METABOLISM AND INTERCONVERSION
OF LABELLED SORBITOL
AND RELATED CARBON SOURCES
BY APPLE TISSUE CULTURES

by

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A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of the requirements
for the degree of Master ès Science

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May 1986

ABSTRACT

METABOLISM AND INTERCONVERSION OF LABELLED SORBITOL AND RELATED CARBON SOURCES BY APPLE TISSUE CULTURES

Radioactive fructose, glucose, sorbitol (D-glucitol), and sucrose were supplied to explants of three apple cultures, Ottawa-3 apple rootstock (O-3), scion cultivar of Macspur apple (MA), and seedlings of Macspur (SM), at both the shoot multiplication (Stage II) and rooting (Stage III) stages of *in vitro* micropropagation to study the uptake and interconversion of the carbon source fraction by these cultures. Using a labelling incubation period of 2 hours, liquid scintillation indicated no significant difference in the uptake of sorbitol compared to the uptake of fructose, glucose or sucrose. Sorbitol, found at an average proportion of 93.1% over all genotypes and stage of culture, was interconverted to the lowest extent, followed in order by glucose (80.5%), fructose (63.0%) and sucrose (62.4%).

RESUME

METABOLISME ET INTERCONVERSION DU SORBITOL ET D'AUTRES SOURCES DE CARBONE, CHEZ LE POMMIER CULTIVE IN VITRO

Dans le but d'étudier l'absorption et l'interconvertion de différentes sources de carbone, du fructose, glucose, sorbitol (D-glucitol) et saccharose ont été fournis à des explants de pommiers de trois différents génotypes: un porte-greffe Ottawa-3 (O-3), un greffon de type Macspur (MA) et des plantules issues de graines de Macspur (SM). L'étude a porté au stade de multiplication et au stade de racinement des explants cultivés *in vitro*. Après une periode d'incubation de deux heures sur les milieux contenant une source de carbone radioactive, le comptage par scintillation liquide n'a pas révèlé de différences significatives entre l'absorption du sorbitol, du fructose, du glucose, ou du saccharose, par les explants. Le sorbitol, représentant en moyenne une proportion de 93.1% pour tous les génotypes et stades de propagation, a été interconverti par les explants dans la plus faible mesure, suivi dans un ordre décroissant par le glucose (80.5%), le fructose (63.0%) et le saccharose (62.4%).

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Dr. C. Chong for suggesting the subject of investigation and for his continued enthusiasm, criticisms, patience, and for his assistance in the elaboration of the manuscript.

My sincere thanks to Drs. N. N. Barthakur and I. Alli, of the Agricultural Chemistry and Physic department, for providing analytical equipment and for their assistance and advice during this project.

Appreciation is extended to Dr. L. M. Sanford of the Animal Science Department, for making available the liquid scintillation counter.

Finally, the Natural Sciences and Engineering Research Council (NSERC) of Canada is acknowledged for financial support of this research project.

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A6PR	aldose-6-phosphate reductase
ATP	adenosine triphosphate
BA	benzyladenine
DNA	deoxyribonucleic acid
DPM	decay per minute
F-6-P	fructose-6-phosphate
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
GA	gibberellic acid
IBA	indolebutyric acid
MA	apple scion cultivar Macspur
MCWF	methanol/chloroform/water/formic acid
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NAD-SDH	NAD-dependent sorbitol dehydrogenase
NADP-SDH	NADP-dependent sorbitol dehydrogenase
$Na_2HPO_4-7H_2O$	sodium phosphate
NO_3^-	nitrate
O-3	apple rootstock Ottawa-3
RNA	ribonucleic acid
SM	seedling of Macspur apple
So-1-P	sorbitol-1-phosphate

So-6-P	sorbitol-6-phosphate
UDP-G	uridine diphosphate-glucose
UTP	uridine triphosphate

CHAPTER I

INTRODUCTION

Sorbitol (D-glucitol) is an important carbohydrate in plants of the Rosaceae, even though it is generally uncommon in other higher plants (Plouvier 1955, Wallaart 1980). In many species of the Rosaceae, sorbitol is the major end product of photosynthesis (Anderson *et al*. 1961, Bieleski 1969, Bieleski and Redgwell 1977, Chong and Taper 1972, Grant and ap Rees 1981, Priestley 1983), and major translocated carbohydrate (Webb and Burley 1962). Sorbitol also serves as a reserve carbohydrate (Whetter and Taper 1963, Grant and ap Rees 1981). In apples, its metabolism has been considered to be a key factor in the mechanism of photosynthate partitioning, *i.e.* sink and source activity (Loescher *et al*. 1982).

Because of the fundamental importance of sorbitol in the physiology and metabolism of rosaceous species, research at Macdonald College over the past 20 years have focused on the role of sorbitol in apples. More recently, this research has been extended to studying the influence of sorbitol in micropropagated apple cultures.

Sucrose has been the standard carbon source used in the nutrient medium for *in vitro* propagation of higher plants, including numerous apple rootstocks and scion cultivars (Jones *et al*. 1979, Lane and McDougald 1982, Zimmerman 1983). However, studies have demonstrated that sorbitol is beneficial as an adjunct or alternate carbon source for *in vitro* culture of apple callus (Chong and Taper 1972, Coffin *et al*. 1976), and shoot tip cultures (Pua and Chong 1985). Pua and Chong (1984) found that during stages of *in vitro* propagation of *Malus robusta* No. 5 apple rootstock, sorbitol was required for initiation of meristem tip explants, and was the most effective carbon source for shoot multiplication. Chong and Pua (1985) and Pua and Chong (1984, 1985) observed morphological differences both within or between cultures of apple due to type and/or composition of the carbon source fraction of the nutrient medium.

The present study attempted to clarify the differential utilisation of sorbitol and related carbon sources by various apple shoot cultures. To this end radioactive carbon sources were fed to cultures during the multiplication and rooting stages (*i.e*. Stages II and III, respectively) of *in vitro* propagation; the relative uptake and interconversion of these labelled carbon sources were determined in the sugar fraction.

CHAPTER II

LITERATURE REVIEW

2.1 ROLE OF CARBOHYDRATES IN *IN VITRO* CULTURES

Carbon Source

Growth of *in vitro* cultures involves the mobilization of many organic compounds of which the carbohydrates are among the most important (Gautheret 1959). As respiratory substrates, carbohydrates were found to be exogenous sources of energy for *in vitro* explants (Gautheret 1959). They also were found to be important carbon backbones, being utilized in the formation of new protoplasmic constituents (Gautheret 1959, Street 1969). For *in vitro* explants, carbohydrates were found to be precursors of many compounds, ranging from organic and amino acids to structural and reserve polysaccharides, including lipids and other secondary products such as anthocyanosides (Gautheret 1959).

Haberlandt (1902) attempted to culture leaf cells *in vitro*. Since he considered these cells to be autotrophic, he did not supply them with an external source of organic carbon and the cultures gradually died. Through photosynthesis, explants *in vitro* can synthesize substances required for growth, but this process generally occurs at an insufficient degree (Gautheret 1959). Thus, cultures *in vitro* are only partially autotrophic and the addition of a carbon source in the nutrient medium is essential (Gautheret 1959).

Studies indicated that cultures of tobacco or sugar cane calli undergoing organogenesis exhibited higher respiration rates (Thorpe and Meier 1972, Thorpe 1978), and enhancement in glycolysis and in pentose phosphate and shikimate pathways (Thorpe and Laishley 1973, Rawal *et al*. 1985, Beaudoin-Eagan and Thorpe 1983, 1984, 1985). Brown and Thorpe (1980) observed a decrease in the NADPH level of tobacco calli during shoot formation. This evidence supported the idea that the shoot-forming process created a great demand for reducing power which could have been partly fulfilled by the enhanced pentose phosphate pathway (Thorpe and Laishly 1973). More recently, Beaudoin-Eagan and Thorpe (1984,1985) showed that enhancement of the shikimate pathway in tobacco calli was mainly associated with tyrosine metabolism and occurred only in the shoot-forming portion (the lower part) of the calli. This evidence supported the concept expressed by Thorpe (1978, 1980) that organized development occurred at specific loci of a plant tissue involving certain metabolic shifts, ultimately reflecting selective gene activity.

Similarly in sycamore cell cultures, fast-growing cells showed high activities in pentose phosphate and glycolysis pathways, whereas in slow-growing cells carbohydrate oxidation mainly occurred through glycolysis (King and Street 1977, Jessup and Fowler 1977). According to Jessup and Fowler (1977), the increase of the pentose phosphate pathway found in fast growing cells of sycamore could be related to the prodution of NADPH, which was apparently required for NO_3^- reduction and amino acid synthesis in these cells.

Osmoticum

During the process of cellular volume increase associated with cell expansion, the intracellular concentration of solutes must be kept more or less constant to maintain a critical turgor pressure required as a driving force (Cleland 1971). Since the major constituent in most tissue culture media is sugar (Gamborg and Shyluk 1981), the sugar fraction is believed to play an important osmotic role (Thorpe 1978). In tissue cultures of radiata pine (*Pinus radiata* D. Don) and alfalfa (*Medicago sativa* L.), a reduction in the media carbohydrate concentration from 3% to 2% or 1% promoted shoot development. Thorpe (1982a) suggested that the inhibition of the shoot development by the higher level of carbon source (3%) could, at least in part, be osmotic in nature. Similarly in apples, high concentrations of carbohydrates (7%) reduced shoot fresh weight and shoot number per explants of different genotypes (Pua and Chong 1985, Chong and Pua 1985). A reduction of root development by high carbohydrate concentrations (7%) was also observed (Chong and Pua 1985, Pua and Chong 1985). Based on these results, and on the fact that in cuttings of Pinus banksiana Lamb. an accumulation of carbohydrates occurred in the basal stems during rooting (Haissig 1984), Chong and Pua (1985) suggested that osmotic ajustment involving carbohydrate levels within the explants could influence the initiation of root primordia.

Another indication of the occurrence of some osmotic adjustment during organogenesis was the finding of a rapid depletion of malate in organ-forming tobacco calli (Plumb-Dhindsa et al. 1979). Amino acids, such as proline, threonine and serine, also have shown different pattern of accumulation during organogenesis (Thorpe 1983). According to Thorpe (1983) :

> " ... it appears that the morphogenic system uses a variety of metabolites colligatively, and furthermore, different metabolites contribute osmotically at different stages of the process."

Osmotic adjustments are among the earliest events correlated with organogenesis in tobacco calli (Brown and Thorpe 1980a). Although their significance on growth and differentiation needs clarification, Thorpe (1982a) indicated that biochemical as well as biophysical events (e.g. membrane properties) could be modified or triggered by osmotic adjustments.

Type of carbohydrates

Sucrose generally is regarded as the most effective carbon source for in vitro cultures (Gautheret 1959, Street 1969), although glucose can be as effective (Gamborg and Skyluk 1981).

These carbohydrates allow optimal growth of most explants. Attempts to use other carbon sources have resulted in little success (Gautheret 1959, Chong 1972, Maretzki et al. 1974, Fowler 1978), although research on the use of sugar alcohols in cultures of Fraxinus (Wolter and Skoog 1966) and of woody Rosaceae (Chong and Taper 1972, 1974, Coffin et al. 1976, Klenovska and Balkova 1983) has shown that these compounds could be effectively metabolized by some callus cultures. In fact, the growth of calli of cv. Cortland apple and of Malus robusta Rehd. No.5 rootstock on sorbitol exceeded those on sucrose (Chong and Taper 1974).

Other carbon sources which normally do not support sustained growth of tissue cultures have been used sucessfully (Maretzki et al. 1974). Cell cultures of sugarcane and Japanese morning-glory have shown adaptability to growth on galactose (Maretzki and Thom 1978) and lactose (Hisajima and Thorpe 1985), respectively. It appears that in metabolizing "unusual" carbon sources, a latent part of the cell genome is utilized by explants in vitro and a normally absent "enzymatic machinery" is expressed (Fowler 1978).

Up to a certain level, increases in carbohydrate concentration in the nutrient medium are accompanied by increases in cell wall material, starch, dry weight, and growth of the explants (Gautheret 1959, Street 1969, Pech et al. 1974). Production of secondary metabolites is also stimulated. According to Ibrahim (1983), phenols, flavonoids, and quinones showed enhanced production following an increase in carbohydrate content of the culture medium. In somatic embryos of Theobroma cacao L., Konowicz and Janick (1984) reported an increase in lipids, anthocyanins, and alkaloids following an increase in sucrose concentration. A similar response was not found when glucose was used, although superior growth of the somatic embryos was related to this carbon source. In callus cultures, Jeffs and Northcote (1967) showed that xylem differentiation was favored by low concentration of sucrose (1%-2%), whereas higher concentration (3%-4%) favored phloem induction. Similarly, Wright and Northcote (1972), using cultures of Acer pseudoplatanus L., observed induced differentiation of xylem, phloem, and roots using different growth regulators in combination with carbohydrates at concentrations ranging from 2% to 3%; roots were not formed at concentrations less than 2% of carbohydrate, although extensive xylem and phloem differentiation occurred.

Carbohydrate supplied to cultures grown in light also influenced photosynthesis of the explants. In Douglas fir (Pseudotsuga menziesii (Mirb.) Franco), high sucrose concentration (4.5%) generally inhibited maximum net photosynthesis (Evers 1982). In carrot (Daucus carota L.), a reduction in chlorophyll synthesis, chloroplast numbers and photosynthetic rate of cultures grown on sucrose also has been observed (Neumann and Raafat 1973, Edelman and Hanson 1972). More recently, Kumar et al. (1984), using carrot root explants, emphasized that chlorophyll synthesis was stimulated by sucrose (2%) in conjunction with inositol, indoleacetic acid (IAA), and kinetin. A hormonal requirement for this biochemical differentiation process might be indirectly related to cellular osmolarity, since plant hormones are known to affect starch accumulation and therefore indirectly affect osmotic pressure (Thorpe 1982a). Morphogenic differentiations in tobacco cell layer cultures also were related to variations in carbohydrates and ratio of growth regulators. Tran Thanh Van (1978) used glucose to induce flowering with an auxin/cytokinin ratio of 1. However, to induce differentiation into calli, shoots, or roots, she used sucrose and ratios of growth regulators different than 1, depending upon the morphological response desired.

Carbohydrates also inhibited enzymes not related to their synthesis. In *Asparagus officinalis* L. cv. Argenteuil cell cultures, glucose concentration of 111 mM decreased activities of glutamate dehydrogenase by 31% and acid phosphatase by 50% (Tassi *et al*. 1984). Galactose, lactose, and raffinose were found in decapitated *Avena* coleoptiles to be possible inhibitors of auxin synthesis (Anker 1974).

Recently, stimulation of ethylene production was demonstrated in tobacco leaf discs with 12 different carbohydrates including sorbitol (Meir *et al*. 1985), although ethylene synthesis was most active with galactose, sucrose, and lactose. The same phenomenon was observed in *Citrus* leaf discs using mannitol as a carbon source. In this case, mannitol, normally considered unmetabolizable, or very little so, was metabolized actively, and converted mainly to sucrose (Riov and Yang 1982).

It is becoming clearer that the polysaccharides forming the cell wall do not serve a purely structural role (Lamport 1978). Recently, Albersheim _et al_. (1983) postulated that these could be biological regulatory molecules. Compounds designated as "oligosaccharins" have been found to be especially important in regulating growth, development, reproduction, and disease resistance in plants. From a tissue culture standpoint, Albersheim _et al_. (1983) further indicated that the plasmolysis requirement for protoplast isolation might not, as generally considered, be required to protect the naked cells from possible damages caused by the cell wall degradative enzymes, but rather to prevent an effective attachment or transport of oligosaccharins capable otherwise of inducing the death of the protoplast. Whether or not oligosaccharins are effectively controlling the plant growth and development _in vivo_ remains to be elucidated (Albersheim _et al_. 1983).

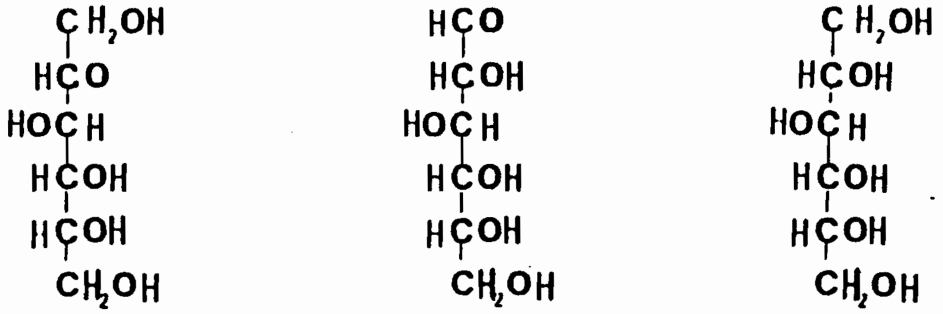
Finally, it can be generalized from the above evidence that sugars affect directly or indirectly developmental events. As postulated by Parr _et al_. (1976), the control of the entry as well as the compartmentation of these compounds within the cells could influence by interaction with hormones morphological and biochemical differentiations of tissues in culture (Tran Thanh Van 1978, 1981).

2.2 SORBITOL

2.2.1 Occurrence and Physiology

Sorbitol is rarely found in lower plants such as algae, fungi, or mosses. However, it occurs in one group of liverworts, the Marsupellaceae (Bourne 1958, Lewis and Smith 1967, Chong 1972, Bieleski 1982). Among other lower plants, brown algae were until recently, known to form mannitol, but not sorbitol (Bieleski 1982). However, using a more specific analytical technique, Quillet *et al*. (1985a) reported the presence of sorbitol in all brown algae so far studied. Even though occurring in lower concentration than mannitol in these lower species, sorbitol appeared to have a physiological significance as a precursor in alginic acid synthesis (Quillet *et al*. 1985b).

In higher plants, sorbitol is commonly found in the Rosaceae family (Lewis and Smith 1967). Found for the first time in 1872 in the fruits of *Sorbus aucuparia* L. by Boussingault (Plouvier 1955), this sugar alcohol was named according to its source of extraction. However, in systematic chemistry, sorbitol is named in terms of its aldose parent as D-glucitol (Bourne 1958) (Figure 1). In the Rosaceae, sorbitol was studied according to its taxonomic significance (Plouvier 1955, 1963, Lewis and Smith 1967, Wallaart 1980). In general,plants of the sub-families Maloideae, Prunoideae, and Spiraeoideae were found to contain sorbitol, whereas in the sub-family Rosoideae, its occurrence was found to be heterogeneous (Plouvier 1955, Wallaart 1980). According to Wallaart (1980), the accumulation of sorbi-



D-fructose D-glucose sorbitol

Figure 1: Fisher representation of the sorbitol (D-glucitol), D-glucose, and D-fructose molecules.

tol in the Rosaceae taxa is more than a character related to an ecological distribution, since a correlation was found between the presence of sorbitol and the chromosome number of the taxa. Members of the Rosoideae with a basic chromosome number of 7 were lacking sorbitol.

There have been reports of sorbitol occurrence in at least 17 plant families (Chong 1972), although the significance of this finding cannot be assessed because of a lack of systematic investigation in this area (Bieleski 1982). Of particular interest in the monocotyledons, is the fact that sorbitol has been reported in the milk of coconut (Cocos nucifera L.). (Pollard et al. 1961). Coconut milk (i.e. liquid endosperm) is often included in the composition of certain tissue culture media, and seems to be especially important in cell cultures grown at a low population density (Kao and Michayluk 1975).

Sorbitol was found to be the major product of photosynthesis in many temperate fruit trees of the Rosaceae, often representing up to 80% of the assimilate translocated from mature leaves (Anderson et al. 1961, DeVillier et al. 1974, Bieleski and Redgwell 1977, Steenkamp et al. 1981, Priestley 1980, Grant and ap Rees 1981, Webb and Burley 1962, Bieleski 1969, Yamaki and Ishikawa 1986). In the Genus Malus, sorbitol has been found in all parts of the plant (Fidler and North 1970, Hansen 1970, Chong 1971, Whetter and Taper 1966), except in seeds of fruits from cold storage (Taper et al. 1972). Nevertheless, germinating seeds and developing seedlings contained sorbitol (Whetter and

Taper 1966).

The content of sorbitol within apple plants varied depending upon factors such as genotype, the state of development or physiological status, and the type of tissue or organ (Berüter 1985, Bieleski 1977, Bieleski and Redgwell 1977, Fidler and North 1970, Ismail et al. 1980, Chong et al. 1972, Taper and Liu 1969, Whetter and Taper 1963, Chong and Taper 1974, Chong 1971, Chong and Taper 1972, Chan et al. 1972). As a general rule, tissues serving as sinks and rapidly utilizing their carbohydrates, had low levels of sorbitol (Loescher et al. 1982, Bieleski 1982, Berüter 1985, Yamaki and Ishikawa 1986). Mature fruits of apples contain less than 8% of their soluble carbohydrates as sorbitol, whereas "source" mature leaves often showed the highest sorbitol concentrations, representing more than 50% of their soluble carbohydrates (Loescher et al. 1982, Bieleski 1982, Berüter 1985, Yamaki and Ishkawa 1986).

The accumulation of sorbitol in apple leaves (Chong 1971), its slow rate of utilization (Grant and ap Rees 1981, Anderson et al. 1962, Hansen and Ryugo 1979), and its occurrence along with starch in many apple tissues (Chong 1971, Chong and Taper 1972, Chong et al. 1972) suggested that this sugar alcohol be considered as a reserve carbohydrate. Furthermore, during dormancy, sorbitol and sucrose were clearly found to be important reserves of storage carbohydrates (Williams and Raese 1974) which were mobilized during spring (Hansen and Grauslund 1978).

Since sorbitol is a compound more reduced than the common hexoses (Figure 1), Lewis and Smith (1967) have suggested that it could be a suitable source of reducing power. Polyhydroxyl compounds such as sugars and sugar alcohols are also known to have stabilizing properties on proteins and on isolated organelles (Shifrin and Parott 1975, Levitt 1972). Therefore, it is possible for sorbitol to play important protective and osmotic roles. In response to osmotic stress, the polyol content of *Plantago maritima* L. (Ahamad *et al*. 1979) was found to correlate with the stress level, suggesting an osmoregulatory role for the sugar alcohol. So far, a similar function involving sorbitol in rosaceous species have not been reported (Bieleski 1982).

In young fruits, two recent studies suggested that sucrose, rather than sorbitol, was the predominant carbon source for cellular growth, since the activity of acid invertase was found to be at a high level during the phase of fruit development characterized by cell division (Berüter 1985, Yamaki and Ishikawa 1986). However at the fruit maturation phase, sorbitol was the major substrate for fructose accumulation (Berüter 1985). In fruit tissue of apple (*Malus pumila* Mill. var. *domestica* Schneid.), more than 80% of the total soluble sugars were found in the vacuole, representing an osmotic pressure of 19.9 atmospheres (2 bars) (Yamaki 1984). This osmotic pressure seemed to play an imporant role at the cell expansion stage of fruit development.

Sorbitol also has been suggested to act as a "cryoprotector" in many temperate fruit trees (Sakai 1966, Ichiki and Yamaya 1982). Chen et al. (1984a) proposed that sorbitol protected cells from freezing injury by lowering the cellular water content. In the sap of apple shoots, high levels of sorbitol were related to cold hardiness (Raese et al. 1978, Ichiki and Yamaya 1982). A sharp increase of sorbitol in the tracheal sap was found to be associated with the onset of low minimum temperatures in autumn (Williams and Raese 1974, Ichiki an Yamaya 1982). During cryopreservation, sorbitol was incorporated into the "conditioning" media of Periwinkle (*Catharanthus roseus* (L.) G. Don.) cell cultures prior to freezing treatment (Chen et al. 1984a, 1984b), and seemed to be an effective "natural" cryoprotector.

Sorbitol occurence in apple fruits has been associated with watercore, internal breakdown and other storage disorders (Chan et al. 1972, Faust et al. 1969). However, Marlow and Loescher (1985) reported that no relationship existed between susceptibility to watercore and the activity of sorbitol dehydrogenase in three different apple cultivars. They suggested that the high concentration of sorbitol "associated to watercore" (Chan et al. 1972, Faust et al. 1969) was more likely related to a leaking of this carbohydrate in the apoplast, than to the absence of intracellular degradative enzymes and cofactors. Thus, the metabolism of sorbitol in fruits suffering from this disorder could be impaired or slowed down.

2.2.2 Metabolism

With the use of isotopic tracers and enzymological methods, the actual knowledge of sorbitol metabolism in the Rosaceae has significantly progressed since the early review made by Touster and Shaw (1962). New hypotheses on the pathways of synthesis and utilization of sorbitol have been suggested (Bieleski and Redgwell 1977, Hirai 1979, 1981, Negm and Loescher 1979, 1981, Grant and ap Rees 1981, Yamaki 1980a, 1981, 1982a, 1984a). These pathways emphasized the central position of this sugar alcohol in the carbohydrate metabolism of woody Rosaceae. The different end products are summarized in Figure 2.

Sorbitol Synthesis

Following exposure of leaves to $^{14}CO_2$, a rapid synthesis of sorbitol was reported in apple, apricot, pear, plum, and cotoneaster (Hutchinson et al. 1959, Bieleski 1982, Anderson et al. 1961). This evidence clearly demonstrated that sorbitol was the major product of photosynthesis in many rosaceous species (Bieleski 1977). In apple seedlings, sorbitol synthesis occurred principally in the leaves, and was not necessarily dependent upon light, since leaf discs supplied with labelled fructose produced labelled sorbitol even in the dark (Grant and ap Rees 1981).

The synthesis of sorbitol was assumed to occur via the hexose monophosphates. Bieleski and Redgwell (1977) and Redgwell and Bieleski (1978) proposed this pathway after finding labelled sorbitol-6-phosphate (So-6-P) and sorbitol-1-phosphate in apricot leaves following a short (10-15 min) $^{14}CO_2$ exposure in

F-6-P : Fructose-6-phosphate
G-1-P : Glucose-1-phosphate
G-6-P : Glucose-6-phosphate
So-1-P : Sorbitol-1-phosphate
So-6-P : Sorbitol-6-phosphate
UDP-G : Uridinediphosphate-glucose
UTP : Uridinetriphosphate

1 : Invertase

2 : UDP-dependent Sucrose synthase

3 : NAD-dependent Sorbitol dehydrogenase

4 : Sorbitol oxidase

5 : Hexokinase

6 : NADP-dependent Sorbitol dehydrogenase

7 : NADP-dependent Aldose-6-phosphate reductase

8 : Sorbitol-6-phosphate phosphatase

9 : Phosphohexoisomerase

10 : Phosphoglucomutase

11 : UDP-glucose pyrophophorylase

12 : UDP-sucrose phosphate synthase and sucrose phosphatase

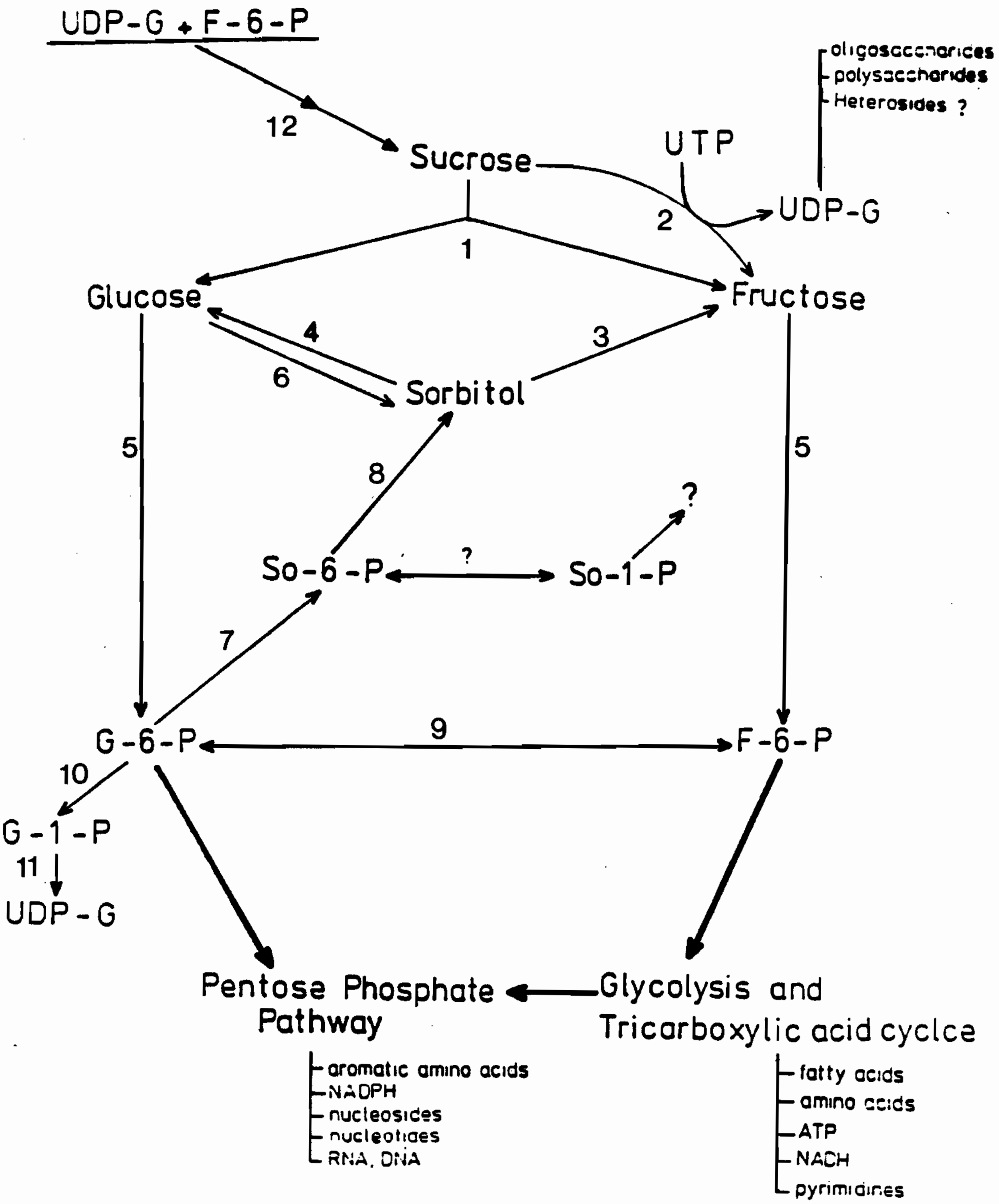
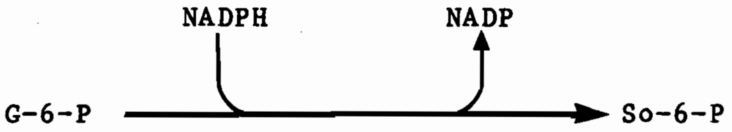


Figure 2: Proposed pathways of sorbitol metabolism and its principal end products.

light (Figure 2). Since fructose-6-phosphate (F-6-P) became labelled much more rapidly than glucose-6-phosphate (G-6-P), these authors suggested the following pathway of sorbitol synthesis :

$$\text{F-6-P} \longrightarrow \text{Sorbitol phosphate} \longrightarrow \text{Sorbitol}$$

However, enzymes reducing F-6-P into So-6-P or So-1-P has yet to be isolated from plant tissue. It is noteworthy that So-6-P dehydrogenase (Figure 2, No. 7) has been found in loquat fruits (Hirai 1979, 1981), in apple fruits and cotyledons (Yamaki 1980, 1980a), and in eight rosaceous species of the Prunoideae, Pomoideae and Spiraeoideae subfamilies (Hirai 1981). The substrate of this enzyme has been found to be G-6-P, and not F-6-P; the enzyme was NADP-dependent and, at neutral pH, was more effective in the reduction of G-6-P than in the reverse reaction (Hirai 1981):



A similar enzyme was detected in mature leaves of apple, pear, peach, and apricot by Negm and Loescher (1981). This enzyme was apparently not specific to G-6-P, but also was able to reduce galactose-6-P, and to a lesser extent mannose-6-P and 2-deoxy-D-glucose-6-P. In view of the range of substrates, the enzyme was designated as an aldose-6-P reductase by Negm and Loescher

(1981). Interestingly, there is yet no report using enzymatic approches dealing with the role of So-1-P in the metabolism of sorbitol in woody Rosaceae.

In the conversion of So-6-P into sorbitol, the activity of a phosphatase was assumed (Bieleski and Redgwell 1977). Such an enzymatic activity was recently detected, in apple seedlings by Grant and ap Rees (1981) (Figure 2, No. 8). A partial purification of this enzyme at neutral pH, resulted in an activity 10 times greater with So-6-P than with the common hexose-6-phosphates. Hence, these results gave more credence to the suggested key role of So-6-P in sorbitol synthesis (Bieleski and Redgwell 1977).

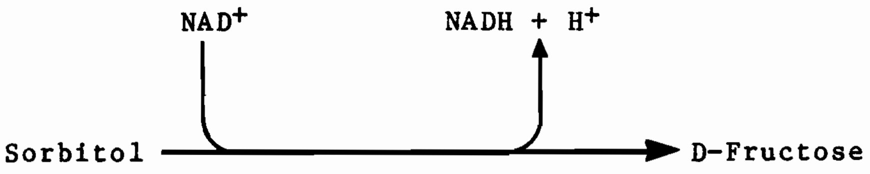
In apple, the activity of the aldose-6-P reductase (A6PR, also designated as S-6-P dehydrogenase) has been found in leaves (Negm and Loescher 1981) and cotyledons (Yamaki 1981). Yamaki (1981) found this activity to be localized in chloroplasts, presumably associated with the thylakoid membrane. A similar association of sorbitol synthesis with chloroplast has been suggested from experiments with plum leaves (DeVillier <u>et al</u>. 1974). However, 35-40% of the A6PR activity in apple was associated with the cytosol (Yamaki 1981), indicating that the synthesis of sorbitol was not restricted to the chloroplast. In loquat leaves, seasonal variations in the activity of A6PR was found to correlated with changes in sorbitol content (Hirai 1983). Similarly, increase in the activity of this enzyme in apple seedlings paralleled the accumulation of sorbitol during germination (Yamaki 1980). The lack of A6PR activity in very young apple leaves and its presence in mature ones suggested the possibility of a close relationship between chloroplast development, sorbitol synthesis, and the mechanism regulating partitioning of photosyntates (Negm and Loescher 1981, Loescher et al. 1982).

Even though there is no final conclusion on the possible pathways of sorbitol synthesis (Bieleski 1982), the enzymatic and thermodynamic considerations, as well as the labelling pattern following $^{14}CO_2$ exposure, strongly suggested that an aldose-6-phosphate was involved in sorbitol synthesis (Bieleski and Redgwell 1977, Grant and Rees 1981, Negm and Loescher 1981). However, it is noteworthy that direct reduction of hexoses also has been suggested as a pathway for sorbitol synthesis (Bieleski 1982). Its synthesis in apples was suggested to occur by direct reduction of fructose (Chong and Taper 1972) or glucose (Hutchinson 1958). These suggested pathways have not been confirmed by enzymological investigations. Moreover, the early finding by Anderson and co-workers (1962) that the conversion of D-glucose into sorbitol occurred without rupture of the carbon chain, did not necessarily suggest that a direct oxidation occurred. The conversion involving hexose monophosphates in sorbitol synthesis also occurred without rupture of the glucose chain.

Sorbitol Utilization

So far in higher plants, three enzymes have been found to catalyze the oxidation of sorbitol : a sorbitol oxidase and two sorbitol dehydrogenases, one being NAD^{+}-dependent (NAD-SDH), the other NADP^{+}-dependent (NADP-SDH).

Sorbitol dehydrogenase was first partially purified from rat liver by Blakley (1951). Since then it has been purified from a variety of mammals and insects (Touster and Shaw 1962). More recently, a NAD^{+}-dependent sorbitol dehydrogenase was detected in calli, fruits, seeds and cotyledonary tissues of apple (Gorrod 1961, Negm and Loescher 1979, 1981, Yamaki, 1980a), and in seeds and calli of pear (Negm and Loescher 1981). The reaction catalyzed by this enzyme was an oxidation of sorbitol to D-fructose, considered by Loescher and co-workers (1982) to be essentially irreversible.



Sorbitol dehydrogenase (NAD-SDH) is a cytoplasmic enzyme having its optimum activity at alkaline pH (9.5) (Yakaki 1980, Negm and Loescher 1979). The presence of this enzyme seemed to be essential for the utilization of sorbitol by a plant tissue or organ.

Recently another sorbitol dehydrogenase, NADP-SDH was identified in apple leaves (Yamaki 1984a). It reversibly catalyzed the conversion of sorbitol to glucose, and was found to be NADP^{+} dependent (Figure 2, No. 6). The partially purified enzyme had optimum activity at pH 9.6 and Km value of 128 mM for sorbitol. However in *Malus domestica* Borkh. cv. Jonnagold, its activity was barely detectable (Yamaki and Ishikawa 1986).

According to Yamaki (1982a), sorbitol oxidase catalyzed the following reaction:

This enzyme had an optimum activity in acidic pH (4.0) and was detected in association with the tonoplast and plasmalemma membranes (Yamaki 1980a, 1982a). The specific activity of sorbitol oxidase reported by Yamaki (1980a) was low, and 10 times lower in apple fruit tissues than that of sorbitol dehydrogenase.

By analogy with the acidic invertase which is associated with the cell membranes (Ricardo and Rees 1970, Maretzki et al. 1974) and which appears to control the sugar form and/or concentration within the cytoplasm (Glasziou and Gayler 1972, Parr et al. 1976, Fowler 1978), Yamaki (1980a, 1982a) suggested that sorbitol oxidase could be directly involved in the transport and oxidation of sorbitol through the cell membranes. Obviously, sorbitol transport also occurred otherwise, since the hypothesis involving a sorbitol oxidase did not explain the fact that sorbitol was found in the vacuole sap of cotyledons and fruit tissues (Yamaki 1982, 1984).

Except for the previously mentioned anabolitic and catabolitic enzymes involved in sorbitol metabolism, very little is known about other enzymes that could operate in the metabolism of this sugar alcohol in higher plants. The finding by Redgwell and Bieleski (1978) of So-6-P and So-1-P in apricot tissues suggested that these compounds could be of some importance in polysaccharide synthesis. Sorbitol or its derivatives could also be linked to another carbohydrate to form a secondary product, namely heteroside (Bieleski 1982). However, little is known about the metabolism and physiological roles of heterosides (Guignard 1974).

CHAPTER III

MATERIALS AND METHODS

3.1 STOCK CULTURES

3.1.1 Origin

Cultures of three apple genotypes, previously initiated and maintained in this laboratory were used in these studies. The first culture was initiated in May 1981, as described by Pua *et al*. (1983), from 1 mm meristem tips excised from buds of Ottawa-3 (O-3) apple (*Malus baccata* (L.) Borkh. x *M. sylvestris* Mill. x Jaune de Metz) rootstock (Spangelo *et al*. 1974). O-3 is a winter hardy dwarfing rootstock that is difficult to propagate by conventional methods. The second culture was initiated in May 1981, as described by Pua and Chong (1985), from 1mm meristem tips excised from buds of a 10-year-old mature fruiting tree of Macspur (MA), a spur-type mutant of *Malus domestica* Borkh. cv. McIntosh. The third culture was initiated in December 1979, as described by Pua and Chong (1985), from 5 mm shoot tips of aseptically germinated open-pollinated seedlings of Macspur (SM).

In experiments described in these studies, the ages of the cultures after establishment were : 0-3, 41 months; MA, 41 months; SM, 57 months.

3.1.2 Culture Conditions

All cultures were grown in a culture room at 26 ± 2°C under 16-hr photoperiod of cool white illumination of 25 ± 2 μE m^{-2} sec^{-1}. Stocks were maintained by monthly transfer of 5 mm shoot tips, four or five in a 500 ml Mason jar with 40 ml of medium, or one in a 25x120 mm test tube with 10 ml of medium. The formulation of the culture medium used for each genotype is presented in Table 1. The pH of the media was adjusted at 5.7 before autoclaving at 1 kg/cm^2 and 121°C for 15 minutes.

Unlike the SM or MA cultures, the 0-3 culture developed contamination in December 1983, and was disinfested by selecting and placing healthy-appearing shoots in 10% Javex solution (0.6% NaOCl) for two minutes and rinsing three times with sterile distilled water. Meristem tips (ca. 1mm) were aseptically excised and placed into test tubes containing 10 ml of multiplication medium (Table 1).

During the summer of 1984, all cultures were multiplied in sufficient numbers to proceed with the isotopic tracer studies. Even though, for 0-3, translucent (vitreous) plants with curled leaf margins appeared (Werner and Boe 1980, Strahlheim 1980, Pua et al. 1983, Kevers et al. 1984, 1985), a

Table 1: Nutrient media[a] used for multiplication and rooting stages (Stages II and III, respectively) of three apple genotypes.

Genotype	SM		MA		0-3	
Stage	II	III	II	III	II	III
MS-Salts[b] strength :	1	1/2	1	1/2	1	1/2
Organics : (mg/L)						
Glycine	2.0	--	2.0	--	2.0	--
Myo-Inositol	100.0	50.0	100.0	50.0	100.0	50.0
Thiamine-HCl	0.1	--	0.1	--	0.1	--
Pyridoxine-HCl	0.5	--	0.5	--	0.5	--
Nicotinic acid	0.5	--	0.5	--	0.5	--
Carbohydrate : (g/L)						
Sucrose	30	30	30	30	30	30
Plant Growth Regulator : (mg/L)						
IBA	--	0.25	1.0	3.0	0.5	6.25
BA	1.0	--	1.0	--	0.5	--
GA_3	--	--	--	--	3.0	--
Agar[c] : (g/L)	7	7	7	7	7	7

[a] pH = 5.7, adjusted before autoclaving of all media.

[b] Murashige and Skoog (1962) macro and micro nutrients.

[c] Difco-bacto.

sufficient number of normal plants of this genotype was obtained by growing and screening a large number of explants.

The sequence in exprimental procedures are shown schematically in Figure 3.

3.1.3 Preparation of Labelled Medium and Plant Material

Labelled nutrient media were prepared as follows :

Stock solutions and carbon source

Media constituents such as Murashige and Skoog (1962) macro and micro nutrients, vitamins and other organics, plant growth regulators, carbohydrates, and agar (Table 1) were prepared to yield a final volume of 100 ml. Initially all constituents, except the carbon source fraction, were mixed, the volume made to 90 ml, and pH adjusted with 1N NaOH to 5.7 (Chong and Taper 1974). This was transferred into a 250 ml Erlenmeyer flask covered with aluminium foil and autoclaved. After autoclaving, 10 ml of 30% stock solution of either fructose, glucose, sorbitol, or sucrose, previously filter sterilized through millipore filters (0.22 µm pore size), were aseptically added and shaken gently to mix the constituents homogeneously.

. Addition of Tracers

The radiochemicals were handled under the supervision of Dr N. Barthakur according to the general advice of the Atomic and Energy Control Board of Canada (Anonymous 1979).

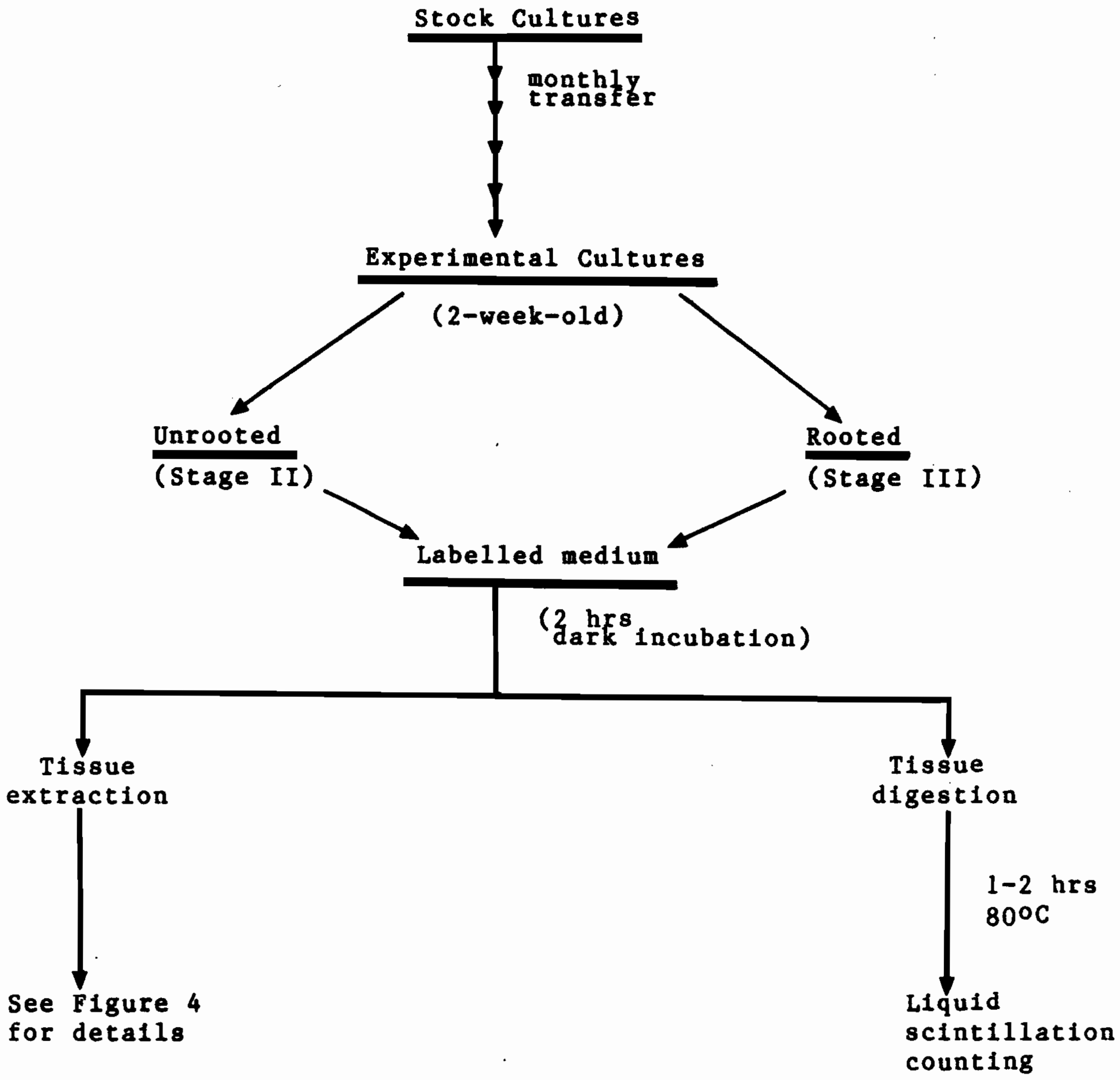


Figure 3: Outline of the sequence in experimental procedures.

Two ml of medium, containing fructose, glucose, sorbitol, or sucrose, as described above, were dispensed aseptically into sterile flat bottom 21x85 mm test tubes each containing respectively 1 μCi (37 KBq) of uniformally labelled [U - ^{14}C] fructose, glucose, sorbitol, or sucrose (ICN Biomedicals Canada Ltd.). This activity (1 μCi) resulted from the addition to each test tube of 20 μl of a labelled sugar stock solution having an activity concentration of 50 μCi per milliliter. The final absolute activity of the labelled medium for each carbon source was therefore 0.5 μCi ml^{-1}. The specific activities of fructose, glucose, sorbitol, and sucrose, were 3.00, 3.00, 3.04, and 5.71 mCi mole^{-1}, respectively. Each test tube was sealed with a rubber stopper and the medium allowed to solidify. The labelled media were kept for seven days at room temperature to ensure that there was no contamination.

Uniform 2-week-old unrooted (Stage II) or rooted (Stage III) sucrose-grown cultures of each genotype were transfered to the labelled media. Each test tube was resealed with a rubber stopper.

3.2 TIME COURSE INVESTIGATION OF ^{14}C-SORBITOL UPTAKE

Labelled [U-^{14}C] sorbitol was supplied to MA shoot explants at Stage II. The origin of the explants and the stock culture conditions were previously described (sections 3.1.1 and 3.1.2). Two-week-old MA shoots (ca. 250 mg FW) were placed one per flat bottom test tube (21x85 mm), containing 2 ml of labelled shoot proliferation medium prepared as described in section 3.1.3, but with an activity of 1 µCi ml^{-1}. The test tube was sealed with a rubber stopper and then placed in a growth room under conditions previously described. A total of 15 Macspur shoot explants was prepared.

Three explants were sampled after each of the following sampling times: 1, 2, 4, 8 and 12 hours. The portion of each explant in contact with the labelled medium was carefully washed with a spray of distilled water. The total fresh weight (_ca_. 220 mg) of each explant, as well as the fresh weight of a sample of 2 to 3 randomly selected leaves (_ca_. 30 mg) from the explant, were recorded. The sample of leaves (herein refered to as leaf sample) and of the remainder of each explant minus 2 to 3 leaves (herein refered as whole explant sample) were each placed separaditely into 8 ml plastic mini-vials (previously described) and digested prior to being evaluated for their ^{14}C-content by liquid scintillation counting (see section 3.3.2). The ^{14}C-activity was determined in decay per minutes per mg fresh weight (DPM/mg FW), and thereafter corrected for the specific activity of the carbon sources used. The uptake was therefore expressed in µmole of sugar per g fresh weight (µmole/g FW) of each sample. The means and standard errors of sorbitol uptake for each sampling time were determined.

3.3 TISSUE SAMPLING AND ANALYTICAL PROCEDURES

Cultures of the three apple genotypes (O-3, MA, and SM), at both multiplication or rooting stages (Stages II and III, respectively) of *in vitro* propagation, were supplied with four different labelled carbohydrates (fructose, glucose, sorbitol, and sucrose) used separately at a concentration of 3%, according to a factorial experimental design. The factor combinations represented 24 treatments for each of which 6 replicates were used.

After a 2-hour dark incubation period at room temperature, the explant from each test tube was removed, the portion of each explant in contact with the labelled medium was carefully washed with a spray of distilled water, and the total fresh weight of the explant was recorded (*ca.* 200 mg). Leaf sample (one to three randomly selected leaves, *ca.* 25 mg) of each explant were removed from each explant and placed into a capped, plastic mini-vial (*ca.* 8 ml capacity, ICN Biomedicals Canada Ltd.) for the digestion procedures. The remaining portion of the explant (*ca.* 175 mg, whole explant sample) was weighed and reserved for extraction. The experimental protocol, adapted from Bieleski (1977) and Peng (1977), allowed an evaluation of the total soluble and insoluble ^{14}C-content and the activity contained in the water soluble fraction of the extracted tissues.

3.3.1 *Digestion*

To each leaf sample within the plastic mini-vial was added a mixture of 60% perchloric acid and hydrogen peroxide

(2/1, v/v). Digestion of the sample was allowed to occur in an oven for 1 to 2 hours at 80°C. Each mini-vial was allowed to cool, then 5 ml of PCS were added prior to liquid scintillation counting. According to Peng (1977), this digestion method is applicable to the preparation of samples containing ^{14}C without any apparent loss of radioactivity, or of luminescent interference during liquid scintillation counting. After a 10 minute counting period per vial, results obtained in DPM/mg FW (Kobayashi and Maudsley 1974) were either used to calculate the percentage of respiration (Section 3.3.1), or adjusted for the specific activity of the carbon source and expressed in µmole of sugar per g fresh weight (µmole/g FW). The latter results (µmole/g FW) were used in an analysis of variance according to a completely randomized experimental design. Comparisons between means were performed with LSD tests.

3.3.2 Extraction

Each whole explant sample (i.e. total explant with 1-3 leaves removed) was extracted four times as illustrated in Figure 5. The first extraction was conducted by placing each whole explant sample in a 15 ml glass vial with 10 ml of methanol / chloroform / water / formicacid (MCWF, 12/5/2/1, v/v/v/v) (Bieleski and Young 1963). The vial was kept at - 18 ± 2°C for a period of at least 24 hours, after which the supernatant extract was transfered into a 100 ml round bottom flask. The second and third extractions were conducted in the same vial at room temperature with 5 ml of MCWF for a period of 1 hour each.

To the combined supernatant of those three extractions in the round bottom flask was added 5 ml of chloroform (CH_3Cl) and 6 ml of distilled water to "split" the extract into two phases. The chloroform phase containing pigments, lipids, and phospholipids was discarded (Bieleski and Turner 1966); the aqueous phase containing amino acids, organic acids, and sugars was reserved for futher procedures.

The fourth extraction was conducted with 20 ml of 70% ethanol added to the residual explant. The plant sample was then homogenized for 1 minute in a micro-Waring Blender according to the method used by Chong (1972). This ethanolic extract was filtered under vacuum through Whatman No. 50 filter paper, pooled with the aquaeous phase previously extracted, and dried at 55°C on a rotary evaporator (Figure 4). The flask was left for 12 hours over KOH pellets in a desiccator to remove any traces of formic acid from the dried sample (Redgwell 1980).

3.3.3 Fractionation

The fractionation method used was adapted from Redgwell (1980). Glass barrels of 5 ml hypodermic syringes, filled with QAE-25 sephadex (Pharmacia Fine Chemicals Ltd. Dorval, Quebec) equilibrated in an ammonium buffer (0.05M NH_4HCO_3/NH_4OH , pH 9.5) and washed to neutrality with distilled water were used as columns (Redgwell 1980). Each column was used only once.

The dried extract (see section 3.3.3) was redissolved in 1 ml of sodium phosphate (0.2M Na_2HPO_4-$7H_2O$, pH 7), and poured through the column described above, followed by three 1 ml rinses of the round bottom flask with distilled water. Additional distilled water was then poured through the column to allow collection of the first 20 ml (neutral) fraction. This fraction contained neutral compounds such as sugars and sugar alcohol. Organic acids, phosphate esters and most amino acids were retained on the column (Redgwell 1980).

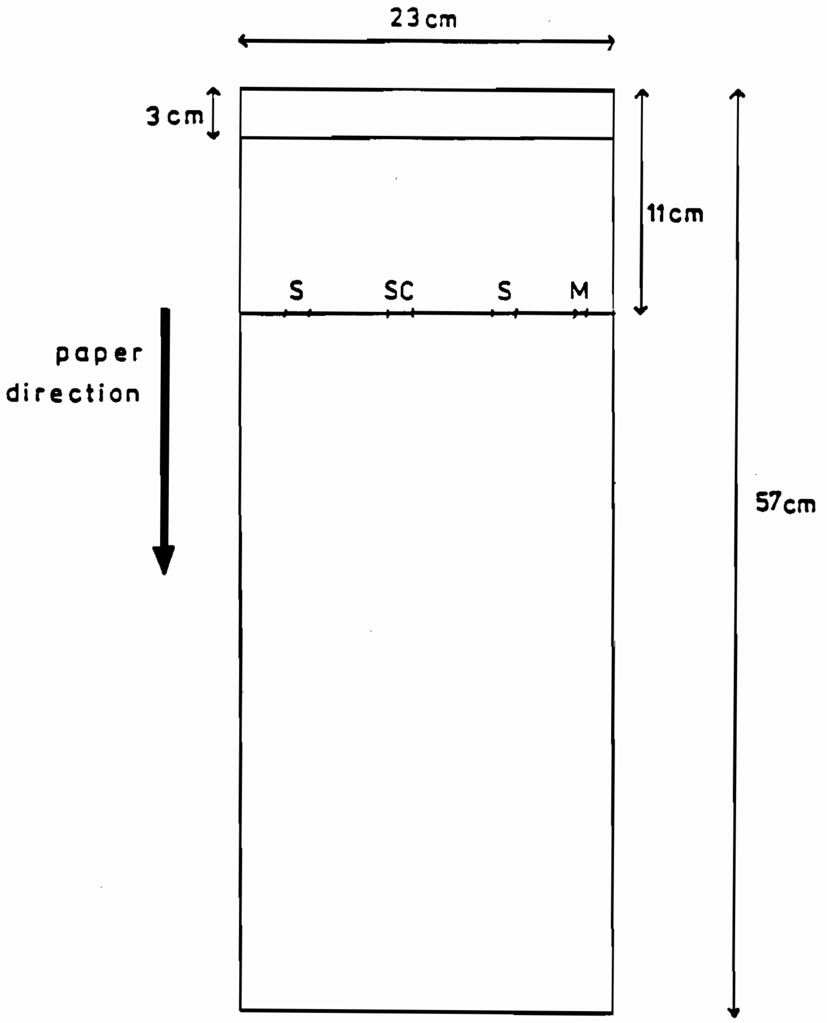
Eighteen milliliters of neutral fraction were transfered into a test tube and reserved for paper chromatographic analysis and liquid scintillation counting to determine relative interconversion of the sugars. Each test tube containing the sugar fractions was frozen and subsequently freeze-dried before being used for analysis.

3.3.4 Paper Chromatographic Procedures

Two of six replicates per treatment were selected randomly for paper chromatographic analysis. At the time of analysis, each freeze-dried sample was redissolved in 500 ul of distilled water. This amount of distilled water was determined by preliminary tests to dissolve the sugars completely in the test tube and yield a satisfactory and reproducible counting rate. Fifty microliters of each sample were spotted along with a standard mixture containing fructose, glucose, sorbitol, and sucrose on half sheet of Whatman No. 1 filter paper (23 x 57 cm), as shown in Figure 5. Using the descending method, and methyl ethyl ketone / acetic acid / water saturated with boric acid (9/1/1, v/v/v) as a solvent system (Webb and Burley 1962), sorbitol, sucrose, glucose, and fructose were separated using a development time of about 44 hours. A standard mixture of 10% sucrose / 10% sorbitol / 10% glucose / 10% fructose (1/1/1/1, v/v/v/v) was used to aid in the identification of the individual sugars after the chromatogram had been separated. The two reference sections as well as that of the standard sugar mixture (Figure 5) were cut from the chromatographic sheet and developed according to the two step procedure of Smith (1960). Firstly, the paper sections were dipped in a solution of 1 ml saturated aqueous $AgNO_3$ dissolved in 200 ml of acetone (Zweig and Sherman 1972), and hung until the acetone dried. Secondly, the paper sections were dipped into an ethanolic NaOH solution (0.5% NaOH in 80% ethanol). The sugar spots became visible within a few minutes, although better resolution (darker contrast) was obtained upon standing for 12 hours (Figure 6). Developed and undeveloped chromatograms were placed side by side to aid in the cutting of zones corresponding to each individual sugar from the undeveloped chromatogram. Each zone was cut into smaller pieces (*ca.* 1 cm^2) and placed into test tubes for elution. The above chromatographic procedures were repeated three times for each sample.

. Elution

Each zone of paper corresponding to an individual sugar was placed in a test tube and eluted three times, once with 10 ml of distilled water (Dubois *et al.* 1956)



S : reference section, used for development of individual sugars

Sc: sample used for liquid scintillation counting

M : standard sugar mixture

Figure 5: Illustration of the paper chromatographic technique used, showing the spotted samples and standard sugar mixture before development

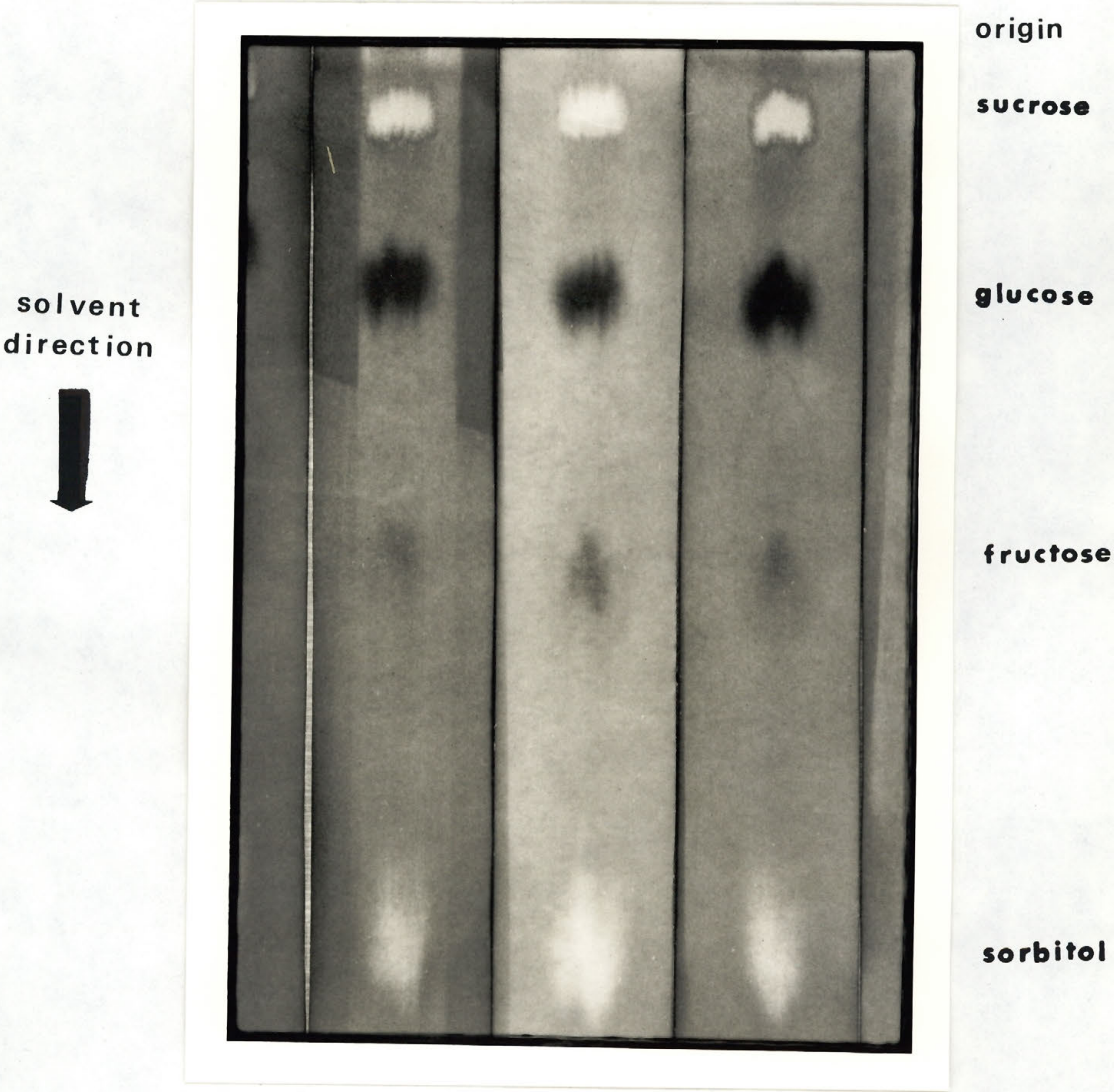


Figure 6: Developed paper chromatograms showing the separation of sucrose, glucose, fructose and sorbitol.

and subsequently twice with 5 ml of 70% ethanol. Each elution was allowed to continue for a period of 1 hour, with periodic gentle agitation of the test tube to facilitate the elution process (Chong 1972). The combined eluate was evaporated to dryness in an oven at 110 °C, after which 2 ml 70% ethanol were added to redissolve the sugar in solution. Ethanol was used because it served as a good solvent for sugars and was more soluble in the liquid scintillation fluid (Huskisson and Ward 1978). From this ethanolic solution, 1.6 ml were transfered to a mini-vial (previously described) to which 5 ml of liquid scintillation fluid (PCS, Amersham, Ontario) were added. The mixture was counted for 10 minutes in the Beckman liquid scintillation counter previously descibed. After correction for quenching and dilution, results were obtained in DPM/mg FW of tissue. For each apple culture and stage of in vitro propagation, result for the interconversion of each carbon source was expressed as a percentage of the total soluble carbohydrates (sum of activities of fructose, glucose, sorbitol, and sucrose). For each treatment, the mean and standard error of the latter percentages of interconversion were determined.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 TIME-COURSE INVESTIGATION OF ^{14}C-SORBITOL UPTAKE

Result

The means and standard errors of ^{14}C-sorbitol uptake for each sampling time by MA shoot explants by leaf (Figure 7a) and whole explant (Figure 7b) samples are presented. The MA shoot explants at Stage II absorbed labelled sorbitol readily (Figure 7). There was a good correlation ($\underline{r}$ = 0.98, significant at $\underline{P}$ = 0.01) between the ^{14}C-activity recorded in leaf (Figure 7a) and that in whole explant (Figure 7b) samples. Multiple regression analyses performed on the data obtained from this study showed that sorbitol uptake by MA shoot explants increased linearly as a function of time at a mean uptake rate (average of leaf and whole explant samples) of 4.5 µmole of sorbitol/g FW per hour (Figure 7). This rate showed no decrease even after a long (12-hour) incubation period and was in the same order of magnitude as that reported for uptake of sorbitol by Bieleski (1977).

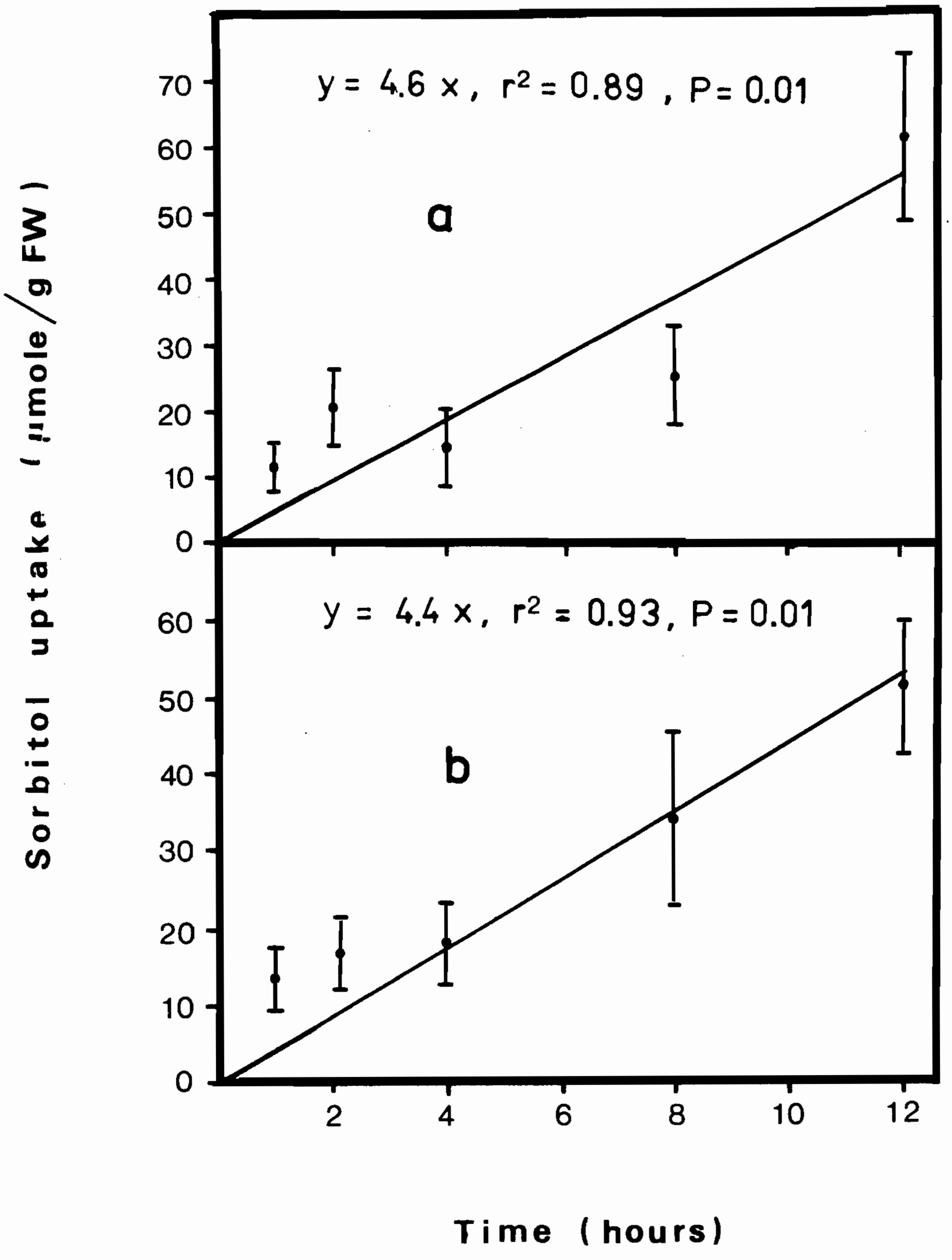


Figure 7: Time course investigation of ^{14}C-Sorbitol uptake by MA shoot explants from (a) leaf sample and (b) whole explant samples. Each datum is the mean of three replicates. Vertical bars represent standard errors.

Discussion

A linear uptake is one of the accepted characteristics of an active transport process (Bieleski 1977). However, in this study, it was not tested whether or not after a long (12-hour) incubation period the uptake of sorbitol into the MA shoots was against a concentration gradient. Nevertheless, in a related study, it was found that O-3 shoots after only two hours of incubation contained sugar concentrations close to those of the initial nutrient media (Renaud, unpublished data). Based on this observation, it is assumed that for MA shoots an incubation period longer than 2 hours is required for establishing a concentration equilibrium between the sugars of the explant and those of the nutrient medium.

Bieleski (1977) investigated the carbohydrate metabolism of pear and rose leaf slices using carbon sources labelled with an activity fo 1 μCi/ml. However, for MA shoot explants, the reproductibility of the results was not affected by a reduction of the ^{14}C-source using an activity of 0.5 μCi/ml. Therefore, this reduced level of activity was used in later experiments since it allowed a larger number of samples to be investigated with the same initial quantity of tracers. Since a shorter incubation period minimized the interconversion and metabolic processes of the absorbed carbon source, thus making less complicated the interpretation of results in regard to the transport processes (Guy et al. 1980), a relatively shorter incubation period of two hours was used in other experiments. Moreover, other experiments were conducted in the dark to avoid any possible secondary fixation of the $^{14}CO_2$ released by the explant (Bieleski and Redgwell 1977).

4.2 UPTAKE OF LABELLED SORBITOL AND RELATED SUGARS

Result

Data for uptake of labelled fructose, glucose, sorbitol, and sucrose by leaf samples of MA, SM, O-3 apple cultures at stages of both multiplication (Stage II) and rooting (Stage III) of *in vitro* propagation are shown in Table 2. Analysis of variance performed on the data (Table 3) indicated no significant difference in the mean uptake of the four carbon sources in each of both stages of culture, although there was a significant genotype x stage interaction. There was greater uptake by each of the three apple cultures at Stage II than at Stage III. While there was no significant difference in uptake of sugars among the three genotypes in Stage III (5.2-6.3 µmole/g FW), in Stage II uptake in SM shoot explants (20.1 µmole/g FW) was significantly greater by 2.5 and 2.9 times than in corresponding explants of O-3 (8.2 µmole/g FW) and MA (7.0 µmole/g FW), respectively.

Discussion

The greater uptake of sugars by SM cultures during the multiplication stage appears to be related to the more rapid proliferation habit of this genotype compared to MA and O-3. As indicated by Pua and Chong (1985) and Zimmerman (1983, 1983a), different apple genotypes are known to respond differently to the same medium during different stages of *in vitro* culture. How-

Table 2: Uptake of ^{14}C labelled fructose, glucose, sorbitol, and sucrose by leaf samples of MA, SM, and O-3 apple cultures at Stages II and III, after 2-hour incubation period.

Carbon Source Supplied	Uptake from cultures of: (μmole/g FW)			MEAN
	MA	SM	O-3	
		Stage II		
Fructose	9.6 ± 2.6	18.0 ± 3.8	5.8 ± 1.0	11.1
Glucose	6.4 ± 1.6	21.4 ± 5.4	6.4 ± 1.4	11.4
Sorbitol	6.6 ± 0.8	20.8 ± 3.8	9.8 ± 2.8	12.4
Sucrose	5.4 ± 1.4	20.0 ± 3.0	10.8 ± 2.8	12.1
Mean:	7.0	20.1	8.2	11.8
		Stage III		
Fructose	6.6 ± 1.6	7.8 ± 2.4	7.8 ± 2.6	7.4
Glucose	2.8 ± 1.0	6.6 ± 2.4	3.6 ± 0.8	4.3
Sorbitol	8.2 ± 2.4	3.8 ± 0.8	6.0 ± 1.6	6.0
Sucrose	3.0 ± 0.8	7.0 ± 3.0	4.0 ± 1.0	4.7
Mean:	5.2	6.3	5.4	5.6
Significant LSD at 0.05:	Genotype (G)=1.2 Carbon source=NS	Stage (S)=1.0 Other interactions = NS	G * S= 1.7	

Each individual figure is the mean of 6 replicates, ± S.E.

Table 3: Analysis of variance for the uptake of ^{14}C labelled fructose, glucose, sorbitol, and sucrose by leaf samples of MA, SM, and 0-3 apple cultures at Stages II and III of *in vitro* propagation.

Sources	DF	Mean Squares
GENOTYPE (G)	2	188.3 **
STAGE (S)	1	344.0 **
CARBON SOURCE (C)	3	3.9
G * S	2	130.8 **
G * C	6	6.4
S * C	3	6.5
G * C * S	6	6.4
ERROR	120	8.7

** Significant at the 0.01 level.

ever, there were important qualitative and quantitative differences with regards to the plant growth regulator composition of the culture media of each stage of development (Table 1). Most importantly, the level of cytokinin was reduced or eliminated from the culture medium to encourage the development of roots at Stage III. Thus, the greater absorption of sugars by all genotypes at the multiplication stage than at the rooting one is obviously related to ontogenic characteristics.

Micropagated apple shoots are dependent upon a carbohydrate source (Zimmerman 1983a) and, by analogy, could be considered as physiological sinks as are fruits. The results presented in Table 2 showed no difference in relative absorption of sorbitol, sucrose, glucose, or fructose by the three apple cultures at the multiplication or rooting stage. This finding contrasts with results of Williams _et al_. (1967), who found that labelled sorbitol was translocated and absorbed by apple fruits at a much faster rate than sucrose. In pear leaf tissue, there were differences in the uptake characteristics of sorbitol and glucose in response to tissue aging, sugar concentration, competing sugars, and darkness (Bieliski 1977). Thus the active transport mechanism of sorbitol uptake is somewhat different from that of glucose (Bieleski 1977). Interestingly, darkness was found in leaf tissue of pear to inhibit glucose uptake significantly more than sorbitol uptake (Bieleski 1977).

4.3 INTERCONVERSION OF SORBITOL AND RELATED SUGARS

Result

Data for percent ^{14}C activity in fructose, glucose, sorbitol, and sucrose analyzed in samples of the three apple cultures supplied separately with each of the four labelled carbon sources at both Stages II and III of *in vitro* propagation are presented in Tables 4, 5, and 6. In view of significant differences found previously in uptake of the labelled carbon sources due to genotype (Section 4.2), the relative amounts (^{14}C activity) of each sugar analyzed within the sample were expressed as a percentage of total soluble carbohydrates (sum of the individual activities recorded in fructose, glucose, sorbitol, and sucrose). This allowed a direct comparison of the relative interconversions with respect to carbon sources supplied, genotype, and stage of *in vitro* propagation.

Notwithstanding differences between genotypes and stage of culture, each supplied carbon source represented a relatively large portion of the ^{14}C activity of the carbohydrate fraction (Tables 4, 5, 6). Sorbitol, found at an average proportion of 93.1% over all genotype and culture, was interconverted to the lowest extent, followed in order by glucose (80.5%), fructose (63.0%), and sucrose (62.4%).

When ^{14}C-fructose was supplied to Stage II MA cultures (Table 4), there was a marked conversion into glucose (49%) followed in order by sucrose (16.9%) and sorbitol (3.5%). When ^{14}C-glucose was supplied, its conversion into sucrose was

Table 4: Percent ^{14}C activity in fructose, glucose, sorbitol, and sucrose analysed in whole explant samples of MA apple culture supplied separately with each of these labelled carbon sources at both Stages II and III.

Carbon Source Supplied	Activity recorded in: (% of the total soluble carbohydrate activity)[a]			
	Fructose	Glucose	Sorbitol	Sucrose
		Stage II		
Fructose	30.7 ± 2.3[b]	49.0 ± 1.0	3.5 ± 0.4	16.9 ± 1.8
Glucose	1.9 ± 0.4	77.2 ± 2.7	0.5 ± 0.2	20.5 ± 2.4
Sorbitol	1.0 ± 0.1	1.6 ± 0.2	93.5 ± 1.0	3.9 ± 0.9
Sucrose	11.0 ± 0.8	32.3 ± 1.3	6.3 ± 2.2	50.4 ± 3.3
		Stage III		
Fructose	57.6 ± 2.4	27.3 ± 0.3	3.0 ± 1.0	12.1 ± 2.0
Glucose	2.6 ± 0.9	76.2 ± 1.8	4.0 ± 1.8	17.2 ± 0.5
Sorbitol	15.5 ± 7.2	0.6 ± 0.3	82.7 ± 7.7	1.2 ± 0.5
Sucrose	11.8 ± 1.6	27.4 ± 2.7	3.3 ± 0.7	57.2 ± 4.0

[a] The total soluble carbohydrate activity is the sum of the individual activities recorded in fructose, glucose, sorbitol, and sucrose.

[b] Each figure is the mean of 6 observations ± S.E.

Table 5: Percent 14C activity in fructose, glucose, sorbitol, and sucrose analysed in whole explant samples of SM apple culture supplied separately with each of these labelled carbon sources at both Stages II and III.

Carbon Source Supplied	Activity recorded in: (% of the total soluble carbohydrate activity)[a]			
	Fructose	Glucose	Sorbitol	Sucrose
		Stage II		
Fructose	62.3 ± 4.7[b]	15.2 ± 4.5	7.4 ± 2.6	15.1 ± 2.7
Glucose	7.4 ± 2.9	69.4 ± 4.3	2.8 ± 1.1	20.4 ± 6.6
Sorbitol	1.2 ± 0.4	0.8 ± 0.4	96.0 ± 1.5	2.0 ± 1.1
Sucrose	5.4 ± 0.2	6.8 ± 0.2	0.5 ± 0.0	87.4 ± 0.2
		Stage III		
Fructose	78.6 ± 2.0	9.1 ± 1.1	6.4 ± 1.5	6.0 ± 0.8
Glucose	5.6 ± 1.6	87.2 ± 1.1	4.2 ± 1.7	2.9 ± 0.4
Sorbitol	1.4 ± 0.2	1.0 ± 0.2	97.0 ± 0.3	0.7 ± 0.2
Sucrose	13.9 ± 2.2	14.8 ± 1.5	0.6 ± 0.0	70.7 ± 3.7

[a] The total soluble carbohydrate activity is the sum of the individual activities recorded in fructose, glucose, sorbitol, and sucrose.

[b] Each figure is the mean of 6 observations ± S.E.

Table 6: Percent 14C activity in fructose, glucose, sorbitol, and sucrose analysed in whole explant samples of 0-3 apple culture supplied separately with each of these labelled carbon sources at both Stages II and III.

Carbon Source Supplied	Activity recorded in: (% of the total soluble carbohydrate activity)[a]			
	Fructose	Glucose	Sorbitol	Sucrose
		Stage II		
Fructose	64.8 ± 7.6[b]	20.2 ± 6.0	4.1 ± 1.2	11.1 ± 0.8
Glucose	0.2 ± 0.2	85.6 ± 3.2	5.3 ± 1.8	8.9 ± 1.3
Sorbitol	0.0 ± 0.0	0.5 ± 0.9	96.3 ± 1.6	3.3 ± 1.4
Sucrose	18.2 ± 2.3	36.1 ± 2.9	4.7 ± 0.5	41.0 ± 1.9
		Stage III		
Fructose	83.2 ± 1.6	7.6 ± 1.6	2.7 ± 0.2	6.5 ± 0.4
Glucose	4.2 ± 0.3	88.5 ± 0.6	0.6 ± 0.1	6.6 ± 0.7
Sorbitol	3.7 ± 0.7	0.6 ± 0.3	93.2 ± 1.1	2.5 ± 0.7
Sucrose	17.9 ± 1.6	15.6 ± 1.3	1.0 ± 0.2	65.6 ± 2.7

[a] The total soluble carbohydrate activity is the sum of the individual activities recorded in fructose, glucose, sorbitol, and sucrose.

[b] Each figure is the mean of 6 observations ± S.E.

greatest (20.5%) followed by small quantities into fructose (1.9%) and sorbitol (0.5%). When ^{14}C-sucrose was supplied, its conversion into glucose was greater (32.3%) followed in order by intermediate quantities into fructose (11%) and sorbitol (6.3%). In Stage III, these trends in interconversions were generally similar.

Although in SM (Table 5) and O-3 (Table 6) cultures the relative order of interconverted sugars tended to be similar to that of the MA cultures, in general, a smaller proportion of each supplied sugar became interconverted. It is noteworthy that SM explants interconverted ^{14}C-sucrose and ^{14}C-sorbitol to the lowest extent (Tables 4, 5, and 6).

Discussion

The results of this study indicated that in the apple shoot cultures, ^{14}C-fructose interconverted predominantly into glucose, ^{14}C-glucose predominantly into sucrose, and ^{14}C-sucrose predominantly into glucose. There was little interconversion of sorbitol.

The presence of normally lower quantities of fructose in plants is reflective of a more rapid turnover of this carbohydrate (Fowler 1978) involving either a direct utilization of fructose into the oxidative and synthetic pathways, or the action of hexoseisomerase (Duffus and Duffus 1984) converting fructose into glucose. In view of the generally lower quantities of unmetabolized fructose present after ^{14}C-fructose is fed to each of the three apple genotypes (Tables 4,5, 6), this suggests a more rapid turnover of the fructose pool than that in glucose in apple shoot cultures. This observation is strenghtened by the fact that when sucrose was supplied and metabolized by the apple culture, more glucose than fructose was found in the tissues. A similar observation has been made when apricot leaves were supplied with $^{14}CO_2$ (Bieleski and Redgwell 1977).

Sorbitol synthesis occurs very rapidly in leaves following a $^{14}CO_2$ exposure (Anderson et al. 1961, 1962; Bieleski and Redgwell 1977) in the light. However, in the present investigations (Tables 4, 5, 6) sorbitol was synthesized, although at a low level in the dark. This result indicates, as previously observed by Grant and ap Rees (1981) in apple seedlings, that the synthesis of sorbitol does not depend directly upon the presence of light.

The fact that sorbitol has remained largely unmetabolized within the explants (Tables 4, 5, and 6) suggests that in micropropagated apple shoots, it could be fulfilling an important reserve and/or osmotic role, rather than being generally used for oxidative pathways. Similar results showing a low metabolism of sorbitol have also been found with apple seedling, mature leaf, or fruit explants (Grant and ap Rees 1981, Priestley 1983, Berüter 1985). For instance, Grant and ap Rees (1981) showed that in apple seedlings 80-90% of the ^{14}C-sorbitol supplied remained unmetabolized after six hours in all parts of the plants, except in root apices where an active utilization of sorbitol ocurred. In plums 82% of labelled sorbitol absorbed by the cut stems was still present as such five hours after treatment (Anderson *et al*. 1962).

CHAPTER V

GENERAL DISCUSSION

To the best of the author's knowledge, this study is the first conducted to elucidate the utilization and metabolism of sorbitol in micropropagated apple shoots. Previously Chong and Pua (1985) found differences when sorbitol, sucrose, glucose or fructose were supplied to cultures of various apple genotypes, and under certain circumstances sorbitol was found to be as good as, or more beneficial to growth of micropropagated apple explants during shoot multiplication and rooting stages. Pua and Chong (1984) have further demonstrated the special requirement for sorbitol during organogenesis of meristem tip cultures of Malus robusta Rehd. No. 5 rootstock.

In view of the findings of the present study, such beneficial effects of the sorbitol cannot be explained by a preferential uptake or a more active interconversion of sorbitol in comparison to related carbon sources. Since it is generally considered that organ formation and development have high-energy requirements (Thorpe and Murashige 1970, Thorpe and Laishley

1973, Thorpe 1978), it was assumed that meristem tips of Malus robusta Rehd. No. 5 possessed the "enzymatic machinery" required for the breakdown of sorbitol (Pua and Chong 1984). In fact, Negm and Loescher (1979) observed that sour and sweet cherry callus tissues which did not grow well when supplied with sorbitol had very low sorbitol dehydrogenase activity. In apple fruits (strong "sinks") normally utilizing sorbitol extensively (Hansen 1967, 1970), the activity of the NAD-SDH was found to be 13 times higher than in cotyledonary ("source") tissue (Yamaki 1980a). According to Loescher et al. (1982), as leaves develop and undergo the transition from "sink" to "source", the activity of sorbitol dehydrogenase decreases and that of aldose-6-phosphate reductase (A6PR) increases. Thus, perhaps, the most important prerequisite for the utilization of sorbitol by apple cultures is the presence of the necessary enzymes of catalysis (Chong and Taper 1974, Thorpe 1982a).

Explants in culture have been shown to become adapted to different carbohydrates. For instance, Golden Delicious apple calli grown on sorbitol have shown noticeable improvement in growth after several subcultures on this carbohydrate (Pech et al. 1974). Similarly, growth of Reliance peach (Prunus persica (L.) Batsh.), previously initiateed on sucrose medium was found to be better on sorbitol medium in comparison with corresponding sucrose medium (Coffin et al. 1976). In cultures of Malus robusta No. 5 there was a delayed rooting response of shoots on sorbitol medium although after a three week period shoots rooted as well on sorbitol medium as on sucrose medium (Pua 1983). In these studies, cultures were exposed for a relatively long period (3 weeks or more) (Pua 1983), unlike what was done in the present study, in which ^{14}C-sorbitol was supplied for only two hours. Thus there was not sufficient time for complete metabolism of the labelled source.

In *in vitro* cultures where the carbon source represents an important part of the medium composition, diffusional translocation is a major pathway for the uptake of the carbon source fraction occurring possibly through the apoplastic path (Thorpe 1982a) and the differentiated xylem and phloem. According to Beevers (1974), the unmetabolized sugar could have remained in the "apparent free space" (extracellular space) and thus was not immediatedly available for metabolic utilization.

In callus of morning glory (*Pharbitis nil* L.), Hisajima (1985) indicated that the supplied glucose or fructose was converted intracellularly to sucrose. Moreover, although some authors consider that carbohydrates can pass through the plasmalemma by simple diffusion (Berüter and Kalberer 1983, Parr and Edelman 1975), others believe that uptake across the plasmalemma is a catalyzed active process (Glasziou and Galer 1972, Maretzki *et al*. 1974, Bieleski 1977, Yamaki 1980a, Komor 1982, Thorpe 1982a). Thus a limited uptake within cells, and a possible absence or low rate of extracellular interconversion, may account for the high concentrations of the source carbohydrate found within apple explants (Tables 4, 5, and 6).

Experiments by Chong and Taper (1974) showed that when sorbitol (unlabelled) was supplied at a concentration of 3% to stem calli of various apple genotypes, there was a greater interconversion into sucrose than amounts unmetabolized. However, at a higher concentration of 6%, marked quantities of sorbitol remained unmetabolized as shown in Tables 4, 5, and 6. This evidence indicates that the physiological significance of sorbitol in micropropagated apple shoots might be dual: as an osmoticum and as a carbon reserve. Brown *et al*. (1979) established that during organogenesis of tobacco calli, only two thirds of the sucrose supplied was likely to fulfil a role as a carbon and energy source. The other third was required as an osmoticum, since this portion could be substituted by mannitol without any detrimental effects on shoot development (Brown *et al*. 1979). In this context, the water potential of the nutrient medium used is also an important factor in *in vitro* organogenesis. Klenovská and Balkavá (1983) have found that by using sorbitol at a higher osmotic potential (-4.8 bars) than sucrose (3%) (-6.5 bars) in the nutrient medium of apricot callus, the cultures demonstrated an increase in water absorption. Such an augmentation in water within callus tissues might interfere with the degree of protein hydration, as suggested by Thorpe and Murashige (1970), and ultimately regulate enzyme and metabolic activities of cells. It is known, for instance, that small variations (*e.g*. 10%) in the intracellular water content of plants have pronounced effects on various metabolic activities such as growth, respiration, photosynthesis, and enzyme synthesis (Hsiao 1973).

The results obtained in this study provided only a limited view of sorbitol metabolism in micropropagated apples. However the physiological significance of a dual role for sorbitol as an osmoticum and a carbon reserve was elucidated.

CHAPTER VI

SUGGESTIONS FOR FUTURE RESEARCH

Based on the "proton symport cotransort system" involved in the cellular uptake of hexoses (Komor et al. 1981), Singh and Maclachlan (1983) proposed that growth, and particularly cell expension, could be regulated differentially according to the cellular uptake of hexose rather than sucrose. The release of proton following the hexose uptake could have some effect on the pH of the cell walls and intercellular spaces and could interfere with cell enlargement and growth (Maclachlan and Singh 1983, Singh and Maclachlan 1983). The same authors have also postulated that differential growth could result from an inhibitory effect of the hexoses, as the latter were found to form insoluble material more readily than sucrose, and could impede cell expansion (Singh and Maclachlan 1983). These hypotheses, used to explain the superiority of sucrose over hexose in the stimulation of growth of pea epicotyls, could be adapted to sorbitol and tested with apple explants.

Pulse and chase studies using isotopic tracers may yield more information about the turnover of sorbitol and related carbohydrates. Cultures supplied with labelled CO_2 may indicate if in vitro synthesis of sorbitol occurs as readily as that in vivo during photosynthesis.

In view of differences due to differential adaptability of cultures to carbon sources, studies should be directed at maintaining stock cultures of one genotype on different carbon sources over several subcultures before studying the extent of variations of the cultures in terms of anatomical, morphological, and biochemical (starch and carbohydrate status) differences. The contribution of the supplied carbohydrates to the oxidative pathways (i.e. glycolysis and pentose phosphate) could also be an interesting area of investigation. Moreover the possible effects of carbon sources on the endogenous hormonal balance of micropropagated apple shoots is another area of investigation which might be fruitfull.

Finally, since little is known about the physiological changes occurring during acclimatization (Stage IV) (Donnelly et al. 1984), it is worthy to examine the possible impact that the presence from previous propagation stages of different carbon sources could have on the acclimatization of the plantlets to ex vitro conditions. Acclimatization is often a major problem in the micropropagation system (Read and Fellman 1985), and thus this type of study could have practical impact.

A time-course study of the carbohydrate composition and content in the media as well as within the explants may aid in the characterization of the role and the fate of these compounds in apple cultures and may help to elucidate the role of sorbitol as osmoticum.

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