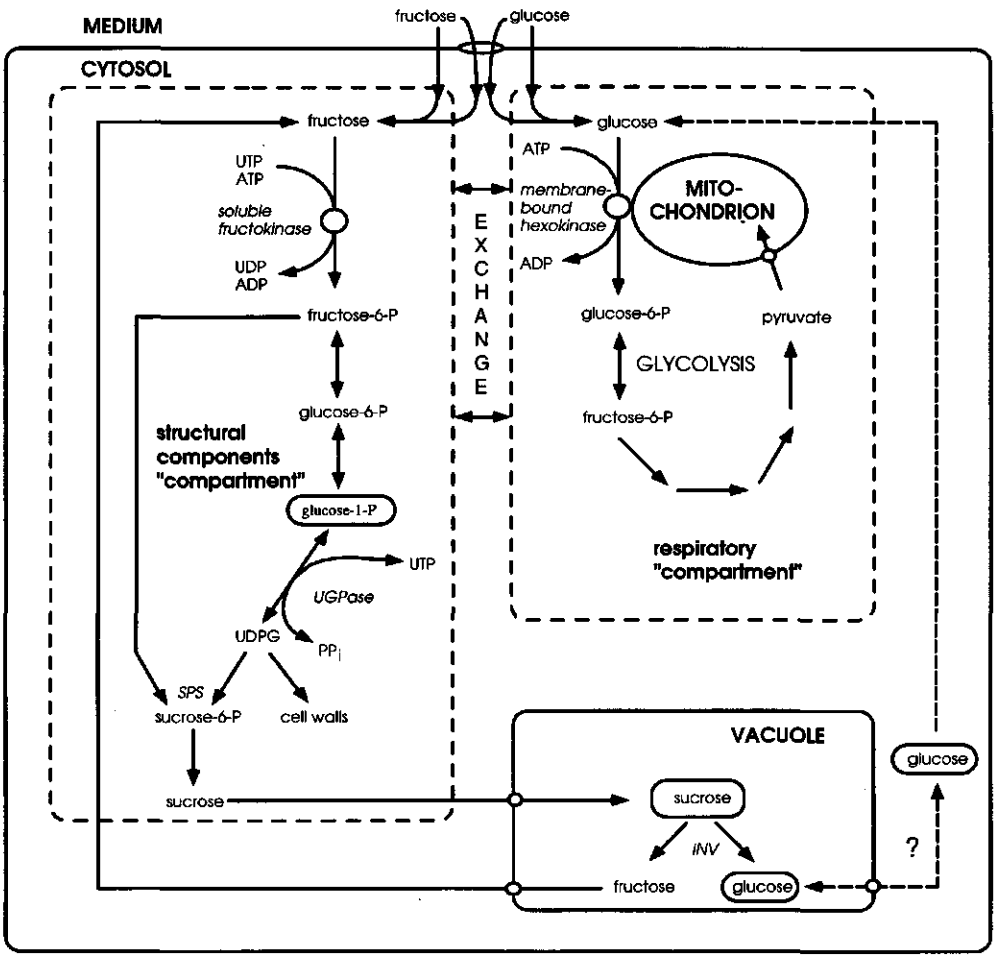

Figure 7 Proposed model for hexose uptake and conversion in *Daucus carota* cells grown in batch culture. Hexoses are taken up by carriers or diffusion. *INV* = invertase, P = phosphate group, PP_i = pyrophosphate, *UGPase* = UDP-glucose pyrophosphorylase, *SPS* = sucrose phosphate synthase. Boxed metabolites depict accumulated sucrose and glucose. The dashed lines surrounding the phosphorylation-reactions show the "compartment" associated with respiration, preferentially using glucose and the "compartment" synthesizing structural components preferentially using fructose.

Both pools might be separated since the mitochondrial volume is only a few percents of the cytosolic volume (Winter *et al.* 1994). Exchange between these two pools is suggested to be limited, probably depending upon the number of mitochondria, their cellular localisation, their connection with the endoplasmatic reticulum and the rate of hexose converting reactions within each pool relative to the exchange rate between them (Fig. 7).

Consequences of the distribution of fructokinase and hexokinase with respect to growth and sugar accumulation

Since the mitochondrial hexokinase is far in excess compared to soluble glucokinase (Figs 6A,C), glucose-grown cells mainly phosphorylate glucose in the respiratory "compartment" and relatively less glucose is phosphorylated in the structural components "compartment". As a result these cells show less biomass production (Figs 1B,C) and accumulated less soluble sugars compared to fructose-grown cells (Figs 5A,B). Since accumulated sugars are known to be located mainly in the vacuole (Preisser *et al.* 1992), it is suggested that less sugar accumulation in glucose-grown cells lead to less osmotic water uptake, thereby explaining the lower fresh weight (Fig 1B) and the lower ratio of fresh weight and dry weight compared to fructose-grown cells (deduced from Figs 1B,C).
In addition, glucose is the best substrate in the first phase of embryogenesis in differentiating cells of embryogenic cultures of *Daucus carota* (Verma and Dougall 1977), *Asparagus officinalis* (Levi and Sink 1992) and *Cucumis sativus* (Callebaut *et al.* 1986); in this phase high energy consumption takes place necessary for cell divisions (i.e. the formation of globular embryos). A correlation between high cellular glucose levels and embryogenic potential was reported for *Daucus carota* cells by Dijkema *et al.* (1988). In the second phase of embryogenesis, fructose was found to be a better substrate in *Asparagus officinalis* cells (Levi and Sink 1992). Higher growth rates when growing on fructose as a substrate were also reported for heterotrophic *Daucus carota* cells (Zwayyed *et al.* 1991) and *Taxus* cells (Wickremesinhe *et al.* 1994), for mixotrophic cells of *Glycine max* (Spilatro and Anderson 1988) and *Carica papaya* shoot explants (Drew *et al.* 1993).

Cellular phosphorylated intermediates

In all three culture types, the level of hexose phosphates increased after inoculation. It is assumed that hexoses were phosphorylated already during the lag phase, while enzymes required for production of structural cell components, cell division and sucrose accumulation still had to be synthesized or activated. After day two the level of hexose phosphates decreased again and growth started.

The high levels of glucose-1-phosphate at the second day after inoculation in fructose-grown cells (Fig. 4B) and to a lesser extent in sucrose-grown cells (Fig. 4C) obviously is a result of the supply of fructose as initial substrate. For an efficient turnover of glucose-1-phosphate the UTP-level should be sufficient to support the reaction by uridinediphosphate-glucose pyrophosphorylase (UGPase) of glucose-1-phosphate into UDP-glucose (UDPG, Fig. 7), an important reaction product in growing plant cells because of its role in cell wall synthesis (Lawson *et al.* 1989) and sucrose synthesis (Wendler *et al.* 1990; Xu *et al.* 1989). As a consequence of the fast start of growth in fructose-grown cells (Figs 1A,B,C), the production of UTP in the lag phase might be insufficient to support the complete conversion of glucose-1-phosphate into UDPG, resulting in a transient accumulation of glucose-1-phosphate (Figs 4, 7). Nakano *et al.* (1989), Vella and Copeland (1990), Sowokinos *et al.* (1993) and Elling (1996) reported that UGPase had K_m values of 74 to 170 μM for UTP. However, the K_m value of *Daucus carota* UGPase was about 400 μM (*results not shown*), making fructokinase and UGPase compete for UTP at low levels of NTPs.

Accumulation and cycling of sucrose and hexoses

In general, hexose levels remained low during growth due to hexose phosphorylating activity (Fig. 6) and sucrose accumulated in the logarithmic growth phase (Fig. 5). However, glucose accumulated to 10-fold higher levels in fructose-grown cells compared to glucose-grown cells. Glucose accumulation followed the pattern of sucrose accumulation and fructose uptake (Figs. 1A, 5B), suggesting that the process of sucrose cycling, i.e. synthesis of sucrose from UDPG and fructose-6-phosphate in the cytosol and degradation of sucrose at the same time is responsible for the production of glucose and

fructose (Fig. 7) as proposed by Dancer *et al.* (1990) and Wendler *et al.* (1990).
The observed retention of glucose was unexpected, because glucose was found to disappear from the medium and was found to be converted easily in glucose- and sucrose-grown cells (Figs 1A, 5). Two explanations might be valid to explain the accumulation of glucose. Firstly, fructose might take over metabolism in fructose-grown cells in such a way that glucose phosphorylation is impaired. Secondly, glucose might be sequestered in the vacuole for osmotic purposes. However, further research should be performed to examine the validity of these hypotheses.

Conclusion

Our results are taken to indicate that two separate pools exist for glycolytic intermediates in *Daucus carota* cells: a pool localised in the cytosol associated with soluble fructokinase producing UDPG which results in the synthesis of sucrose and cell wall material and a pool surrounding the mitochondrial-bound hexokinase preferentially using glucose, serving as substrate for respiration. As a consequence, a different balance between production of biomass, sucrose synthesis and energy production was observed between fructose-grown and glucose-grown cells.

Acknowledgements

This project was financially supported by the Foundation for Life Science (SLW), which is subsidized by the Netherlands Organization for Scientific Research (NWO). The authors are indebted to Sacco C. de Vries and Marijke Hartog of the Laboratory of Molecular Biology, Wageningen University for supplying cell suspensions of *Daucus carota*.

Chapter 4

Uptake of ^{13}C-glucose by cell suspensions of carrot (*Daucus carota*) measured by in vivo NMR: Cycling of triose-, pentose- and hexose-phosphates

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submitted to Physiologia Plantarum

Abstract

After a lag phase of two days batch-cultured, sucrose-grown suspension cells of carrot (*Daucus carota* L.) cv. Flakkese entered the exponential growth phase and started to accumulate sucrose and hexoses. Short-term feeding ^{13}C-glucose in this period resulted in only minor labelling of sucrose or fructose. CO_2 production from [1-^{13}C]- and [6-^{13}C]-glucose revealed, that at least 40% of the added glucose passed through the oxidative pentose phosphate pathway (OPPP), up to 40% through the respiratory pathway while about 20% was incorporated in various cell components in the exponential growth phase. After about 11 days of culture the medium sugars were exhausted, cells entered the stationary growth phase and consumed stored sugar. Both neutral and acid invertase (EC 3.2.1.26) and sucrose synthase (EC 2.4.1.13) increased 50% from day 0 to day 11-13, thereafter their levels decreased again. Short-term feeding ^{13}C-glucose resulted in the accumulation of labelled sucrose and fructose during the stationary growth phase. Sucrose labelling was transient, i.e. after 6 h its level started to decrease again. Labelled fructose, however, evolved slower and increased even after 8 h. In sucrose and fructose up to 20% of the ^{13}C-label was exchanged from C-1 to C-6 carbons, indicating intensive cycling of at least 40% of the carbon between hexoses and triose phosphates. In the stationary phase only 10% of the labelled glucose passed through the OPPP and about 30% passed through the respiratory pathway; the remaining 60% was incorporated in cell constituents and sugars. Comparing the various cycles showed that the regulation of the OPPP operated relatively independently from cytosolic cycling of hexoses phosphates through sucrose and between hexose phosphates and triose phosphates.

Key-words: metabolic cycles, oxidative pentose phosphate pathway, *Daucus carota* L. (cell suspensions), cytosol, vacuole, respiration

Introduction

In heterotrophically growing plant cell suspensions sucrose is usually hydrolysed extracellularly by a cell-wall-bound invertase and the resulting hexoses are taken up (Kanabus *et al.* 1986; Dijkema *et al.* 1988, 1990). After phosphorylation the imported hexoses will be used for biosynthesis and for energy production. A common feature of plant cell suspensions grown in batch culture is a 10-14 days biphasic growth cycle (Kanabus *et al.* 1986; Dijkema *et al.* 1988, 1990; Wendler *et al.* 1990; Kubota and Ashihara 1993). In the first phase sugar uptake and conversion result in logarithmic growth and concomitant storage of sucrose and starch. In the second phase, when medium sugars are depleted, cells enter the stationary phase: cell growth declines and stored sucrose and starch are consumed for cell maintenance. Next to this growth-cycle related conversion of carbohydrates short-term cycling of carbohydrates has also been shown: futile cycles are a common feature of plant cell metabolism. Cycling of hexoses through the sucrose pool, cycling of hexose phosphates through the oxidative pentose phosphate pathway (OPPP) and cycling between hexose- and triose phosphates are reported in both cell suspensions and different kinds of plant tissues. Sucrose is subject to cycling by a continuous process of synthesis and degradation as was found in *Saccharum* cells (Dancer *et al.* 1990; Wendler *et al.* 1990). The activities of sucrose phosphate synthase, invertase and sucrose synthase and the compartmentation of sucrose and hexoses determine the net accumulation or breakdown of sucrose as was found in *Acer pseudoplatanus* cells (Huber and Akazawa 1986), *Daucus carota* roots (Lee and Sturm 1996) and *Saccharum* stem tissue (Zhu *et al.* 1997).

The OPPP removes the C-1 carbon from glucose phosphates. Hexoses labelled at the C-1 carbon with either ^{13}C or ^{14}C therefore produce $^{13}CO_2$ and $^{14}CO_2$, respectively, when going through the OPPP while C-6 labelled hexoses do not. OPPP activity is reported in a range of species and tissues as *Solanum tuberosum* callus (Hemrika-Wagner 1985), isolated *Pisum sativum* plastids (Emes and Fowler 1983) and *Capsicum annum* chloroplasts (Thom and Neuhaus 1995). The OPPP is able to cycle the resulting pentose-phosphates back to hexose-phosphates and triose-phosphates; as a result glucose-6-phosphate can be fully oxidised, thereby producing reducing equivalents (NADPH) for biosynthetic purposes (Kang and

Rawthorne 1996; Hartwell *et al.* 1996). Alternatively, the OPPP may be used as a source of pentose, erythrose and heptulose sugars for the biosynthesis of nucleic acids, aromatic amino acids and secondary metabolites (Hagendoorn *et al.* 1991). The OPPP is localised both in the cytosol and the plastids in some tissues as was postulated for *Zea mays* root tips by Frehner *et al.* (1990) and Averill *et al.* (1998). Krook *et al.* (1998) concluded that also in *Daucus carota* cell suspensions OPPP activity is present both in the cytosol and in the plastids.

The combined actions of glycolysis and gluconeogenesis give rise to cycling of carbon between triose phosphates and hexose phosphates in which label is exchanged from C-1 to C-6 carbons as was reported for *Catharanthus roseus* cells, *Solanum tuberosum* tubers and *Zea mays* endosperm by Hatzfeld and Stitt (1990) and germinating *Triticum aestivum* seeds (Keeling *et al.* 1988). This label exchange was supposed to occur at the level of triose phosphates (Hatzfeld and Stitt 1990); concomitant gluconeogenesis results in C-6 labelled hexose (phosphates). As a consequence, plants fed with C-1 labelled glucose also produce C-6 labelled hexoses, sucrose and starch. Label exchange percentages up to 50% were reported in cells and tissues of various species (Keeling *et al.* 1988; Viola *et al.* 1991; Kosegarten *et al.* 1995; Krook *et al.* 1998).

In all the reports published before, only one cycle was studied. Here, we report on different cycles operating together during a 14 days culture period of batch-grown cell suspensions of *Daucus carota*. Cells were fed with ^{13}C-labelled glucose in short-term labelling experiments in an 'airlift-system', i.e. airlifted cells in a NMR-tube (Fox *et al.* 1989), to study the simultaneous cycling of hexose phosphates through the sucrose pool, the OPPP and between hexose phosphates and triose phosphates in vivo in relation to total carbohydrate consumption. Cells from the lag, exponential and stationary phase were used to measure the changes in relative contribution of each cycle during different growth phases.

Materials and Methods

Cell suspensions

After being initiated from hypocotyl-derived callus of *Daucus carota* L. cv. Flakkese (Zaadunie, Enkhuizen, The Netherlands) "Line 10" cell suspensions were kindly provided by Sacco C. de Vries and Marijke Hartog (de Vries *et al.* 1988). Cells were subcultured every 14 days by diluting 2 ml of packed cells in 50 ml Gamborg's B5 medium (Gamborg *et al.* 1968) supplemented with 2.3 μM 2,4-D and 50 mM sucrose. Cells were grown at 25°C in 250 ml erlenmeyer flasks at an Innova 2300 orbital shaker (New Brunswick Scientific, Nijmegen, The Netherlands) at 100 rpm.

NMR experiments

Cells of day 1 (lag phase), day 5 (exponential growth phase) and day 11, 13 and 15 (stationary growth phase) were harvested from batch cultures for NMR experiments. About 6.6 ml of packed cells were washed two times with Gamborg's B5 medium without sugar and diluted in 16.5 ml total volume. Cells were pipetted into a 20-mm diameter NMR tube containing an airlift-system aerated with 100% O_2 for oxygen supply and to maintain cell suspending (Fig. 1, Fox *et al.* 1989). At time zero 500 μmol 99.9% enriched [1-^{13}C]-glucose (g dry weight)$^{-1}$ was added after which acquisition of the NMR spectra was started immediately. ^{13}C-labelled compounds were purchased from Isotec Inc. (Miamisburg, OH, USA). In addition, experiments in which [6-^{13}C]-glucose was added to the cells were performed. The outward gas flow was led through a 10 mm diameter tube containing 7.0 ml of 10% KOH solution which was sufficient to trap all the released $^{13}CO_2$ (Fig. 1). The KOH solution was refreshed every 2 h so release of $^{13}CO_2$ could be followed in time. Control experiments in which unlabelled glucose was used were also performed to determine natural abundance (1.1%) $^{13}CO_2$ production during the experiments.

^{13}C-NMR of cells and CO_2

^{13}C-labelled sugars were analyzed using a wide bore AMX-300 spectrometer equipped with a 20 mm internal diameter ^{13}C-probe (Brucker, Germany). ^{13}C-labelled CO_2 was analyzed

using a 10 mm internal diameter ^{13}C-probe; samples were prepared by mixing 2.20 ml $^{13}CO_2$-containing KOH with 200 μl D_2O for field lock and 50 μl 500 mM [1-^{13}C]-glucose as internal standard. The Waltz sequence and two-level proton decoupling were applied, 7200 and 3600 FID's were accumulated in 8k data points using a 45° and 30° pulse and a pulse repetition time of 0.5 s and 2.0 s for spectra of cells and CO_2 samples, respectively. A line broadening of 3 Hz was used and zero-filling to 16k data points was applied prior to Fourier transformation. Peak areas at 164.5 ppm (CO_3^{2-}), 96.8 ppm (ß-glucose C-1), 64.8 ppm (fructose C-1), 64.2 ppm (fructose C-6), 63.3 ppm (sucrose-fructosyl C-6) and 62.0 ppm (sucrose-fructosyl C-1) were integrated. Integral values out of spectra of standard solutions containing 50 mM gluose, fructose and sucrose or 10 mM 99.9% enriched $NaH^{13}CO_3$ recorded under similar experimental conditions were used for quantification of the C-1 and C-6 carbons.

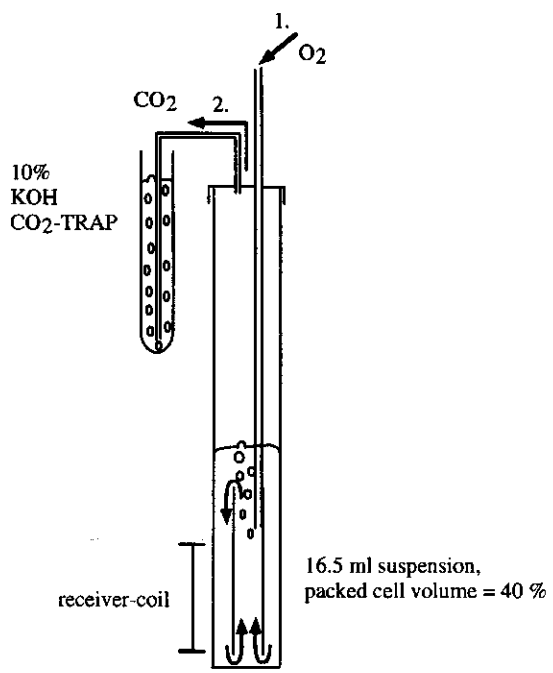

Figure 1 Schematic drawing of the airlift-system. 1. The oxygen supply (100 ml.min^{-1}) causes circulation of cells providing a homogeneous suspension. 2. Outward gas flow is led through a 10% KOH solution in order to trap the released CO_2.

The exchange of label from C-1 to C-6 carbons was expressed as percentage C-6 labelled carbons of total labelled (C-1 and C-6) carbons. The amounts of labelled C-1 and C-6 carbons were added and divided by the total concentration of sugar measured by HPLC in order to calculate labelling percentages. Glucose, fructose and sucrose concentrations were determined from samples taken at 0, 4 and 8 h after starting the NMR-experiments by filtering cells over a Büchner funnel and washing 2 times with Gamborg's B5 medium without sugar, after which they were frozen in liquid nitrogen and stored at -80°C until freeze-drying in a Modulyo 4k (Edwards, Crawley, Sussex, England).

Invertase and sucrose synthase activity

Invertase (EC 3.2.1.26) and sucrose synthase (EC 2.4.1.13) were assayed in freshly made extracts from freeze-dried material. Samples of 20 mg were extracted in 1.2 ml buffer containing 50 mM HEPES at pH 7.5, 5 mM DTT, 5 mM Mg-acetate and 1 mM EDTA at 4 °C. Low molecular mass components were removed on a Biogel P6 column (BioRad, Veenendaal, The Netherlands) (modified after Appeldoorn *et al.* 1997). Neutral and acid invertase assays were performed in a final volume of 300 μl containing 20 mM Na-phosphate/citrate and 25 mM sucrose at pH 7.5 and pH 5.2, respectively. After incubation for 45 min at 30°C, reactions were terminated by boiling for 4 min. Glucose units were measured by means of HPLC as described in 'sugar determinations'.

The sucrose synthase reaction was performed in an assay with a final volume of 100 μl containing 100 mM MES at pH 8.0, 3 mM UDP and 200 mM sucrose. Samples were incubated for 30 min at 30°C, after which reactions were stopped by boiling for 4 min. UDP-glucose was determined in an assay with a final volume of 975 μl containing 20 mM MES at pH 8.0, 0.13 mM NAD^+ and 0.02 U UDP-glucose dehydrogenase (Boehringer, Mannheim, Germany). NAD^+ conversion was measured at 30°C using a double beam - spectrophotometer operating at 340 nm (Shimadzu, Kyoto, Japan). One Unit represents the amount of enzyme hydrolysing one μmol sucrose min^{-1} at 30°C.

Dry weight and sugar determinations

Cells were harvested from batch culture and washed 2 times with Gamborg's B5 medium

after which cells were frozen in liquid nitrogen. After freeze-drying dry weight was determined. Soluble sugars were extracted by boiling 20 mg freeze-dried material in 1.0 ml 80% methanol for 15 min at 76°C. Methanol was evaporated in a Speedvac (Savant Instruments Inc. Farmingdale, NY, USA) and the samples were dissolved in ultra pure water (Millipore Intertech, Bedford, USA).

Soluble sugars were measured with a Dionex HPLC system (Dionex Corporation, Sunnyvale, CA, USA) using a Carbopac PA-1 (guard)column coupled to a pulsed amperometric detector Tetteroo *et al.* 1995). Isocratic elution was performed with 100 mM NaOH for 15 min to separate glucose, fructose and sucrose. Peak areas were quantified using standard sugar solutions.

Respiration measurements

Oxygen uptake was determined by transferring 2.5 ml of cell suspension directly from the batch culture or after washing and NMR-experiments into an oxygen electrode (Rank Bros., Bottisham, Cambridge, UK) equilibrated with air. Oxygen uptake was followed for about 10 min at 25°C while stirring the suspension. The amount of hexoses respired per flask was calculated by integrating oxygen consumption divided by six against the dry weight expressed as mg per flask.

Differences between experimental series

The growth rates of cell suspensions, and the exact timing of the consecutive growth phases differed considerably between various experimental series. The data for sucrose and fructose labelling and respiration rate show comparable differences. Therefore, calculating mean values of labelling of sucrose and fructose and respiration rate, in replicate experiments would not be meaningful since the physiological age of the cells is not the same for each time point of different experimental series.

Results

Cell growth, sugar content and respiratory activity

Figure 2A shows the cumulative weights of total medium sugars, respired sugars and dry weight of the carrot cells. Medium sugars were exhausted after 11 days. During the first 8 days about 50% of the total amount of hexose consumed was used for respiration and hexose and sucrose levels remained high, at levels of about 400 and 250 μmol (g dry weight)$^{-1}$, respectively (Fig. 2C).

After day 8, cells entered the stationary growth phase: dry weight decreased while respiration continued. At day 15 about 75% of the initially supplied hexoses were used in respiration. Concomitantly, internal sugar levels dropped to about 20 μmol.(g dry weight)$^{-1}$ (Fig. 2C). Respiration rate was 3 μmol O_2.(g dry weight)$^{-1}$.min^{-1} initially and increased up to 5 μmol O_2.(g dry weight)$^{-1}$.min^{-1} during the lag phase (day 1-4). From day 4 to day 8 the respiration rate remained constant at 5 μmol O_2.(g dry weight)$^{-1}$.min^{-1}, whereas from day 8 it gradually decreased towards 3 μmol O_2.(g dry weight)$^{-1}$.min^{-1} (Fig. 2B, open symbols). When cells were transferred to an airlift-system, respiration rate increased 1.5-4-fold (Fig. 2B, closed symbols), depending on the growth phase of the cell culture.

Figure 2D shows the time course of the activities of sucrose-degrading enzymes: sucrose synthase, neutral and acid invertase increased gradually by about 50% towards day 11-13; thereafter activities of all three enzymes decreased again.

Short-term labelling of the cells

Batch cultured cells were harvested, washed with fresh medium lacking sugar and incubated with 500 μmol [1-^{13}C]-glucose.(g dry weight)$^{-1}$ in order to follow the incorporation of label into other soluble sugars. Lag phase 1-day-old and exponentially growing 5-days-old cells showed less than 15 and 5 μmol.(g dry weight)$^{-1}$ incorporation of label into sucrose and fructose, respectively (Figs 3A,B). In the stationary phase (11-13 days-old-cells) labelled sucrose accumulated up to 60 μmol.(g dry weight)$^{-1}$ at 3 h after adding the labelled glucose and its level remained high until 5-6 h. After 5-6 h the applied ^{13}C-glucose had disappeared from the medium (*data not shown*) and the level of labelled sucrose started to decrease again

(Fig. 3A). In cells of 15 days old a more or less steady level of 35 μmol.(g dry weight)$^{-1}$ was reached.

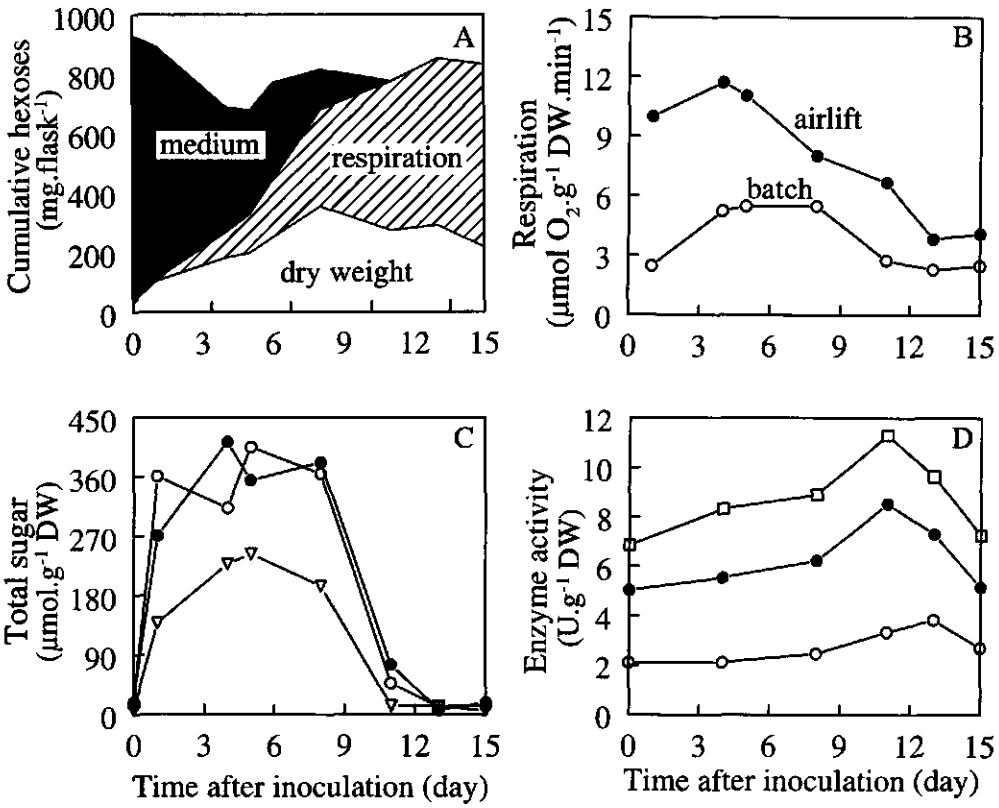

Figure 2 (A) Partitioning of carbon during the course of growth of *Daucus carota* cells grown in batch culture expressed in mg.flask^{-1} Cumulative representation of total medium sugar (black area), dry weight (white area) and respiration (hatched area). The latter was calculated by dividing the respiration rate by 6 as a function of dry weight in mg.flask^{-1}, (B) the respiration rate of cells taken directly from batch (○) or after a 4 h-treatment in the airlift-system (●), expressed in µmol O_2.g^{-1} DW.min^{-1}, (C) cellular content of glucose (○), fructose (●) and sucrose (▽) in µmol.g^{-1} DW and (D) the amounts of soluble acid invertase (□), neutral invertase (●) and sucrose synthase (○) expressed as U.g^{-1} DW. One Unit represents the amount of enzyme which hydrolyses 1 µmol sucrose.min^{-1}. Data of a representative series of experiments.

The rate of fructose labelling was only half of that of sucrose labelling and was increasing even after 8 h (Fig. 3B). Labelled glucose is not depicted since no discrimination can be made between internal and externally supplied glucose in this experimental set-up (Fig. 1). Total sugar levels changed during the 8 h labelling period: 1 to 5-days-old cells showed initially high sugar levels, that gradually decreased, while the initially low levels of sugars in 11 to 15-days-old cells rose slightly (Figs 3C,D).

Labelling percentages of sucrose and fructose during the NMR experiments were calculated for time points 4 and 8 h after starting the experiment. Figure 3E shows that the labelling percentage of sucrose in logarithmically growing cells of 5 days old did not exceed 8%. Cells of 1 day old showed a slightly higher labelling percentage up to 20%. Sucrose labelling percentage was about equal for cells from day 11, 13 and 15 and was maximally 80-95% after 8 h. Fructose labelling percentage (Fig. 3F) was correlated with age: maximally 20% in cells of 1 day old towards 85% in cells of 15 days old. Cells of 5 days old (logarithmic growth phase) showed a very low labelling of about 2 μmol.(g dry weight)$^{-1}$ (2%).

The percentage of C-6 labelled carbons in fructose was calculated (Tab. 1). For cells of 1 and 5 days old the fructose C-6 signal at 64.8 ppm was not detectable; for cells of 11, 13 and 15 days old the labelling percentage of C-6 carbons was constant at 18-20%.

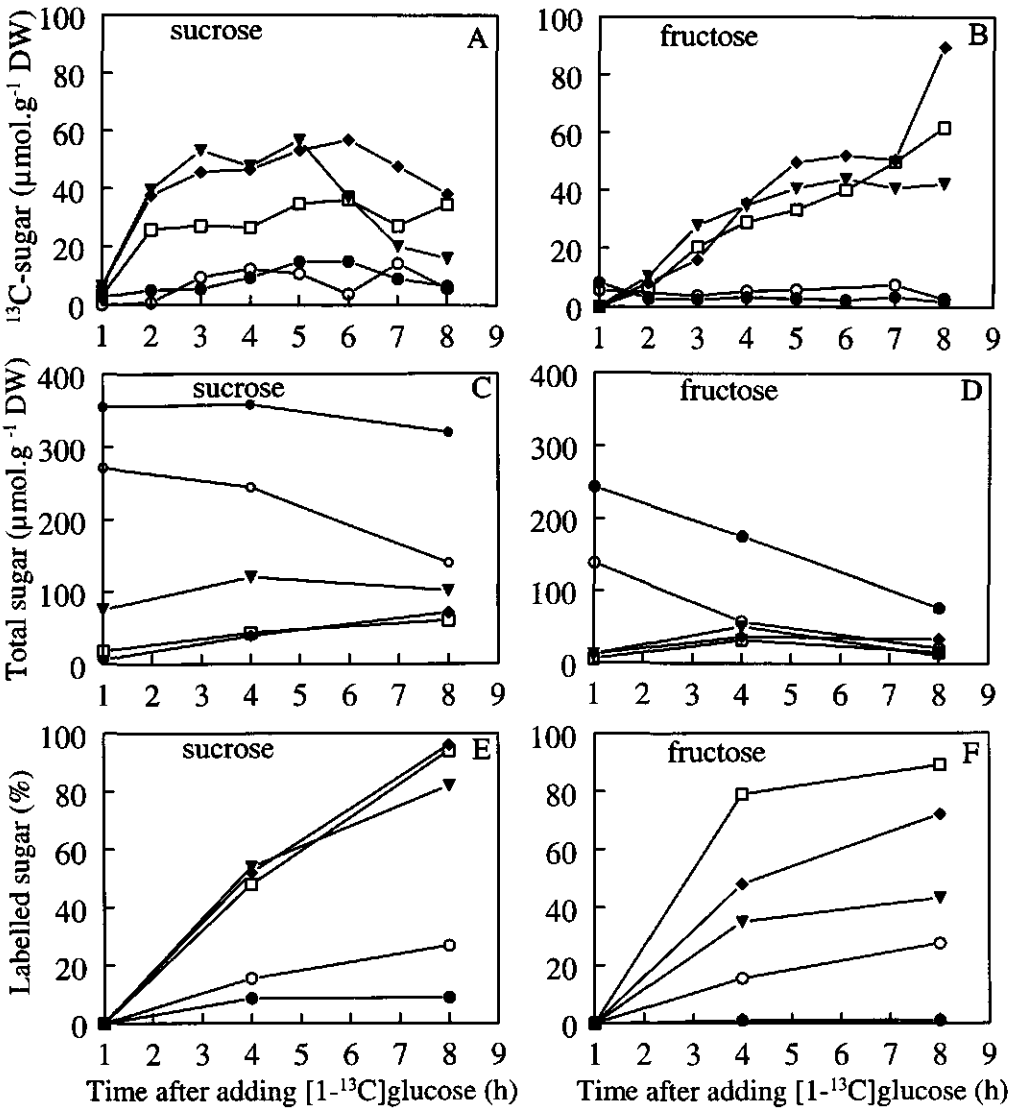

Figure 3 Profile of ^{13}C-labelled sucrose (A) and fructose (B) and changes in total sucrose (C) and total fructose (D) expressed as µmol.g^{-1}DW, and changes in labelling percentage calculated as the sum of labelled C-1 and C-6 carbons of sucrose and fructose divided by total (labelled and unlabelled) sucrose (E) and fructose (F) during an 8-h treatment with ^{13}C-glucose in the airlift-system of 1 (○) 5 (●), 11 (▼), 13 (◆) and 15 (□) days-old-cells of *Daucus carota* grown in batch culture with sucrose as carbon source. Data of a representative series of experiments.

Table 1 Percentage labelled C-6 of total labelled (C-1 and C-6) carbons of free fructose in batch cultured cells of different age, after incubation with 500 μmol [1-^{13}C]-glucose g^{-1} DW in an airlift-system. n.d.: C-6 carbons were not detectable in the spectra. Values are means of time-points 3,4,5 and 6 h ± S.D.

Day	Percentage C-6 in fructose
1	n.d.
5	n.d.
11	18.3 ± 2.5
13	18.1 ± 4.6
15	19.8 ± 5.1

Respiration measured by O_2 consumption

In Fig. 4 the respiration rate of cells of 1 day old taken directly from batch is compared to that of cells during the NMR experiment. Absolute values of respiration were half of that of 1 day old cells presented in Fig. 2B, that were from a different experimental series; apparently there is a considerable variation in basal respiration rate in-between experiments. However, increase of respiration after washing the cells quickly for 2 times in fresh medium was 4-fold in both experiments (Figs 2B, 4). During the period of the NMR experiments respiration was about constant. The O_2 concentration used to aerate the cells in the NMR-tube (20% or 100%) did not significantly influence the respiration rate as determined in the oxygen electrode at 20% O_2.

Respiration measured by $^{13}CO_2$ production

Incubation of cells with [1-^{13}C]-glucose or [6-^{13}C]-glucose resulted in production of $^{13}CO_2$ which was trapped by leading the outgoing gas through a 10% KOH solution for 8 h (Fig. 1). This $^{13}CO_2$ production was corrected for natural abundance ^{13}C which was measured

using unlabelled glucose and accounted for 19-39% of total trapped $^{13}CO_2$.

During the first week cells consuming [6-^{13}C]-glucose released 40% of the added label as $^{13}CO_2$, decreasing to about 30% in cells in the stationary phase. Cells fed with [1-^{13}C]-glucose released up to 80% of the label as $^{13}CO_2$ in the first week decreasing to 40% in the second week (Fig. 5).

In Fig. 6 the rate of CO_2 release during the NMR experiment for cells of 7 and 14 days old is depicted. The difference in CO_2 release between cells fed with [1-^{13}C] and [6-^{13}C]-glucose occurred mainly from 2 to 6 h. After 6 h, when labelled glucose was completely taken up (*data not shown*) the rate of $^{13}CO_2$ production remained constant for both labelling conditions.

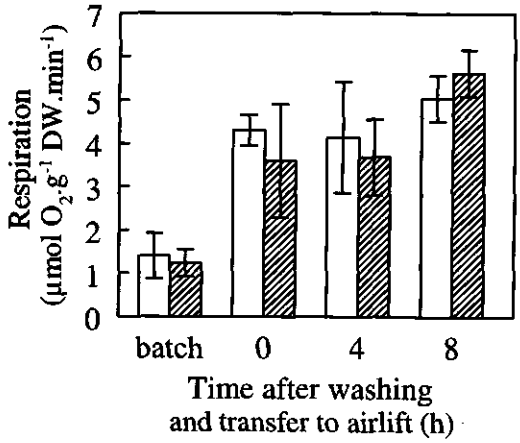

Figure 4 Respiration of 1-day-old cells of *Daucus carota* during the 8 h- incubation of the cells in an airlift-system aerated with 20% (open bars) or 100% oxygen (hatched bars). T=0 is directly after washing the batch cultured cells 2 times in fresh medium. Means of 3 replicates ± S.D. out of a representative series of experiments.

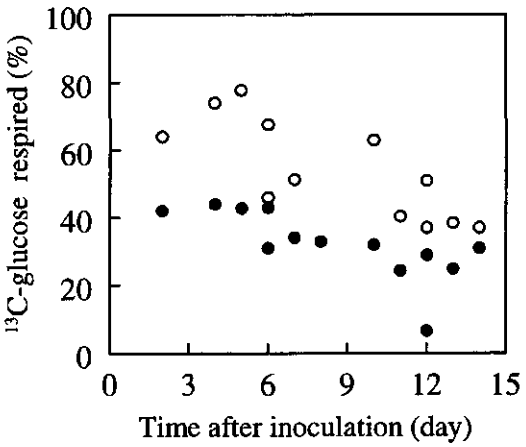

Figure 5 Percentage of added [1-^{13}C] (○) or [6-^{13}C]-labelled (●) glucose recovered as $^{13}CO_3^{2-}$ in a 10% KOH solution during 8 h-incubation in an airlift-system of *Daucus carota* cells originally grown in batch culture on sucrose. Pooled data of 2 series of experiments.

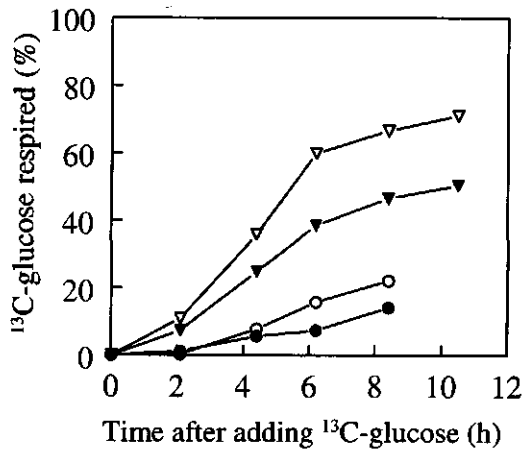

Figure 6 Respired $^{13}CO_2$ during uptake and conversion of 500 μmol.g^{-1} DW [1-^{13}C]-(open symbols) or [6-^{13}C]-glucose (closed symbols) by 7-days-old (▽, ▼), and 14-days-old (○, ●), batch-grown cells of *Daucus carota* incubated in an airlift-system. $^{13}CO_3^{2-}$ was trapped in a 10% KOH solution in 2-h intervals.

Discussion

Cell growth and long-term sucrose cycling in batch culture

Hexose levels might be low in the cytosol since active kinases are present (Chapter 3, Fig. 6) and hexoses might be channelled into the vacuole as was shown for *Nicotiana tabacum* leaves (Heineke *et al.* 1994) and *Saccharum* cell suspensions (Preisser *et al.* 1992). Sucrose is thought to be taken up in the vacuole by a passive carrier-mediated process as found in vacuoles of *Saccharum* cells (Preisser and Komor 1991) or as an ATP/PP_i-dependent process taking place under well-energized conditions parallel to the uptake of hexoses as shown for *Pisum sativum* mesophyll cells (Guy *et al.* 1979) and *Beta vulgaris* roots (Getz 1991).

It was shown by Zhu *et al.* (1997) that sucrose accumulation is determined by the difference in sucrose phosphate synthase (SPS) and invertase activity. Goldner *et al.* (1991) showed in *Saccharum* stem tissue that sucrose accumulation was positively correlated to cytosolic SPS and inversely correlated to vacuolar acid invertase activity, indicating that accumulated sucrose might be localised in the vacuole. Huber and Akazawa (1986) showed that the activities of sucrose synthase and neutral invertase are about the same in *Acer pseudoplatanus* cells. In *Daucus carota* cells, Stommel and Simon (1990) reported about twice as much acid invertase protein compared to neutral invertase. In our *Daucus carota* cells, all three sucrose hydrolysing enzymes were present and neutral and acid invertase activities were twice and three times higher than that of sucrose synthase (SUSY), respectively (Fig. 2D). For *Daucus carota*, a K_m-value of 20 mM sucrose was reported for both neutral and alkaline invertase, while fructose and glucose are inhibitors with K_i values of 15 and 30 mM, respectively (Lee and Sturm 1996). Fructose was also found to be an inhibitor of *Solanum tuberosum* acid invertase (Isla *et al.* 1991, 1998). Glucose and fructose concentrations of 360 μmol.(g dry weight)$^{-1}$ at day 1-9 were found in *Daucus carota* cells (Fig. 2C) corresponding to about 25 μmol.(g fresh weight)$^{-1}$ (assuming a mean dry matter percentage of 7.5%) which might be equivalent to at least 25 mM, depending on the intracellular localisation. As a consequence of the high hexose concentration in the vacuole, acid invertase will not be active under these conditions and sucrose accumulates.

Since hexose levels were supposed to be low in the cytosol, cytosolic neutral invertase might

permit sucrolysis giving rise to cytosolic sucrose cycling as was observed by Dancer *et al.* (1990) and Wendler *et al.* (1990). SUSY shows a higher K_m value of 87 mM for sucrose and lower K_i values of 17 mM for fructose and 4 mM for glucose compared to invertases in *Daucus* roots (Sebková *et al.* 1995). Furthermore, the level of UDP-glucose (UDPG) might be high in the logarithmic growth phase as was found for *Nicotiana tabacum* cells by Meyer and Wagner (1985); therefore sucrolysis catalysed by SUSY will be inhibited, making invertase a more likely candidate for cytosolic sucrose cycling in the logarithmic growth phase. In addition, high levels of UDPG in this period might stimulate sucrose synthesis by SPS (Fig. 7). It is suggested that both inhibition of acid invertase in the vacuole by high levels of hexoses and a high rate of sucrose synthesis by SPS due to high levels of UDPG are responsible for sucrose accumulation during the logarithmic growth phase.
After day 8 fructose and glucose levels declined to 20 μmol.(g dry weight)$^{-1}$ (Fig. 2C), corresponding to about 1.5 mM; this is considerably lower than the K_i values of SUSY and invertases for glucose and fructose. Both cytosolic sucrolysis by neutral invertase and by SUSY and vacuolar sucrolysis by acid invertase might take place now.

Short-term labelling of cells: sucrose cycling, triose phosphate cycling and respiratory activity
Five days old cells (logarithmic growth phase) showed labelling percentages of less than 8% for sucrose (Fig. 3E) and less than 2% for fructose (Fig. 3F) when incubated with 500 μmol [1-^{13}C]-glucose.(g dry weight)$^{-1}$. The calculated respiration rate of 880 μmol hexoses.(g dry weight)$^{-1}$.(8 h)$^{-1}$ (derived from Fig. 2B) was consistent with the disappearance of this 500 μmol added hexose and the decreasing sucrose level of about 380 μmol hexose units.(g dry weight)$^{-1}$ (deduced from Fig. 3C). In view of the decrease of the level of sucrose and the low labelling of sucrose and fructose it is concluded that most of the phosphorylated hexoses were used in respiration rather than for sucrose synthesis; sucrose cycling as reported by Wendler *et al.* (1990) and Dancer et al. (1990) therefore might be limited in this period.
Stationary phase cells (11-15 days old) showed an increase of labelled and total sucrose up to about 60 μmol.(g dry weight)$^{-1}$. When medium glucose was exhausted after 6 h, total sucrose levels started to decline again. After 8 h nearly the complete pool of sucrose was labelled (up to 80-95%, Fig. 3E). Increase of labelled fructose was slower but labelling

increased even after 8 h, suggesting that sucrolysis was responsible for the increasing labelling of fructose. The retention of labelled fructose (Fig. 3B) after 8 h suggests compartmentation of hexoses inside the vacuole since hexose phosphorylating capacity is high (Chapter 3, Fig. 6). In our view sucrose is subject to simultaneous synthesis in the cytosol, transport to the vacuole and subsequent breakdown by acid invertase. The evolving hexoses remained in the vacuole for several hours under well energized conditions (i.e. sugar consumption coupled to high respiratory activity (Fig. 7)). At day 13 respiration was 180 and 320 μmol hexose.(g dry weight)$^{-1}$.(8 h)$^{-1}$ in batch and airlifted cells, respectively (deduced from Fig. 2B). This indicates that batch-grown cells with a depleted sugar pool in the medium respired internal substrates; in airlifted cells fed with 500 μmol.(g dry weight)$^{-1}$ [1-^{13}C]-glucose 320 μmol.(g dry weight)$^{-1}$ was respired and 180 μmol.(g dry weight)$^{-1}$ remains for conversion into biomass and storage sugars during that 8 h experiment.

Cells of 1 day old showed 25% labelling for both sucrose and fructose (Figs 3E,F), indicating that lag-phase cells were intermediate between the logarithmic phase (low labelling) and stationary phase (high labelling) with respect to respiration and sugar synthesis. About 18-20% of the C-1 label was exchanged to C-6 carbons (Tab. 1), indicating that up to 40% of the hexoses cycled between triose phosphates and hexose phosphates (Fig. 7) before they were incorporated in sucrose and hexoses. This is consistent with reports about *Chenopodium rubrum* cell suspensions (Hatzfeld and Stitt 1990), *Triticum aestivum* seeds (Keeling *et al.* 1988), *Solanum tuberosum* tubers and seeds of *Vicia faba* (Viola *et al.* 1991) which were labelled for 1-4 h. However, it is considerably lower than found during long-term labelling of cell suspensions of *Solanum tuberosum* and *Daucus carota*: labelling percentages up to 50% were reported by Kosegarten *et al.* (1995) and Krook *et al.* (1998, Chapter 2), respectively. Treatment of cells or tissues during short-term labelling experiments often causes severe stress responses like an increased respiration rate (Fig. 2B). It is suggested that under these conditions the flow from triose-phosphates is in the direction of glycolysis; as a consequence of the resulting lower levels of fructose-1,6-bisphosphate and triose phosphates less cycling between triose- and hexose phosphates might occur and the rate of sucrose synthesis might decrease.

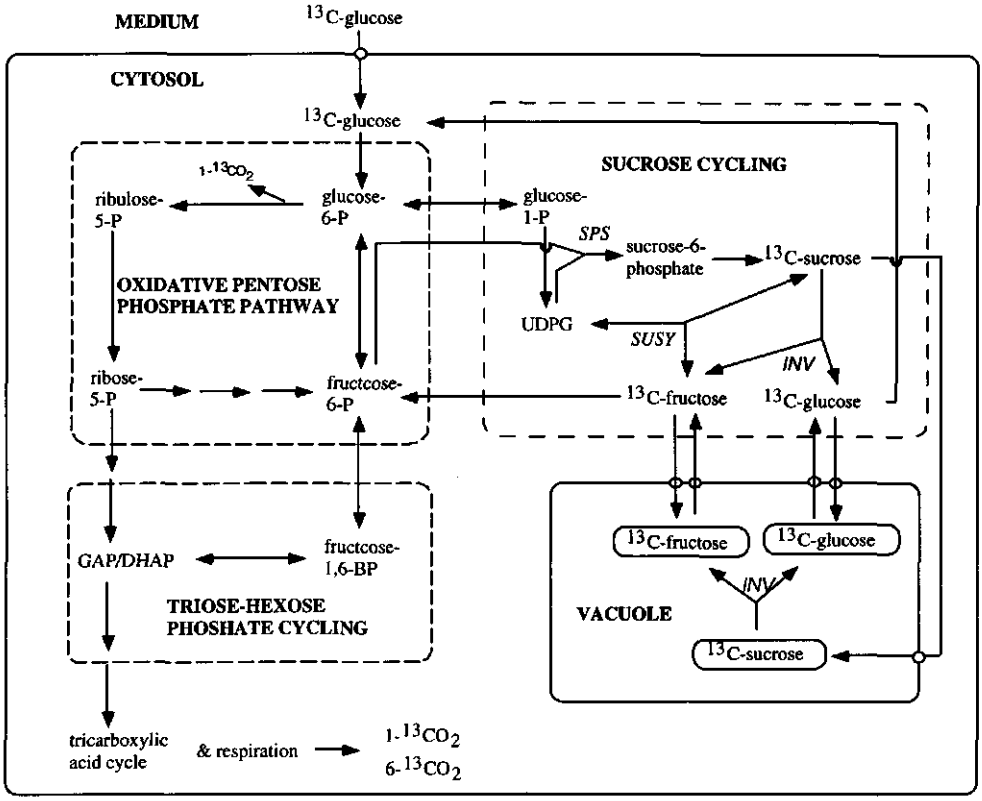

Figure 7 Flow of [1-^{13}C]-glucose through the triose-hexose phosphate, the sucrose cycle and the oxidative pentose phosphate pathway in *Daucus carota* cells grown in batch culture. Hydrolysis of sucrose takes place in the cytosol in the logarithmic growth phase and both in the cytosol and vacuole in stationary phase cells. Metabolic cycles are encircled by dashed lines. Glucose, fructose and sucrose, accumulating in the vacuole during the logarithmic phase are encircled. Small circles on membranes indicate transmembrane-carriers. P = phosphate group, *SPS* = sucrose phosphate synthase, *INV* = invertase, *SUSY* = sucrose synthase, UDPG = UDP-glucose, GAP = glyceraldehyde-3-phosphate, DHAP = dyhroxyacetone phosphate.

Cycling through the OPPP and respiration

When cells were washed and supplied with 500 μmol glucose.(g dry weight)$^{-1}$, respiration rate increased within 5 min (Fig. 4); this increase was higher in cells of one day old (about four times) than in cells of 13-15 days old (about one and a half times, Fig. 2B). During the subsequent NMR-experiment respiration was almost constant, irrespective of the oxygen concentration of 20 or 100% (Fig. 4). Such a sudden increase is suggested to occur via activation of already present respiratory pathway components; the capacity of this pathway is often higher than its actual activity as was found for e.g. *Petunia hybrida* cells (van Emmerik *et al.* 1994). The capacity was maximal in lag phase cells suggesting that the availability of one or more nutrients in the medium is responsible for the synthesis of extra components of the respiratory pathway. In continuous cultures of *Petunia hybrida* cells grown at low cell density respiration remains high, suggesting that maintenance costs were high due to this low cell density (de Gucht and van der Plas 1995). It is suggested by van Emmerik *et al.* (1994) that at low cell densities cells are prepared for oncoming cell divisions which require high energy production by respiratory activity. Although the *Daucus* cells used in our experiments were kept at high density, i.e. a packed cell volume of about 40%, the lack of cell-cell contacts or signal-compounds in the first hours after resuspension of the cells in fresh medium may cause comparable effects.

In the oxidative pentose phosphate pathway (OPPP) $^{13}CO_2$ is primarily released from [1-^{13}C]-glucose, while during complete degradation of glucose in glycolysis and the tricarboxylic acid cycle $^{13}CO_2$ is released from both [1-^{13}C]-glucose and [6-^{13}C]-glucose (Fig. 7). Therefore, $^{13}CO_2$ released from [6-^{13}C]-glucose was taken as a measure for glucose respired via glycolysis and $^{13}CO_2$ released from [1-^{13}C]-glucose minus [6-^{13}C]-glucose was assigned to *in vivo* OPPP activity. In the first week both glycolysis and OPPP used 40% of the added sugar leaving 20% that is incorporated in NMR-invisible compounds. In stationary phase cells 30% was used in glycolysis, 10% by the OPPP and 60% of the ^{13}C-label was built into soluble sugars (Figs 3C,D) and NMR-invisible compounds. This was consistent with reports for *Solanum tuberosum* callus (Hemrika-Wagner 1985), who also found a higher OPPP-dependent $^{14}CO_2$ production in the induction phase of callus growth, parallel to a higher in vitro activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase

activity. Similar increases in enzymes of the OPPP were found in *Catharanthus roseus* cells during the logarithmic growth phase and were explained by the higher demand of pentose sugars and NADPH for the synthesis of nucleic acids (Ishida and Ashihara 1993). Also, synthesis of lipids (Kang and Rawthorne 1996) and the reduction of nitrite (Ishida and Ashihara 1993; Hartwell *et al.* 1996) during the logarithmic growth require high activity of the OPPP. When cells enter the stationary phase as a result of sugar-exhaustion of the medium, the pentose phosphate pathway activity decreased more than did glycolysis, parallel with the decreased need for the synthesis of cell components (Fig. 2A). Glycolysis continued to fulfil its role in energy production necessary for cell maintenance.

The results presented in Fig. 6 show that a short-term pulse-labelling results in non-linear reactions: $^{13}CO_2$ evolved slowly during the first 2 h, and the difference between [1-^{13}C]- and [6-^{13}C]-glucose was only small. It was concluded that labelled glucose entered the cytosol, became phosphorylated and saturated the pool of cytosolic hexoses in a period of 2 h. From 2 to 6 h different amounts of $^{13}CO_2$ evolved from [1-^{13}C]- and [6-^{13}C]-glucose, indicating that labelled hexose phosphates cycled intensely through the OPPP. From 6 h onwards, the rate of $^{13}CO_2$ production is the same for [1-^{13}C]- and [6-^{13}C]-glucose. All labelled glucose has been taken up and it was concluded that label between C-1 and C-6 carbons has equilibrated completely. Only ^{13}C-label derived from the degradation of ^{13}C-labelled sucrose and hexoses (in 11-15 days old cells, Figs 3A,B) as well as from NMR-invisible compounds like lipids and proteins contribute to the continuing release of $^{13}CO_2$.

However, a few considerations have to be made about the interpretation of these measurements. From Tab. 1 it was concluded that up to 20% of label was exchanged from C-1 to C-6 carbons in hexoses and sucrose. If at the same time 20% of the C-6 label was transferred to C-1 carbons of hexoses and only one pool of hexose (phosphate) was present in the cells (Fig. 7), the actual OPPP activity would be considerably underestimated. Although earlier work on batch-grown *Daucus* cells showed OPPP activity in the plastids as well (Chapter 2, Figs 2,3) only minute quantities of labelled starch were found after labelling for 8 h indicating that nearly no labelled hexoses entered the plastids in these short-term experiments (*results not shown*), suggesting that only a cytosolic-localised pool of hexose phosphates is playing a role in OPPP activity during the experiments. However, the presence

of more than one pool of hexose phosphates in the cytosol (Chapter 3) might further complicate the interpretation of the data of [1-^{13}C]- and [6-^{13}C]-glucose conversion.

Conclusion

It is proposed that, as a consequence of the increased respiration in the airlift-system, less hexose phosphates are available for cycling between hexoses and sucrose and between triose phosphates and hexose phosphates. In contrast, the presence of hexose phosphates cycling through the OPPP seemed to be relatively unaffected by a high respiration rate. Label exchange from C-1 to C-6 carbons in hexose phosphates might even lead to an underestimation of the measured OPPP activity.

These metabolic cycles are suggested to play a role in intact plants in adaptations to quickly changing environmental conditions: the activities of metabolic pathways are higher than their actual needs and the produced metabolites are cycled back as long as the need for them is limited (Wendler *et al.* 1990).

Acknowledgements

We wish to thank Sacco C. de Vries and Marijke Hartog of the Laboratory of Molecular Biology of the Wageningen University for supplying cell suspensions of *Daucus carota* L.. This work was financially supported by the Foundation of Life Science (SLW) subsidized by the Netherlands Organisation of Scientific Research (NWO).

Chapter 5

The triose-hexose phosphate cycle and the sucrose cycle in carrot (*Daucus carota* L.) cell suspensions are controlled by respiration and PP_i: fructose-6-phosphate phosphotransferase

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submitted to Journal of Plant Physiology

Abstract

Short-term ^{13}C-labelling was applied to two different lines of *Daucus carota* L. cell suspensions. The A10-line, containing 10% proembryogenic masses (PEMs) and 90% large, vacuolated cells (VACs), showed a 2 times higher label exchange from C-1 to C-6 carbons within sucrose and hexoses than the A+-line, containing 80% PEMs. This label exchange is known to be caused by cycling of carbon from hexose phosphates to triose phosphates and *vice versa*, in which ATP-dependent phosphofructokinase (PFK, EC 2.7.1.11) catalyses the glycolytic reaction and PP_i-dependent phosphofructokinase (PFP, EC 2.7.1.90) the gluconeogenetic reaction. The ratio of extractable PFP/PFK was 3 times higher in the A10-line compared to the A+-line. However, PEMs and VACs from one line showed identical PFP/PFK ratios and identical label exchange. It is concluded that the level of PFP is genetically determined and that this level influences the amount of label exchange from C-1 to C-6 carbons in hexoses and sucrose in *Daucus carota* cells. High levels of the reversible enzyme PFP might give plants the advantage to respond adequately to quickly changing demands for substrates for either glycolytic or gluconeogenetic reactions.

Both triose-hexose phosphate cycling and respiration were higher when suspensions were aerated with 100% O_2 instead of 6% O_2. It is concluded that high respiratory activity both stimulated the flow of hexose phosphates into the respiratory pathway and the back-flow from triose to hexose phosphates. However, total labelled sucrose was higher at 6% than at 100% O_2, indicating that more hexose phosphates were available for sucrose synthesis at 6% O_2. Furthermore, PEMs accumulated higher levels of sucrose than VACs, indicating that sugar metabolism was differently regulated in PEMs and VACs.

Keywords: ATP-dependent fructose-6-phosphate phosphotransferase (PFK), carbon-13 nuclear magnetic resonance (^{13}C-NMR), *Daucus carota* L. (cell suspensions), PP_i-dependent fructose-6-phosphate phosphotransferase (PFP), respiration, triose-hexose phosphate cycling

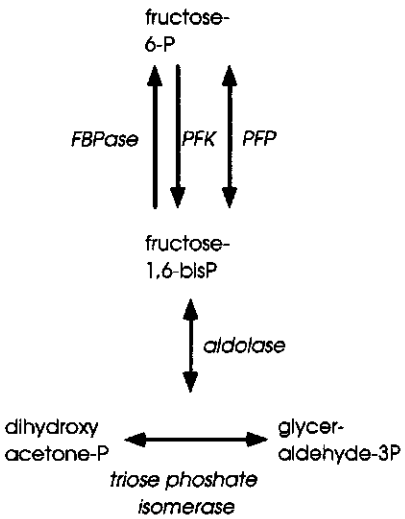
Introduction

Futile cycling of metabolites is a well known property of plant cell metabolism: cycling of hexose phosphates through the oxidative pentose phosphate pathway (Wagner *et al.* 1987; Hartwell *et al.* 1996), cycling between hexoses and sucrose (Wendler *et al.* 1990; Zamski and Barnea 1996) and cycling between hexose monophosphates and triose monophosphates (Hatzfeld and Stitt 1990) have been reported. In the triose-hexose phosphate cycle exchange of label from C-1 to C-6 carbons occurs at the level of triose phosphates (Hatzfeld and Stitt 1990). After feeding [1-^{13}C]-hexoses simultaneous glycolytic and gluconeogenetic activity results in [1-^{13}C]-labelled and [6-^{13}C]-labelled sucrose and hexoses. C-1 and C-6 labelled carbons can be observed in a ^{13}C Nuclear Magnetic Resonance (^{13}C-NMR) spectrum *in vitro* (Krook *et al.* 1998) and *in vivo* (Keeling *et al.* 1988; Dijkema *et al.* 1990; Viola *et al.* 1991). The irreversible conversions between fructose-6-phosphate and fructose-1,6-bisphosphate are catalyzed by the glycolytic enzyme ATP:fructose-6-phosphate phosphotransferase (PFK) and the gluconeogenetic enzyme fructose-1,6-bisphosphatase (FBPase). FBPase plays a role in photosynthetic starch synthesis but was reported to be absent in the cytosol of non-green tissue (Entwistle and ap Rees 1990; Hatzfeld and Stitt 1990). After the discovery of a PP_i-dependent phosphofructokinase in bacteria in the seventies (Reeves *et al.* 1974; O'Brien *et al.* 1975), its existence in plants was discovered in 1979 in pineapple leaves (Carnal and Black 1979). This enzyme catalyses the reversible reaction between fructose-6-phosphate and fructose-1,6-bisphosphate with PP_i as a phosphoryl donor in the glycolytic reaction and P_i as a phosphoryl acceptor in the gluconeogenetic reaction.

Hatzfeld and Stitt (1990) proposed that this enzyme, called PP_i:fructose-6-phosphate phosphotransferase (PFP), is responsible for the observed cycling of carbon between triose phosphates and hexose phosphates in plants. Since this reaction is in near-equilibrium *in vivo*, the direction of the reaction will be determined by the concentration of its substrates fructose-6-phosphate, fructose-1,6-bisphosphate, PP_i and P_i (Stitt 1989; Hatzfeld and Stitt 1990). In addition, the activity will be determined not only by the amount of enzyme, but also by its activator fructose-2,6-bisphosphate (van Schaftingen *et al.* 1982; Hatzfeld *et al.* 1990).

The amino acid sequence of PFP is homologous to that of PFK from plants and mammals

(Carlisle *et al.* 1990). PFK of mammals is stimulated by fructose-2,6-bisphosphate in a similar way as PFP of plants, while PFK of plants is not affected by fructose-2,6-bisphosphate (Hue and Rider 1987).



PFP is thought to play a role when high metabolic activity requires tuning of different pathways and during unfavourable circumstances like low temperature in roots (Black *et al.* 1987). PFP is abundant in sink-tissues, e.g. developing buds or (young) roots during sucrose import and degradation. Hatzfeld and Stitt (1990) found the level of PFP to correlate with the level of sucrose synthase (SUSY) in *Chenopodium rubrum* cell suspensions during the logarithmic growth phase when sucrose was simultaneously synthesized by sucrose phosphate synthase (SPS) and degraded by SUSY, the latter yielding fructose and UDP-glucose (UDPG). When PFP is active in the gluconeogenetic direction, the PP_i produced can subsequently be used by uridinediphosphate glucose pyrophosphorylase (UGPase) to convert UDPG into glucose-1-phosphate and UTP (Huber and Akazawa 1986; Zhu *et al.* 1997).

A role of PFP in glycolysis is also suggested. Botha *et al.* (1992) described that logarithmically growing cell suspensions of *Phaseolus vulgaris* show a high respiration rate; the calculated PFK activity was insufficient to account for the observed high glycolytic activity. Therefore, they suggested that PFP may act in the glycolytic direction during high respiratory activity in these cells.

In addition to the findings that PFP might determine the label exchange between C-1 and C-6 carbons by cycling triose phosphates back to hexose phosphates, it was suggested in earlier experiments on *Daucus carota* cell suspensions that respiratory activity may also have an effect. The oxygen concentration might influence label exchange from C-1 to C-6 carbons by determining the respiratory activity and as a consequence the flow of hexose phosphates to triose phosphates through glycolysis and the subsequent consumption of glycolytic end-products by the citric acid cycle in the mitochondria

Here, we report observations on two embryogenic carrot (*Daucus carota* L.) cell lines, differing in the relative amounts of clustered small cytoplasm-rich cells named proembryogenic masses (PEMs) and single, large vacuolated cells (VACs) (Steward 1958; de Vries *et al.* 1988; Toonen *et al.* 1994). PEMs are thought to be meristematic, actively growing cells which store sucrose and starch while VACs are elongated, metabolically less active cells (Halperin and Jensen 1967; Wurtele *et al.* 1988). One line contained 10% PEMs (A10-line) and a second line 80% PEMs (A+-line) (de Vries *et al.* 1988). The A10-line produced a higher percentage of labelled C-6 carbons in sucrose and hexoses after feeding [1-^{13}C]-glucose than the A+-line (Dijkema *et al.* 1990); these authors suggested that this was due to the high abundance of VACs which might show higher label exchange from C-1 to C-6 carbons than PEMs. However, this was inconsistent with the findings for *Acer pseudoplatanus* cells (Huber and Akazawa, 1986) and for developing seeds of *Phaseolus lunatus* (Xu et al., 1989) that meristematic cells and tissues often show higher levels of PFP. Hatzfeld and Stitt (1990) showed that high levels of PFP were accompanied by high percentages of label exchange from C-1 to C-6 carbons in hexoses and sucrose in logarithmically growing batch-cultured cells of *Chenopodium rubrum*.

In order to investigate these apparently contradictory results, PEMs were separated from VACs and uptake of [1-^{13}C]-glucose and conversion into [1-^{13}C]- and [6-^{13}C]-labelled sucrose and fructose was monitored and compared to the ratio of *in vitro* PFP/PFK activity. Furthermore, the A10 and A+-line were aerated with high and low O_2, to monitor the effect of the O_2 concentration on the respiration rate and label exchange from C-1 to C-6 carbons.

Materials and Methods

Cell suspensions

Cell suspensions were initiated from hypocotyl-derived callus of *Daucus carota* L. cv. Flakkese ("A10-line") and cv. San Valery ("A+-line") (de Vries *et al.* 1988). Cells were subcultured every 14 days by diluting 2 ml of packed cells in 50 ml Gamborg's B5 medium (Gamborg *et al.* 1968) supplemented with 2.3 μM 2,4-D and 50 mM sucrose. For the A+-line 2 μM BAP was added which was necessary to maintain the large amount of PEMs. When BAP was omitted from the medium, the amount of PEMs decreased to 10%, similar to that of the A10-line.

Separation of proembryogenic masses and vacuolated cells

Cells of day 7 were harvested from batch cultures and sieved over 200, 125 and 50 μm nylon filters. Ten ml of cells (with a packed cell volume of about 40%) between 50-125 μm were put on top of a percoll gradient consisting of 5 ml 40% percoll, 10 ml 30% percoll, 10 ml 20% percoll and 10 ml 10% percoll (Ulmer and Flad 1979). Gradients were centrifuged at 200g for 20 minutes with low-acceleration and low-brake in a MSE Mistral 3000*i* swing-out centrifuge (Fisions Scient. Equipm., Crawley, West Sussex, UK). After centrifugation the fractions of 10 and 20% percoll, mainly consisting of vacuolated and isodiametrically shaped cells, were pooled ("VACs") as well as the fractions of 30 and 40% percoll, consisting of mainly proembryogenic masses ("PEMs"). The procedure was carried out under sterile conditions in a laminar flow cabinet. After washing 3 times with fresh medium lacking sugar to remove the percoll, the cells were given the opportunity to recover overnight on an orbital shaker at 25°C in the same medium without sugar.

NMR experiments

Separated cells of 7 days old (20-30% packed cell volume) or cells of 14 days old washed with fresh medium lacking sugar and sieved over a 500 μm nylon filter (40% packed cell volume) were pipetted into a 20-mm diameter NMR tube containing an airlift-system for oxygen supply and suspending the cells (Chapter 4, Fig. 1; Fox *et al.* 1989). Cells were

aerated with 100% or 6% O_2 (10% CO_2, 84% N_2) to determine label exchange percentages from C-1 to C-6 carbons in sucrose and fructose. At time point zero 500 μmol 99.9% [1-^{13}C]-labelled glucose (g dry weight)$^{-1}$ was added after which acquisition of the NMR spectra was started immediately. ^{13}C-labelled glucose was purchased from Isotec Inc. (Miamisburg, Ohio, USA). Total respiration during a 9 h incubation period was measured by feeding a parallel sample 500 μmol unlabelled glucose.(g dry weight)$^{-1}$ during aeration with 1/99. 6/94, 20/80 or 100/0% O_2/N_2. Evolving CO_2 was trapped in 10% KOH solution (w/v) after which the $^{13}CO_2$ was determined by NMR. Total respired ($^{13}CO_2$ + $^{12}CO_2$) CO_2 was calculated by multiplying the amount of $^{13}CO_2$ by 91, since natural abundance ^{13}C is 1.1%.

In vivo ^{13}C-NMR of cells and determination of $^{13}CO_2$

^{13}C-labelled sugars were analyzed *in vivo* using a wide bore AMX-300 spectrometer (Bruker, Germany) equipped with a 20 mm internal diameter ^{13}C probe. ^{13}C-labelled CO_2 was analyzed using a 10 mm internal diameter ^{13}C probe; samples were prepared by mixing 2.20 ml $^{13}CO_3^{2-}$-containing KOH with 200 μl D_2O for field lock and 50 μl 500 mM [1-^{13}C]-glucose as internal standard. The Waltz sequence and two-level proton decoupling were applied, 7200 and 3600 FID's were accumulated in 8k data points using a 45° and 30° pulse and a pulse repetition time of 0.5 s and 2.0 s for spectra of cells and CO_2 samples, respectively. A line broadening of 3Hz was used and zero-filling to 16k data points was applied prior to Fourier transformation. Peak areas at 164.5 ppm (CO_3^{2-}), 96.8 ppm (ß-glucose C-1), 93.0 ppm (α-glucose C-1 and sucrose-glucosyl C-1), 64.8 ppm (fructose C-1), 64.2 ppm (fructose C-6), 63.3 ppm (sucrose-fructosyl C-6), 62.0 ppm (sucrose-fructosyl C-1) were integrated. Spectra of standard solutions containing 50 mM of sucrose, glucose and fructose or 10 mM 99.9% labelled $NaH^{13}CO_3$ and 10 mM 99.9% labelled [1-^{13}C]-glucose as internal reference recorded under similar experimental conditions were used for quantification of the C-1 and C-6 carbons. The exchange of label from C-1 to C-6 carbons was expressed as the percentage labelled C-6 carbons from total labelled (C-1 and C-6) carbons.

Sugar determinations

Soluble sugars were extracted by boiling 20 mg freeze-dried material in 1.0 ml 80 % methanol for 15 minutes at 76 °C. Methanol was evaporated in a Speedvac (Savant Instruments Inc. Farmingdale, NY, USA) and the samples were dissolved in ultra pure water (Millipore Intertech, Bedford, USA) (modified after Tetteroo *et al.* 1995).

Soluble sugars were measured with a Dionex HPLC system (Dionex Corporation, Sunnyvale, CA, USA) using a Carbopac PA-1 (guard)column coupled to a pulsed amperometric detector. Isocratic elution was performed with 100 mM NaOH for 15 minutes to separate glucose, fructose and sucrose. Peak areas were quantified using standard sugar solutions.

Enzyme determinations

PP_i-dependent fructose-6-phosphate phosphotransferase (PFP, EC 2.7.1.90) and ATP-dependent fructose-6-phosphate phosphotransferase (PFK, EC 2.7.1.11) were assayed in freshly made extracts from freeze dried material. Ten to twenty mg samples were extracted in 1.2 ml buffer containing 50 mM HEPES at pH 7.5, 5.0 mM dithiotreitol, 5.0 mM Mg-acetate and 1.0 mM EDTA at 4°C. Low molecular mass components were removed on a Biogel P6 column (BioRad, Veenendaal, The Netherlands) (modified after Appeldoorn *et al.* 1997). Enzyme assays were performed in a final volume of 1.2 ml containing 100 mM Tris/acetic acid at pH 8.0, 0.15 mM NADH, 5.2 mM fructose-6-phosphate, 0.8 U aldolase, 0.8 U glycerol phosphate dehydrogenase and 0.8 U triose phosphate isomerase. Reactions were started by the addition of 1.0 mM PP_i for PFP (modified after Hatzfeld *et al.* 1990) or 1.0 mM ATP for PFK. For PFP an additional 4.3 μM fructose-2,6-bisphosphate and 0.5 mM Mg-acetate and for PFK 0.5 mM $MgCl_2$ were added. NADH conversion was measured at 30°C using a double beam spectrophotometer operating at 340 nm (Shimadzu, Kyoto, Japan).

Respiration measurements

Oxygen uptake was determined by transferring 2.5 ml of cell suspension directly from the batch culture or after washing and NMR-experiments into an oxygen electrode (Rank Bros., Bottisham, Cambridge, UK) equilibrated with air. Oxygen uptake was followed for about 10 min at 25°C while stirring the suspension. The amount of hexoses respired per flask was

calculated by integrating oxygen consumption divided by six against the dry weight expressed as mg per flask.

Differences between experimental series

The growth rates of cell suspensions, and the exact timing of the consecutive growth phases differed considerably between various experimental series. Since sugar accumulating capacity, enzyme levels and respiration rate are growth phase-dependent, sugar labelling and absolute PFP and PFK-values show also differences between the different experimental series when measurements of the same day are compared. As a consequence, calculating mean values in replicate experiments would not be meaningful since the physiological age of the cells is not the same for each time point of different experimental series. Therefore, representative data are shown for each experiment.

Results

Effects of O_2 concentration on uptake and conversion of glucose, and on triose-hexose phosphate cycling.

Oxygen concentrations of 6% and 100% were used to aerate cells in the airlift-system. After addition of 500 μmol [1-^{13}C]-glucose.(g dry weight)$^{-1}$ cells started to take up glucose at a rate of about 50-200 μmol glucose.(g dry weight)$^{-1}$.h^{-1}, depending on the oxygen concentration and the cell line. At 6% O_2 the A+-line took up and converted the glucose about twice as fast as the A10-line. At 100% O_2 nearly all labelled glucose had been taken up after 5-6 h (A10-line) or 4 h (A+-line) while the final level of glucose in the A+-line was slightly lower than in the A10-line. At 100% O_2, the rate of glucose uptake was 2-3 times higher than that at 6% O_2 (Figs 1A,D).

At 6% O_2 at least two times more ^{13}C-label was built into sucrose and fructose compared to 100% O_2 (Figs 1 B,C,E,F). Generally more labelled sugars were found in the A+-line (Figs 1E,F) than in the A10-line (Figs 1B,C). Cellular glucose could not be measured since cellular and medium-localised glucose are observed as one signal in the NMR experiment.

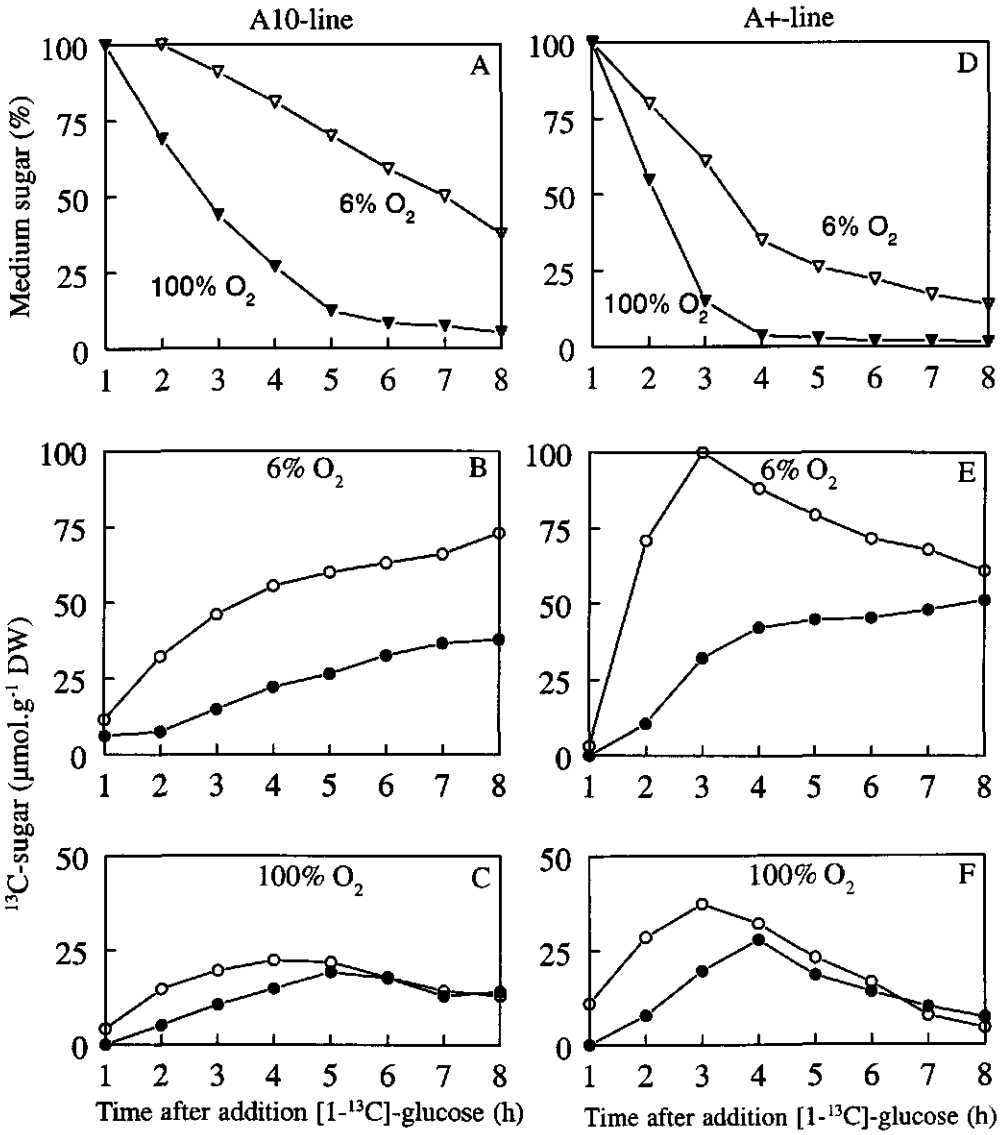


Figure 1 Uptake (A, D) and conversion (B, C, E, F) of 500 µmol [1-^{13}C]-glucose.g^{-1} DW in an air-lift-system by 14-days-old *Daucus carota* cells originally grown in batch culture on sucrose. Disappearance of glucose from the medium at 6% (▽) and 100% (▼) O_2 (A, D), conversion into sucrose (○) and fructose (●) at 6% O_2 (B, E) and at 100% O_2 (C, F). Representative data of different series of experiments.

The difference in label exchange from C-1 to C-6 carbons between the A10- and A+-line was most pronounced at the start of the experiment when cells were aerated with 6% O_2: the percentage C-6 labelled carbons of total labelled carbons in the fructose moieties of sucrose was about 2 times higher in the A10-line at that time (Fig. 2). Label exchange in the glucose moieties of sucrose and in free fructose was similar (*data not shown*). The percentage of C-6 labelled carbons was constant for the A10-line at 6% O_2 (about 18%); at 100% O_2 the percentage labelled C-6 carbons increased after 4 hours from 15 to 23% for this cell line (Fig. 2A). In the A+-line the percentage of labelled C-6 carbons increased at 6% and 100% O_2: from 7 to 14% and 13 to 17%, respectively (Fig. 2B). At 100% O_2 the percentage C-6 carbons was higher compared to that at 6% O_2 (Figs 2A,B), except in the A10-line for the first 4 hours (Fig. 2A).

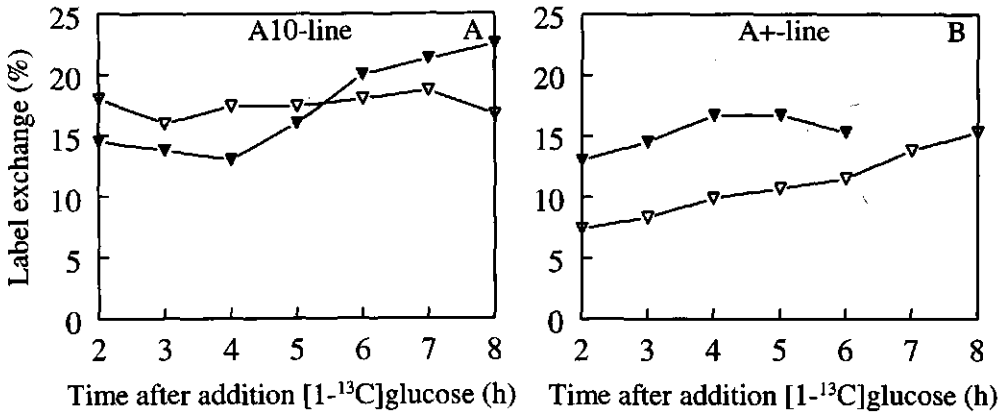


Figure 2 C-6 labelled carbons as percentage of total labelled (C-1 and C-6) carbons of the fructose moiety of sucrose after addition of 500 µmol [1-^{13}C]-glucose.g^{-1} DW in an airlift-system to 14 days old *Daucus carota* cells orginally grown in batch culture on sucrose. Cells of the A10-line (A) and A+-line (B) were aerated with 6% O_2 (▽) or 100% O_2 (▼). Representative data of different series of experiments.

Total respired carbon during the NMR-experiments was determined by measuring the total amount of $^{13}CO_2$ produced during 9 h incubation of cells of the A10-line with 500 μmol.(g dry weight)$^{-1}$ natural abundance glucose in an airlift-system aerated with different oxygen concentrations. These values were multiplied by 91 to calculate total ($^{13}CO_2$ + $^{12}CO_2$) CO_2 production. Figure 3 shows that respiration in the A10-line at 6% O_2 was about half of that at 100% O_2, being 1.7 and 3.4 mmol CO_2.(g dry weight)$^{-1}$.9 h^{-1}. From these data it was calculated that in a 9 h incubation period about 285 and 570 μmol hexoses were respired at 6% and 100% O_2, respectively.

Conversion of [1-^{13}C]-glucose and total sugar levels in PEMs and VACs.

Density centrifugation of the 50-125 μm fraction yielded separated PEMs and VACs populations; VACs of both the A10 and A+-line contained no proembryogenic masses. PEMs contained a 20-fold purified population of proembryogenic masses as was observed microscopically. The purity of PEMs of the A+-line was somewhat higher than that of the A10-line (*data not shown*).

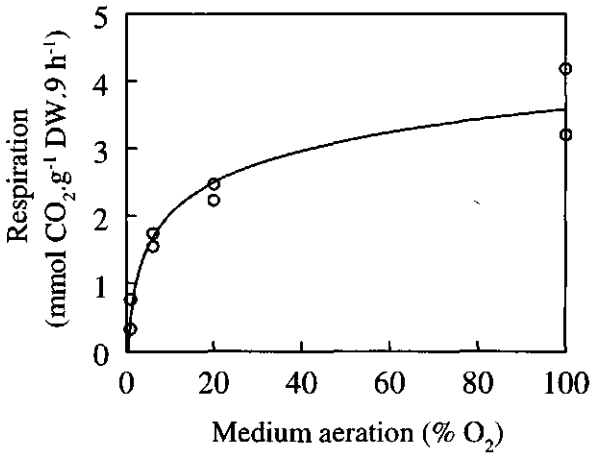


Figure 3 Total respiration during an 9 h incubation period expressed in mmol $CO_2.g^{-1}$ DW.9 h^{-1} of batch-cultured, sucrose-grown *Daucus carota* cells of the A10-line in an airlift-system aerated with 1, 6, 20 or 100% O_2 after addition of 500 μmol. g^{-1} DW natural abundance glucose.

Figure 4 shows the conversion of [1-^{13}C]-labelled glucose into labelled sucrose and fructose in separated PEMs and VACs in an airlift-system aerated with 100% O_2. In general, maximal labelling of sucrose was much higher in PEMs than in VACs on a dry weight basis. A tendency to peak-labelling between 4-7 hours was observed in PEMs of the A10-line (150 μmol.(g dry weight)$^{-1}$), after which labelling decreased again (Fig. 4A). PEMs of the A+-line showed a more or less steady level of labelled sucrose between 4-8 h (Fig. 4C). Sucrose labelling in VACs was much lower than in PEMs, reaching levels of 40-60 μmol.(g dry weight)$^{-1}$ (Figs 4B,D).

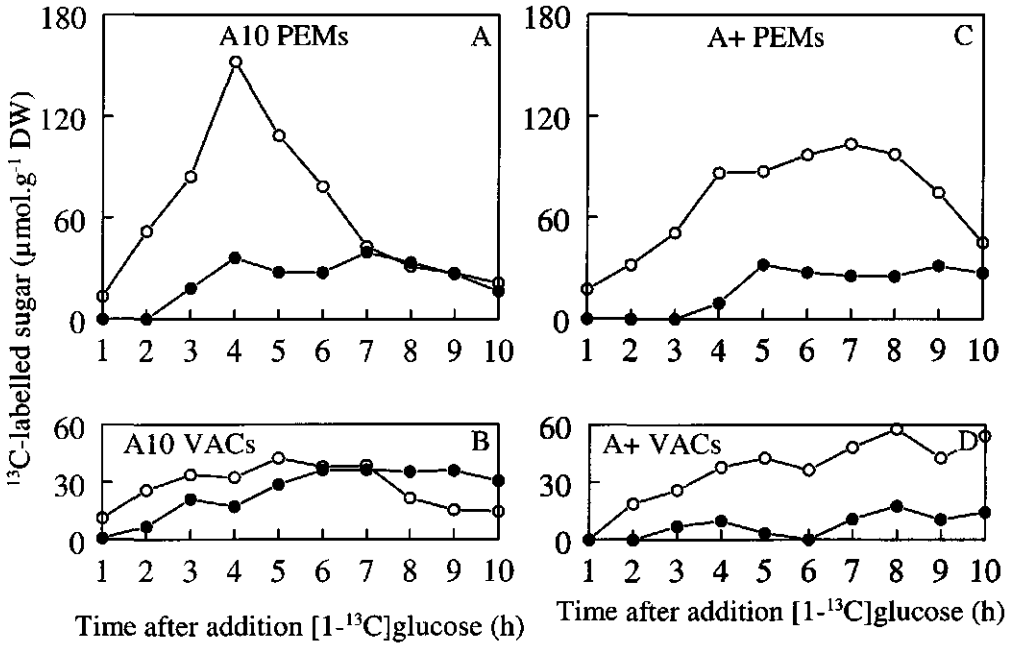


Figure 4 Accumulation of ^{13}C-labelled sucrose (○) and fructose (●) expressed in µmol.g^{-1} DW after addition of 500 µmol [1-^{13}C]-glucose.g^{-1} DW to separated proembryogenic masses (PEMs) (A, C) and vacuolated cells (VACs) (B, D) of the A10-line (A, B) and A+-line (C, D) in an airlift-system aerated with 100% O_2. Isolated PEMs and VACs were given the opportunity to recover overnight in Gamborg's B5 medium without sugar, at 25°C at an orbital shaker after separation from 7-days-old, batch-cultured cells of *Daucus carota* grown on sucrose. Data of representative series of experiments.

The rate of fructose labelling was slower than that of sucrose and reached a more or less stable level in all cell populations after a lag phase of 1-3 h. In PEMs fructose labelling was much lower than sucrose labelling and levelled off at about 40 μmol.(g dry weight)$^{-1}$. In VACs fructose accumulation showed a different pattern: in the A10-line fructose reached a level similar to that of sucrose (about 30-40 μmol.(g dry weight)$^{-1}$, Fig. 4B) and in the A+-line fructose labelling was much lower than sucrose labelling reaching only 10 μmol.(g dry weight)$^{-1}$ (Fig. 4D).

In Fig. 5 the total sugar levels are depicted directly after separation of the PEMs and VACs, before the NMR-experiment. In general, PEMs contained 1.5-2 times more sucrose than VACs. PEMs and VACs of the A+-line contained relatively high ratios of sucrose/fructose and glucose/fructose compared to the A10-line. Both types of cell populations of the A10-line contained more sugar per g dry weight than those from the A+-line.

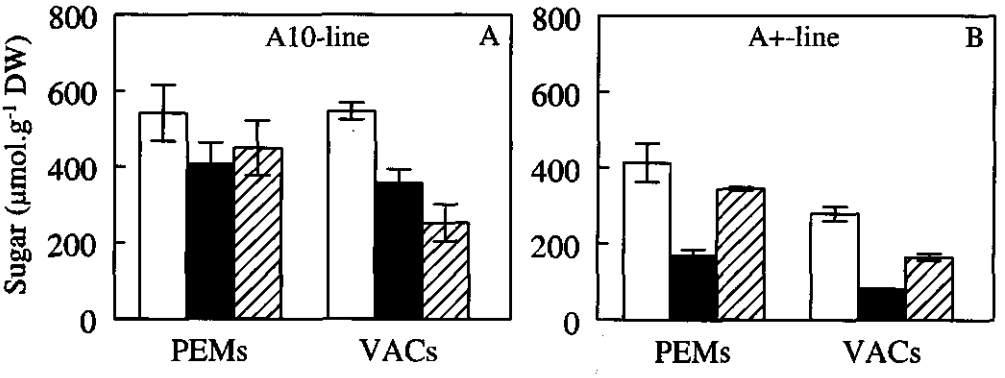


Figure 5 Total concentration of glucose (open bars), fructose (black bars) and sucrose (hatched bars) expressed in μmol.g^{-1} DW in separated proembryogenic masses (PEMs) and vacuolated cells (VACs) of the A10-line (A) and the A+-line (B) of 7-days-old batch-cultured *Daucus carota* cells grown on sucrose. Sugar concentrations were measured directly after separation of the PEMs and VACs at 4°C. Means of 3 determinations ± S.D.

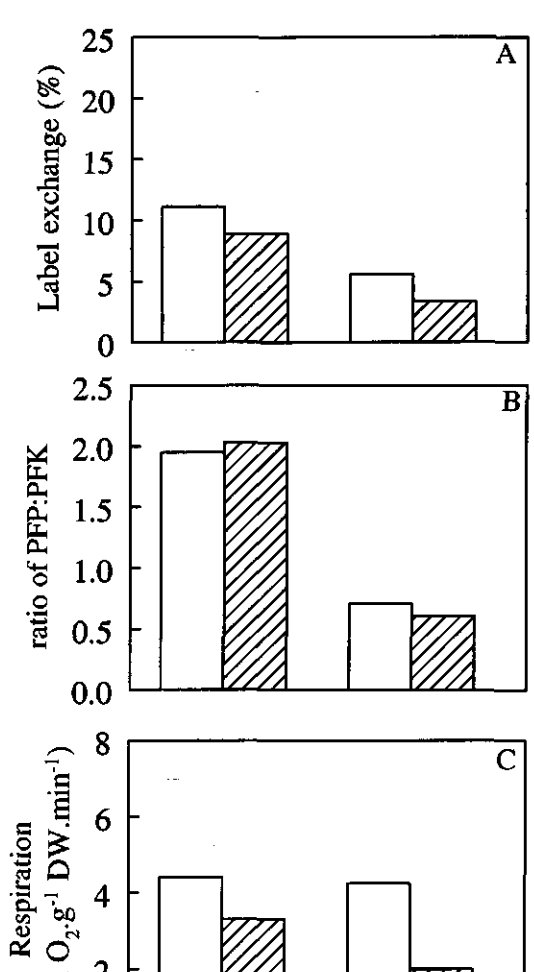


Figure 6 C-6 labelled carbons as percentage of total (C-1 and C-6) labelled carbons of the fructose moieties of sucrose after addition of 500 µmol [1-^{13}C]-glucose.g^{-1} DW to PEMs and VACs in an airlift aerated with 100% O_2 (A). The ratio of *in vitro* PFP and PFK activities directly after separation of the cells at 4°C (B). The respiration rate in µmol O_2.g^{-1} DW.min^{-1} directly after the labelling-experiment (C). Proembryogenic masses (PEMs, open bars) and vacuolated cells (VACs, hatched bars) were isolated from 7-days-old batch cultures of the A10-line and the A+-line of *Daucus carota* grown on sucrose. Means of time points 3, 4, 5, 6, 7 h (A), means of duplicate determinations (B, C).

Label exchange, the ratio of PFP/PFK, and respiration rate in PEMs and VACs.

During the logarithmic growth, the percentage of C-6 labelled carbons of total labelled (C-1 + C-6) carbons in the fructose moieties of sucrose was about 10 for PEMs and VACs of the A10-line and about 4 for PEMs and VACs of the A+-line, respectively (Fig. 6A). This was 2-4 times lower than the label exchange for non-separated, stationary phase cells (Fig. 2). The corresponding ratios of PFP/PFK were similar for PEMs and VACs from each cell line being about 2.0 and 0.7 for the A10 and A+-line, respectively, indicating that the A10-line contained much more PFP relative to PFK than the A+-line (Fig. 6B). This difference was mainly caused by the 3-fold higher level of PFP in the A10-line while the levels of PFK were similar (*data not shown*). The respiration rates of isolated PEMs and VACs were determined after the NMR-experiment. Figure 6C shows that the respiration rate expressed as μmol O_2.(g dry weight)$^{-1}$.min^{-1} was higher for PEMs than for VACs, especially in the A+-line. Respiration of PEMs of the A10- and A+-line was similar while the respiration rate in VACs was about 1.5 times higher in the A10-line compared to the A+-line.

Discussion

Effect of the oxygen concentration on triose phosphate cycling in the A10 and A+-line

An oxygen concentration of 6% resulted in at least two times more sucrose and fructose labelling (Figs 1B,C,E,F) in both lines than 100% O_2. Figure 3 showed that at a concentration of 100% O_2 respiration was about two times higher than at 6% O_2. In parallel, glucose utilization was about 2 times higher at 100% than at 6% O_2 (Figs 1A,D). Apparently, glucose phosphorylation and the concomitant glycolytic activity and the synthesis of sucrose were highly affected by the medium oxygen concentration, possibly by determining the amount of hexoses used in glycolysis: 100% O_2 resulted in less hexose phosphate available for sucrose synthesis while more sugar was channelled towards the respiratory pathway. This indicates, that the cells in the airlift were still not saturated with O_2 at an aeration with 100 ml.min^{-1} 6% O_2 and perhaps even at aeration with pure O_2.

However, the cytochrome c and alternative oxidase of *Petunia hybrida* were reported to be saturated at about 12 μM and 60 μM O_2, respectively, which corresponds to 1 and 5% O_2 (Hoefnagel *et al.* 1992). Since cultures of *Daucus carota* consist of relatively large cell clumps (up to 500 μm in diameter), intercellular and intracellular diffusion of O_2 in this system might limit the respiration rate.

The label exchange from C-1 to C-6 carbon in the A10-line ranged from 15-23%; this is consistent with reports for *Chenopodium rubrum* cells (Hatzfeld and Stitt 1990), *Triticum aestivum* (Keeling *et al.* 1988), *Solanum tuberosum* tubers and *Vicia faba* seeds (Viola *et al.* 1991), labelled for 1-4 h. Label exchange in the A+-line was lower, ranging from 7-17%. During incubation of A+-line cells at 100% O_2 label exchange from C-1 to C-6 carbons was higher than at 6% O_2. Aeration of the A10-line with 100% O_2 finally also led to a higher label exchange than with 6%. Apparently, a higher rate of respiration at 100% O_2 leads at the same time to a high flow of triose phosphates in the glycolytic direction and a high gluconeogenetic backflow of triose phosphates towards hexose phosphates. In the A10-line, label exchange increased from 14 to 20% between 4 and 6 h after start of the incubation at 100% O_2, concomitant with the exhaustion of medium sugar (Figs 1A, 2A). Obviously, the continued high respiration caused increasing label exchange from C-1 to C-6 carbons within a decreasing labelled hexose (phosphate) pool due to continuing sucrose cycling and triose-hexose phosphate cycling (Fig. 7).

In separated cell populations from 7-days-old cultures the percentage of C-6 labelling was about 10% in fractions of the A10 and 4% in fractions of the A+-line, respectively (Fig. 6A). This was 2-4-fold lower than in 14 days-old-cells, reaching values of 17-23% (Fig. 2A). Cultures of 7 days old consist of logarithmically growing cells which possess a higher respiration rate (Chapter 4, Fig. 2B) and a higher capacity of sucrose accumulation (Chapter 4, Fig. 2C,D) than 14 days old cultures. Therefore, the partitioning of hexose phosphates between triose phosphates and sucrose, and partitioning of triose phosphates between glycolysis and gluconeogenesis might be differently regulated in these different types of experiments.

Since fructose-1,6-bisphosphatase is absent in many non-green tissues (Entwistle and ap Rees 1990), PFP is supposed to account for the observed C-1 to C-6 label exchange in sucrose and

hexoses in *Daucus carota* cells as was suggested in earlier work on *Chenopodium rubrum* cells (Hatzfeld and Stitt 1990). The A10-line which contained more VACs showed in general higher levels of label exchange from C-1 to C-6 carbons compared to the A+-line (Fig. 2), which led to the working hypothesis put forward in the introduction, that the level of PFP might be higher in VACs than in PEMs in *Daucus carota* cell suspensions.

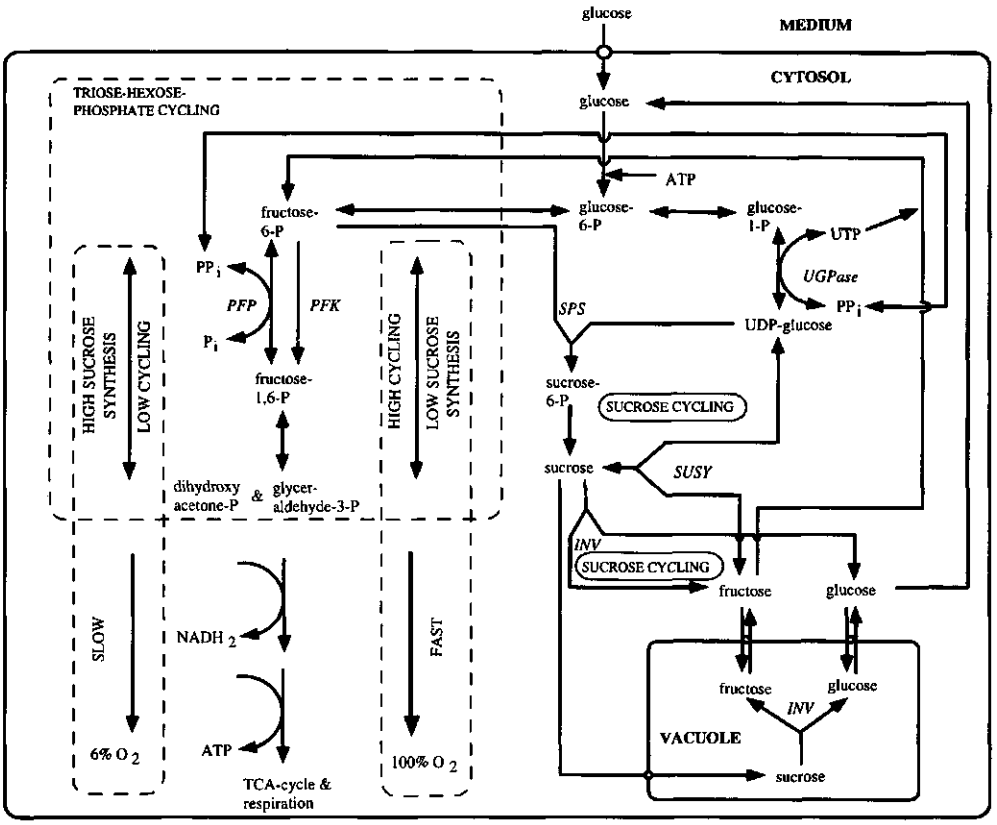


Figure 7 Uptake and conversion of [1-^{13}C]-glucose by PEMs and VACs of *Daucus carota* cells originally grown in batch culture on sucrose. Effects of PFP and oxygen concentration on the triose-hexose phosphate cycling and sucrose cycles are shown. *INV* = invertase, *PFK* = ATP:fructose-6-phosphate phosphotransferase, *PFP* = pyrophosphate:fructose-6-phosphate phosphotransferase, *SPS* = sucrose phosphate synthase, *SUSY* = sucrose synthase, UGPase = UDP-glucose pyrophosphorylase, UDPG = UDP-glucose. Sucrolysis might take place both in the cytosol and, after uptake of sucrose, in the vacuole.

PFP and C-1 to C-6 label exchange in PEMs and VACs.

Comparing the two cell lines, PFP/PFK ratios were higher in the A10-line in parallel with a higher percentage of labelled C-6 carbons than in the A+-line, suggesting that the different levels of PFP are connected with the differences in triose-hexose phosphate cycling. The A+-line, normally cultured in the presence of BAP, contained a high number of PEMs, while both PEMS and VACs of this cell line showed a low percentage label exchange from C-1 to C-6 and a low PFP/PFK ratio compared to PEMs and VACs of the A10-line (Figs 6A,B). When the A+-line was grown in the absence of BAP, this resulted in a culture which resembled the A10-line with respect to the amount of PEMs (10% PEMs), but showed the same low labelling percentage of C-6 carbons as the A+-line cultured in the presence of BAP (*results not shown*). It is concluded that the label exchange from C-1 to C-6 carbons in hexoses and sucrose is connected with the level of PFP and is genetically determined. Therefore, label exchange from C-1 to C-6 carbons is not related to the presence of PEMs or VACs in *Daucus carota* cell suspensions. The postulated positive correlation between sucrose accumulation and PFP activity in meristematic tissues like logarithmically growing *Acer pseudoplatanus* cells (Huber and Akazawa 1986), *Chenopodium rubrum* cells (Hatzfeld and Stitt 1990) and *Saccharum* stem tissue (Zhu *et al.* 1997) was not found when comparing PEMs and VACs of *Daucus carota* of either the A10 or A+-line, as PEMs from both lines showed high sucrose accumulation despite the large difference in PFP activity (Figs 4, 6). Although the ratio of PFP/PFK was different for cells of the A10 and the A+-line (Fig. 6B), PFK levels were similar. PFK is thought to act as a "maintenance" enzyme producing substrates for respiration; therefore, this enzyme is expected to reach similar levels in identical tissues of related plant species and cell lines. PFP is thought to play an important role as an "adaptive" enzyme, since its reversible action is known to be in near-equilibrium *in vivo* (Sung *et al.* 1988; Ashihara and Sato 1993). It might be responsible for adjusting the equilibrium between the different pools of hexose phosphates, triose phosphates, P_i and PP_i during periods of high metabolic activity (Black *et al.* 1987; Stitt 1989; Hatzfeld and Stitt 1990). Therefore, PFP activity might differ largely between tissues and species which show differences with respect to biosynthetic capacity or stress-resistance (Black *et al.* 1987). The observed ratio of PFP/PFK of about 2 in the A10-line coincided with that reported for

Chenopodium rubrum cells (Hatzfeld and Stitt 1990), *Zea mays* kernels (Tobias *et al.* 1992) and *Vigna mungo* seeds (Ashihara and Sato 1993). In the A+-line the ratio of PFP/PFK was relatively low compared to the A10-line. Therefore, it is suggested that the maintenance pathway and adaptive pathway are more closely coupled in this line giving rise to less metabolic flexibility.

PFP is activated by fructose-2,6-bisphosphate (van Schaftingen *et al.* 1982; Stitt 1990). Except from the role of fructose-2,6-bisphosphate in photosynthetic tissues (Stitt *et al.* 1983; Scott and Kruger 1995), its concentration was found to be about 0.1-0.3 nmol.(g fresh weight)$^{-1}$ in non-green tissues like *Solanum tuberosum* tubers and *Daucus carota* roots (Hajirezaei and Stitt 1991) and *Glycine max* cell suspensions (Spilatro and Anderson 1988) which might be 1-3 μM in the cytosol supposing a cytosolic localisation and a cytosolic volume of 10%. This is more than 100 times higher than its K_e-value of 4-22 nM (Kombrink *et al.* 1984; Spilatro and Anderson 1988; Trevanion and Kruger 1991; Hajirezaei and Stitt 1991) suggesting that the PFP enzyme is fully activated by fructose-2,6-bisphosphate. PFP activity, therefore, might be determined by the levels of its substrates. Stitt (1990) and Hajirezaei *et al.* (1994) showed that PP_i, P_i, fructose-6-phosphate and fructose-1,6-bisphosphate levels might be saturating *in vivo*, making both the glycolytic and the gluconeogenetic reaction possible.

To explain the simultaneous increase in the rate of glycolytic and gluconeogenetic reactions at high levels of O_2, it is suggested that the level of phosphoenolpyruvate decreases as a consequence of the high respiratory activity at 100% O_2, leading to a decrease of the allosterical inhibition of PFK by phosphoenolpyruvate (Dennis and Greyson 1987; Huppe and Turpin 1994). This stimulates the glycolytic conversion of fructose-6-phosphate into fructose-1,6-bisphosphate mediated by PFK. These higher levels of fructose-1,6-bisphosphate might not only support a higher respiration rate (Fig. 3), but also stimulate the gluconeogenetic conversion of fructose-1,6-bisphosphate and P_i into fructose-6-phosphate and PP_i via PFP (Fig. 7). For *Daucus carota* storage root fructose-1,6-bisphosphate aldolase a K_m value of 6 μM is reported for fructose-1,6-bisphosphate (Moorhead and Plaxton 1990). The K_m values of PFP for fructose-1,6-bisphosphate are reported to be 5, 20 and 23 μM in *Solanum tuberosum* tubers (Stitt 1989), *Camellia* pollen (Nakamura *et al.* 1992) and *Ricinus communis*

seedlings (Kombrink *et al.* 1984), respectively, making PFP and aldolase compete for fructose-1,6-bisphosphate, probably leading to increases in the reaction products of both enzymes and to both glycolytic and gluconeogenetic conversions.

Gluconeogenetic PFP activity produces PP_i, which is supposed to be used for the cytosolic conversion of UDPG into glucose-1-phosphate and UTP by UGPase after hydrolysis of sucrose by sucrose synthase (SUSY, Huber and Akazawa 1986; Xu *et al.* 1989; Fig. 7). UTP, in turn, might be used to phosphorylate the released fructose since fructokinases are known to be active with UTP in addition to ATP (Yamashita and Ashihara 1988; Schnarrenberger 1990; Chapter 3). Therefore, high PFP activity might be connected with SUSY-mediated cycling of sucrose (Huber and Akazawa 1986; Hatzfeld and Stitt 1990). It is hypothesized, that the A10-line, showing high levels of PFP, might contain higher levels of SUSY than the A+-line, showing low levels of PFP.

At low O_2 concentrations the respiration rate is lower (Fig. 3) and less hexose phosphates are converted to fructose-1,6-bisphosphate and triose-phosphates: as a consequence more hexose phosphates are available for sucrose synthesis (Figs 1B,C,E,F). In addition, lower PFP activity might produce less PP_i and, as a consequence, sucrose degradation by SUSY and UGPase might also be lower (Fig. 7) and sucrose labelling might reach higher levels at those low O_2 concentrations (Figs 1B,C,E,F).

In vivo labelling of PEMs and VACs of the A10 and A+-line.

PEMs and VACs of both the A10 and A+-line were separated from 7 days old cultures on density gradients. Glucose, fructose and sucrose levels were still high at the start of the NMR-experiments (Fig. 5) since sugar levels decreased only slightly during recovery overnight in Gamborg's B5 medium without sugar (*results not shown*).

After addition of 500 μmol [1-^{13}C]-glucose.(g dry weight)$^{-1}$, fructose labelling started later and with a lower rate than sucrose labelling in both PEMs and VACs (Fig. 4); therefore, it is suggested that fructose evolved from sucrolysis. Fructose can only accumulate when protected from the high activity of fructokinase in *Daucus* cells (Chapter 3). Therefore, simultaneous synthesis and hydrolysis of sucrose only in the cytosol, would probably not result in accumulation of labelled fructose. It is concluded that sucrose is at least partially

transported to the vacuole after being synthesised in the cytosol, in both PEMs and VACs of the A10 and A+-line (Fig. 7).

PEMs of both the A10 and A+-line showed higher steady-state levels of sucrose than VACs (Figs 5A,B) and also accumulated higher levels of labelled sucrose in the airlift (Figs 4A,C). Parallel to a higher level of sucrose, higher levels of starch were also observed in PEMs (*results not shown*), which was reported earlier by Wurtele *et al.* (1988). It is concluded, therefore, that PEMs have a higher sucrose and starch accumulating capacity than VACs, indicating that these cells resemble meristematic, carbon importing tissues. Apparently, PEMs and VACs are different with respect to primary metabolism, although these differences were not related to the ratio of PFP/PFK (Fig. 6).

Conclusion

Both PFP and the O_2 concentration were found to be related with label exchange from C-1 to C-6 carbons within sucrose and hexoses. PEMs accumulated higher levels of sucrose than VACs, indicating that sugar metabolism was differently regulated in both cell types. However, PEMs and VACs of one cell line were identical with respect to label exchange from C-1 to C-6 carbons and the ratio of PFP/PFK, indicating that these properties are genetically determined in the investigated *Daucus carota* cell lines. High levels of PFP are supposed to enable plants to adjust the equilibrium between the flow of carbon to glycolysis, the pathway leading to synthesis of sucrose and to structural cell components when environmental conditions change strongly.

Acknowledgements

This research was financially supported by the Foundation of Life Science (SLW) which was subsidized by the Netherlands Organisation of Scientific Research (NWO). We are grateful to Marijke Hartog and Sacco C. de Vries of the Laboratory of Molecular Biology, Wageningen University for supplying different types of embryogenic cell lines of *Daucus carota* L.

Chapter 6

General Discussion

A model for uptake and conversion of glucose and fructose, and for cycling and storage of hexoses, sucrose and starch in carrot suspension cells.

Introduction

This thesis presents a study of the primary carbon metabolism in plant cells using different techniques. Besides the classical way of measuring enzyme activities, and sugar and starch levels by invasive techniques, *in vivo* and *in vitro* ^{13}C-NMR were used to follow the pathway of sugar molecules and of certain atoms within these molecules.

With this combined approach, information was gathered about metabolic cycles in the lag-, logarithmic- and stationary growth phase of batch-grown suspension cells of carrot (*Daucus carota*). Differences between glucose and fructose as a carbon source were studied. We were able to qualify and quantify the uptake and conversion of glucose and fructose into glycolysis, the oxidative pentose phosphate pathway (OPPP) and respiration. Short-term ^{13}C-labelling was applied to suspension-cultured cells of *Daucus carota* to get information on the flexibility and dynamics of plant metabolism. Cycling of hexoses through sucrose, i.e. synthesis of sucrose in the cytosol and concomitant hydrolysis in either the cytosol or the vacuole was analyzed. Furthermore, cycling of hexose phosphates through the OPPP in cytosol and plastids and cycling between hexose and triose phosphates in the cytosol were observed. Long-term labelling was applied to get information on sucrose and starch metabolism occurring during a 14 days growth period in batch culture.

The relationship between the observed metabolic cycles and sugar uptake, biomass production and respiration, and the cellular compartmentation of these metabolic cycles was analyzed. The observed characteristics will be discussed in this chapter in the context of plant-specific metabolic pathways, properties and regulations.

Glucose and fructose uptake and conversion

Chapter 3 describes the difference between glucose and fructose as a substrate for growth and energy production in carrot cells. Because of the high activity of the abundant enzyme phosphoglucoisomerase, interconverting glucose-6-phosphate and fructose-6-phosphate (Ashihara *et al.* 1988; Schnarrenberger *et al.* 1995), it was hypothesized that both sugars would have an equal impact on growth and energy production. However, when glucose and fructose were supplied together, glucose was converted preferentially in *Daucus* cells. On the contrary, it was shown that fructose given as the sole carbon source resulted in a shorter lag

phase and higher biomass production as compared to glucose. Although different hexose and sucrose transporters are known, which are often tissue and development-specific (Weig *et al.* 1994; Burkle *et al.* 1998), it is assumed that cell suspensions represent one type of tissue with respect to sugar uptake. *Daucus* suspension cultures grown on glucose or sucrose for 6 months showed the same higher biomass production when transferred to fructose-medium, indicating that probably the same set of membrane carriers and phosphorylating enzymes was already present or rapidly induced after inoculation, irrespective of the carbon source on which the cultures had been grown before. Reports of Spilatro and Anderson (1988), Zwayyed *et al.* (1991), Levi and Sink (1992) and Drew *et al.* (1993) also showed that fructose gave more biomass compared to glucose. In Chapter 3, it was shown that the maximal uptake rates of glucose and fructose were about the same when supplied separately. Although at low concentrations (up to 2 mM), glucose and fructose competed for the hexose carrier, this could only partially explain the difference between glucose and fructose conversion, suggesting that phosphorylation should be differently regulated in a competitive way.

Daucus cells showed two distinct fractions of sugar phosphorylating activities: a soluble fraction converting mainly fructose and a membrane-bound fraction capable of converting both glucose and fructose. However, the membrane-bound enzymes showed a 10-fold higher glucose phosphorylating activity next to a 2-fold higher fructose phosphorylating activity than the soluble enzymes. Furthermore, the membrane-bound enzymes showed a 10-fold higher affinity towards glucose as compared to fructose. It is concluded that the soluble fraction consists of mainly specific fructokinases, while the membrane-bound fraction consists of a different set of one or more unspecific hexokinases. It was shown by several authors that a significant part of the hexokinases are attached to the mitochondrial outer membrane (Steward and Copeland 1993; Galina *et al.* 1995, 1999). As a consequence, glucose as a carbon source might provide a pool of glycolytic intermediates surrounding the mitochondria and might therefore be a better substrate for respiration than fructose. Tubers of *Solanum tuberosum* cotransformed with a yeast invertase and a bacterial glucokinase, which showed higher levels of especially glucose, also showed a markedly increased respiration rate. However, this glucokinase was assumed to be a soluble enzyme and not specifically localised at the

mitochondrial site (Trethewey *et al.* 1998).

The first eukaryotic algae might have been developed by a process of "assimilation", i.e. uptake of bacteria by other bacteria. The assimilated bacteria developed to specialized organelles, i.e. mitochondria and chloroplasts (endosymbiontic theory, Whatley and Whatley 1981). The resulting unicellular algae used the plastids for sugar degradation, and lost the ability of cytosolic glycolysis and starch synthesis. Possibly as a result, nowadays many unicellular algae do not posses a cytosolic glycolytic pathway and do not synthesize sucrose. Only the chloroplast shows a complete glycolytic pathway in which starch rather than sucrose is the substrate for glycolysis (Whatley and Whatley 1981; Huppe and Turpin 1994). Plastids and chloroplasts still take up glucose phosphates as their carbon source, also in higher plants. The conversion of glucose into glucose-6-phosphate by mitochondrion-bound hexokinase might result in a pool of hexose phosphates in the respiratory "compartment"; the resulting pyruvate will be used in mitochondrial respiration.

For fructose a pathway might have developed leading to structural components (cell wall components) and sucrose in the cytosol. In this view, sucrose might have been the key to the development of higher plants, since it is involved in energy production by glycolysis and respiration (Sung *et al.* 1988), synthesis of storage carbohydrates (Whittaker and Botha 1997) and transport of carbon from source to sink tissues (van Bel *et al.* 1994). It is suggested that glucose alone is insufficient to fulfil all these jobs simultaneously, while the occurrence of fructose and glucose supplied the plant cell with parallel regulatory mechanisms to coordinate these different tasks at the same time.

Geiger *et al.* (1998) also found for slices of growing potato tubers that feeding ^{14}C-glucose led to an increase in respiration, while ^{14}C-sucrose led to an increase in starch synthesis within 20 minutes. Since ADP-glucose (ADPG) and 3-phosphoglycerate levels did not increase, they proposed that the imported sucrose was involved in a specific signal-transduction pathway leading to stimulation of starch synthesis. However, this explanation was not based on the demonstration of the existence of such a signal-transduction pathway. An alternative explanation might be that sucrose taken up by potato tuber slices, which is degraded by sucrose synthase (SUSY), results in UDP-glucose and fructose rather than in free glucose (and fructose). These products of sucrolysis might be available throughout the

cytosol and used for starch synthesis, while externally fed ^{14}C-glucose might be primarily phosphorylated by mitochondrion-bound hexokinase, leading to glucose-6-phosphate in the mitochondrial environment which resulted in an increased respiration.

Next to the described localization of hexokinase in the respiratory "compartment" and fructokinase randomly in the cytosol, cell compartments separated by biological membranes such as the plastids and the vacuole are essential to independently regulate glycolysis, OPPP, and sucrose and starch metabolism within one single cell as will be discussed in the next paragraphs.

Sucrose cycling

Chapter 4 describes short-term effects *in vivo*, during labelling of cells of different developmental stages in an airlift-system. Respiration was 1.5-fold higher in the stationary growth phase and 4-fold higher in the logarithmic growth phase compared to the corresponding cells from batch culture. It was shown that sucrose cycling occurred in stationary phase cells when growth and respiration rates were relatively low, i.e leaving enough hexose phosphates for sucrose synthesis. In short-term experiments with logarithmically growing cells respiration was high and no hexose phosphates were available for sucrose synthesis and cycling. However, during long-term labelling experiments sucrose cycling also occurred in logarithmically growing cells, as appeared from the production of (labelled) sucrose and fructose in experiments in which cells were grown on ([1-^{13}C]-labelled) glucose. It is concluded, that during the whole period of batch culture cycling of sucrose was an integral part of metabolism of *Daucus* cells.

Regulation of sucrose metabolism is complex, since synthesis and degradation might take place in the same compartment as well as in separate compartments (Chapter 7, Figs 1,2). Synthesis is assumed to be localized in the cytosolic compartment and to be catalyzed by sucrose phosphate synthase (SPS, Goldner *et al.* 1991; Zhu *et al.* 1997), but cellular hydrolysis of sucrose might be catalyzed by 3 different enzymes: in the cytosol by neutral invertase or sucrose synthase (SUSY), and in the vacuole by acid invertase. All three enzymes increased in *Daucus carota* cells in the logarithmic growth phase when sucrose was stored and during the stationary growth phase when sucrose was degraded. When all sucrose

was consumed, the levels of the sucrose-degrading enzymes decreased again.

Hexoses and sucrose are transported into the vacuole (Preisser *et al.* 1992, Heineke *et al.* 1994). Guy *et al.* (1979), Thom and Komor (1984), Briskin *et al.* (1985), Getz (1991), Rausch (1991) and Davies (1997) found that ATP stimulates this uptake by means of a H^+/antiport-system as might be expected to be functional in well-fed cells. Inside the vacuole sucrose is hydrolysed to a minor extent since the resulting glucose and fructose strongly inhibit further acid invertase action permitting sucrose storage in the vacuole (Lee and Sturm 1996).

SUSY and neutral invertase in the cytosol are also strongly inhibited by fructose *in vitro* (Sebková *et al.* 1995; Lee and Sturm 1996). However, as a consequence of the uptake of hexoses into the vacuole and the strong hexose phosphorylating activity in the cytosol, the level of hexoses in the cytosolic compartment might be low. Furthermore, we suggest that UDP-glucose (UDPG) levels are high in the logarithmic growth phase as was found for *Nicotiana tabacum* cell suspensions by Meyer and Wagner (1985), well above the K_m for UDPG in the sucrose synthesizing direction of SUSY (Vella and Copeland 1990; Elling 1996). Furthermore, feeding [1-^{13}C]-fructose resulted in nearly equal labelling of the fructose and glucose moieties within sucrose, therefore SPS rather than SUSY must be responsible for sucrose synthesis. In addition, feeding fructose as the sole carbon source resulted in cellular glucose, indicating that sucrolysis probably took place by invertase action and not by SUSY. Although SUSY was found to be present during the whole culture period, it is concluded that neutral invertase is responsible for sucrose cycling in the cytosol and that SUSY probably shows very low activity *in vivo* in both directions in the logarithmic growth phase.

In the stationary phase lower levels of ATP (Meyer and Wagner 1985; Kubota and Ashihara 1993) might cause lower H^+/ATPase and H^+/PPase activities on the tonoplast resulting in a lower membrane potential. As a consequence, hexoses are mobilised to the cytosol to sustain the need for carbon necessary for cell maintenance. The inhibition of acid invertase in the vacuole then will be abolished, resulting in further hydrolysis of stored sucrose. Concomitant with the decrease of total cellular sugars and NTPs, UDPG levels will decline (Meyer and Wagner 1985) and as a result, sucrose degradation in the cytosol might also

occur by SUSY. It is concluded that additional to sucrose cycling by neutral invertase also SUSY in the cytosol and acid invertase in the vacuole become active in the stationary growth phase, resulting in activation of the SUSY-mediated and "vacuolar" sucrose cycles. This is consistent with short-term labelling of stationary phase cells which are sugar-starved in which enough hexose phosphates are available for synthesis of sucrose. Labelled fructose was retained, while sucrose labelling was found to be transient. Obviously, hydrolysis of sucrose took place in the vacuole, since sucrolysis in the cytosol would not permit fructose accumulation due to the abundant hexose phosphorylating activity. It is proposed that if sucrose reaches a certain level in the cytosol, transport into the vacuole takes place where sucrolysis might occur, if hexose levels are low enough to permit invertase activity, leading to limited accumulation of fructose (and glucose) in the cytosol.

Since embryogenic cell suspensions undergo changes in time, with respect to embryogenic potential and sugar accumulation, suspensions were newly initiated every 6 months. The ratio of sucrose:hexoses shows profound differences between the different experimental series (Chapter 3, Fig. 5 *versus* Chapter 4, Fig. 2C). Dijkema *et al.* (1988) showed that this changing ratio of sucrose:hexose correlated with the life-span of embryogenic cell suspensions. The embryogenic potential of a just initiated suspension of one month is maximal, and in parallel it shows high amounts of PEMs and high levels of intracellular glucose (and to a lesser extent fructose). As the suspension ages, the ratio of hexoses/sucrose decreases. However, it appeared that the maximal amount of hexose units within glucose, fructose and sucrose taken together was between 1000-1200 μmol.(g dry weight)$^{-1}$ in all experiments, suggesting that total sugar accumulation did not change. Sucrose accumulation is determined by the difference in SPS and invertase activity in *Saccharum* stem tissue (Zhu *et al.* 1997), in carrot roots (Zamski and Barnea 1996) and in cell suspensions of *Saccharum* (Wendler *et al.* 1990, Goldner *et al.* 1991). Since it is suggested that young, just initiated carrot lines containing relatively large numbers of PEMs resemble meristematic tissues with high levels of glucose (Dijkema *et al.* 1988), it is suggested that they have relatively high levels of sucrose hydrolysing enzymes. In other words: embryogenic cells might show a large flow of metabolites through the sucrose cycle which may provide the cells with a continuous source of glucose (phosphates) for maintaining the high respiratory activity, necessary for

the primary events in embryogenesis (Kikuta and Masuda 1981). However, at the moment we can only speculate on differences in the ratio between SPS and invertase in the various lines used in this work.

Oxidative pentose phosphate pathway activity

In chapter 2 experiments are described in which batch cultures were grown on a mixture of glucose and fructose; either the glucose was [1-^{13}C]-labelled and the fructose unlabelled or the fructose was [1-^{13}C]-labelled and the glucose not labelled. One might expect that glucose would be used primarily in the respiratory "compartment" and fructose would be used in the structural cell components "compartment". However, labelling with [1-^{13}C]-glucose resulted in a 2 times higher labelling percentage of sucrose compared to labelling with [1-^{13}C]-fructose in the first week of batch-culture. Obviously, glucose was taken up and converted preferentially to fructose, not only for respiratory metabolism but also for synthesis of sucrose in the structural cell "compartment". It is concluded that in this experimental set-up exchange of hexose phosphates between the respiratory "compartment" and the structural component "compartment" is considerable, or that glucose phosphorylation by the soluble phosphorylating enzymes is relatively large.

Chapter 2 showed that feeding [1-^{13}C]-glucose or [1-^{13}C]-fructose to batch-cultured cells both resulted in [1-^{13}C]-labelling of the glucose and fructose moiety of sucrose. The C-1 labelling of the fructose moiety was always slightly lower than of the glucose moiety. This could be explained by cytosolic OPPP activity which consumes glucose-6-phosphate, removes its C-1 label and, via transaldolase and transketolase reactions, finally produces triose phosphates. The triose phosphates can be shuttled back to fructose-6-phosphate via the gluconeogenetic pathway resulting in a lower C-1 labelling than in the initial glucose-6-phosphate. Apparently, the flow of carbon through the OPPP is quick compared to the phosphoglucoisomerase reaction, the latter being responsible for the equilibration of glucose-6-phosphate and fructose-6-phosphate.

Furthermore, if fructose is phosphorylated in the structural component "compartment" there is a close link primarily between the pool of hexose phosphates in the cytosol and the enzymes of the OPPP, localized in the same compartment; as a result fructose is a better

substrate for cycling through the OPPP than glucose, thus, [1-^{13}C]-fructose gives lower labelling than [1-^{13}C]-glucose.

The same hexose-phosphate pool as used for synthesis of sucrose is assumed to be used by the plastids to synthesize starch. In general, it is assumed that glucose-6-phosphate is transported from the cytosol into the plastids (Kang and Rawthorne 1996). Inside the plastids at least half of the hexose phosphates flows through the OPPP before being incorporated into starch resulting in a much lower labelling percentage of starch as compared to sucrose (especially at the C-1 carbons). It is concluded that conversion from hexose phosphate into ADPG took place in the plastid as was found for all dicots studied, but not for cereals (Villand and Kleczkowski 1993; Thorbjørnsen *et al.* 1998).

Thus, *Daucus carota* cells show separate OPPP activities in the plastids and the cytosol. Separated OPPP activities were also found in *Catharanthus roseus* cells by Ishida and Ashihara (1993): they found two isozymes of 6-phosphogluconate dehydrogenase, one in the cytosol and one in the plastid. Cytosolic 6-phosphogluconate dehydrogenase-deficient genotypes from *Zea mays* showed inhibited plastidic nitrite reduction, suggesting that cytosolic NADPH can be transported into the plastids in this tissue (Averill *et al.* 1998). However, Kang and Rawthorne (1996) showed that externally supplied NADPH does not affect lipid synthesis in isolated *Brassica napus* plastids. Thus, cytosolic OPPP might cooperate in plastidic biosynthetic reactions by supplying NADPH, depending on the kind of tissue and species.

The flux through the OPPP might be determined by regulation of the activity of the key-enzymes by the NADPH/NADP ratio (Fahrendorf *et al.* 1995) or by the affinity of the enzyme glucose-6-phosphate dehydrogenase towards NADPH which might inhibit its activity (Bredemeyer and Esselink 1995). Glucose-6-phosphate was found to achieve levels of at least 5 μmol.(g dry weight)$^{-1}$ in carrot cells, which might be at least 4 mM assuming a dry matter percentage of 7.5% and a cytosolic volume of 10%. Hexose phosphate levels therefore should not be limiting for starch synthesis and plastidic OPPP activity, since a level of 4 mM hexose phosphates is high enough to saturate the hexose phosphate carriers in the plastid membranes (Thom *et al.* 1998) and the key enzymes of the OPPP (Borchert *et al.* 1993; Bredemeyer and Esselink 1995).

In Chapter 4 it was shown that the 4-fold increased respiration of logarithmically growing cells in an airlift, sincerely affected sucrose synthesis and/or hydrolysis: 40% of the added labelled glucose was used by glycolysis in short-term labelling experiments of exponentially growing cells and no labelled sucrose was measured. However, about the same amount of labelled glucose flows through the OPPP at that time. In stationary phase cells, although the respiration rate increased only 1.5-fold in airlifted cells, still 30% of the labelled glucose was used by glycolysis while only 10% entered the OPPP. These results were taken to indicate that the increased respiration in logarithmically growing cells did not reduce OPPP activity to a large extent, suggesting that the levels of the key-enzymes of the OPPP regulated its activity rather than availability of its substrates or the ratio of NADP/NADPH as was also described for *Solanum tuberosum* callus by Hemrika-Wagner (1985) and in *Catharanthus roseus* cells (Ishida and Ashihara 1993).

From the much lower labelling percentage of starch as compared to sucrose in batch grown cells, it was concluded that the OPPP-cycles in the cytosol and plastids function independently, at least in logarithmic phase cells. Since in airlift experiments nearly no ^{13}C-labelled starch was found, the question arose whether all [1-^{13}C]-hexose phosphates that were transferred into the plastids cycled through the OPPP, losing their label, or that no hexose phosphates were transported into the plastids at all in this type of experiments. Therefore, it was unclear if the OPPP activity may be confined to the cytosol during short-term labelling experiments or was also active in the plastids.

In isolated *Brassica oleracea* bud plastids Neuhaus *et al.* (1995) showed that carbohydrates appearing from starch degradation do not enter the OPPP, suggesting that plastidic OPPP activity might be confined to periods of starch synthesis in the logarithmic growth. In this view, the OPPP might be down-regulated if the availability of nutrients like phosphate, sugar and nitrate is low. During the logarithmic growth phase of batch cultures nutrients are present in excess, while phosphate and sugar are known to be exhausted in the stationary growth phase. Thus, OPPP activity might be regulated by coarse control rather than by metabolic control of its enzymes.

During a batch culture of 14 days the profile of starch (labelling) fluctuated less than that of sucrose. Stitt and Heldt (1981) and Stitt and Steup (1985) suggested cycling of starch, i.e.

a simultaneous synthesis and degradation of starch in *Spinacia oleracea* leaves. However, Hargreaves and ap Rees (1988) concluded for *Pisum sativum* roots that turnover of starch was much lower than turnover of sucrose. This might be connected with the difference in localisation of storage starch and storage sucrose: (insoluble) granules in the plastids and random distribution in the cytosol or vacuole, respectively. Keeling *et al.* (1988) showed that starch degradation was confined to the surface of the starch granules of developing *Triticum aestivum* grains. The latter was concluded from experiments in which starch granules were isolated after labelling with [1-^{13}C]-glucose for 4 h and were treated with amyloglucosidase. Only the outer layers yielded labelled glucose units, while the inner layers were still unlabelled. Apparently, starch cycling might occur but was limited to the easily accessible outer-layers of the granules. Cycling of starch might be relatively low in "storage" starch in typical storage tissues like plastids of logarithmic growth phase cells of *Daucus carota* suspensions, compared to "transient" starch formed in chloroplasts of photosynthetic tissues during day time, since storage starch shows a higher density, more α-(1,6) branches and larger granules than transitional starch (French 1975; Stitt and Steup 1985).

Triose phosphate cycling and PFP

Chapter 5 considers a metabolic reaction which is also present in many bacteria and fungi but not in warm-blooded animals: the reversible reaction from fructose-6-phosphate to fructose-1,6-bisphosphate. It was suggested before in *Chenopodium rubrum* cells that PP_i-dependent fructose-6-phosphate phosphotransferase (PFP) was responsible for the observed label exchange (Hatzfeld and Stitt 1990) and it was demonstrated with *Solanum tuberosum* tubers that antisense-PFP plants showed less label exchange (Hajirezaei *et al.* 1994). In a cell line of *Daucus carota* showing a high label exchange from C-1 to C-6 carbons in sucrose and hexoses (A10) also more PFP was present compared to a line with less label exchange (A+). Label exchange from C-1 to C-6 carbons was found to be about 14-24% in cells of the A10-line in the airlift-system after 8 h labelling. This was consistent with the results reported by other authors applying short-term labelling to a variety of plant tissues (Keeling *et al.* 1988; Hatzfeld and Stitt 1990; Viola *et al.* 1991). Oxygen concentrations of 100% led to a higher

percentage of label exchange during the short-term labelling of cells in an airlift-system than 6% O_2. It was concluded that at higher O_2 concentrations the glycolytic reaction from fructose-6-phosphate to fructose-1,6-bisphosphate and (triose phosphates) was stimulated in the airlift. The K_m values of PFP for fructose-1,6-bisphosphate and aldolase are similar (Stitt 1989; Moorhead and Plaxton 1990), resulting in competition of the gluconeogenetic and glycolytic reactions for fructose-1,6-bisphosphate. As a result of the higher level of fructose-1,6-bisphosphate the gluconeogenetic reaction, leading to a higher percentage of C-6 labelled hexose (phosphates) and sucrose increased. At the same time, the carbon flux through glycolysis increased to fulfil the need for respiratory substrates, as was concluded from the 2-fold higher respiration in the presence of 100% O_2 compared to 6% O_2.

Label exchange during long-term labelling of batch cultured cells was found to achieve values of 45% in *Daucus carota* and 50% in *Solanum tuberosum* cell suspensions (Kosegarten *et al.* 1995). Respiration rate was lower in batch culture than in airlift-experiments. Therefore, next to the respiration rate and the level of PFP, a third parameter apparently influences the observed label exchange from C-1 to C-6 carbons. Figure 3 from Chapter 2 showed, that batch-cultured cells grown on [1-^{13}C]-glucose and unlabelled fructose gave these high values of label exchange especially after day 8, when medium glucose was already completely taken up by the cells. Continued label exchange within the cellular pools of intermediates by the various metabolic cycles, will lead to a further increase of the C-6 labelling percentage finally resulting in equal labelling of C-1 and C-6 carbons. It is therefore concluded that the percentage label exchange is determined by the level of PFP, the respiration rate and the incubation period.

In Chapter 5 we already concluded that the extractable amount of PFP will be fully activated by fructose-2,6-bisphosphate. Dancer and ap Rees (1989) suggested, that PFP is extremely powerful in maintaining the desired concentration of cytosolic PP_i, due to its high activity. However, Hajirezaei *et al.* (1994) showed that antisense-PFP plants had no visible phenotype and had identical PP_i levels but lower levels of starch and glycolytic intermediates downstream of triose-phosphates. Also, label-exchange from C-1 to C-6 carbons in sucrose and hexoses was much lower. They concluded, that PFP is probably catalyzing a gluconeogenetic reaction and glycolytic reaction at the same time, but is not exclusively

responsible for maintaining the desired level of PP_i.

PP_i may also evolve from other reactions than gluconeogenetic PFP activity. By activation of building blocks for the biosynthesis of macromolecules, e.g. UDPG for sucrose and cell walls and ADPG for starch, PP_i is produced in the reaction of hexose phosphates with ATP or UTP. In animal physiology these reactions are known to be irreversible due to pyrophosphatase activity, resulting in low or undetectable cytosolic PP_i-levels. PP_i, therefore, might be seen as a by-product of the activation of precursors for biosynthetic processes. However, in *Spinacia oleracea* leaves PP_i levels of about 0.3 mM were found in the cytosol (Gross and ap Rees 1986); therefore the reaction of glucose-1-phosphate and UTP into UDPG and PP_i is thought to take place in both directions (in different growth stages). Plastids contain pyrophosphatases; as a result PP_i is absent and the reaction from glucose-1-phosphate and ATP into ADPG and PP_i is unidirectional, resulting in rapid synthesis of starch (Weiner *et al.* 1987). Cytosolic pyrophosphatases are also shown in *Solanum tuberosum* leaves (Rojas-Beltrán *et al.* 1999) and tubers (*Niek J.G. Appeldoorn, personal communication*) although their function is still unknown. Transformed plants of *Nicotiana tabacum* and *Solanum tuberosum* containing a bacterial pyrophosphatase expressed in the cytosol, showed much lower levels of PP_i accompanied by much lower growth rates and metabolite levels (Jelitto *et al.* 1992), indicating that PP_i is an essential and integral part of plant metabolism (Sung *et al.* 1988; Stitt 1998). In conclusion, although the gluconeogenetic flux of metabolites mediated by PFP is considerable, it is not exclusively responsible for determining the level of PP_i in plants.

PP_i might be used in alternative reactions in primary metabolism next to the ATP-dependent counter-enzyme at different places in the cell, e.g. PP_i- and ATP-dependent fructose-6-phosphate phosphotransferase in the cytosol and H^+-PPase and H^+-ATPase at the tonoplast (Stitt 1998). It is supposed by different authors, that although the PP_i-enzymes often catalyse reversible near-equilibrium reactions and may play a role in restoring the equilibrium between the various pools of intermediates, these enzymes may play a role in PP_i consumption during periods of phosphate limitation. Indeed, ATP levels were found to decrease about 4-fold at low levels of P_i (Hoefnagel *et al.* 1994), suggesting that PP_i-dependent enzymes might play a role during such circumstances. Some PP_i-dependent

enzymes are reported to be induced by low levels of P_i, such as PFP in *Brassica nigra* seedlings (Theodorou and Plaxton 1994). Furthermore, PP_i is a by-product of activation of building blocks produced for anabolic processes, i.e. the reaction of ATP or UTP with glucose-1-phosphate resulting in ADPG for starch and UDPG for sucrose and cell walls (Huppe and Turpin 1994). PP_i then might just be a waste product which, in plants, developed to an extra energy buffer next to ATP. PP_i might built up during periods of sufficient nutrients as was found during the logarithmic growth of *Catharanthus roseus* cells (Kubota and Ashihara 1993); utilization might take place during (short) periods of anoxia or phosphate limitation to maintain (high) metabolic activity.

Chapter 7
Summarizing conclusions

In the work described in this thesis, uptake and conversion of sugar by cells of batch-grown suspensions of *Daucus carota* L. were studied. Invasive techniques (measurements of enzyme activities and sugar and starch levels) and non-invasive techniques (^{13}C-NMR) were used to follow the pathway of sugar molecules and of certain atoms within these molecules to analyze "futile" cycles between hexoses and sucrose, between hexoses and pentoses using the oxidative pentose phosphate pathway (OPPP) and between triose and hexose phosphates.
The activities of the various metabolic cycles were analyzed in logarithmic phase cells in relation to sugar uptake and storage of carbohydrates and in stationary phase cells in relation to consumption of stored carbohydrates. Plant cell metabolism appeared to excel both in metabolic cycling and in substrate conversions by parallel enzymes catalyzing similar reactions like PP_i- and ATP-dependent fructose-6-phosphate phosphotransferase (PFP/PFK) and invertase/sucrose synthase (SUSY).
Figure 1 summarizes the events in the lag and logarithmic growth phase including their cellular compartmentation. Sugar metabolism starts with uptake of hexoses, since high levels of cell wall-bound invertase hydrolyse all the external sucrose. It was concluded that at the start of the logarithmic growth phase sugars are present in excess resulting in a fully active glycolytic, respiratory and oxidative pentose phosphate pathway. More ATP and building blocks than necessary for maintenance of the cells give rise to synthesis of new enzymes and precursors necessary for growth. After a few days cells start dividing and enter the logarithmic growth phase. Production of biomass and synthesis of sucrose and starch are coupled processes.
Fructose-6-phosphate is synthesized in the cytosol by soluble fructokinase activity, and acts as a substrate for the invertase-mediated sucrose cycle and the triose-hexose phosphate cycle in the cytosol. Via glucose-6-phosphate, a substantial part of the fructose is substrate for synthesis of UDP-glucose necessary for sucrose and cellulose synthesis in the cytosol and apoplast, respectively. Furthermore, glucose-6-phosphate is used in the OPPP-cycle in the cytosol and in the plastids, and for starch synthesis in the plastids. Glucose will be phosphorylated by the mitochondrial-associated hexokinases and supply substrates to the respiratory pathway. Exchange between glucose-6-phosphate in the respiratory "compartment" and structural component "compartment" also occurs, although limited.

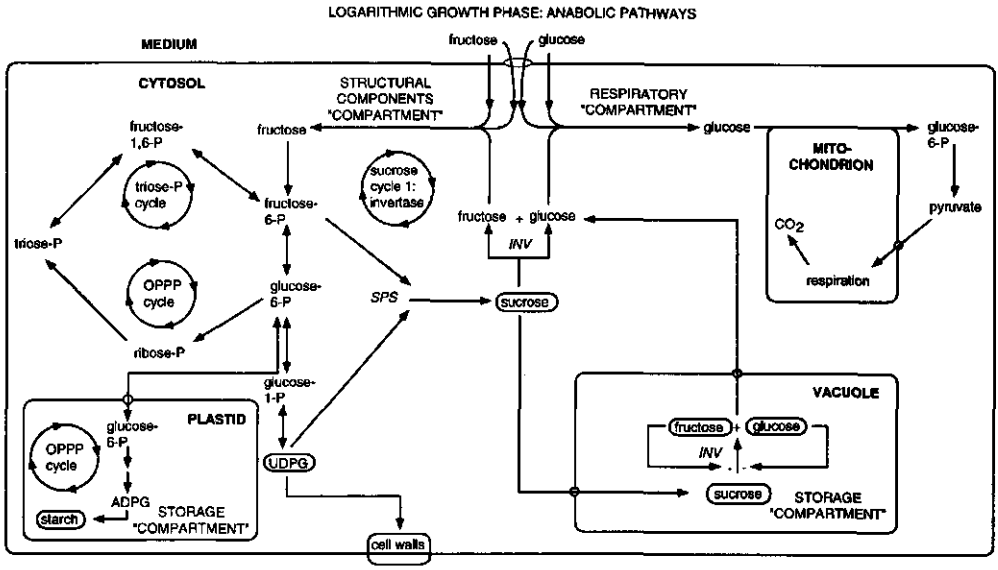


Figure 1 Uptake and conversion of glucose and fructose in logarithmically growing cells. Cytosolic and plastidic OPPP cycles are present and cycling between triose phosphates and hexose-phosphates in the cytosol takes place. Invertase-mediated cycling between hexoses and sucrose takes place only in the cytosol. Sucrose is stored in the vacuole and starch in the plastids. Only major metabolites are shown. *PFP* = PP_i-dependent fructose-6-phosphate phosphotransferase, *INV* = invertase, *SPS* = sucrose phosphate synthase.

Due to the high hexose phosphorylating activity, the levels of hexoses are relatively low and the levels of hexose phosphates and UDPG are relatively high in the cytosol; as a result sucrose is synthesized and the sucrose cycle mediated by invertase is active, while the sucrose cycle mediated by SUSY is inactive. Excess sucrose will be taken up into the vacuole, where it is protected from hydrolysis as long as the levels of hexoses are high enough to inhibit acid invertase activity. Cycling of carbon between triose and hexose

phosphates was observed by the occurrence of [6-^{13}C]-labelled sucrose and hexoses after feeding [1-^{13}C]-glucose or fructose (Fig. 1).

In the logarithmic growth phase high activity of the OPPP was observed, even when respiration was increased 4-fold by dilution of the cells. Therefore, it is concluded that OPPP activity was not subject to extensive metabolic regulation. It is suggested, that OPPP activity takes place in the cytosol during the whole culture period; the OPPP in the plastids probably was only active in the logarithmic growth phase (Fig. 1).

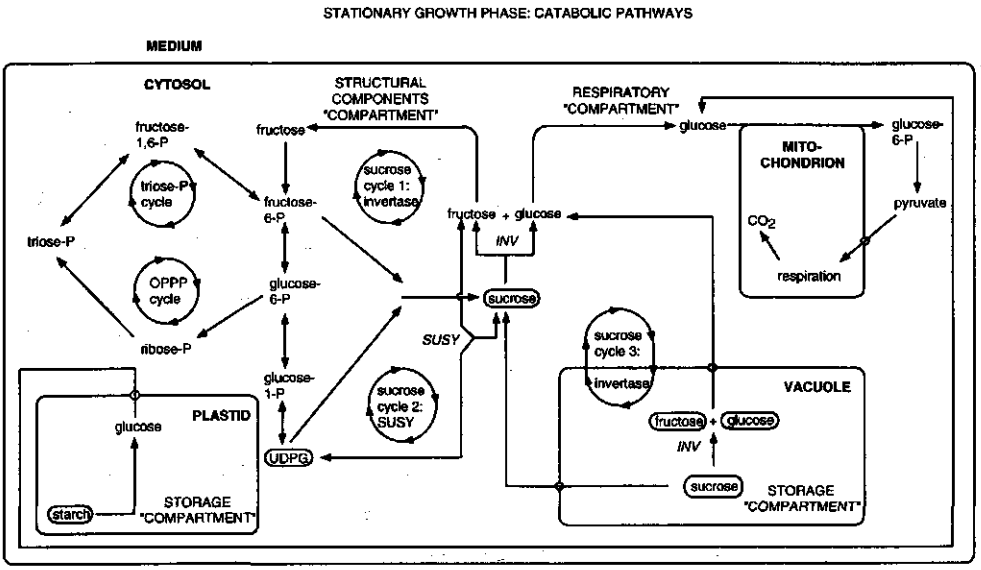


Figure 2 Mobilization of stored sucrose and starch in stationary phase cells. Cycling of OPPP and cycling between triose phosphates and hexose phosphate in the cytosol and cycling of sucrose in both the cytosol and the vacuole take place. The plastidic OPPP cycle probably is not active. Accumulated metabolites are boxed. Only major metabolites are shown. *PFP* = PP_i-dependent fructose-6-phosphate phosphotransferase, *INV* = invertase, *SUSY* = sucrose synthase, *SPS* = sucrose phosphate synthase.

As soon as the external supply of carbon is exhausted, cytosolic carbohydrates and, via respiratory control, the resulting ATP decrease. Reverse reactions with respect to stored sucrose and starch occur. In the stationary growth phase, hexose units released from stored sucrose and starch are used in glycolysis to fulfil the demand of substrates for cell maintenance. Both the OPPP activity and the growth rate decline in this period because of the lack of carbon for the production of new cells (Fig. 2).

If external hexoses are supplied to these cells, low OPPP cycling and high sucrose cycling are observed (Fig. 2), indicating that the OPPP activity is down-regulated by coarse control of its enzymes rather than by availability of sugar. Furthermore, (cytosolic) triose-hexose phosphate cycling was observed, which was related to the level of PP_i-dependent fructose-6-phosphate phosphotransferase (PFP) which catalyzes the gluconeogenetic reaction from fructose-1,6-bisphosphate into fructose-6-phosphate. Hydrolysis of sucrose by invertase and SUSY in the cytosol and by acid invertase in the vacuole takes place in the stationary growth phase and result in activity of the "cytosolic" and the "vacuolar" sucrose cycles.

The above described phenomena imply that plants have multiple ways to control carbon metabolism and carbon partitioning in cells and tissues. "Environmental" conditions in batch cultured cells with respect to humidity and temperature will be rather constant, and changes in availability of nutrients and oxygen will only occur gradually. The meaning of the described high activities of metabolic cycles might thus be redundant under the controlled conditions in the laboratory. However, in field-grown plants these properties might be essential to survive adverse environmental conditions.

Chapter 8
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Hoofdstuk 9
Samenvatting

In dit proefschrift is de dynamiek van de suikerhuishouding van planten beschreven. De opname en verwerking van de meest-voorkomende suikers, de enkelvoudige hexoses glucose en fructose en het dubbelsuiker sacharose, zijn onderzocht en hun weg door de verschillende stofwisselingsroutes is gevolgd. Opname van suikers is bepaald met behulp van ^{14}C-gemerkte suikers en kolom-chromatografie. De verwerking van suikers is gemeten aan de capaciteit van sleutelenzymen van de verschillende routes. Met behulp van niet-destructieve NMR- ('kernspinresonantie') metingen kon de weg van ^{13}C-gemerkte suikers gevolgd worden waardoor opeenvolgende stappen in de stofwisselingsroutes bepaald konden worden. Een groot voordeel van NMR is dat de techniek toepasbaar is op levend, intact materiaal zonder dat dit geëxtraheerd hoeft te worden, zodat het lot van de gemerkte suiker aan één monster in de tijd vervolgd kan worden.

De activiteiten van de verschillende stofwisselingsroutes zoals de glycolyse, de gluconeogenese, de oxidatieve pentose-fosfaat-route en de ademhaling zijn aan celsuspensies gemeten tijdens de logaritmische groeifase, waarin snelle groei plaatsvindt, en tijdens de stationaire fase, waarin geen groei meer optreedt. Met deze benadering kon de relatie tussen de suikeropname, de groei en de activiteit van de verschillende stofwisselings-routes worden vastgesteld.

In dit onderzoek zijn celsuspensies als proefsysteem gebruikt, omdat hierbij de samenstelling van het voedingsmedium en de milieucondities goed controleerbaar zijn; bovendien zijn celsuspensies ook min of meer homogene 'weefsels'. Celsuspensies van de peen (*Daucus carota* L.) werden gekweekt in voedingsmedium met glucose, fructose of sacharose als koolstofbron. De sacharose in het voedingsmedium werd door celwand-invertase binnen één dag gesplitst in glucose en fructose. Glucose werd sneller opgenomen en verwerkt dan fructose; dit kon gedeeltelijk worden verklaard doordat de transport-eiwitten in de celmembraan een hogere affiniteit voor glucose hebben. Verder bleek dat er twee 'pools' van hexose-fosforylerende enzymen zijn. Deze verzorgen de omzetting van glucose en fructose in glucose-6-fosfaat respectievelijk fructose-6-fosfaat. In het cytoplasma van peen-cellen komen hexose-fosforylerende enzymen voor die voornamelijk fructose omzetten; de gevormde intermediairen bevinden zich vrij in het

cytosol en worden in de eerste plaats gebruikt om sacharose en bouwstenen voor celwanden te vormen. De tweede 'pool' van hexose-fosforylerende enzymen is geassocieerd met membranen en bezit 2-20 keer zoveel fosforylerende activiteit; uit de literatuur blijkt dat deze enzymen waarschijnlijk met mitochondriën geassocieerd zijn. Ze hebben een 10 keer hogere affiniteit voor glucose dan voor fructose; hieruit is geconcludeerd, dat glucose bij voorkeur wordt gefosforyleerd door deze 'mitochondriële' fractie. Dit leidt tot een aparte 'pool' van metabolieten binnen de cel, die makkelijk in de mitochondriën worden opgenomen. Deze resultaten leidden tot de hypothese dat er 2 'pools' van glycolytische intermediairen zijn: één afgeleid van fructose(-fosfaat) resulterend in biomassa en één afgeleid van glucose(-fosfaat) resulterend in ademhaling en energie-productie.

Eenmaal gefosforyleerd vindt er ook uitwisseling plaats tussen hexose-fosfaten uit beide 'pools'. Bovendien kunnen glucose-6-fosfaat en fructose-6-fosfaat binnen één 'pool' in elkaar worden omgezet door het enzym fosfoglucoïsomerase. Hierna zijn glucose-6-fosfaat en fructose-6-fosfaat equivalent en leggen dezelfde biochemische routes af.

Tijdens de fase waarin logaritmische groei optreedt, worden hexoses uit het voedingsmedium opgenomen, gefosforyleerd en omgezet in nieuw celmateriaal en reservestoffen: sacharose wordt gemaakt in het cytosol en opgeslagen in de vacuole en zetmeel wordt gemaakt en opgeslagen in zetmeelkorrels in de plastiden. Een deel van de koolstof wordt verademd in de mitochondriën. Wanneer de suiker in het medium uitgeput is en geen groei meer optreedt, worden opgeslagen zetmeel en sacharose gehydrolyseerd en gebruikt voor de celademhaling.

Tijdens beide groeifases worden hexose(-fosfaten) veelvuldig onderworpen aan snelle, kortdurende cycli: koolstof-intermediairen worden omgezet van A in B, waarna B weer via een andere route wordt omgezet in A. Dergelijke omzettingen worden 'futiele cycli' genoemd: ze lijken op het eerste gezicht geen functie te hebben, maar algemeen wordt aangenomen dat ze planten(cellen) flexibel maken: allerlei stofwisselings-routes zijn reeds actief ook als de producten nog niet nodig zijn. Indien de behoefte aan een product plotseling toeneemt als gevolg van zich wijzigende omstandigheden kan de cel hier snel op inspelen.

^{13}C-gemerkte suikers maken het mogelijk om de verschillende reactie-producten van zo'n cyclus zichtbaar te maken en van elkaar te onderscheiden. De verdeling van de '^{13}C-label' over de verschillende suikers, en over verschillende atomen van die suikers leert iets over de activiteit van de verschillende cycli, en hun plaats in de cel. De NMR-techniek is dan ook bij uitstek geschikt om dergelijke futiele koolstof-cycli te meten. Zo zijn er cycli tussen hexoses en sacharose en tussen hexose-fosfaten en triose-fosfaten. De oxidatieve pentose-fosfaat-route gebruikt hexose-fosfaten, en zet deze via pentose-fosfaten weer om in triose-fosfaten en hexose-fosfaten. De activiteit van deze cycli is afhanklijk van de ontwikkelingsfase van de plantencellen.

In de logaritmische groeifase werden een sacharose-cyclus, de oxidatieve pentose-fosfaat-route en de hexose-triose-fosfaat-cyclus waargenomen in het cytosol. Daarnaast was de oxidatieve pentose-fosfaat-route ook actief in de plastiden, zoals werd geconcludeerd uit de ^{13}C-verdeling in zetmeel die duidelijk afweek van die in sacharose.

Tijdens de stationaire fase was er niet alleen een stroom van koolstof-intermediairen in de richting van suikerafbraak en ademhaling: toevoeging van een kleine hoeveelheid ^{13}C-glucose resulteerde ook dan in activiteit van een sacharose-cyclus en de hexose-triose-fosfaat-cyclus in het cytosol. Twee extra sacharose-hydrolyserende enzymen, één in het cytosol en één in de vacuole werden actief. Zetmeel-synthese en activiteit van de oxidatieve pentose-fosfaat-route in de plastiden werd niet meer waargenomen in de stationaire fase.

Voor één van de futiele cycli, de hexose-triose-fosfaat-cyclus, kon worden aangetoond dat de activiteit afhankelijk is van de hoeveelheid van één van de sleutelenzymen, PFP, die genetisch bepaald is. Verder wordt de activiteit van de triose-hexose-fosfaat-cyclys bepaald -via de ademhalingssnelheid- door de beschikbaarheid van koolstof-intermediairen. Voor de oxidatieve pentose-fosfaat-route kon worden aangetoond dat ze relatief ongevoelig is voor de ademhalingssnelheid en waarschijnlijk alleen gereguleerd wordt door de hoeveelheden van de enzymen die betrokken zijn bij deze cyclus.

Uit deze resultaten is geconcludeerd, dat planten(cellen) veel stofwisselings-processen via parallelle enzym-systemen aan- en uit- kunnen schakelen. Een aantal futiele cycli is voortdurend actief, terwijl andere afhankelijk zijn van de groeifase. Zowel de parallelle

enzymsystemen als de futiele cycli maken permanent onderdeel uit van de basale stofwisseling in planten; dit is waarschijnlijk essentieel voor een snelle en soepele regulatie van de koolstof-stromen onder sterk wisselende milieucondities, zoals die onder natuurlijke omstandigheden veelvuldig voorkomen.