

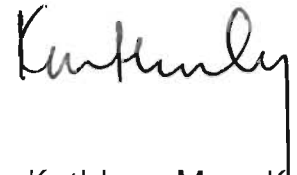
CARBOHYDRATE METABOLISM
IN
***PARTHENIUM ARGENTATUM* GRAY**

BY
KATHLEEN MARY KELLY

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PREFACE

The experimental work described in this thesis was carried out in the Department of Botany, University of Natal, Pietermaritzburg from January 1987 to December 1990 under the supervision of Professor Van Staden. These studies except where the work of others has been acknowledged, are the results of my own investigation.

A handwritten signature in black ink, appearing to read 'Kathleen Mary Kelly', with a long vertical line extending downwards from the end of the signature.

Kathleen Mary Kelly

December 1991

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Having spent ten post graduate years in the Botany Department it becomes very difficult to know whom to thank. However there remains one person who has encouraged, reprimanded, advised and supervised my work over these years. So it is Professor Van Staden whom I must first thank. Then to my research committee (past and present) for their advice and guidance. Then no project work is ever possible without the excellent service offered by the Technical staff of the Botany Department, Electron Microscope Unit and the Library. So to all those concerned, many thanks.

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ABSTRACT

The metabolism of carbohydrates in guayule is a subject which has not been considered with respect to its role in *cis*-polyisoprene synthesis. It has been suggested that acetate or sucrose act as the distal, and isopentenylpyrophosphate as the immediate precursor of the isoprenoid biosynthetic pathway.

Application of radioactive precursors to the leaves of guayule plants in Winter and Summer showed that the fate of the carbohydrate depends on the chemical structure of the carbohydrate and the time of application. [^{14}C] Sucrose was incorporated into the acetone (resin) fraction during the Summer and petroleum ether (rubber) fraction during the Winter. The amount of radioactivity that was translocated in Winter and Summer was similar. The loss of leaves during Winter reduced the area for photosynthesis, while the loss of carbon from the leaves during Summer, probably due to photorespiration, decreased the amount of available photosynthates. These two phenomena did not disadvantage the plant as far as the allocation of carbon was concerned. No plant components were acting as sinks during the Winter. The pith of the crown area incorporated the most radioactivity in Summer.

[^{14}C] Fructose was more readily translocated than [^{14}C] sucrose during a 12 hour experiment. When fructose was applied and plants were left for 48 hours, more radioactivity was translocated to the stems and roots during the Summer. The [^{14}C] from fructose was incorporated into the acetone (resins) rather than the petroleum ether (rubber) fraction in Winter therefore apparently having a different fate to [^{14}C] sucrose.

The principal reserve carbohydrates in guayule are fructans. Two types of fructans were detected and are referred to as water-soluble or ethanol-soluble fructans. The ethanol-soluble fructan polymers apparently played an active role

in metabolism of guayule and showed cyclic patterns of accumulation. The water-soluble fructans seem to be true reserve carbohydrates, depolymerizing when the carbon supply decreased at the end of Winter, and the demand for carbon increased at the inception of budbreak. Fructans provide carbon for budbreak and exposure of plants to longer days and higher temperatures did not seem to alter this role. It is proposed that fructans are providing carbon for budbreak and renewed growth and are utilized for flowering when required.

Starch production occurs during the warmer months in the leaves and young stems. Starch is synthesized from the immediate photosynthetic supply and it is this source of carbon which is utilized for the synthesis of *cis*-polyisoprene (rubber). Sucrose in the cytosol is sequestered for *cis*-polyisoprene synthesis while fructose, which can enter the plastid, is providing carbon for the synthesis of isoprenoids. Compartmentation of resin and rubber production ensures that the supply of carbon is adequate for both processes.

As *cis*-polyisoprene synthesis occurs at a time when the plant is not disadvantaged by insufficient carbon, induction of rubber transferase enzymes would not depend on excess substrate, but would use a more reliable cue like temperature or daylength. Any attempt therefore to increase the carbon supply in guayule during the winter months would not necessarily lead to partitioning into *cis*-polyisoprene, but may be stored as fructan to ensure that, at budbreak, the plant has an adequate and utilizable carbon supply.

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GENERAL INTRODUCTION

Parthenium argentatum Gray, a desert shrub from Mexico and Southwest Texas is commonly known as guayule and has been a commercial source of rubber since 1888. During the Second World War, with the Japanese invasion of Malaysia, the source of much of the natural rubber tapped from *Hevea brasiliensis* (A.Juss) Muell. Arg., stimulated the establishment of the Emergency Rubber Project in the United States of America. A number of plants, including *Parthenium argentatum* were investigated as sources of natural rubber. Much of this early research forms the basis of our present knowledge concerning the cultivation and rubber production in guayule. Unfortunately the project was terminated in 1946, only to be resumed in 1975 when the potential of guayule as a plant for cultivation on marginal land was realised, and pressure from the Organisation of Petroleum Exporting Countries once again emphasised the vulnerability of this strategic commodity.

Although synthetic rubber has been able to substitute for some of the domestic functions of natural rubber, it is susceptible to oxidative degradation and requires the protection of additional antioxidants (ALLEN AND BLOOMFIELD, 1963). It is also unsuitable where heat build-up is a problem, especially in aircraft, heavy vehicle tyres and in tank pads (BONNER, PARKER AND MONTERMOSO, 1954). Synthetic rubber is derived from oil and the price is therefore governed by the price of oil. In addition, the use of oil contributes to the depletion of the world's oil and gas reserves. The western world is largely dependent on the Middle East for these oil supplies. The January 1991 Middle East War and the resulting destabilisation in this region further strengthened the need for alternative sources of hydrocarbons.

The increase in oil prices during the 1970's led to a change in attitude concerning energy resources, resulting in considerable discussion on long-term reserves of fossil fuel. There was also a reduction in world energy consumption, renewed research into non-fossil sources of power, and renewed

interest in the power of plants as sources of fuels and materials (ALLEN AND JONES,1988), especially *Parthenium argentatum*, which would enable the cultivation of rubber to extend into the semi-arid regions of the world.

CALVIN (1984,1987) promulgated the merits of plants as "petrochemical" feedstocks. All plants reduce carbon dioxide to carbohydrate, yet there are many which go further and reduce carbon dioxide to hydrocarbons (fatty acids, glycerides and terpenes). Rubber is a renewable energy source and a good source of hydrocarbon.

Parthenium argentatum belongs to the tribe Heliantheae of the family Asteraceae and the genus *Parthenium* includes seventeen species ranging from woody to small herbaceous shrubs. A comprehensive taxonomic treatment of the genus was provided by ROLLINS (1950). All seventeen species are native to the sub-tropical and temperate parts of north and south America (NAQVI AND HANSON,1983; NAQVI, YOUNGNER AND RODRIGUEZ,1984), including the Chihuahuan desert in southwestern Texas and northern Mexico (LLOYD,1911).

Guayule is the only temperate-zone plant that produces linear, high molecular weight *cis*-polyisoprene rubber (BACKHAUS AND NAKAYAMA,1988). Other *Parthenium* species produce low yields of low molecular weight oligomers. The molecular weight and molecular weight distribution can vary with cultivar. The rubber content of guayule varies between three to seventeen per cent (GOSS,1991). Guayule may produce one kilogram of rubber and 0.75 kilograms of resin for every six kilograms of defoliated plant. The remaining 4.25 kilograms although a potential source of bioenergy (HALL AND OVEREND,1987), has until now been regarded as waste .

Guayule appears to be ideal for South Africa as it has a wide environmental tolerance. The plant can be cultivated in arid regions and requires limited irrigation, although it can be killed by freezing temperatures and is not salt-tolerant (HINMAN,COOKE AND SMITH,1985). Use could therefore be made of the

increasing marginal farming land without the added expense of irrigation. Rubber is regarded as a strategic material for defence purposes and stocks are always maintained in excess of that which is required for local needs. A local source of rubber would ensure control of the supply and price of the product, reducing both dependence on imports and foreign debt. Another advantage is that cultivation involves labour intensive practises which could form the basis of a major upliftment of the third world component by agricultural and industrial developments.

The guayule project was established in South Africa during the Second World War and in 1978 the University of Stellenbosch was asked to investigate guayule in South Africa for a private company, Environtech (Pty.) Ltd.. In 1979, the collaborative programme administered by the Cooperative Scientific Programmes now incorporated into the Foundation for Research and Development of the South African Council for Scientific and Industrial Research (CSIR) was established to determine whether rubber could be produced economically in this country. During the 1980's South Africa had become the focus of a variety of sanctions and required local sources of strategic commodities. If these initial findings were successful then the skills required to establish an agrochemical industry could be developed.

The economic feasibility of cultivating this shrub is very controversial. In order for the project to become profitable the low percentage of rubber per plant would have to be increased, possibly by breeding and selection (NAQVI, YOUNGER AND RODRIGUEZ, 1984), the use of bioregulators (YOKOYAMA, HSU AND HAYMAN, 1984) or a combination of these factors. Appropriate cultivars would have to be selected for the cultivation of marginal land in view of the great variability which exists in the resin and rubber contents of guayule cultivars (NAQVI, 1986). The costs of farming practises would need to be reduced, and lastly, the extraction and processing would have to be adapted for local conditions.

CHAPTER 1

REVIEW OF THE LITERATURE PERTAINING TO RUBBER BIOSYNTHESIS

1.1 **cis-POLYISOPRENE SYNTHESIS**

1.1.1 INTRODUCTION

Rubber or *cis*-1,4-polyisoprene is synthesised and accumulated by over 2000 species of plants (ARCHER AND AUDLEY,1973). Three types of *cis*-polyisoprenes are produced, of which rubber has the highest molecular weight and has elastic properties. Rubber is not only composed of *cis* bonds but also has three or four *trans* bonds (TANAKA,1984,1985). Gutta percha is a low molecular weight *trans*-polyisoprene, and is harder than rubber, while chicle is an equal mixture of low molecular weight *cis*- and *trans*-polyisoprenes (HAGER AND MEYER-BERTENRATH,1966) (Table 1).

A number of reviews have been published on the phenomenon of rubber production. Topics covered included the factors affecting, and the physiology of rubber production (BONNER AND GALSTON,1947), the biochemistry of rubber formation (ARCHER AND AUDLEY,1967), the nature and role of rubber in plants (ARCHER,1980), the activity of the enzymes in relation to rubber production (BENEDICT,1983), the regulation of rubber synthesis (BACKHAUS,1985) and an informative book on the investigations of the American researchers published by the Office of Arid Land Studies,U.S.A (WHITWORTH AND WHITEHEAD,1991).

In most cases the *cis*-polyisoprene is mixed with resins in a latex which is difficult to use when the content of the resin is high. Latex is a fluid, generally milky in appearance, which is produced and stored, rarely , in parenchyma cells or more frequently in laticifers. The families containing latex include the

Table 1: A list of the polyisoprene-producing plant families and species and the distribution of the polyisoprene in the plant (modified from ARCHER AND AUDLEY,1973).

Family	Plant	Location
RUBBER PRODUCERS		
Apocynaceae	<i>Alstonia</i> species <i>Dyera</i> species <i>Funtumia elastica</i> Stapf. <i>Landolphia</i> species <i>Hancornia speciosa</i> Gomez	Laticifers in bark and in some species of <i>Alstonia</i> and <i>Dyera</i> , in the medullary rays
Asclepiadaceae	<i>Cryptostegia grandiflora</i> R.Br.	Branched laticifers in all organs of plant, 80% leaf rubber in collenchyma, also in parenchyma of stem and root
Compositeae	<i>Parthenium argentatum</i> Gray	Mainly in the parenchyma of the stem and root
	<i>Scorzonera tau-saghyz</i> Lipschitz & Bosse	Anastomosing laticifers throughout plant, mainly in secondary phloem of root
	<i>Solidago</i> species	Leaf parenchyma
	<i>Taraxacum kok-saghyz</i> Rodin	Anastomosing laticifers throughout plant, mainly in secondary phloem of root
Euphorbiaceae	<i>Hevea brasiliensis</i> (A.Juss) Muell. Arg.	Anastomosing laticifers present in the secondary tissues of bark, pith and leaves
	<i>Manihot glaziovii</i> Muell. Arg.	Anastomosing laticifers in phloem of stem and leaf, at margin of pith and primary cortex
Moraceae	<i>Castilla</i> species	Single elongate laticifers in bark,pith and leaves
	<i>Ficus elastica</i> Roxb.	Branched laticifers in bark,pith and leaves
GUTTA PRODUCERS		
Celastraceae	<i>Euonymus europaeus</i> L.	Secretory sacs in secondary phloem of old stems
Eucommiaceae	<i>Eucommia ulmoides</i> Oliver	Unicellular laticifers in bark and leaves
Euphorbiaceae	<i>Cnidoscolus elasticus</i> Lundell <i>Cnidoscolus tepiquensis</i> (Costantine & Gallaud) Lundell	Laticifers
Sapotaceae	<i>Mimusops balata</i> Gaertn.	Laticiferous sacs present in bark,pith and leaves
	<i>Palaquium gutta</i> Burck.	
CHICLE PRODUCER		
Sapotaceae	<i>Achras sapota</i> L.	Laticifers in bark,pith and leaves.

Compositae, Apocynaceae, Asclepiadaceae, Euphorbiaceae, Moraceae, Celastraceae, Eucommiaceae and Sapotaceae (ARCHER AND AUDLEY,1973).

Hevea is the most exploited of the rubber-producing plants. Guayule and *Hevea* produce predominantly high molecular weight polymers when compared to plants which produce rubber of an inferior quality (HAGER, MacARTHUR, McINTYRE AND SEEGER,1979). Only a small number of rubber-producing plants can be exploited and few cultivated species are economically important.

Hevea, as the most exploited source of natural rubber, has been extensively studied at the horticultural, physiological and biochemical levels. In *Hevea* the rubber is located in the latex which is tapped from the trees. The extracted latex has been utilised to determine rubber synthesis *in vitro* as it has not been possible to study rubber synthesis *in situ*. Thus experimentation has looked at yield as an indicator of rubber transferase activity and rubber production. However, yield is governed by factors which do not affect rubber synthesis directly. High rainfall, for example, decreases the viscosity of the latex during the Summer, making tapping possible. During Winter the latex has a high viscosity due to the low rainfall making tapping difficult or impossible (JACOB,SERRES, PRÉVÔT,LACROTTE,VIDAL,ESCHBACH AND D'AUZAC,1988). It has not been determined if this increased viscosity could be due to increased rubber transferase activity and rubber production during the Winter.

The biochemical system of rubber synthesis in *Hevea* and guayule may be fairly similar although anatomically the sites of rubber deposition differ. *Hevea* rubber particles are suspended in latex in laticifers while guayule rubber is found in the vacuoles and cytoplasm of the parenchyma cells of the cortex, pith and vascular rays of the stems and roots (BAUER, 1982). Thus, in contrast to *Hevea*, guayule has to be harvested destructively before processing can take place. This means that new fields of guayule must be planted every three or four years.

1.1.2 ELUCIDATION OF THE ISOPRENOID BIOSYNTHETIC PATHWAY

About one per cent of all plant species has the capacity to synthesize *cis*-polyisoprenoids (MANN,1987). Rubber or *cis*-polyisoprene is synthesized at the end of the isoprenoid biosynthetic pathway which includes the acetate - mevalonic acid (MVA) pathway (**Figure 1**). This pathway gives rise to the terpenes which are the most abundant and widely distributed group of natural products in plants (HASLAM,1986). All have the isoprene carbon skeleton as the structural basis and are derived from isopentenylpyrophosphate (IPP) (**Figure 2**). Many terpenes play vital roles in the metabolism and development of plants and are involved in the synthesis of abscisic acid, gibberellins, chlorophylls, and sterols. All these are produced by a common biosynthetic pathway, and for this the plant must have fine control mechanisms to ensure the necessary compounds are in the right place at the right time (GRAY,1987). This will be discussed in the section dealing with secondary metabolism.

THE PRECURSORS

It was realised a long time ago that the leaves play an important role in the synthesis of rubber. Defoliation of guayule prevented assimilates from the leaves reaching other organs and no rubber was formed (SPENCE AND McCALLUM,1935). Yet, defoliated guayule plants supplied with $^{14}\text{CO}_2$ are able to incorporate the isotope into rubber (GILLILAND,VAN STADEN AND MITCHELL,1985). This implies that the stems are able to fix CO_2 . A relationship therefore exists between CO_2 assimilation and rubber formation or between rubber and carbohydrate metabolism (BONNER,1949,1975).

A lack of precursors or substrate from the leaves seems to be the limiting factor in the synthesis of rubber (BONNER,1949). Using aseptic tissue culture techniques, it was shown that only the leaves from plants involved in active rubber production were able to promote rubber production in isolated stem

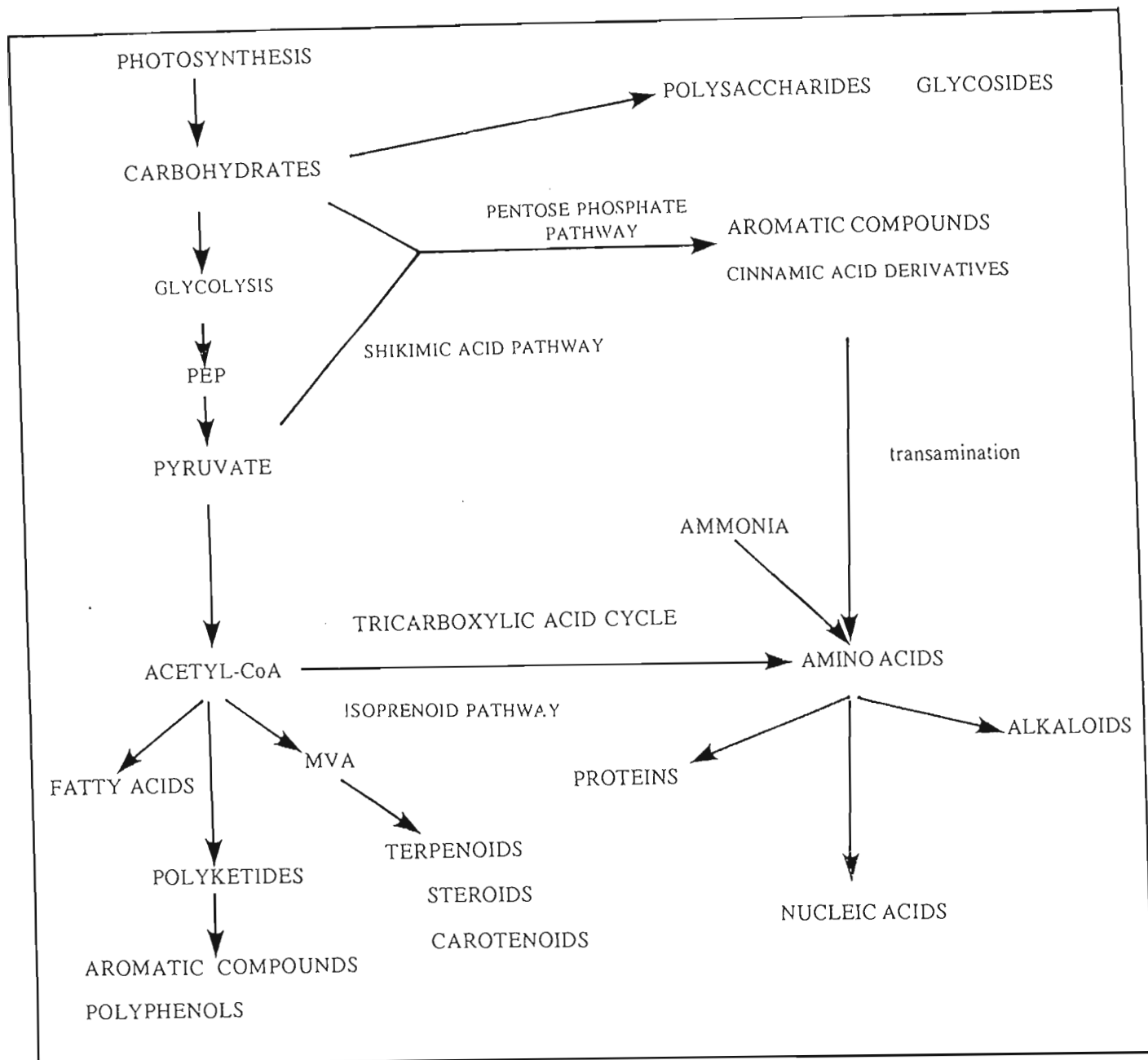


Figure 1.

A general representation of the biosynthetic pathways in plants to show the relationship between the isoprenoid and the other biosynthetic pathways.

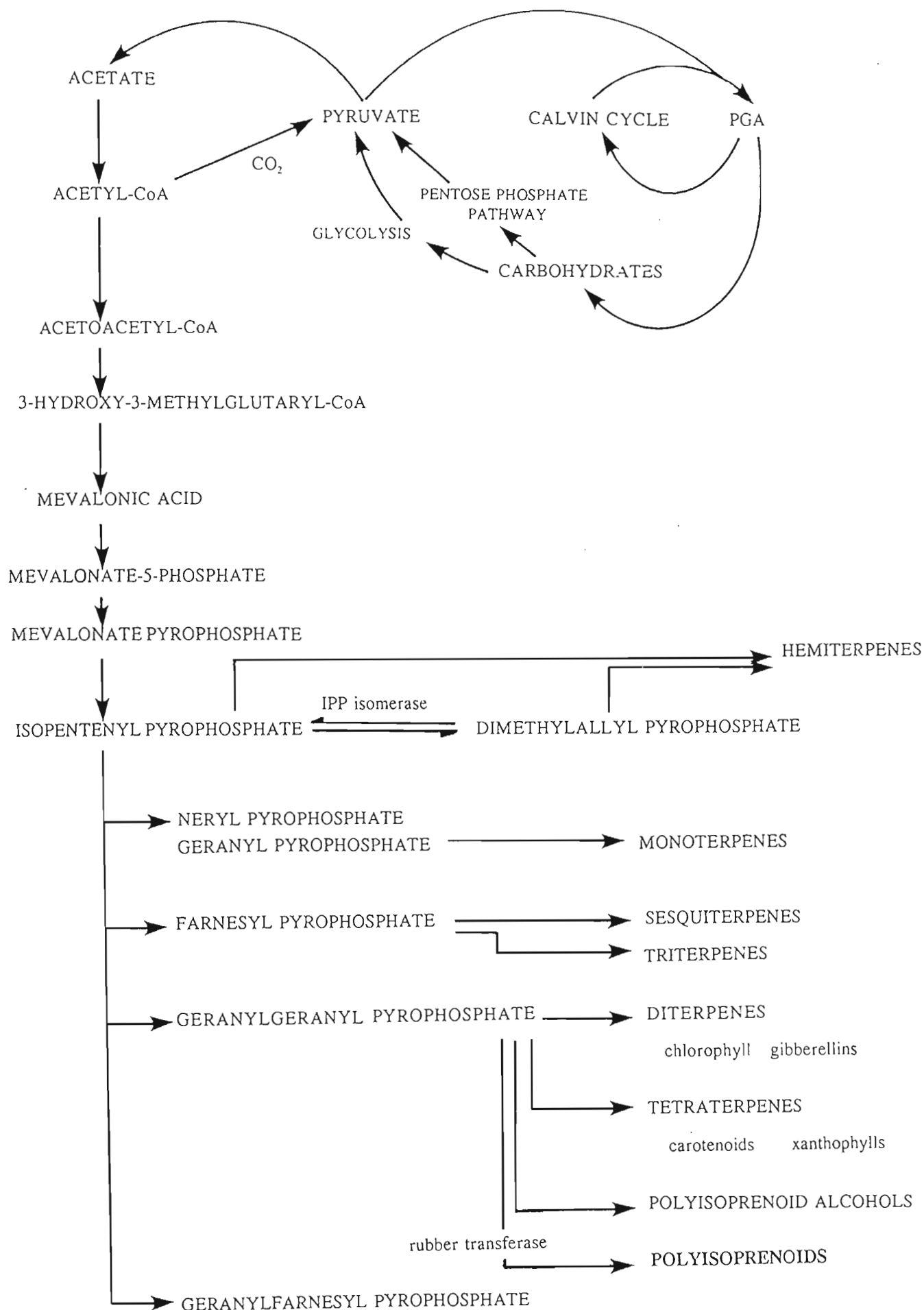


Figure 2.

The isoprenoid biosynthetic pathway including the contribution of the carbohydrates.

samples of guayule. The addition of acetate or substances metabolically related to acetate also promoted rubber production (BONNER AND ARREGUIN,1949). Acetate was therefore thought to supplement available carbohydrate as an additional carbon source if extensive rubber formation was to take place (ARREGUIN,BONNER AND WOOD,1951).

The findings of BONNER AND ARREGUIN (1949) that acetate-related substances promoted rubber synthesis in guayule led to similar investigations in *Hevea*. [1-¹⁴C] Acetate was incorporated into rubber in *Hevea* latex *in vitro*. However, pyruvate incorporation was slower while sucrose and carbon dioxide were not incorporated (TEAS AND BANDURSKI,1956). Subsequently, however, a slow incorporation of [¹⁴C] sucrose into *Hevea* rubber was reported (BANDURSKI AND TEAS,1957). PARK AND BONNER (1958) were unable to substantiate this and suggested that the uptake was dependent on the condition of the trees, which would influence the quality of the latex. The rate of utilization of sucrose by *Hevea* latex is only one tenth the rate of rubber production (TUPÝ,1969). BEALING (1969) concluded that the low glucose and fructose levels in the latex of *Hevea* were due to competition for carbohydrate precursors between glycolysis and the α -inositol pathway.

Rubber may be formed by other substances which enter the latex vessels from the surrounding cells. As the extracted latex does not contain nuclei and mitochondria, these organelles are situated in the parietal cytoplasm which is not expelled on tapping (BENEDICT,1983), rubber synthesis is not likely to improve when more substrate is added (BEALING ,1976). In guayule, however, where the rubber is located in the parenchyma cells, KELLY AND VAN STADEN (1987) were able to show rapid incorporation of [¹⁴C] sucrose into guayule rubber. Sucrose is the basic molecule for all plant syntheses. Isoprenoids and *cis*-polyisoprene are therefore directly or indirectly derived from sucrose.

THE ROLE OF ACETATE

Acetate is only metabolized once it had been transformed to acetyl-Coenzyme A (acetyl-CoA) (LYNEN AND HENNING, 1960). The idea that the acetate required transformation was confirmed by STUMPF, BROOKS, GALLIARD, HAWKE AND SIMONI (1967) who found that although acetate was the most effective substrate for incorporation into rubber and long chain fatty acids, it probably does not occur in the free form in leaf tissues.

Acetate is not the sole source of acetyl-CoA for terpenoid synthesis (SHAH, ROGERS AND GOODWIN, 1967): it may also arise via pyruvate from glycolysis (DENNIS AND MIERNYK, 1982) and via the glycolate pathway in peroxisomes (SHAH AND ROGERS, 1969). The β -oxidation of fatty acids (in peroxisomes) and the metabolism of branched chain amino acids will, although not proven, also yield acetyl-CoA (BACKHAUS, 1985). Most polyisoprene research has emphasized that acetate and pyruvate are major sources of acetyl-CoA (BENEDICT, 1983).

Although anatomically the site of rubber synthesis differs in guayule and *Hevea*, biochemically the systems are presumed to be similar. Any discussion of the isoprenoid pathway therefore cannot be based on guayule work alone but must draw on the extensive *Hevea* research. The biosynthesis of rubber can be divided into three stages:

1. the generation of acetyl-CoA ;
2. the conversion of acetyl-CoA to isopentenylpyrophosphate (IPP) via mevalonic acid (MVA) and ;
3. the polymerization of IPP into rubber (Figure 2)

THE GENERATION OF ACETYL-CoA

There are two theories relating to the intracellular organization of the isoprenoid pathway. GOODWIN AND MERCER (1963) proposed that there are spatially separate pathways from acetyl-CoA to the isoprenoid compounds, located in the

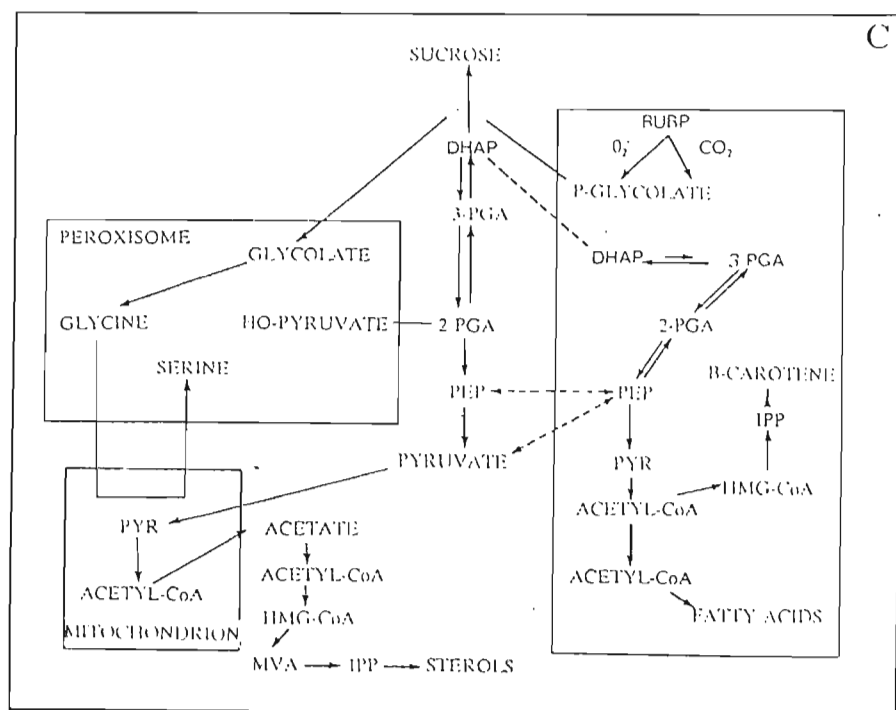
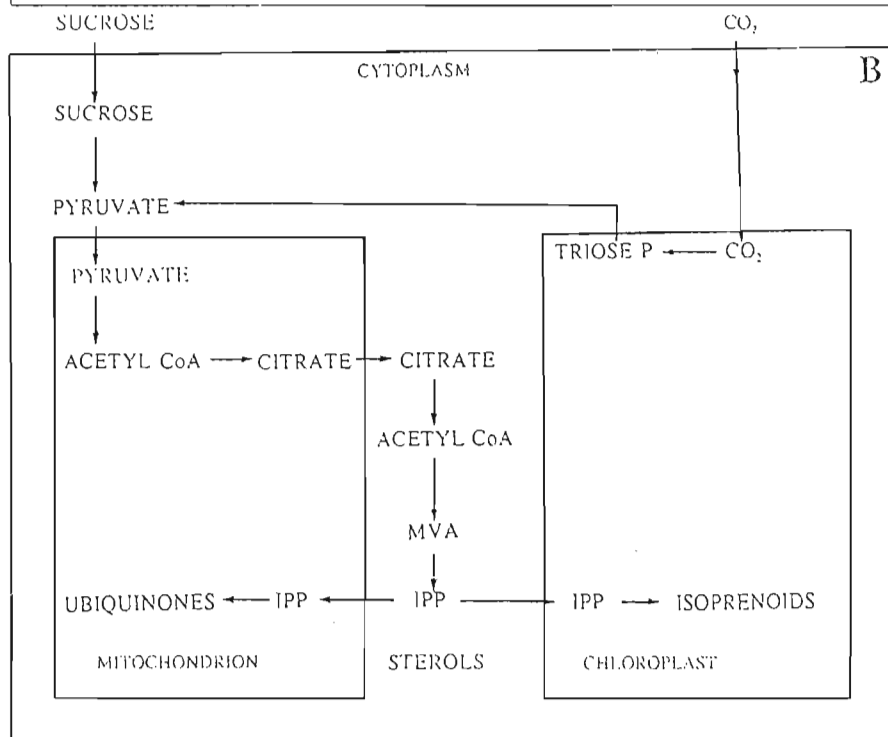
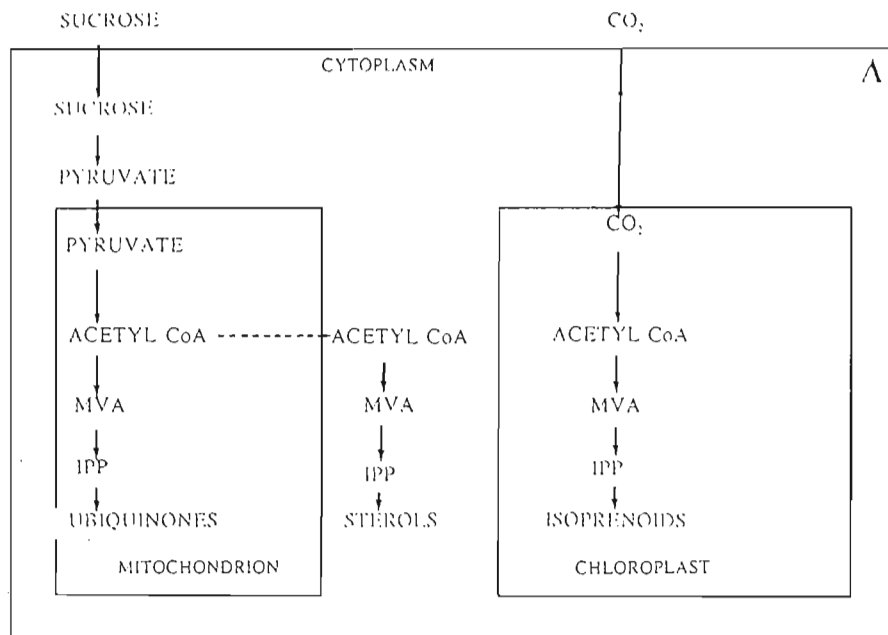
cytosol, plastids and mitochondria. The different compartments are separated by membranes which are impermeable to the intermediates of the pathway (GOODWIN,1965ab)(Figure 3A).

The second theory (KREUZ AND KLEINIG,1984), was based on the fact that the enzymes of IPP synthesis could not be found in plastids and mitochondria. In this case, IPP was ascribed a pivotal role as a common and exclusive precursor molecule of all prenyl-lipids, independent of their intracellular localization. Accordingly, IPP was synthesized in the cytosol and was translocated to the different subcellular compartments for the synthesis of specific compounds. In this theory, all organelles are completely dependent upon the cytoplasmic capacity for MVA and IPP synthesis (Figure 3B). The validity of the first theory was tested on the basis of the sub-cellular location of enzymes and the permeability of the organelle membranes to intermediates of the pathway (GRAY,1987) and the second theory on the purity of the chloroplast preparations in which hydroxymethylglutaryl-Coenzyme A reductase (HMGR) activity had been assayed (BACH,WEBER AND MOTEL,1990).

Acetyl-CoA, according to the first theory must be located in the three separate compartments, and the chloroplasts are required to convert carbon dioxide to IPP (GOODWIN,1965a). The incorporation of radioactive carbon dioxide by isolated chloroplasts was reported by GRUMBACH AND FORN (1980) who supplied [^{14}C] carbon dioxide, phosphoglycerate, phosphoenolpyruvate (PEP), acetate and MVA to isolated intact chloroplasts of spinach (*Spinacia oleracea* L.). All the supplied substrates were capable of crossing the chloroplast envelope, and all except PEP were incorporated into β -carotene thus converting the applied substrates to IPP. However, the incorporation of the radioactivity from the [^{14}C] carbon dioxide was so low (0.008% of the applied [^{14}C] carbon dioxide), that any cytoplasmic contamination would have negated the conclusion of a chloroplastic location of the biosynthetic pathway.

Figure 3.

A diagrammatic representation of the theories pertaining to the intracellular organization of the isoprenoid biosynthetic pathway. A: GOODWIN AND MERCER (1963) B: KREUZ AND KLEINIG (1984) C: SCHULZ-SIEBERT AND SCHULTZ (1987)



Young chloroplasts, however, were able to form isoprenoid compounds from carbon dioxide although this ability disappeared once the chloroplasts had matured (SCHULZ-SIEBERT AND SCHULTZ, 1987; HEINTZE, GÖRLACH, LEUSCHNER, HOPPE, HÄGELSTEIN, SCHULZE-SIEBERT AND SCHULTZ, 1990), suggesting that the dependence of organellar isoprenoid synthesis on cytoplasmic IPP formation might be a function of age.

In some plants, including guayule (REDDY AND DAS, 1987), acetyl-CoA is generated via a pyruvate dehydrogenase complex (PDC) in the chloroplast, with a direct flow of carbon from photosynthate to the PDC and further along the pathway (LIEDVOGEL AND STUMPF, 1980). The rate at which acetyl-CoA is produced is adequate for isoprenoid synthesis (SCHULZE-SIEBERT, HEINTZE AND SCHULTZ, 1987), but does not fulfil the substrate requirement for fatty acid synthesis. The missing intermediates are supplied from the cytosol by importation across the membranes supporting the KREUZ AND KLEINIG (1984) theory. This was also the case for mature chloroplasts where IPP formed from MVA on the endoplasmic reticulum was imported into the chloroplasts (HEINTZE, GÖRLACH, LEUSCHNER, HOPPE, HÄGELSTEIN, SCHULZE-SIEBERT AND SCHULTZ, 1990).

Isopentenylpyrophosphate and acetate are imported into the plastids for the synthesis of plastidic isoprenoids (KREUZ AND KLEINIG, 1984) and for fatty acid synthesis respectively (ROUGHAN, HOLLAND, SLACK AND MUDD, 1979). A separate sub-cellular location for the synthesis of acetyl-CoA for isoprenoid and fatty acid synthesis would simplify the control mechanism needed to regulate the provision of acetyl-CoA for two separate biosynthetic pathways.

The mitochondria also possess a PDC which can generate acetyl-CoA for the Tricarboxylic Acid Cycle (SCHULZE-SIEBERT, HEINTZE AND SCHULTZ, 1987), although no one has established if this acetyl-CoA can be used directly for isoprenoid synthesis within the mitochondria. However, pyruvate dehydrogenase and a PDC are not located in the chloroplasts of all plants and acetyl CoA must be

derived by other pathways (ROUGHAN,HOLLAND AND SLACK,1979; ROUGHAN,HOLLAND,SLACK AND MUDD,1979; MURPHY AND STUMPF,1981).

Direct movement of acetyl-CoA from the mitochondria or plastids into the cytosol as envisaged by GOODWIN (1965a) is unlikely as the boundary membranes of these organelles are impermeable to acetyl-CoA. In the KREUZ AND KLEINIG (1984) model, ATP-citrate lyase (EC 4.1.3.8) has sufficient activity to provide acetyl-CoA for the synthesis of isoprenoids (GRAY,1987).

Enzymes which can convert MVA to IPP must exist both inside and outside the chloroplast for the first theory (GOODWIN AND MERCER,1963). BACH (1987), proposed that plastids and mitochondria (BROOKER AND RUSSELL,1975) possess their own HMGR and do not rely on cytoplasmic IPP, again emphasizing the independence of the plastid. However, the association of the HMGR with the membrane has yet to be demonstrated using immunocytochemical localization (GRAY,1987).

The chloroplast does not appear to be able to form IPP from MVA, although the necessary enzymes are present in the cytosol. In accordance with the second theory, LIEDVOGEL (1986) believes that the IPP is formed solely in the cytosol and that it is this IPP that is channelled to the different IPP-utilizing compartments, namely the endoplasmic reticulum, plastids and mitochondria.

The permeability of membranes was also used to determine which theory was the most suitable (TREHARNE,MERCER AND GOODWIN,1966). Aspartate, malate, glycolate, hexose phosphates, hexoses, phosphoglyceric acid, inorganic phosphate and ATP are all membrane-permeable substances (KURSANOV,1984). Malonate, malonyl-CoA and acetyl-CoA penetrate the membrane with difficulty, while the membrane may be uni-directionally permeable to pyruvate, that is, pyruvate enters the plastid readily but is unable to pass to extra-chloroplastic sites (SHAH AND ROGERS,1969). The plastid and the mitochondrial membranes are relatively permeable to IPP (KREUZ AND KLEINIG,1984). However, it is not known

how IPP moves across the plastid envelope membranes and if a transporter is involved. There is evidence for and against both theories.

The problem with the KREUZ AND KLEINIG (1984) theory is whether cytoplasmic IPP can serve as a substrate for the multi-branched, compartmentalized isoprenoid pathway. Various prenyltransferases and translocators would have to compete for IPP or its isomer dimethylallylpyrophosphate (DMAPP). Such sequential enzyme systems would have to possess substrate affinities which would differ in order of magnitude to allow for an unchanged substrate flow to each end product (BACH,1986; GRAY,1987).

The question of plastid autonomy is pertinent to guayule as the resin canal epithelial cells contain plastids which have reduced thylakoids (GILLILAND,APPLETON AND VAN STADEN,1988). The resin canal epithelial cells are the first cells in which rubber is deposited in the Winter and these cells secrete resin into the canals throughout the year. The resin canal epithelial cells are well connected with the surrounding parenchyma through plasmodesmata. The plastids in the resin canal epithelial cells have been implicated as the source of IPP for rubber synthesis (GILLILAND,APPLETON AND VAN STADEN,1988) although this has not been verified and the idea supports neither the GOODWIN AND MERCER (1963) nor KREUZ AND KLEINIG (1984) theory.

If IPP was synthesized in the individual cellular compartments of guayule, the carbon supply for *cis*-polyisoprenoid production would be located in the cytosol and *trans*-terpenoid synthesis in the plastids. If the autonomy of the chloroplast is a function of plastid age, then during the early stages of plastid development, acetate synthesized in the mitochondria could be transported into the plastid via the cytoplasm. This acetate would be converted to acetyl-CoA within the plastid and utilized for the *de novo* synthesis of fatty acids leaving the internally-produced acetyl-CoA free to serve as the substrate for isoprenoid synthesis (**Figure 3C**, SCHULZE-SIEBERT AND SCHULTZ,1987).

During the Winter months, the supply of carbon to the plastids would be reduced, limiting *trans*-terpenoid production. In the Summer months, carbon supply to the plastids is unlimited as *cis*-polyisoprene production is very low.

THE CONVERSION OF ACETYL-CoA TO IPP VIA MVA

The intermediates between acetate and MVA were elucidated once MVA had been identified as part of the cholesterol biosynthetic pathway (LYNEN,1967; HEPPER AND AUDLEY,1969). 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) is the intermediate following acetyl-CoA and acetoacetyl-CoA (BONNER,1955). HMG-CoA is incorporated into *Hevea* latex and was thought to be the rate limiting step as the process showed seasonal variation (HEPPER AND AUDLEY,1969). REDDY AND DAS (1986) demonstrated that HMGR, the enzyme which catalyses the reduction of HMG-CoA to mevalonate, could be isolated from guayule leaves. Two separate forms of HMGR occurred inside and outside the chloroplast of guayule respectively.

Using an inhibitor of HMGR, mevinolin, BACH (1987) showed that plastids and mitochondria are independent of cytoplasmic MVA synthesis. The intermediates between MVA and IPP were identified as 5-phosphomevalonate and 5-pyrophosphomevalonate (ARCHER, AUDLEY, COCKBAIN AND McSWEENEY,1963). MVA was phosphorylated by MVA-kinase (WILLIAMSON AND KEKWICK,1965) to produce mevalonate-5-phosphate which was followed by a second phosphorylation catalysed by MVA-phosphokinase (SKILLETER,WILLIAMSON AND KEKWICK,1966), to produce MVA pyrophosphate (MVA-PP). This MVA-PP is decarboxylated and dehydrated to IPP. Adenosine triphosphate from the carbohydrate degradation pathway provides energy for these reactions (LYNEN,1969).

Mevalonic acid has two functions in the cell cycle. It acts as a precursor for cholesterol which is required for cell membrane synthesis; and it has an

important initiating function in DNA replication (BACH,1987). It is poorly incorporated in *in vivo* tracer studies, and it has been suggested that MVA arises at the site of synthesis from sugars (LOOMIS AND CROTEAU,1973) and is synthesised endogenously in the chloroplast (GRUMBACH AND FORN,1980). [2-¹⁴C] Mevalonic acid is incorporated into *Hevea* latex very rapidly (PARK AND BONNER,1969).

THE POLYMERIZATION OF ISOPENTENYLPYROPHOSPHATE TO RUBBER

Guayule produces all the usual isoprenoid compounds including chlorophyll, gibberellins, carotenoids, xanthophylls, polyprenols, *trans*-terpenes (resins) and *cis*-polyisoprene (rubber). All these compounds rely on the primary carbohydrate substrate and IPP as precursors for this pathway. Resin and rubber are not the only sinks for carbon in the isoprenoid biosynthetic pathway. The resins are low molecular weight *trans*-terpenoids which diverge early on in the isoprenoid pathway (Figure 2). The levels of resin in guayule remain consistently high throughout the year (APPLETON AND VAN STADEN,1989). The resin composition varies with cultivation site, shrub strain and harvest date (SCHLOMAN,GARROT,RAY AND BENNETT,1986). Rubber biosynthesis has been reviewed by ARCHER (1980), ARCHER, AUDLEY AND BEALING (1982), BENEDICT (1983), BACKHAUS (1985) and ARCHER AND AUDLEY (1986).

INITIATION OF POLYMERIZATION

Isopentenylpyrophosphate is the building block for the isoprenoid biosynthetic pathway (Figure 2). The IPP molecule has to be polymerized to form rubber. There are two processes which have to take place: if there are no existing rubber particles then new rubber particles must be formed, or; in cells where rubber particles exist, IPP molecules have to be attached to the surface of these particles.

Isopentenylpyrophosphate is isomerized to 3,3-dimethylallyl pyrophosphate (DMAPP) by IPP isomerase (EC 5.3.3.2) (LYNEN AND HENNING, 1960). Isomerase activity has been detected but not characterized. This is followed by the head-to-tail condensations with IPP units to yield geranylpyrophosphate (GPP) or neryl pyrophosphate (NPP) (BEYTIA AND PORTER, 1976). GPP has a *trans* configuration and NPP a *cis* configuration. The chain continues by the transfer of a prenyl unit of a pyrophosphate donor to phosphate to generate a new pyrophosphate donor with one additional C5 prenyl unit in the chain. The mechanisms involved have been detailed by POULTER AND RILLING (1981).

Dimethylallylpyrophosphate and NPP were regarded as the starter molecules to initiate the formation of new rubber chains. Geranylpyrophosphate, a *trans* molecule was found to be as effective as NPP in initiating rubber synthesis (AUDLEY AND ARCHER, 1988). In fact, *trans,trans* farnesylpyrophosphate (FPP); *trans,trans,trans* geranylgeranylpyrophosphate (GGPP); and *cis,trans,trans* GGPP all stimulated rubber synthesis (ARCHER, AUDLEY AND BEALING, 1982; BENEDICT, 1986). This was unexpected as rubber had been regarded as a *cis* molecule with no *trans* bonds. TANAKA, SATO AND KAGEYU (1983) and TANAKA (1984, 1985) showed that there were three or four *trans*-isoprene units at the ω -end of the molecule in *Solidago altissima*, *Ficus elastica*, guayule and *Hevea*. Rubber synthesis was therefore similar to the biosynthesis of *cis,trans* oligoprenols (BABA AND ALLEN, 1978, 1980; HEMMING, 1983). Rubber differs from the other polyprenols in that 5000 to 7000 IPP units are added to the surface of existing molecules (TANAKA, 1984).

The initiation step for the synthesis of rubber molecules remains unknown. The "starter" molecule could be DMAPP which is required before chain elongation can occur (ARCHER AND AUDLEY, 1987). DENNIS AND LIGHT (1989) observed small particles, about 100 nm long, in *Hevea* serum-free rubber particle preparations which were thought to be composed mainly of protein "starter" molecules.

THE RUBBER TRANSFERASE ENZYME

The enzyme rubber transferase (EC 2.5.1.20) is thought to exist on the surface of existing rubber molecules and in the latex (McMULLEN AND McSWEENEY,1966). The rubber transferase was partially purified from *Hevea* and guayule and farnesyl pyrophosphate (FPP) synthase activity was observed (ARCHER AND COCKBAIN,1969; MADHAVAN AND BENEDICT,1984). Purified rubber transferase from *Hevea* latex co-purified with FPP synthase activity (LIGHT AND DENNIS,1989). CORNISH AND BACKHAUS (1990) disputed these claims by LIGHT AND DENNIS (1989) and maintained that their purified enzyme was not rubber transferase.

The rubber transferase requires a divalent cation (Mg^{2+}) to catalyse thousands of 1-4 *cis* condensations (Z-oligomerization) of IPP, the prenyl acceptor, to the allylic pyrophosphate acceptor (MADHAVAN, GREENBLATT, FOSTER AND BENEDICT,1989), the prenyl donor, before random termination occurs. Other prenyltransferases terminate after a characteristic and low number of additions of IPP to the allylic pyrophosphate substrate. For example, solanesyl (nonaprenyl) pyrophosphate synthase catalyzes 6 to 7 *trans* additions to GPP (SAGAMI, OGURA AND SETO,1977), undecaprenyl pyrophosphate synthase catalyzes 7 to 8 *cis* additions to *trans,trans*-FPP (MUTH AND ALLEN,1984), and dolichol pyrophosphate synthase catalyzes 13 to 18 *cis* additions to *trans,trans*-FPP (ADAIR, CAFMEYER AND KELLER,1984). In liver tissue, these terminal additions are located on the endoplasmic reticulum (ERICSSON, EKSTRÖM, CHOJNACKI AND DALLNER,1988). The nature of the allylic prenyl donor (BABA AND ALLEN,1978) or the Mg^{2+} concentration (FUGII, SAGAMI, KOYAMA, OGURA, SETO, BABA AND ALLEN,1980) affects the chain length made by other prenyltransferases by a few isoprene units. Product analysis shows loss of termination precision with increasing chain length. Enzymes may remove the terminal pyrophosphate group and be specific about chain length or the supply of initiator relative to monomer may be important (AUDLEY AND ARCHER,1988).

All early rubber transferase assays required a fresh preparation of washed rubber particles (WRPs). The existence of an insoluble form of rubber transferase associated with rubber particles was established by ARCHER, AUDLEY, COCKBAIN AND McSWEENEY (1963). The WRPs would form new rubber if an initiator DMAPP was added to the mixture (ARCHER, AUDLEY, COCKBAIN AND McSWEENEY, 1963; BERNDT, 1963; LYNEN, 1969). Evidence for a soluble form of rubber transferase was also presented (McMULLEN AND McSWEENEY, 1966) and a method for its purification was published (ARCHER AND COCKBAIN, 1969). However, these soluble extracts could not synthesize rubber when used alone: they have an absolute requirement for WRPs. When it was established that this soluble factor could be replaced by a series of initiators (BERNDT, 1963; ARCHER AND AUDLEY, 1987; AUDLEY AND ARCHER, 1988; MADHAVAN, GREENBLATT, FOSTER AND BENEDICT, 1989), the possibility arose that the soluble enzyme system was part of the initiating system synthesizing allylic pyrophosphate instead of polyisoprene.

The rubber transferase enzyme is firmly associated with guayule WRPs and exists as an insoluble form similar to the *Hevea* enzyme (CORNISH AND BACKHAUS, 1990). Additions of soluble enzyme extracts are not required for IPP-polymerization to occur in *Hevea*.

The utilization of soluble protein extracts combined with easily isolated WRPs from other plant sources, may have lead to erroneous conclusions (MADHAVAN AND BENEDICT, 1984; REDDY AND DAS, 1988). These soluble protein extracts may have supplied the enzyme(s) necessary to synthesize initiator molecules rather than supplying rubber transferase itself.

The activity of the rubber transferase observed in the WRPs was dependent on the guayule line tested: the line that produced more rubber had higher rubber transferase activity (CORNISH AND BACKHAUS, 1990). Isopentenylpyrophosphate substrate was found not to be the limiting factor and FPP (starter molecule) is implicated as the limiting substrate.

LIGHT AND DENNIS (1989) believe that it is the number of competent end groups in the rubber particle that limit rubber production. Active ends may not be distributed evenly among all particles, as large particles may have no growing ends and smaller particles may have multiple growing ends. This is not in accordance with the proposals of LYNEN (1969) who suggested that maximum levels of enzymes for isoprenoid synthesis may impose limitations on the speed of end-product formation. The main criticism with this work is that these workers are dealing with FPP-synthase and that the controls in the set of experiments were inadequate.

THE RUBBER PARTICLE

Electron microscopy has revealed that rubber particles are coated with a glycoprotein coat of unknown function (ARCHER,1980) which may be a major polypeptide and an integral membrane protein (BACKHAUS AND BESS,1986). This glycoprotein extends into the cytoplasm and could not be washed from the rubber particles with simple aqueous buffers. This glycoprotein layer contained a number of rubber molecules. Rubber transferase activity was associated with the glycoprotein layer. Washing rubber particles revealed a few predominant polypeptides, of which the 48500 M_r polypeptide is the most abundant "rubber protein" in all the guayule lines and has been tentatively identified as rubber transferase (BACKHAUS AND BESS,1986). This is not the same as the 38000 M_r polypeptide which is characteristic of FPP synthase in *Hevea*.

The glycoprotein is referred to as the rubber elongation factor (REF) in *Hevea* (LIGHT,LAZARUS AND DENNIS,1989). In this case it is found bound to serum-free rubber particles. The REF is 137 amino acids long, has a molecular mass of 14600 Daltons, and lacks the amino acids cysteine, methionine, histidine and tryptophan. The amine terminus is highly charged, containing only acidic residues and the amount of REF in whole *Hevea* latex is proportional to the rubber content (DENNIS,HENZEL,BELL,KOHR AND LIGHT,1989).

BACKHAUS AND BESS (1986) proposed that IPP is taken from the cytoplasm and condensed to a growing rubber pyrophosphate or prenylpyrophosphate starter molecule in the interior of the rubber particle. The molecule increases in size until it dissociates from the rubber transferase. There are a finite number of molecules that the particle may contain. In time the particle increases in size and the enzyme activity is reduced.

Elongation ceases upon removal of the REF, and is inhibited by antibodies raised to the REF. The REF is probably responsible for the orientation of the prenyl transferase on the rubber molecule, in accordance with the model proposed by BACKHAUS AND BESS (1986). This may involve a reorientation of the binding sites for IPP and the allylic primer, or the REF may position and protect the growing pyrophosphate ends on the rubber molecule.

The stereochemistry is such that the 2-pro(R)-hydrogen of IPP is lost to form a new *trans* double bond. Similarly, the 2-pro(S)-hydrogen is lost when forming a new *cis* double bond, as in the biosynthesis of natural rubber. The rubber particles have been found to alter the stereo selective removal of the 2R-prochiral proton in favour of the removal of the 2S-prochiral proton. This apparent inversion of C₂ of IPP during the proton abstraction step represents a novel example of a switch in enzyme stereospecificity (LIGHT, LAZARUS AND DENNIS, 1989). The stereochemical outcome of the reactions may be a consequence of different conformations in which IPP may be held at the active site of the enzyme (or enzymes) responsible. The factors that control the molecular weight of rubber and the mechanism by which the initiation and rate of rubber synthesis is controlled remain unknown.

THE REGULATION OF THE BIOSYNTHESIS OF RUBBER

It was thought that higher temperatures promoted resin production while lower temperatures favoured rubber production. The temperature required for the synthesis of rubber was specified by BONNER (1967) as 27 °C day and 7 °C night.

There has been no consensus as far as the minimum temperature is concerned. DOWNES AND TONNET (1985) advocate temperatures above 11 °C. It was at these lower temperatures that the expression of the genes coding for the enzymes involved in rubber synthesis was induced (BONNER,1975). No molecular work has been done on this aspect. It was found that the minimum temperatures, in combination with the duration and extent to which they fall below a threshold value (7 to 9 °C), induce the enzymatic potential for *cis*-polyisoprene synthesis (APPLETON AND VAN STADEN,1989). GOSS, BENEDICT,KEITHLY,NESSLER AND STIPANOVIC (1984) proposed that the low night temperature of 7 °C stimulated the incorporation of acetate and MVA into rubber, thus accounting for the increase of rubber synthesis. A reduction in light intensity was associated with a 30% reduction in rubber production and reduced the dry weight of the plant (MITCHELL,WHITING AND BENEDICT,1944). This has been investigated by APPLETON AND VAN STADEN (1991) who found that light intensity over a period of 24 hours had no effect on rubber production. Nutritional aspects have also been implicated in rubber biosynthesis, with nitrate promoting growth and rubber accumulation while a phosphate deficiency limited growth and rubber production (BONNER,1944). In both these situations, growth and rubber production are related to one another.

It has been speculated that rubber is produced in response to excess photosynthate, and it is an excess of the IPP substrate that switches on the genes coding for rubber transferase (PATERSON-JONES,GILLILAND AND VAN STADEN,1990). No measurements of the levels of any of the photosynthates or IPP have been made. The levels of the primary substrates (acetate or carbohydrates), will affect the level of the secondary substrate, IPP and all the molecules synthesized along the pathway. Concentrations of metabolites are usually kept at a low level, yet there are times when the plants or microorganisms accumulate or overproduce substantial quantities of metabolic intermediates (HASLAM,1986).

If the model of KREUZ AND KLEINIG (1984) (**Figure 3B**) is to be followed, then the IPP synthesized in the cytosol moves into the mitochondrial and plastidic

compartments. Resin and fatty acid synthesis would utilize chloroplastic IPP, while rubber synthesis could utilize cytosolic IPP. As resins are produced throughout the year IPP must be made available for rubber synthesis in the Winter months and this may be the reason why the synthesis of rubber is regulated by the activity of the rubber transferase enzyme rather than by the presence of available substrate (IPP).

1.1.3 REGULATION OF THE ISOPRENOID PATHWAY

Regulation of the pathway takes place at two levels: a coarse control achieved by the amounts of enzyme present, and a fine control of the enzymatic activities modulated by the metabolic concentrations and possible reversible covalent modification of enzymes (GRAY,1987). The most important factor for this study is the fine control achieved by the metabolic flux through the pathway. The flux of metabolites through the pathway will be greater before any branching takes place than through the individual pathways. This branched nature of the pathway implies that there will be competition between enzymes utilizing the same substrate. For example, the level of GGPP influences the synthesis of chloroplastic isoprenoid compounds (KLEINIG,1989). Development can be regarded as a coarse control because the amounts and types of isoprenoid compounds found in the plants change during development as a consequence of altered activities of the associated isoprenoid metabolic pathways (GRAY,1987).

1.2 TRANSLOCATION OF PRECURSORS

No actual model has been formulated for the translocation of assimilates in guayule, although a great deal of anatomical evidence has defined areas of active metabolism. The carbohydrate precursor is presumably translocated from the leaves to the various sinks. Rubber is synthesized in young and mature stems and roots in the Winter months. Resin production continues throughout

the year and is not thermosensitive (APPLETON AND VAN STADEN,1989). The carbon that is required by this sink is supplied continuously with exudation of resin occurring through fissures in the bark of the stems in the Summer months. Physiological evidence will be used to devise a model for the translocation and fate of carbohydrates in guayule.

Considerable information on the translocation of assimilates in *Hevea* has been published. As the girth of the tree increases, the mantles of older laticifers are pushed progressively outward by new cambial growth and sclerified parenchyma cells (stone cells), which disorganize phloem rays and destroy laticifer rings. The inner bark tissue lying between the new cambium tissue and hard bark is therefore productive. Wood also accumulates reserves (starch), as well as transporting water and mineral ions (HÉBANT AND DE FÄY,1980). The vascular rays appear to be a major zone of horizontal translocation between wood and phloem, which contains the laticifers. The latter and the companion cells are metabolically active (HÉBANT AND DE FÄY,1980). Sugars are probably transported symplastically (in the cytoplasmic continuum) between the parenchyma cells of the vascular rays and the parenchyma cells adjacent to the laticifers, as there are numerous plasmodesmata between these cells. The lack of plasmodesmata in mature laticifers of *Hevea* implies that the metabolites must penetrate the wall apoplastically and then be actively transported across the plasmalemma of the laticifer. The spatially connected parenchyma cells of phloem rays and laticifers are cytoplasmically connected by numerous plasmodesmata (DE FÄY,SANIER AND HÉBANT,1989). Parenchyma transport of sucrose in *Hevea* may follow both a symplastic and apoplastic pathway with metabolites and other nutrients translocated in the apoplast.

1.3 THE SITE OF RUBBER SYNTHESIS IN GUAYULE

1.3.1 THE LEAVES

The leaves of guayule have a prominent midrib with three to five vascular bundles which continue into the petiole. Lateral veins diverge from the midrib of the leaf forming an essential reticulation of minor veins (GILLILAND, VAN STADEN AND BRUTON, 1984). There is only palisade mesophyll present. All veins are surrounded by a thin-walled, single layered bundle sheath. There are adaxial and abaxial resin canals inside the bundle sheath running parallel to the mid rib. The five to six transfer cells associated with the two sieve tubes of the phloem in the tertiary veins all possess wall ingrowths, active mitochondria, chloroplasts and ribosome-rich cytoplasm. In the secondary veins, the wall ingrowths have nearly all disappeared in the companion cells, but some of the sieve elements have nacreously thickened walls.

Sucrose is thought to be loaded from the leaf mesophyll cells surrounding the secondary and tertiary veins into the phloem sieve tubes (GILLILAND, pers.com.). Modified companion cells called transfer cells are found in the tertiary veins. These transfer cells have cell wall ingrowths which facilitate apoplast transfer by increasing the plasmalemma surface area (TURGEON, 1989). Associated with the secondary veins are sieve tubes with nacreous thickening which are thought to contribute to the efficacy of apoplastic loading of assimilates (GILLILAND, pers.com.).

1.3.2 THE STEM

PRIMARY TISSUE

The primary vascular tissue in the stem occurs in bundles separated by interfascicular parenchyma. The vascular rays of the xylem and phloem are also continuous. Resin canals are produced in the phloem of the stem and in the pith. Xylem is differentiated before the phloem by the cambium.

SECONDARY TISSUE

The mature stem of guayule contains a well developed periderm. Beneath the periderm are the peripheral parenchyma cells of the cortex which contain chloroplasts. These plastids contain stroma and little grana and may contain starch. Plasmodesmata occur between the cells.

THE PHLOEM

As the stem has an eccentric pith, development of the secondary phloem is uneven (ARTSCHWAGER,1943). Secondary phloem is produced by the cambium which is continuous with the original bundles which are capped with phloem fibres. The old phloem becomes sclerified before the new phloem cells appear. The processes are synchronized and the quantity of active phloem remains constant (WESTERLING,1984). A growth increment of phloem always preceeds the appearance of secondary cortical resin canals (WESTERLING,1984). The vascular cambium produces secondary phloem to the periphery and secondary xylem towards the centre and ray cells in both directions.

Secondary phloem is divided into an axial and ray system. The axial system is composed of sieve elements, companion cells, phloem parenchyma and phloem fibres which participated in long distance transport. The companion cells and sieve tubes occur in the ratio of 1:2-3 (GILLILAND,VAN STADEN AND BRUTON,1984). As the plant matures the companion cells develop wall ingrowths, dense cytoplasm and numerous mitochondria (GILLILAND,VAN STADEN AND BRUTON,1984).

The sieve tubes of the secondary phloem have pronounced nacreous thickening but the companion cells do not have wall projections. As the tissue matures, the phloem sieve tubes become impregnated with lignin and become non-functional. The companion cells collapse when the sieve tubes become non-functional.

Once again, transfer cells are associated with the sieve tubes. In this case the transfer cells may enhance the uptake of assimilate from the nacreously

thickened walls of the sieve tubes to the companion cells and parenchyma. Sieve tubes are connected via sieve plates. Movement from the phloem region probably takes place apoplastically, and then symplastically through the parenchyma of the interfascicular rays into the pith and cortex, although there is no proof to substantiate this.

Ray initials give rise to parenchymatous cells which extend radially, facilitating the translocation and exchange of assimilates between the bark and pith, and may provide additional storage capacity. Communication between the axial and ray systems is achieved via the plasmodesmata of the parenchyma cells.

THE XYLEM

In the primary xylem of the stem the parenchyma cells also exhibit transfer cell modifications. The secondary xylem is mainly comprised of vessels and little parenchyma.

1.3.3 THE RESIN CANAL SYSTEM

The resin canals appear in layers alternating with the phloem, when seen in cross section (WESTERLING AND HEALEY, 1984). The resin canals are produced by the cambium with the new canals appearing as a ring within the concentric growth rings. Canals of each new growth increment only branch and anastomose with canals in the same growth increment and do not anastomose between growth increments (WESTERLING, MEHTA AND HEALEY, 1983). A single layer of epithelial cells surrounds each resin canal in guayule, although in most cases two layers of cells occur around the canals, with the internal layer being secretory (BUVAT, 1989). The resin canals differentiate during the late Summer, after the period of active growth has been completed (WESTERLING, 1984).

The resin canal epithelial cells are regarded as secretory or gland cells. In addition to rubber, large quantities of terpenes, mainly α and β -pinene are synthesised in epithelial cells and deposited in the resin ducts of guayule

(HAAGEN-SMIT AND SIU,1944). The composition of the resin showed seasonal differences (SCHLOMAN,1985). The resin canal epithelial cells contain smooth endoplasmic reticulum and poorly developed plastids. These plastids are indicative of active secretory cells (SCHNEPF,1974).

Non-chlorophyllous plastids, such as those found in guayule, require carbon and energy to be imported for their biosynthetic activity. The range of activities include fatty acid synthesis, primary assimilation of nitrite into glutamate, biosynthesis of a number of amino acids, starch synthesis, and pigment biosynthesis (DENNIS AND MIERNYK,1982). In addition to the anabolic processes, plastids are also able to catabolize hexose phosphates via glycolysis and the oxidative pentose phosphate pathway (STITT AND AP REES,1979). These plastids are capable of converting hexose and hexose phosphates as far as pyruvate, and in some cases when a PDC is present, to acetyl-CoA.

The predominant metabolic pathway will differ depending on the tissue in which the plastid is found. These differences may be the result of differential gene expression occurring both as a function of the plant species and tissue location. The development is under the control of the nucleus (DENNIS AND EMES,1990). The least studied aspect of this work is how the plastids interact with the other compartments of the cell (DENNIS AND EMES,1990).

During active rubber formation the resin canal epithelial cells contain large numbers of mitochondria, peroxisomes, several plastids and a prominent smooth endoplasmic reticulum which is sometimes aggregated into complexes of tubules (BACKHAUS,1985). Resin canals also increase in size during active rubber formation (WESTERLING,1984). The epithelial cells lose their spherical appearance, becoming distended and elliptical with age.

Whether the epithelial cells become non-functional as they fill with rubber is debatable. GILLILAND, APPLETON AND VAN STADEN (1988) support non-functionality while WESTERLING (1984) believes that the epithelial cells remain active after the

peripheral and central phloem has sclerified. In *Mangifera indica* L. the epithelial cells disintegrate and the neighbouring parenchyma cells assume an epithelial function without a significant change in anatomy (JOEL AND FAHN,1980). In *Lannea coromandelica* (Houtt.) Merril. both the epithelial and neighbouring cells disintegrate following the secretion of gum into the lumen (VENKAIAH AND SHAH,1984).

There has been no record of plasmodesmata between the epithelial cells and the resin canals in guayule. It has been suggested that resin seeps through the cell wall into the canal (GILLILAND,APPLETON AND VAN STADEN,1988). In *Commiphora wightii* (Arnott) Bhandari plasmodesmata have been recorded between the epithelial cells and the parenchyma cells but are absent in the area between the epithelial cells and the resin ducts (BHATT,1987).

The structure and development of the resin canal epithelial cells in guayule substantiates the idea that it is in these cells that the precursors for isoprenoid biosynthesis are produced (BACKHAUS,1985; GILLILAND,APPLETON AND VAN STADEN,1988). In the surrounding parenchyma, rubber synthesis and accumulation eventually takes place, however, no monoterpenoid synthesis occurs. Rough endoplasmic reticulum is present in these cells but not smooth endoplasmic reticulum, with fewer mitochondria and peroxisomes. Vacuolation also becomes more prominent (BACKHAUS AND WALSH,1983).

1.3.4 RUBBER SYNTHESIS

Rubber in guayule is first observed in resin canal epithelial cells of two to five-month-old plants (BACKHAUS AND WALSH,1983). The resin canal cells are relatively small at this stage, isodiametric and highly vacuolated. The organelles present included mitochondria, plastids, microbodies and dictyosomes. No rubber particles are discerned at this stage in the vacuoles of the young resin canal epithelial cells.

As the resin canal epithelium matures, large rubber particles accumulate. The endoplasmic reticulum has specialized membrane tubules which are folded and coalesced into a regular repeating pattern (BACKHAUS AND WALSH,1983). Additionally, the endoplasmic reticulum is spatially associated with the plastids which have reduced thylakoids. This is an accepted feature of resin-forming cells (DELL AND McCOMB,1974,1977).

When the guayule plants are eight months old, rubber particles are observed in the distal parenchyma cells and the parenchyma cells adjacent to the epithelial cells have rubber in the cytoplasm and vacuoles. The epithelial cells show reduced vacuolation and the cytoplasm is very different to the adjacent parenchyma and younger epithelial cells.

In the three-year-old plants, the number of rubber particles increases greatly, as do the proportion of large rubber particles in all the parenchyma cells. Vacuolar rubber deposits are greater than those of the cytoplasm, in keeping with the increased volume of the vacuole (BACKHAUS AND WALSH,1983).

1.3.5 CONCLUSION

The problem remains that our understanding of the processes that control and initiate rubber biosynthesis are incomplete. Once the factors affecting rubber production in guayule are identified, it may be possible to regulate rubber yield. STEWART, DOWNES, FERRARIS, GARTSIDE, NIX AND RAWLINS (1986) recommended that improved models of growth and rubber formation be established by determining the relationships between temperature, radiation and water stress with growth, photosynthate partitioning and water use efficiency. The conditions under which rubber rather than resin is produced, should be established and the possibility of selecting lines capable of converting a large proportion of the photosynthates to rubber should be examined. The possible link between the

photosynthates and resin and rubber production shall be discussed in greater detail in the following section.

1.4 SECONDARY METABOLISM

1.4.1 INTRODUCTION

The isoprenoid pathway, as previously mentioned, is strictly compartmentalized. Most secondary metabolite production relies on compartmentation to ensure control and integration. Compartmentation refers to the spatial control of the location of enzymes, precursors, intermediates and products (LUCKNER, DIETRICH AND LERBS, 1980). Spatial organization of organelles is an important feature in the regulation of metabolism. It makes the orderly synthesis of secondary products from intermediates and co-substrates of primary metabolism possible as a high local concentration of precursors and intermediates can arise, favouring certain sequences of reactions (OAKS AND BIDWELL, 1970). Large amounts of toxic secondary products can be accumulated within or in close proximity to the cell without disruption. Secondly, the controlled release of stored secondary products, which is of significance if they are physiologically important, is possible.

Investigations on the isoprenoid biosynthetic pathway have thus far characterized enzymes from different plant tissues; these appear to be predominantly membrane-bound. The association of enzymes with membranes is advantageous as the membranes form microcompartments favouring certain sequences of reactions, and thus facilitating the formation and function of metabolic chains (ROGERS, SHAH AND GOODWIN, 1968).

It has been argued the accumulation of a secondary product like rubber may be pointless. Initially it was thought that rubber protected the plants from animal attack in addition to functioning as a food reserve. The latter notion is unlikely,

as it is not metabolized any further (BONNER AND GALSTON,1947). However, more recent thinking suggests that the product of secondary metabolism is not important (rubber), but rather that it is the processes that occur during secondary metabolism which are of greater value. The process may be utilizing excess substrate with rubber acting as a detoxification product. Lastly, the onset of unfavourable conditions may again result in excess substrate which will be utilized for secondary metabolism thus allowing for adjustment to the changing environmental circumstances.

Although the isoprenoid pathway commences with IPP, it does rely on carbohydrates to provide the carbon to synthesize isoprenoids. Carbohydrates are regarded as primary metabolites and rubber as a secondary metabolite. Therefore it is important that the boundaries between primary and secondary metabolism be defined. BONNER AND GALSTON (1952) pointed out " that studies of secondary metabolism are ultimately best viewed in the context of the metabolic matrix in which they are enmeshed ".

1.4.2 PRIMARY AND SECONDARY METABOLISM

Primary metabolism comprises both catabolism and anabolism. All organisms synthesize and utilize certain essential chemical species, sugars, amino acids, fatty acids, nucleotides and the polymers formed from them (polysaccharides, proteins, lipids, RNA and DNA). These are primary metabolites and they are essential for the survival of the organism. The processes included in this category are glycolysis, the tricarboxylic acid pathway (Kreb's cycle), oxidative phosphorylation and photosynthesis. In most cases these processes are characterized by a rapid turnover of metabolites with very little accumulation of intermediates occurring (HASLAM,1985,1986; MANN,1987). The formation of products is either enzyme or substrate limited (BU'LOCK,1980).

Secondary metabolites are produced along pathways which diverge from primary metabolism. The process is characterised by the accumulation of these

metabolites in particular organs and tissues. Autotoxicity is usually avoided by physical removal of metabolites through aerial discharge, precipitation or by isolation in the cell vacuole. The term secondary may be regarded as an oversimplification and can be misleading.

As to how metabolism can be divided, the following quote will suffice, " the differentiation between primary and secondary metabolites may thus on occasion appear, if not illogical, then at least arbitrary " (HASLAM,1986).

However, attempts have been made to remedy the above situation. BONNER AND GALSTON (1952) viewed metabolism holistically with the essential pathways analogous to all organisms, and side branches resulting in products which differed between species.

Primary and secondary metabolism is separated by particular groups of molecules. BEYTIA AND PORTER (1976) see three building blocks for secondary metabolism :

- a) shikimic acid, the precursor to many aromatic compounds,
- b) amino acids, leading to the alkaloids; and
- c) acetate, the precursor of the polyacetylenes, prostagladins, macrocyclic antibiotics, polyphenols, and the isoprenoids (terpenes, steroids and carotenoids) via two different pathways. The isoprenoid pathway involves a number of compounds, which have arisen from a number of similar or identical reactions.

The tricarboxylic cycle (TCA) may be used as the critical link between primary and secondary metabolism (NEIJSEL AND TEMPEST,1979). Secondary metabolism is then related to a change in metabolism from normal growth to that in which there is decreased uptake of acetyl-CoA into the TCA cycle (HASLAM,1985). The level of metabolites involved in glycolysis reaches a critical level when there is less demand from the TCA cycle. As the demand from the TCA cycle decreases so a surplus exists of phosphoenolpyruvate, pyruvate, acetyl-CoA,

mevalonic acid , and a number of protein α -amino acids, which are then available for secondary product metabolism. The intermediates could then be translocated or utilized before entry into the TCA cycle (HASLAM,1986).

As primary metabolism provides basic molecules for secondary metabolism, any and all fluctuations in the primary metabolism, enzyme activity of primary-yielding reactions and photoperiodic phenomena are likely to affect the rate of secondary metabolism. Therefore when measuring the accumulation of any molecule, it is not possible to determine whether this is due to changes in the rate of synthesis or the rate of catabolism.

1.4.3 THE ROLE OF SECONDARY METABOLITES

Secondary products are the products of the plant genome, the stage of growth and differentiation, nutritional status, and the environment (MANN,1987). This variation would suggest a dynamic role in plants, and diurnal fluctuations in levels indicate that there is great variability in the rates of synthesis and subsequent catabolism.

Obviously hypotheses abound as to the function of secondary metabolism in plants, but evidence to support the hypotheses do not. A great number of ideas have been put forward. For example, secondary metabolism had/has a role at some point (HASLAM,1986); it rids the plant of waste/detoxification products (MANN,1987); it may be seen as a means of adjusting to changing circumstances, allowing primary metabolic enzymes to function until circumstances are propitious for renewed activity and growth, and it is the processes that are more important than the products, although the products may have acquired a functional role (HASLAM,1986); or secondary products are seen to play a dynamic role in the coexistence and coevolution of species (TORSSELL,1983). Lastly, they are regarded as mutations which are neither beneficial or harmful, persisting for many generations (KREBS,1954).

The only function that has been ignored is that of secondary products repelling or attracting other organisms, thus giving an idea of the fitness of the organism to survive. WILLIAMS, STONE, HAUCK AND RAHMAN (1989) view this theory as the one with the greatest applicability, and that the "repel or attract" theory must be extended to any result in which the survival of the producer A is enhanced when its product acts on organism B. They envisage that many more natural product / receptor sites will be found which will display the same sophistication as the known substrate / enzyme interactions which back up their theory.

Much of the secondary metabolite work pertains to antibiotic production which appears to be the norm for microbial growth. WOODRUFF (1966) envisaged an overflow process where growth terminates in microbial cultures, followed by an accumulation of metabolic intermediates which induce the synthesis of secondary metabolites and leads to further metabolism resulting in a variety of secondary products or products of minor side routes which are referred to as shunt metabolites. These metabolites reduce the abnormal concentration of normal cellular constituents. BU'LOCK AND POWELL (1965) proposed a metabolic grid where a key intermediate is induced to form the secondary metabolite which is then transformed to give rise to an array of other secondary metabolites; characteristic patterns of metabolites would thus arise. The selective advantage of secondary metabolite production is that the mechanisms essential to cell multiplication are kept operative while multiplication is no longer possible (BU'LOCK, 1980). WOODRUFF (1966) believes that there is no advantage in producing secondary metabolites, rather it is the cell that acts upon excess intermediates (overflow), which are of no direct value to the cell and are thus expelled into the surrounding medium. Even microbiologists are questioning the use of the term "secondary" to apply to the over-production of metabolites which serve a function and are of primary importance to the organism (NEIJSSSEL AND TEMPEST, 1979).

1.4.4 THE CONTROL OF SECONDARY METABOLISM

There are four areas in which controls may be active in secondary metabolism (LUCKNER,1980). Secondary metabolism may require cell(s) maturity, and the synthesis of the secondary metabolic enzymes. The activity of the enzymes may in turn be limited by the accessibility of precursors and by the accumulation of the products. In most living systems, enzymes and products are separated by compartmentalisation such that the enzymes of one metabolic pathway may be located in different tissues and organs or the secondary products may be synthesised and stored in different cells, so maintaining a gross level of control.

1.4.5 THE REGULATION OF SECONDARY METABOLISM

Secondary product metabolism is integrated into the differentiation and development of the cells during high metabolic activity and when growth is arrested. The action of a signal, usually non-substrate orientated, as the response of the target cells, is usually dependent on the state of differentiation and not on the stage of secondary product formation (LUCKNER,1980).

During growth plant cells become morphologically and chemically specialized. The synthesis of enzymes and the level of activity in secondary pathways, compartmentalization and channelling of enzymes, precursors, intermediates, products and cell specialization regulates secondary metabolism (LUCKNER,1984), in contrast to primary metabolism which is governed by substrate levels (HASLAM,1986). In a living system, where a multiplicity of alternative metabolic pathways may be possible for a certain substrate, it is steered along a particular path by a series of kinetically controlled enzyme-catalysed reactions (HASLAM,1986).

1.4.6 CONCLUSION

The terpenes are the most abundant and widely distributed group of natural products. Many play vital roles in the metabolism and development of plants. Primary metabolism includes photosynthesis, and photosynthesis requires chlorophyll which is produced along a secondary metabolic pathway. Therefore the practise of dividing metabolism using processes or molecules as divisors is inadequate. The terpenes cannot be regarded as shunt metabolites ridding the cells of excess substrate, if one bears in mind the fine control mechanisms that are functional, and the compartmentation that is utilized to segregate the processes. Perhaps in pathways rather than cycles, it is the molecules, for example rubber, which are not re-utilized or broken down in any way that may be regarded as secondary metabolites. However, the existence in the case of guayule of an inferred enzyme that is activated when the plant is exposed to 21 days of minimum temperatures (APPLETON AND VAN STADEN, 1989) would suggest that the process is an important one, of some adaptive value to the plant.

Most of the definitions and functions of secondary metabolism have originated in experiments on microorganisms which produce antibiotics, for example *Penicillium*. It is therefore unrealistic to seek explanations for higher plant secondary metabolism from research based on the simpler microbial systems. The author is therefore in agreement with BONNER AND GALSTON (1952) who believe that secondary metabolism must be viewed in context to appreciate the complexity of the metabolic matrix of plants.

1.5 THE PROPOSED DIRECTION OF RESEARCH

It has been proposed that four factors are essential for the production of *cis*-polyisoprene in guayule. Firstly, plants require a period of minimum temperatures during which time the rubber enzymatic potential increases before

rubber production begins (APPLETON AND VAN STADEN,1989). Secondly, the process appears to be strictly localized within the resin canal epithelial cells, phloem and adjacent parenchyma. Thirdly, cells need to reach a certain degree of maturity before they are able to respond to the temperature stimulus. Lastly, rubber production takes place at a time when growth has slowed down, although resin (*trans*-terpenoid) production continues and a certain amount of substrate must be partitioned into *cis*-polyisoprene production. Sinks and sources must influence the translocation and accumulation of assimilate in the plant and it is this substrate which is the focus of this thesis.

With this in mind, this study was initiated to ascertain the metabolism of carbohydrates in guayule and their possible role in the rubber biosynthetic pathway. The objectives of the study were to establish :

- 1) the levels of carbohydrates in the plant throughout the year;
- 2) whether the distribution and metabolism of precursors of rubber biosynthesis were influenced by seasonal factors;
- 3) whether the fructans are providing carbon for the isoprenoid biosynthetic pathway, and
- 4) whether budbreak and flowering are acting as sinks for assimilated carbon during Spring preventing the carbon from being utilized for rubber production.

CHAPTER 2

THE PARTITIONING AND METABOLISM OF THE DISTAL PRECURSORS OF THE ISOPRENOID BIOSYNTHETIC PATHWAY

2.1 INTRODUCTION

It is generally accepted that the carbohydrates and acetate are the distal precursors and isopentenylpyrophosphate the immediate precursor of the isoprenoid pathway (MANN,1987). However, a number of other compounds which include carbohydrates, carbon dioxide, acetate, glyoxylate, leucine, valine and serine have been reported as effective precursors. The carbohydrate source is thought to be metabolized to the organic acid pyruvate and then to acetate. Pyruvate and acetate are precursors for a number of other metabolic pathways. It is not known what proportion of the substrate is destined for the isoprenoid pathway, nor the factors which influence substrate partitioning in guayule (**Figure 2**). There have been no investigations as to the efficacy of isoprenoid and *cis*-polyisoprene synthesis in sequestering either the distal or the immediate precursors. The carbohydrate, derived from photosynthesis, is translocated from the leaves to the site of rubber synthesis, for example the stems, and metabolized to form isoprenoids and *cis*-polyisoprene.

2.2 PARTITIONING AND ASSIMILATE TRANSPORT

The division of assimilates following carbon fixation is known as partitioning. The assimilate is transported from the source (area of synthesis) to the sink (area of utilization) by the phloem. Source organs are those that produce excess assimilate for export and sink organs are net importers of assimilate. Partitioning during the vegetative growth phase is required for growth, maintenance and storage. In this case, the roots, stems and leaves are

growth is minimal, ensuring a high reproductive yield. Flowers are poor competitors for assimilate, while fruits and seeds are more dominant. In some instances assimilate is stored or deposited in one area and then remobilized from the area of deposition or storage to an area where it can be re-utilized. In spite of environmental conditions and plant development, there is a remarkable degree of regulation in partitioning and export of photosynthetic material (GEIGER,1979).

The transport of assimilate from the source leaves to the rubber-producing sink in guayule would therefore include a series of steps: phloem loading, long distance translocation, phloem unloading, and transfer into the resin canal epithelial or parenchyma cells, partitioning between storage carbohydrate and mobile sucrose, the ability to compete with other potential sinks for translocated sucrose, and in this case, the channelling of the assimilate into the isoprenoid pathway and lastly, into rubber (WARDLAW,1976).

Carbohydrates are readily synthesized from the primary products of photosynthesis or from the re-mobilization of storage products such as starch, structural carbohydrates and lipids (GIAQUINTA,1980). In most cases the carbohydrate moves as sucrose (MILTHORPE AND MOORBY,1969) which is electroneutral, chemically inert and extremely soluble in water (ESCHRICHT,1989).

Partitioning begins in the source leaves with carbon fixation. Sucrose availability for loading is an important factor controlling the rate of export from the leaves and translocation. Regulation of phloem loading is achieved by chemical partitioning between sucrose and starch, the release of sucrose from the mesophyll cells, by the membrane transport of sugar into the phloem, and by hormones. Assimilates may be translocated between the parenchyma cells, companion cells and the sieve tubes, symplastically or apoplastically or by a combination of the two modes of transport.

Symplastic movement, regarded as more likely by GIAQUINTA (1983), would require numerous plasmodesmata in the transit zone between mesophyll and phloem cells. The plasmodesmata establish a symplastic pathway interconnecting the cytosol of the mesophyll to the long distance pathway of the phloem (ROBARDS AND LUCAS,1990). The plasmodesmata are composed of desmotubules running from cell to cell and connecting at each end with the endoplasmic reticulum (ROBARDS,1968). Assimilates may pass via the pore through the desmotubule or through the cytoplasmic sleeve between the two cytoplasmic compartments. In most herbaceous plants, plasmodesmata are either undeveloped or even absent (KURSANOV,1984).

Solutes which diffuse apoplastically from the mesophyll cells to the sieve tubes move along a concentration gradient. The formation of transfer cells facilitates the loading of assimilates apoplastically as the walls of these companion cells possess cellulose ingrowths which increase the surface area of the walls and hence of the plasmalemma of the companion cells. The phloem has a higher sucrose content than the mesophyll cells. Once the sucrose is loaded, the role of the source leaf, which functioned to control the timing and supply of sucrose for export and to provide energy for sucrose loading into the phloem, is over (FONDY AND GEIGER,1983).

The phloem sap moves along a water potential gradient between the source and the sink (low solute content) (FONDY AND GEIGER,1983). The translocation rate does not limit the movement of the assimilates towards the sink(s).

The final carbohydrate allocation patterns depend on the competitive ability of the sinks and their mobilizing abilities. The released assimilates leave the sieve element / companion cell complex through plasmodesmata into vascular parenchyma elements (symplastic unloading) or directly across the plasmalemma into the apoplast (apoplastic unloading) (OPARKA AND DAVIES,1985). If an apoplastic step mediates unloading, then the sucrose in the free space becomes important in controlling the rate of unloading. A low apoplastic

sucrose concentration is achieved by moving sucrose into storage tissue without hydrolysis, or in cases where low apoplastic sucrose concentrations are maintained, through hydrolysis by a cell wall invertase.

Glucose and fructose are then the major transport sugars present in the apoplast. This is the case in sugar cane. Cell wall invertase ensures low apoplastic sucrose levels but also prevents sucrose from reloading and increasing the osmotic concentration in the free space. Where storage sinks convert sucrose into starch or fructans, the concentration gradient is altered between the sieve tubes and the parenchyma cells and unloading is maintained (ESCHRICH,1989). In sugar beet, leaf blade parenchyma transport proceeds with an intermediate breakdown of certain amounts of sucrose, while in the roots, sucrose penetrates the cells without hydrolysis (KURSANOV,1984). The involvement of the plasmodesmata in unloading remains to be elucidated. Unloading may be achieved by the reversal of the loading process, and may be viewed as a change in balance between sucrose influx and efflux.

The uptake, metabolism and storage of carbohydrates by sink cells is the final step in carbon partitioning. Some sinks (sugar cane, sugar beet and carrot) do store simple sugars. Sucrose is stored in vacuoles of the parenchyma cells in these tissues. The sugars are transported across the plasmalemma and then into the vacuole thus crossing the tonoplast. A negative electric potential exists across the plasmalemma with the inside pH being higher. A low pH and more positive potential is found inside the vacuole relative to the cytoplasm. The pH differential is maintained by the ATPase proton-ion pump (BENNETT AND SPANSWICK,1983). Sucrose uptake into the vacuoles may involve a K^+ stimulated, proton antiport system (LEIGH,1984). This step may present a control point for carbon allocation in some species.

In this Chapter the seasonal morphological development of guayule with the associated anatomical and ultrastructural changes will be detailed. The most suitable precursor and the translocated form of carbohydrate in guayule will be

identified in a short term (12 hour) experiment during which time the uptake, distribution and metabolism of [^{14}C] acetate, [^{14}C] fructose, [^{14}C] glucose and [^{14}C] sucrose during the Winter and Summer months, and the contribution of these substrates to rubber production will be considered. This is followed by a longer experiment (48 hours) in which the uptake, distribution and metabolism of [^{14}C] sucrose and [^{14}C] fructose is investigated. The seasonal effects on these phenomena will also be considered. Lastly, the possibility of [^{14}C] serine or [^{14}C] glycine acting as precursors for the rubber biosynthetic pathway will be investigated using the radiochemical bioassay developed by MACRAE, GILLILAND AND VAN STADEN (1986).

2.3 MATERIALS AND METHODS

2.3.1 CLIMATIC DATA

The experimental period spanned July 1986 to December 1988 at the experimental site in Pietermaritzburg (altitude 762 m ; 29°37'S : 30°24'E). The longest daylength was recorded in January (825 minutes), and the shortest day occurred in June (610 minutes). The daily maximum and minimum temperatures were recorded on a thermohygrograph at the site. The Winter and Spring temperatures from 1986 to 1989 are also included as a comparison (Figure 4).

2.3.2 LEAF TRICHOME ULTRASTRUCTURE

Leaves were collected from plants on a monthly basis and prepared for scanning electron microscopy. Whole leaves were critically point dried in a Hitachi HCPZ critical point drier. The leaves were then mounted on copper stubs and sputter coated with gold palladium before examining in a Hitachi 5570 scanning electron microscope (KELLY, GILLILAND AND VAN STADEN, 1986).

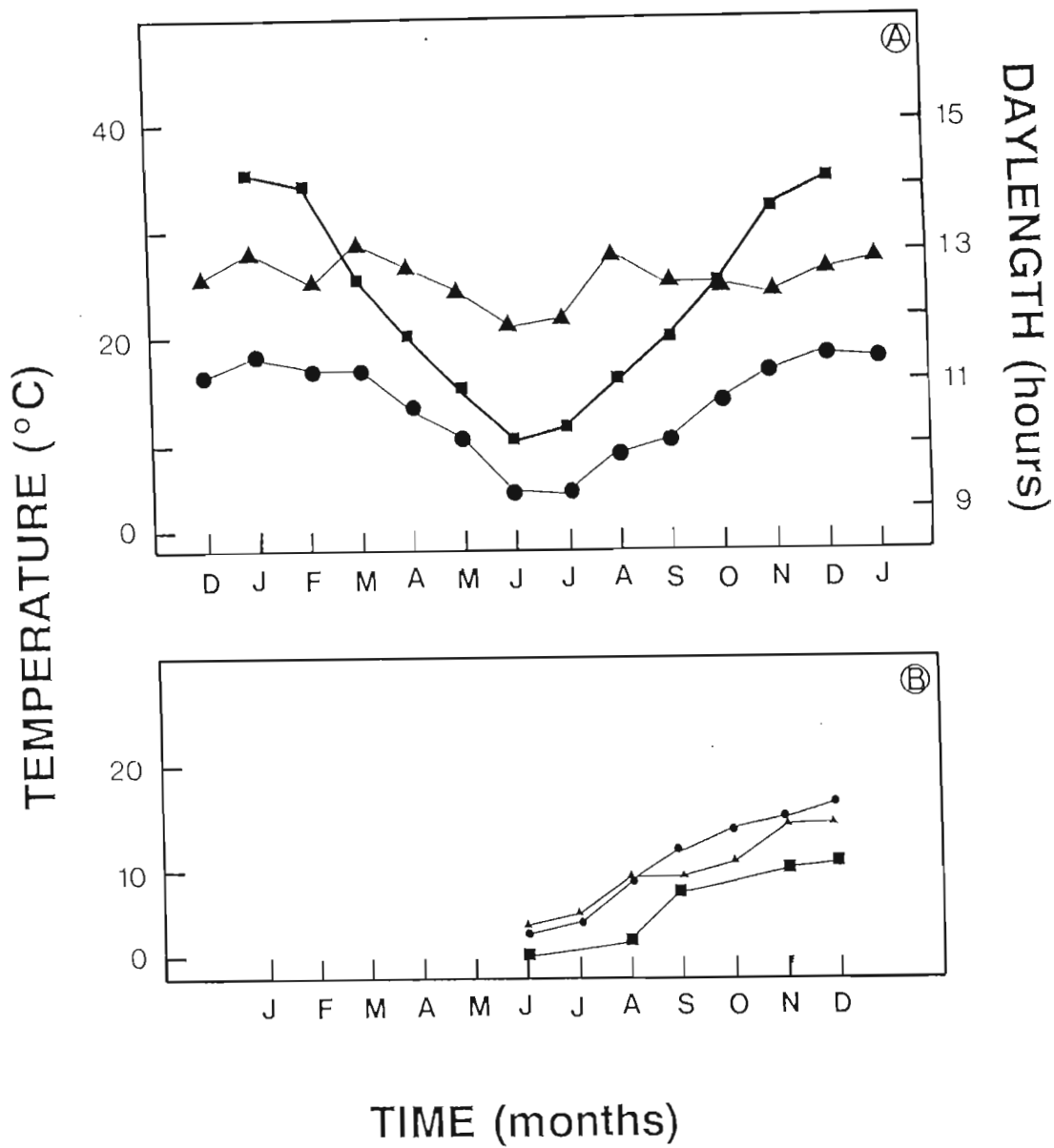


Figure 4. The maximum (▲) and minimum (●) temperatures and daylength (■) recorded for 1988 and 1989 (A), and the minimum temperatures recorded for the winters from 1986 (▲), 1987 (■) and 1988 (●) (B).

2.3.3 ISOPRENE AND cis-POLYISOPRENE SYNTHESIS

At the end of each month, for 12 months, stem samples 10 cm in length were taken from two-year-old plants grown in pots. These stem sections were dried at 60°C for 24 hours and ground to homogeneous powders in a Wiley mill with a 20 mesh sieve. Resin and rubber were extracted in a Soxhlet apparatus with acetone and petroleum ether (40 to 60°C) as the respective solvents.

2.3.4 CHLOROPHYLL DETERMINATION OF WINTER AND SUMMER LEAVES

Leaves were collected from five, two-year-old field-grown guayule plants at 09 00 hours in the morning at the end of each month from January to December 1989. Plants were chosen at random and the leaf samples were thoroughly mixed. A 5 g sample was immediately homogenized in 50 ml methanol, covered with aluminium foil, and left in the dark at 10°C overnight. The following morning samples were filtered through Whatman No. 1 filter paper and the filtrate evaporated to dryness. Samples were redissolved in 10 ml n-butanol and were stored at -10°C in the dark until required.

The butanol samples were dried under a fan in the dark, and the residue resuspended in 5 ml methanol (90%). After dilution (500 μ l of the plant extract in 5 ml methanol (90%)) the sample was filtered through a 0.45 μ m Millipore filter and then scanned between 400 and 700 nm on a Beckman Spectrophotometer (DU Series 60). The specific absorption (extinction) coefficient of the methanol was calculated as follows (HOLDEN, 1965).

$$\text{Total chlorophyll mg/g tissue} = 25.5D_{650 \text{ nm}} + 4.0D_{665 \text{ nm}}$$

$$\text{chlorophyll a mg/g tissue} = 16.5D_{665 \text{ nm}} + 8.3D_{650 \text{ nm}}$$

$$\text{chlorophyll b mg/g tissue} = 33.8D_{650 \text{ nm}} + 12.5D_{665 \text{ nm}}$$

2.3.5 AMINO ACID ANALYSIS

Leaves and primary stem material (5 cm from apex) were harvested from two-year-old guayule plants in December (mid-Summer) and June (mid-Winter), and before and after budbreak in August and September. Ground material (0.5 g) of the leaves and stem were placed in cellulose thimbles for Soxhlet extraction. Samples were extracted in 180 ml, 80 % ethanol (v/v) for eight hours. The pH of the filtrate was adjusted to 2.5. Free amino acids were isolated by passing the ethanolic filtrate through a glass column packed with 30 g Amberlite IR-120 resin in the H⁺ form. The amino acids were eluted from the resin with 200 ml 2N NH₄OH followed by 100 ml distilled water. The eluant was evaporated under vacuum at 35 °C, and then re-suspended in 5 ml *iso*-propanol (10%). A 50 µl aliquot was injected into a Waters Amino Acid Analyzer, with the internal standard nor-leucine.

2.3.6 PROBLEMS ASSOCIATED WITH RADIOCHEMISTRY

The advent of radiochemicals provided a sensitive technique for tracing metabolic pathways in intact functioning plants. The use of isotopically labelled molecules does present problems with respect to the incorporation, translocation, extraction and recovery of radioactive or stable isotopes.

Poor incorporation of the labelled precursors may be due to the impermeability of the tissues to the precursor, poor translocation of the precursor or the degradation of the applied molecule *en route* (MANN,1987). The substrate may be utilized *en route*, especially if it serves as a precursor for other metabolic pathways. Extraction of the compound may involve the loss of a percentage of the isotope at each extraction step. In order to avoid this loss, high levels of radioactivity are often applied initially to the intact plant. However, these artificially high exogenous levels may alter the endogenous metabolism of the plant.

Although radiochemicals are an effective means for tracing partitioning there are disadvantages associated with the technique. Analysis of the radioactive plant material involves digestion and bleaching of the radioactive plant tissues. Oxidation colours the medium and the scintillant and the colour quenching is reduced by bleaching the samples before adding the scintillant. Quenching occurs when the energy transfer process is interfered with at any point. Quenching may be optical, chemical or physical in nature. In most cases it is dealt with by retaining the samples in the dark and cold for a number of days prior to determining the radioactivity.

2.3.7 THE RADIOCHEMICAL EXPERIMENTS

PLANT MATERIAL

All the present radiochemical experiments were conducted on uniform, intact, W10 (R.S.A.) (A48118; U.S.A.) guayule plants. These were maintained in 30 cm plastic pots in bark medium and watered daily. There were four replicates for each harvest time. During harvesting, plants were washed free of the growing medium and divided into root, shoot and leaf components and weighed to determine fresh mass. Samples were then frozen in liquid nitrogen and lyophilized on a freeze drier to ensure that no further metabolic activity took place. After 24 hours, the samples were weighed and the dry mass recorded. The samples were maintained at -20°C.

During this chapter reference will be made to Winter or Summer plants and Winter or Summer leaves, stem and roots. This is acceptable terminology with respect to guayule where Winter plants are plants which are harvested during the period of rubber deposition and Summer plants are plants which have minimum rubber deposition and maximum resin production.

CHEMICALS

The radiochemicals were obtained from Amersham in England. The initial specific activities and labelling of the molecules were as follows : D-[U- ^{14}C] fructose, 330 mCi mmol $^{-1}$; D-[U- ^{14}C] glucose, 290 mCi mmol $^{-1}$; [U- ^{14}C] sucrose, 434 mCi mmol $^{-1}$; and [U- ^{14}C] acetic acid (sodium salt), 58.10 mCi mmol $^{-1}$.

The radioactivity concentration of each substrate was ascertained prior to application. The specific activity of each radioisotope was not the same for all the experiments and therefore it is included in section 2.3.9 under amendments.

The radioactivity extracted from the plant tissues was initially expressed as a percentage of the total radioactivity recovered from each plant. This proved to be unsatisfactory as the actual amount of radioactivity present in each plant component extracted then became obscured. Instead, the radioactivity was expressed as disintegrations per minute (DPM) per gram plant material.

ROUTINE EXPERIMENTAL PROCEDURE FOR THE RADIOCHEMICAL EXPERIMENTS

As a number of radiochemical experiments were conducted over a period of six years, the investigations were extended as interesting facts emerged. The amendments to the routine procedure are described below. These are presented in section 2.3.9.

APPLICATION OF THE RADIOACTIVE COMPOUND

Each isotope was applied to four source leaves (not the apical leaves), located towards the top of each plant, on twenty plants. The most suitable mode of application for radiochemicals in guayule had been tested previously (KELLY, GILLILAND, VAN STADEN AND PATERSON-JONES, 1987). These modes included injecting

the radioactivity into the leaf or stem, painting the adaxial or abaxial leaf surface or the stem with the radiochemicals and assessing how much of the radioactivity was retained on the surface of the leaf.

The most effective mode of application was achieved by painting the abaxial surface of the leaves with the radioactivity. Thus the four source leaves were marked and a wetting agent (Tween 20) was applied to the abaxial surface of the leaf with a paint brush. One μl of the isotope was applied to the abaxial surface of the selected leaves, each plant received 4 μl of the selected isotope. The leaves to which radioactivity was applied are referred to as treated leaves.

Four plants were harvested for each of the experimental times (6, 12, 24, 48, and 168 hours after isotope application). The plants were divided into plant components consisting of treated leaves, all other leaves, young or primary stems (1 to 5 cm from the shoot apex), older or secondary stems (5 to 17 cm from the apex) and roots. The fresh mass of the samples was determined. The various plant components were lyophilized in a freeze drier for 24 hours. Dry mass was then established. These plant components were ground to homogenous powders in a Wiley Mill using a 20 mesh sieve.

EXTRACTION AND DETERMINATION OF RADIOACTIVE COMPOUNDS FROM PLANT SAMPLES

A number of techniques were investigated in order to determine which would produce the most stable reproducible results. In the first technique, 10 mg sub-samples were weighed into glass scintillation vials and then digested with a mixture of hydrogen peroxide (H_2O_2) and perchloric acid (HClO_4) (1:1; v/v) for 24 hours (COOK, 1985). The lids were applied and the samples were incubated at 60°C for three hours and then cooled. Seven ml of Beckman Ready Solve HP (scintillation cocktail), was added to each sample, and the radioactivity recorded using a Beckman LS 3800 scintillation counter. This technique was unsuitable for guayule as the radioactivity was found to decrease with time. As

incubation of samples in the cold and dark reduced chemiluminescence and photoilluminescence (SMITH AND LANG,1987), the samples were stored at 10°C for 24 hours. However, this did not prevent the quenching.

Seven ml of glacial acetic acid was then added to 1 l Ready Solve HP as the addition of glacial acetic acid to the cocktail was reputed to reduce chemiluminescence (Beckman Bulletin). However, this did not solve the problem of quenching.

An alternative method to the hydrogen peroxide digestion was then attempted. Plant material was digested in BTS 450, a quaternary ammonium hydroxide (0.5N) in toluene (Beckman). It is a rapid and economical digester of proteinaceous tissues and tissue homogenates (SIMONNET,1990).

Ten mg of the dried sub-sample were weighed into plastic scintillation vials. The samples were incubated in BTS 450 (1 ml) for five hours at 50°, and then allowed to cool in the dark at 10°C. Thirty per cent H₂O₂ (0.2 ml) was added to the digested sample. The vials were left loosely capped for two hours at 50°C. The amount of hydrogen peroxide added to the mixture and the loosely capped lids are critical factors in this technique, as the exothermic nature of the reaction blows the caps off the samples. After cooling at 10°C for 24 hours, 8 ml Ready Solve EP was added to the digested and bleached sample. Although Ready Solve MP is the recommended cocktail for the BTS 450, Ready Solve EP can be substituted successfully. The samples were kept in the dark for 24 hours at 10°C, whereafter the radioactivity was determined using a Beckman LS 3800 scintillation counter.

This became the standard technique used to determine the incorporation of radioactivity into guayule.

2.3.8 THE PARTITIONING OF THE APPLIED RADIOACTIVE COMPOUNDS

THE ROUTINE EXTRACTION PROCEDURE

Ground material (0.5 g) of the treated leaves, untreated leaves, stem and root samples was placed in cellulose thimbles for Soxhlet extraction. Samples were extracted in 180 ml 80 % ethanol (v/v), acetone and petroleum ether (40 to 60°BP), for eight hours in succession. These three fractions represented the ethanolic, isoprene (resins) and *cis*-polyisoprene (rubber) components of metabolism.

The three fractions obtained in this manner were then brought to constant volume (10ml) by flash evaporation. Aliquots (1ml) of each fraction, were placed in plastic scintillation vials and dried overnight. Four ml of scintillation cocktail (Beckman Ready Solve HP or EP), was added to each vial. The vials were left in the dark at 10°C overnight and radioactivity determined using a Beckman LS 3800 scintillation counter.

THE EXTRACTION OF AMINO ACIDS, CARBOHYDRATES AND ORGANIC ACIDS

Once the radiochemical analysis of the ethanolic fraction had been completed, the remaining 7 ml was diluted in 100 ml 80 % ethanol (v/v). The pH of the solutions were adjusted to 2.5. Free amino acids were isolated by passing the ethanolic extract through a glass column packed with 30 g Amberlite IR-120 (H⁺)(200-400 mesh) resin and the effluent was retained (referred to as filtrate B). The resin was then washed with 100 ml 80 % ethanol (v/v), followed by 100 ml distilled water, and these were combined with filtrate B. The pH of this filtrate was adjusted to 8.2 with 1.5N (NH₄)₂ CO₃.

The amino acids (basic solution) were eluted from the resin with 200 ml 2N NH_4OH followed by 100 ml distilled water. The eluant was evaporated under vacuum at 35 °C, and then re-suspended in 5 ml *iso*-propanol (filtrate A).

Filtrate B was passed through a glass column packed with 20 g Amberlite IRA-400 resin (Cl^-) (200-400 mesh) which was converted to the carbonate form, to separate the sugars from the organic acids. The unretained extract, filtrate B, contained the sugars. This filtrate was brought to dryness under vacuum at 35 to 40 °C, and re-suspended in 5 ml 10 % *iso*-propanol.

To elute the organic acids (acidic fraction) from the IRA-400 resin, 150ml 1.5N $(\text{NH}_4)_2 \text{CO}_3$ was passed through the resin, followed by 100 ml distilled water. The eluant was brought to dryness under vacuum at 55 °C, and resuspended in 5 ml 10 % *iso*-propanol (filtrate C).

Five aliquots (1 ml) were removed from filtrates A, B and C, representing amino acids, carbohydrates and organic acids respectively. These were dried down under a fan, and 5 ml of Beckman Ready Solve HP or EP was added to each. The radioactivity of each filtrate was determined using a Beckman LS 3800 scintillation counter. It was not necessary to add the 0.6 ml H_2O_2 to bleach these samples as described by GILLILAND, VAN STADEN AND MITCHELL (1985) as this caused unnecessary quenching.

2.3.9 AMENDMENTS TO THE ROUTINE EXPERIMENTAL PROCEDURES

AMENDMENTS TO THE ROUTINE PROCEDURE WHERE [^{14}C] ACETATE, [^{14}C] FRUCTOSE, [^{14}C] GLUCOSE AND [^{14}C] SUCROSE WERE SUPPLIED AS PRECURSORS TO THE ISOPRENOID BIOSYNTHETIC PATHWAY.

The choice of precursor is very important in secondary metabolic studies. The precursor must be translocated to the site of secondary product synthesis even if this site is compartmentalised. Thus, it was decided to investigate the

potential of four substrates (acetate, fructose, glucose and sucrose) as precursors of the isoprenoid biosynthetic pathway. In order to include a suitable number of plant replicates, it was decided to limit the harvest time to twelve hours. Once the most suitable substrate had been established, this precursor could be used for further studies.

Fourteen-month-old plants were used for this experiment which was conducted in June (Winter) and February (Summer). The isotope was applied to four leaves, the specific activity of each radioisotope was as follows 1.320 mCi fructose; 1.160 mCi glucose; 1.736 mCi sucrose and 0.232 mCi acetate. All plants were harvested after 12 hours. Treated leaves were washed in 10 ml distilled water. The water was evaporated and 4 ml scintillation cocktail was added to each vial, and the radioactivity then determined.

In this experiment, the division of the ethanolic fraction into amino acids, carbohydrates and organic acids was extended. Once the carbohydrate fraction had been reduced to 10 ml and the radioactivity determined, 500 μ l of the 10% *iso*-propanol sample was applied to Whatman No 1 chromatography paper, in order to separate the carbohydrates using descending paper chromatography. The upper phase of n-butanol: ethanol: water (45:5:50 ; v/v) was utilized. The tank was equilibrated for one day and the chromatograms equilibrated for three hours before the separation began. Sugar standards (sucrose, glucose and fructose) were included on each chromatogram. The separation took four days at 20°C. The chromatograms were removed and allowed to dry.

The chromatogram containing applied standards was sprayed with aniline diphenylamine phosphate (ERVIN AND SYPERDA,1971), which was prepared as follows:

ANILINE DIPHENYLAMINE PHOSPHATE SPRAY

REAGENTS

Solution A. Mix 5 ml aniline, 5 g diphenylamine and 100 ml acetic acid.

Solution B. Acetone.

Solution C. Mix 20 ml 85% phosphoric acid in 100 ml water.

METHOD

A, B and C were combined in that order (1:1:1, v/v) (ZWEIG AND SHERMA, 1972). The chromatograms were sprayed with the reagent and were heated to 100°C for 2 to 5 minutes and the spots were inspected under ultra violet light.

Once the R_f of the standards had been established, the corresponding sections of the chromatogram were marked and cut out. The sugars were eluted by placing the chromatogram pieces in 50 ml distilled water in Erhlenmeyer flasks and heating to 40°C for 20 minutes. Samples were shaken vigorously and the solution was filtered and the volume reduced under vacuum at 45°C. One ml of each solution was transferred to scintillation vials, dried down and 4 ml scintillation cocktail added. Samples were left in the dark at 10°C overnight and the radioactivity of the individual sugars was determined using a LS 3800 scintillation counter.

AMENDMENTS TO THE ROUTINE PROCEDURE WHERE [¹⁴C] SUCROSE WAS SUPPLIED AS THE PRECURSOR TO THE ISOPRENOID BIOSYNTHETIC PATHWAY.

Two-year-old plants were used for this experiment which was conducted in July (Winter) and December (Summer). For the partitioning experiment, in addition to the plants being divided into the components described in section 6, the root crown was divided into two sub-samples, bark/cortex (including the phloem) and the xylem/pith (central parenchyma core enclosed by the vascular tissues) Radioactivity was applied to four leaves. The specific activity applied

to each plant was 1.736 mCi. Plants were harvested after 6, 12, 24 and 168 hours. For the metabolic studies the radioactivity of the ethanolic, acetone and petroleum ether extracts was determined.

AMENDMENTS TO THE ROUTINE PROCEDURE WHERE [^{14}C] FRUCTOSE WAS SUPPLIED AS THE PRECURSOR FOR THE ISOPRENOID BIOSYNTHETIC PATHWAY.

Two-year-old plants were used for this experiment which was conducted in June (Winter) and February (Summer). Flowering stalks were still present and these were combined with the young stem sample during harvesting. Radioactivity was applied to 6 leaves which allowed for analysis of the treated leaves. Specific activity applied to each plant was 1.980 mCi. These treated leaves were washed in 10 ml distilled water after harvesting, the water was evaporated and the radioactivity remaining on the leaves was determined. Thus it was possible to estimate the uptake of the radioactive compound. The ethanolic fraction was analyzed for the incorporation of radioactivity into amino acids, carbohydrates and organic acids.

2.3.10 [^{14}C] SERINE AND [^{14}C] GLYCINE AS POSSIBLE PRECURSORS FOR RUBBER PRODUCTION

As serine and glycine are not translocated readily in the plant, another approach was attempted to determine if these two compounds could be acting as precursors of rubber (REDDY AND DAS,1987). The non-destructive radiochemical assay (MACRAE,GILLILAND AND VAN STADEN,1986), utilizes uniformly cut sections of primary guayule stem (1 to 5 cm from the apex) which are incubated in excess substrate (usually [^{14}C] acetate), and the incorporation of the radioactivity into rubber is then determined. This assay was used as an indirect assessment of the ability of the rubber transferase enzyme to utilize substrate for the formation of *cis*-polyisoprene.

Stem slices of two-year-old primary stems (2 mm thick) were taken in September. These stem slices were incubated in [^{14}C] serine (specific activity 1.501 mCi), [^{14}C] glycine (specific activity 1.266 mCi), and [^{14}C] acetate (specific activity 0.2324 mCi) as described by MACRAE, GILLILAND AND VAN STADEN (1986).

2.4 RESULTS

2.4.1 The growth habit of guayule

Guayule is a multi-branched plant with an upper layer of leaves. The main shoot and all subsequent branches terminate in inflorescences (**Figure 5**). New branches develop from one to three lateral buds in the upper leaf axils, although not all the branches survive (LLOYD, 1911). Guayule plants may reach a height of one metre.

Guayule has a defined morphological sequence in the southern hemisphere, although there are differences between plants that have been grown in the field and those grown in plastic pots (**Figure 6**). In the field, budbreak takes place in August, with the majority of plants flowering by the end of September. A second flush of flowers occurs in January which may last until the end of March. The flowering stalk is a compound cyme with the main peduncle and secondary peduncles bearing heads about one centimetre in diameter. Each head has five ray florets around the outside that produce seed and numerous disc florets in the centre that produce pollen. Flowering begins in Spring and may continue until Autumn. The mature fruit is a cypsela which consists of an embryo enclosed in two seed coats. Leaf fall in these plants is not prolific and the plants retain a great deal of leaves throughout the Winter (**Figure 7**).

In the pots, budbreak occurred in August, with the first reproductive buds appearing on or within a few days of the 10th September every year. By 25th

September, new stem growth equalled or exceeded five centimetres. Flowering stalks were observed. Plants in pots flowered once and produced a limited number of flowering stalks (**Figure 8**). By the end of January, flowering was completed and the large-lobed leaves began to fall from the plants. Leaf fall continued until the end of April, leaving only a cluster of grey leaves at the end of each branch and some old flowering stalks (**Figure 9**). The plants then entered a period of rest, in which no further growth was observed until the end of July. The root system consisted of a modified taproot which loses dominance and the roots become fibrous. The development of the root system is affected by soil texture, water holding capacity, aeration and penetrability. This poses problems for the potted experimental plants which become root-bound very quickly.

2.4.2 The leaf morphology and anatomy and the ultrastructure of the trichomes of guayule.

The leaves are spatulate to narrowly lanceolate with long petioles, possessing entire to severally-lobed margins depending on the season (**Plate 1A**). During the Autumn, leaves were seen to contain starch grains, while in Summer, leaves contained numerous peroxisomes (KELLY, GILLILAND AND VAN STADEN, 1985). The entire surface of the leaves is covered with trichomes (**Plate 1B**). The trichomes are predominantly T-shaped with the stalk attached centrally to the cap cell (**Plate 1C**). The cap cells have one blunt end and one hooked or pointed end. This could mean that W10 guayule can not be classified as true guayule but rather as the result of introgression between guayule and mariola (GOSS, 1991). Also present are one-celled and 2 or 3-celled stalked trichomes (**Plate 1D**). The combination of trichomes is characteristic of each *Parthenium* species (HEALEY, MEHTA AND WESTERLING, 1986). The guard cells in W10 are thick walled, have a waxy epidermis and there is no spongy mesophyll, only a compacted palisade mesophyll (KELLY, GILLILAND AND VAN STADEN, 1986). This is



Figure 5. The growth habit of *Parthenium argentatum* in the field.

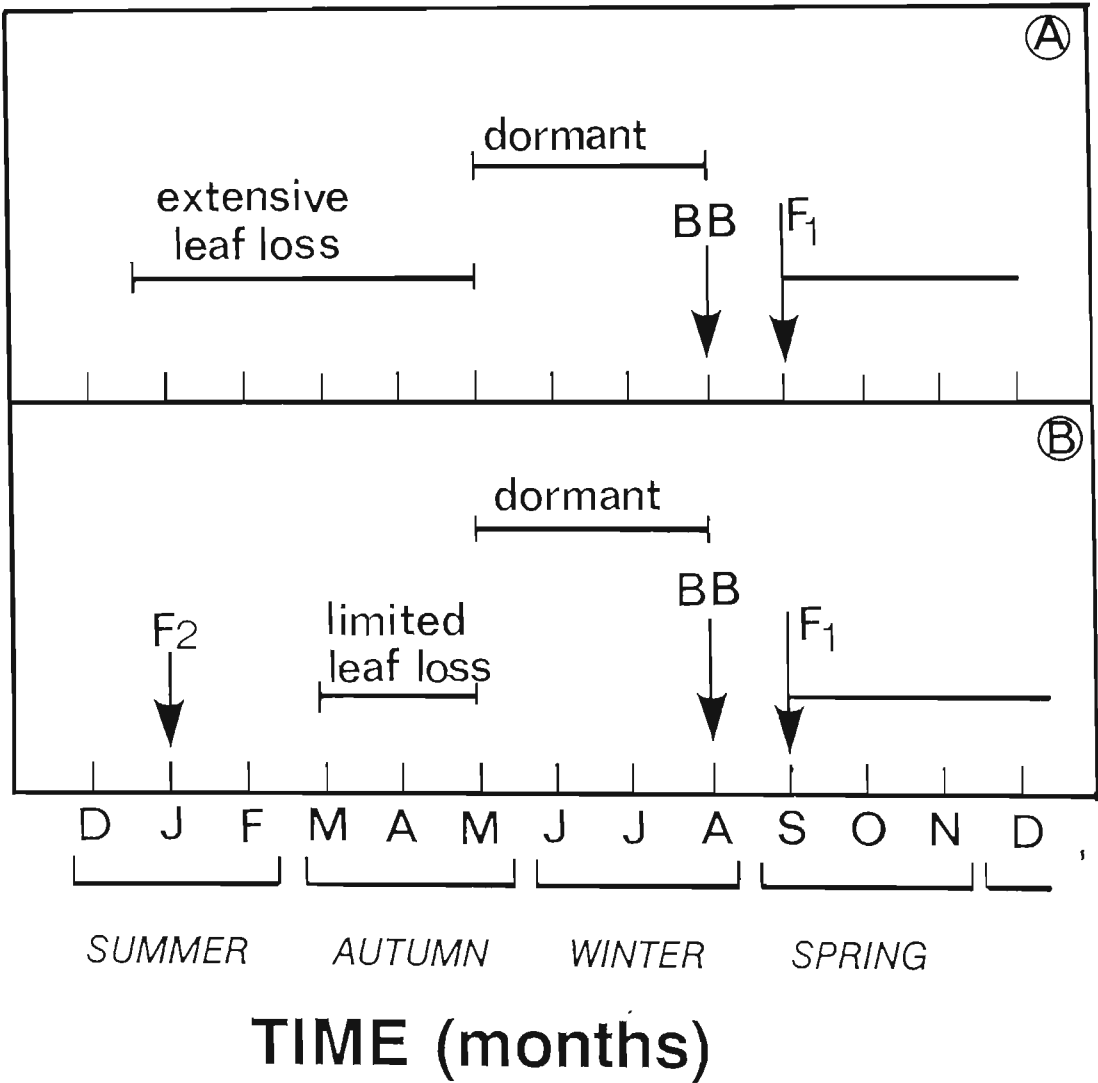


Figure 6. The phenology of pot (A) and field-grown (B) guayule plants. BB = budbreak, F₁ = first flowering flush, F₂ = second flowering flush



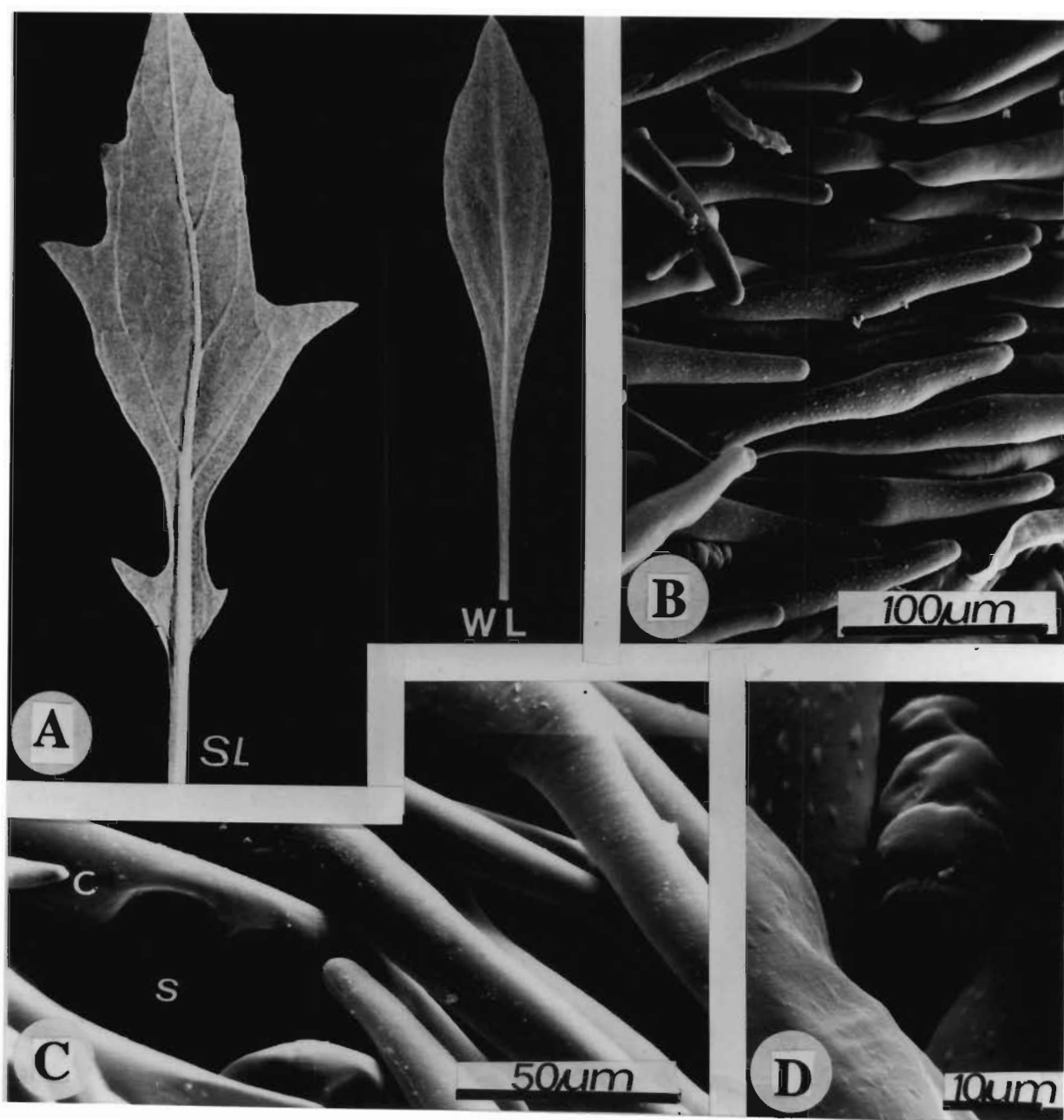
Figure 7. Guayule plants flowering in the field.



Figure 8. Guayule plants grown in pots.



Figure 9. A guayule plant in the winter condition in a pot.



not unusual for a desert plant as total leaf surface can be reduced at a time when water is scarce. The loss of leaves, the ability to become dormant in times of drought, and the appearance of trichomes on the leaves all constitute xeromorphic characteristics (MULLER,1946).

2.4.3 Isoprenoid and *cis*-polyisoprene production in guayule

Chlorophyll, resins and rubber were synthesised by the same precursors. During extraction the chlorophyll and the resins formed a component of the acetone fraction which is referred to as the isoprenoid fraction.

Resin was produced throughout the year in the stem reaching its highest levels during the Spring months, while rubber synthesis reached the highest level during September, after budbreak and as flowering begins (Figure 10). The levels of resins in the leaves were comparable with those in the stem although the leaves contained negligible levels of rubber (GILLILAND AND VAN STADEN,1986).

The levels of chlorophyll a and b showed a seasonality, with chlorophyll a decreasing in May while chlorophyll b decreased in June and July (Figure 11A). In the yellow leaves, analyzed prior to leaf fall, a similar pattern was noted although it occurred three months earlier than the pattern observed in the green leaves (Figure 11B).

2.4.4 The identification and quantification of free amino acids in guayule

The endogenous levels of serine and alanine were high in the Summer leaves (Figure 12A). In Winter leaves the level of serine decreased and the glycine/alanine levels were high. Prior to bud break the levels of proline and alanine were high (Figure 12C), but these decreased after budbreak, and serine increased with the production of the new Summer leaves (Figure 12D). The proline levels were the highest (Figure 12D).

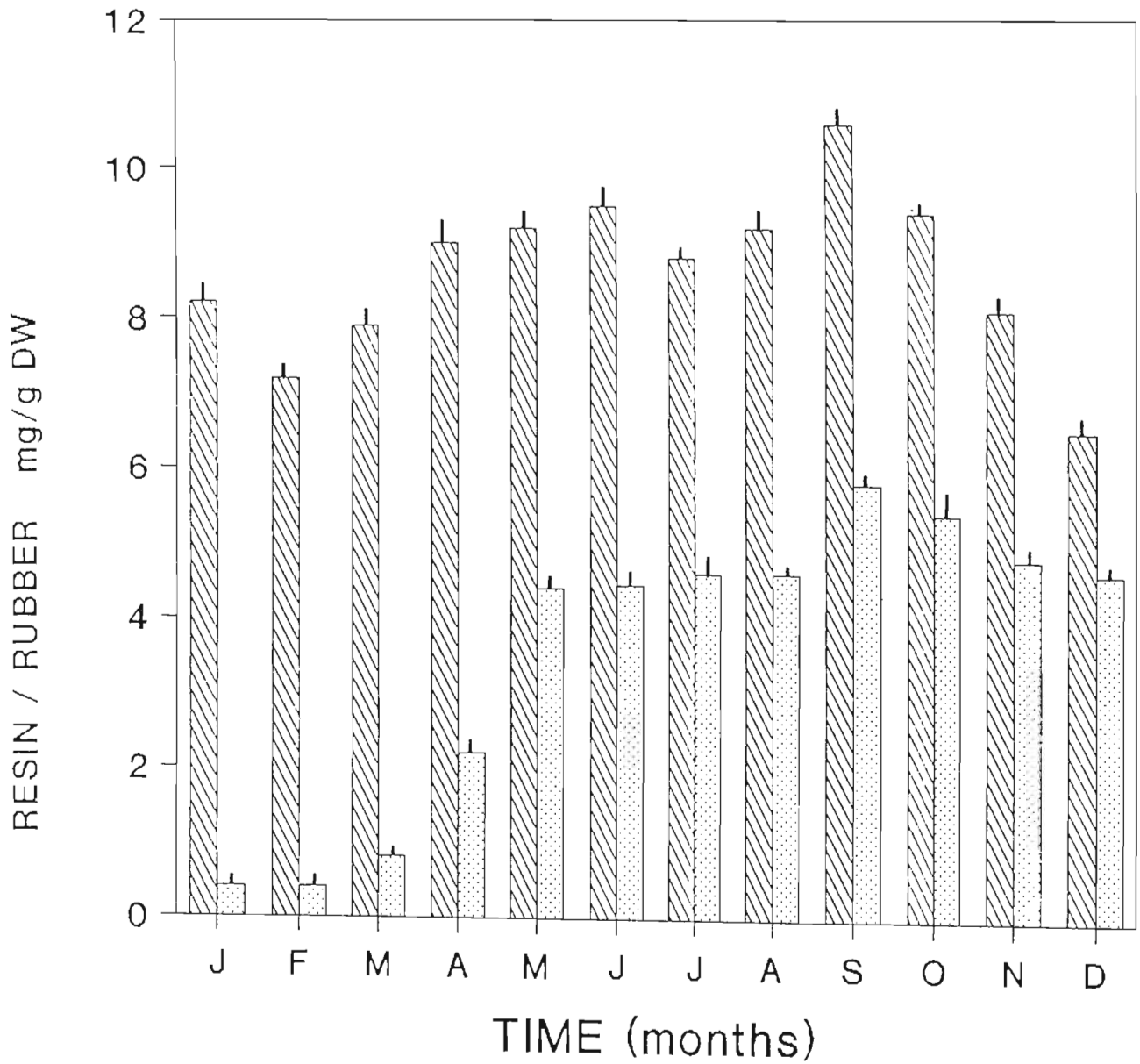


Figure 10. The endogenous levels of resin (▨) and rubber (▤) over a period of twelve months. Bars represent the standard error.

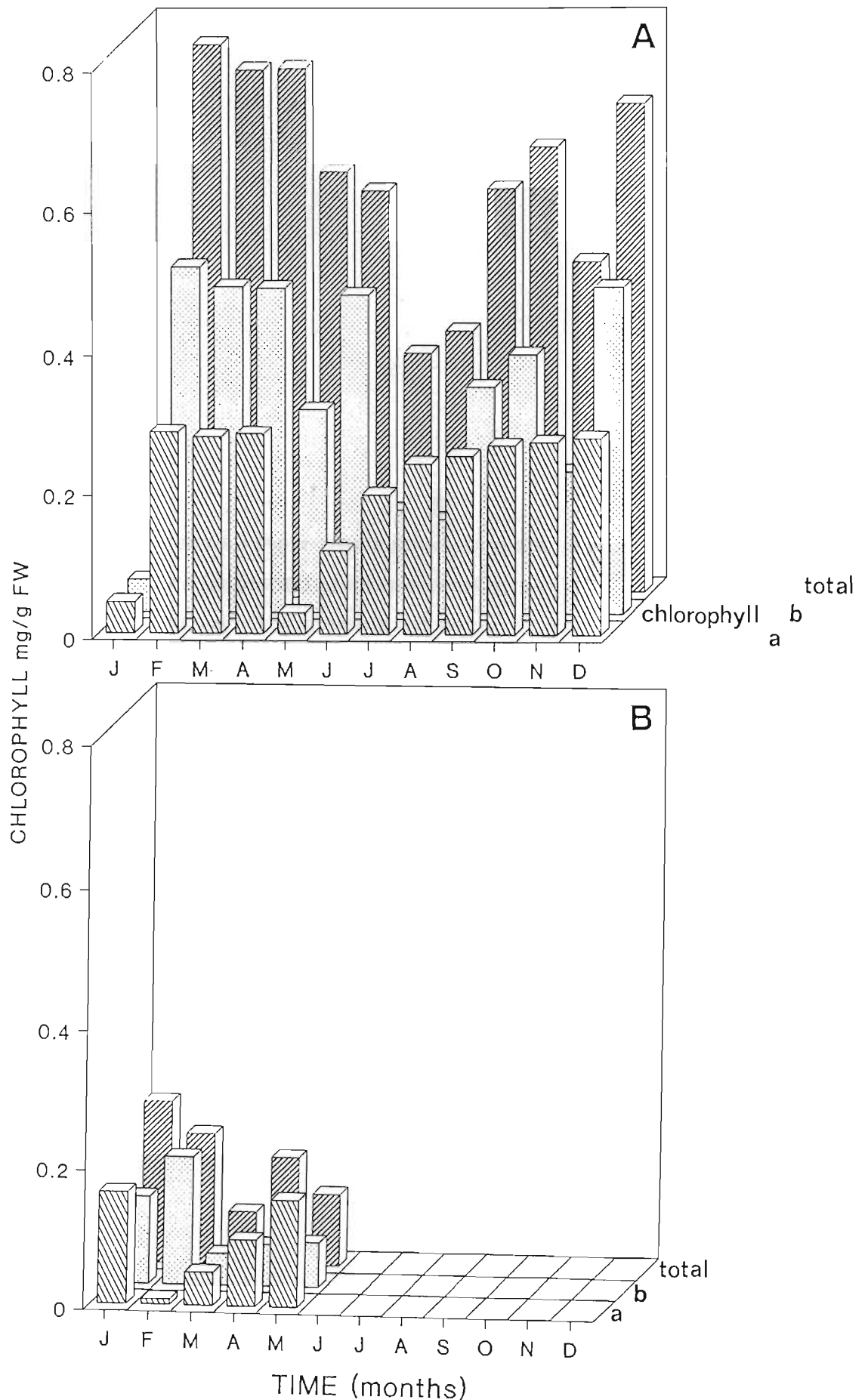
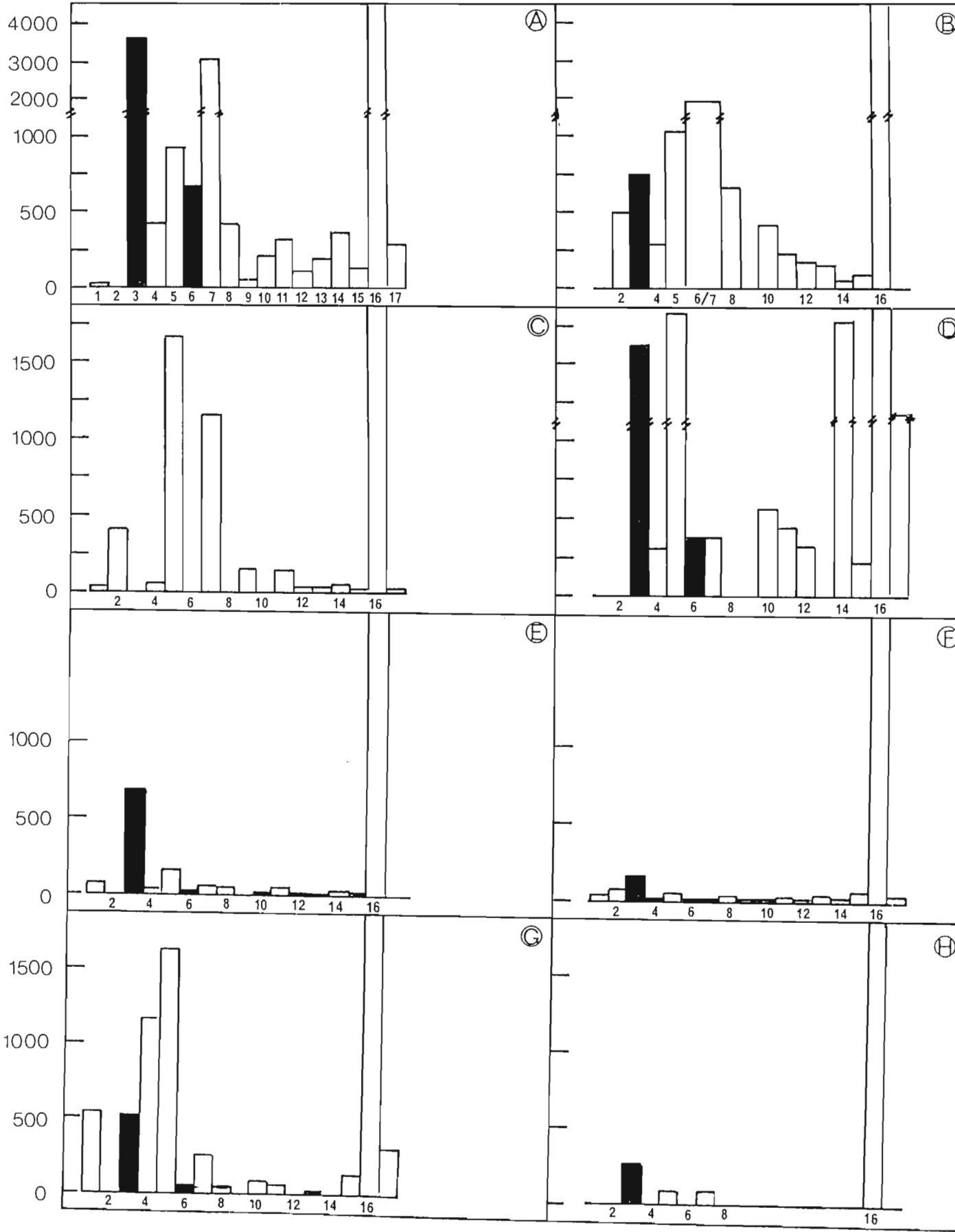


Figure 11. Chlorophyll was extracted from the green leaves of guayule plants grown in the field from January to December 1989 (A). During the period of leaf fall (Figure 6), the senescing yellow leaves were collected and the chlorophyll extracted (B).

AMINO ACIDS nmoles/g DW



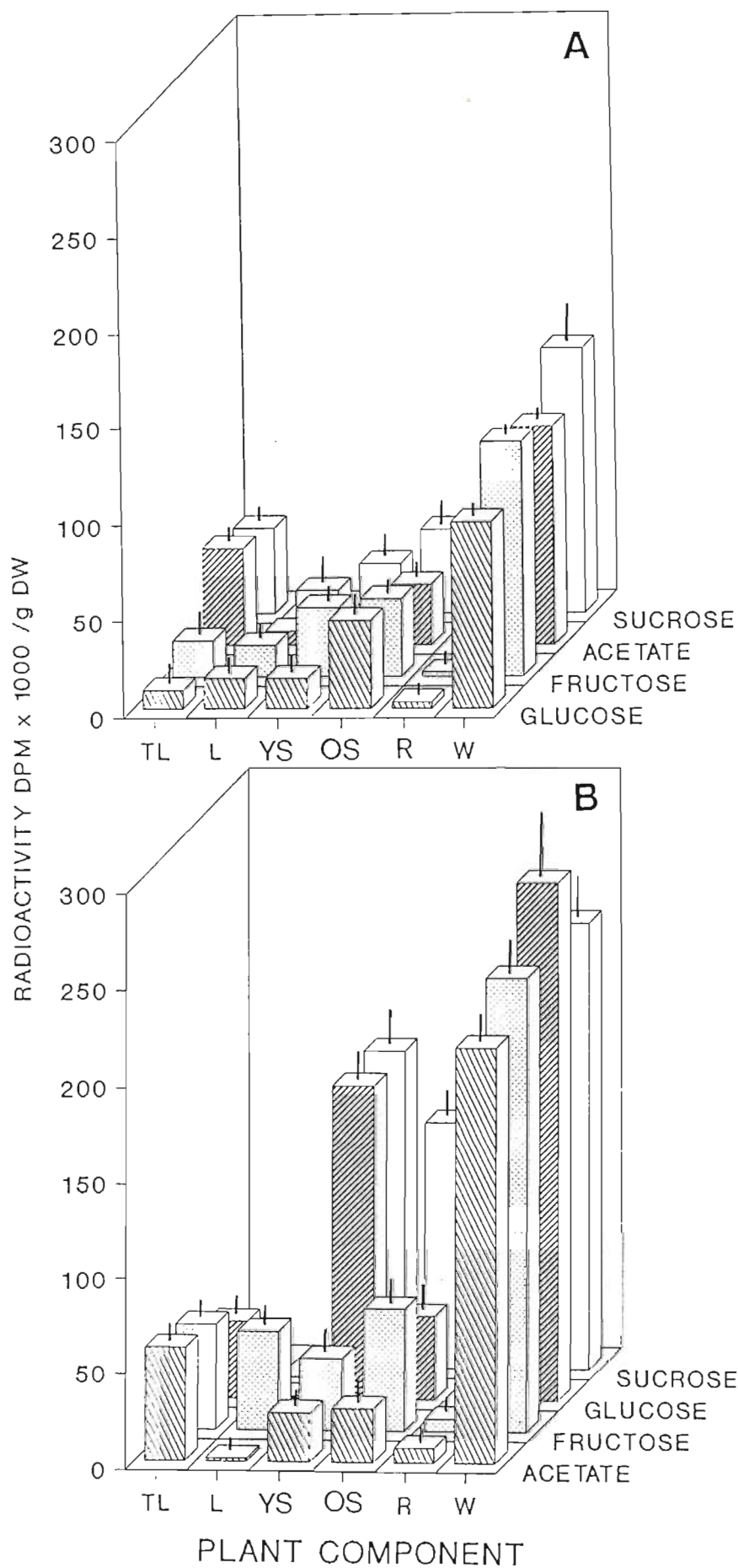
In the stem, the free amino acid levels were lower than those in the leaves, and serine was present in levels five-fold than those in the Summer stem (**Figure 12E**). In Winter, the amino acid levels in the stem were very low (**Figure 12F**). Before budbreak serine levels increased in the stem (**Figure 12G**), but decreased thereafter (**Figure 12H**).

2.4.5 A twelve hour study of the distribution and metabolism of radioactive acetate, fructose, glucose and sucrose in guayule.

In the experiment lasting twelve hours, the treated Summer leaves did not retain as much radioactivity as the Winter treated leaves (**Figure 13**). The radioactivity remaining on the treated leaves (in the wash), was significantly higher in the Winter than in the Summer plants and the total radioactivity extracted from the Winter plants was greater than that extracted from the Summer plants (**Figure 13**). The radioactivity in the Summer plants was detected in all the plant components (**Figure 13A**), while it was concentrated in the stems of the Winter plants (**Figure 13B**). The low levels extracted from the roots may have been due to the short duration of the experiment or due to the difficulty experienced in separating the roots from the growing medium.

During the Summer and Winter, the radioactive carbohydrates were translocated more freely than the acetate to all the plant components (**Figure 13A,B**). There appeared to be no major sink operative in the Summer plants, while in the Winter plants the radioactivity was concentrated in the primary and secondary stems when applied as [^{14}C] sucrose or [^{14}C] glucose (**Figure 13A,B**). [^{14}C] Fructose was evenly distributed through the plant components in Summer and Winter (**Figure 13A,B**).

During Winter, irrespective of the molecular structure of the applied substrate, the radioactivity was highest in the carbohydrate fraction of the treated leaves and young stem (**Figure 14A-D**). When the carbohydrate fraction from each plant component was extracted and the amount of radioactivity incorporated



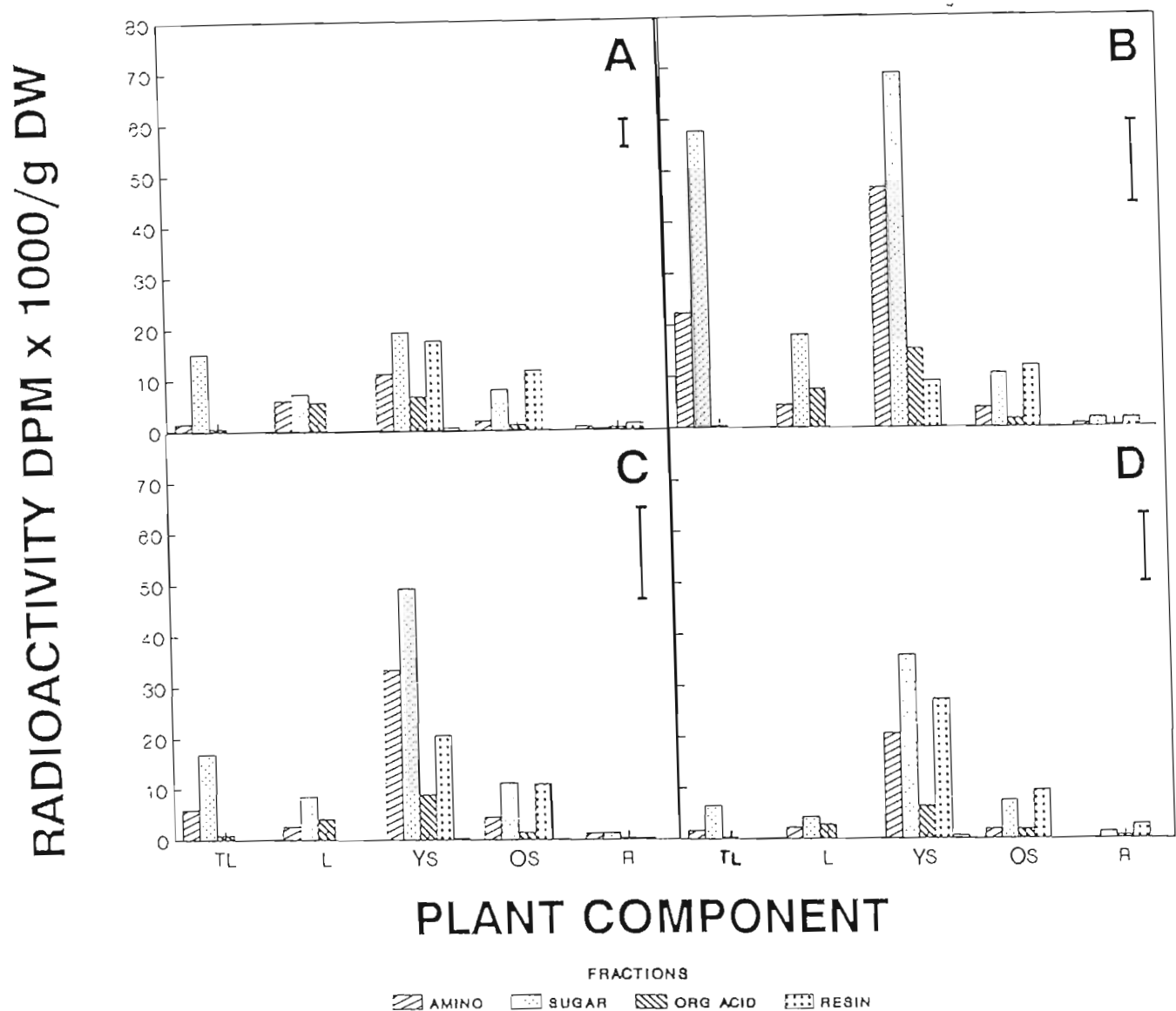


Figure 14. The radioactivity recovered in the amino acid (▨), carbohydrate (▤), organic acid (▩), acetone (▧) and petroleum ether (□) fractions of the treated leaves (TL), untreated leaves (L), young stems (YS), mature stems (OS) and roots (R) of plants where the substrate was applied as [^{14}C] acetate, [^{14}C] fructose, [^{14}C] glucose and [^{14}C] sucrose, in the Winter. The bars represent the least significant difference (L.S.D.).

into fructose, glucose and sucrose determined, the most radioactivity co-eluted with sucrose in the treated leaves, leaves, mature stems and roots whether the substrate was applied as fructose, glucose, sucrose or acetate (**Figure 15**). The young stem incorporated the radioactivity into fructose when the substrate was applied as [^{14}C] acetate, fructose and glucose but not when applied as [^{14}C] sucrose, in which case the highest radioactivity was associated with sucrose (**Figure 15**).

The young stem appears to be a metabolically strong sink (**Figure 14A-D**). As this had been observed in all the long term experiments, it is unlikely to be an artefact of the short experimental time. The labelled substrates were concentrated in the amino acid, carbohydrate and isoprenoid fractions. In the mature stem, the labelled substrates were metabolized and the highest activity was recorded in the carbohydrate and isoprenoid fractions (**Figure 14A-D**). The roots incorporated the least radioactivity (**Figure 14A-D**).

In the Summer, the labelled substrates were incorporated primarily into the carbohydrate and isoprenoid fractions (**Figure 16A-D**). The treated leaves and the rest of the leaves channelled the radioactivity into the carbohydrate fractions while in the young and mature stems significantly higher activity was measured in the resin fraction (**Figure 16A-D**).

The actual amount of radioactivity recovered was greater in the Winter (250 000 dpm) than in Summer (40 000 dpm)(**Figure 13**).

2.4.6 The distribution and metabolism of [^{14}C] sucrose in guayule.

There was a marked difference between the distribution of the [^{14}C] sucrose in the Winter and Summer plants (**Figure 17**). During the Winter, the majority of radioactivity was retained by the treated leaves. This did not change with time (**Figures 17A-D**). The translocated radioactivity was evenly distributed

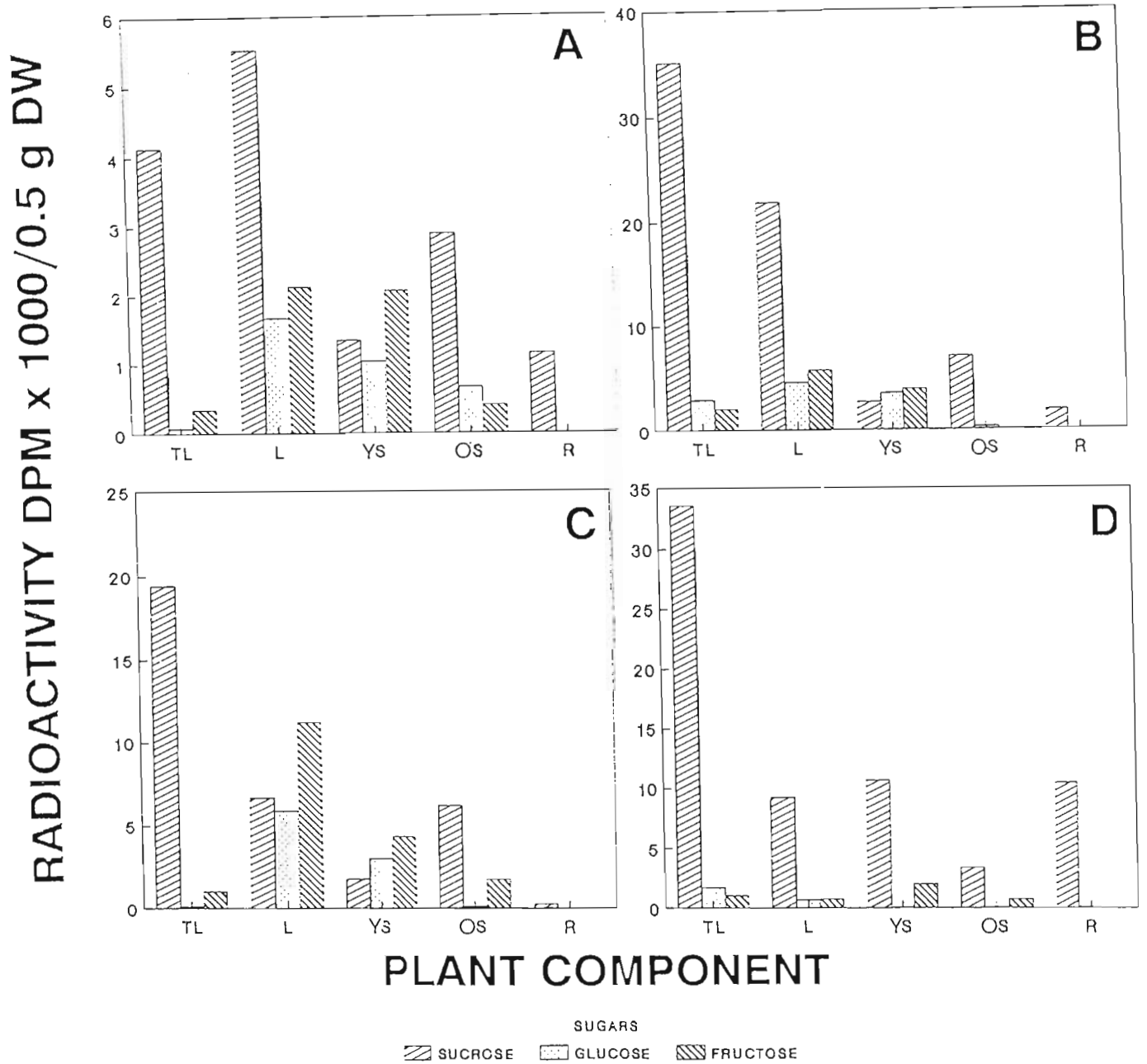


Figure 15.

Analysis of the radioactivity incorporated into glucose (▤), fructose (▥) and sucrose (▧) in the carbohydrate fraction from the treated leaves (TL), leaves (L), young stem (YS), mature stem (OS) and roots (R) of Winter plants following the application of $[^{14}\text{C}]$ acetate (A), $[^{14}\text{C}]$ fructose (B), $[^{14}\text{C}]$ glucose (C) and $[^{14}\text{C}]$ sucrose (D).

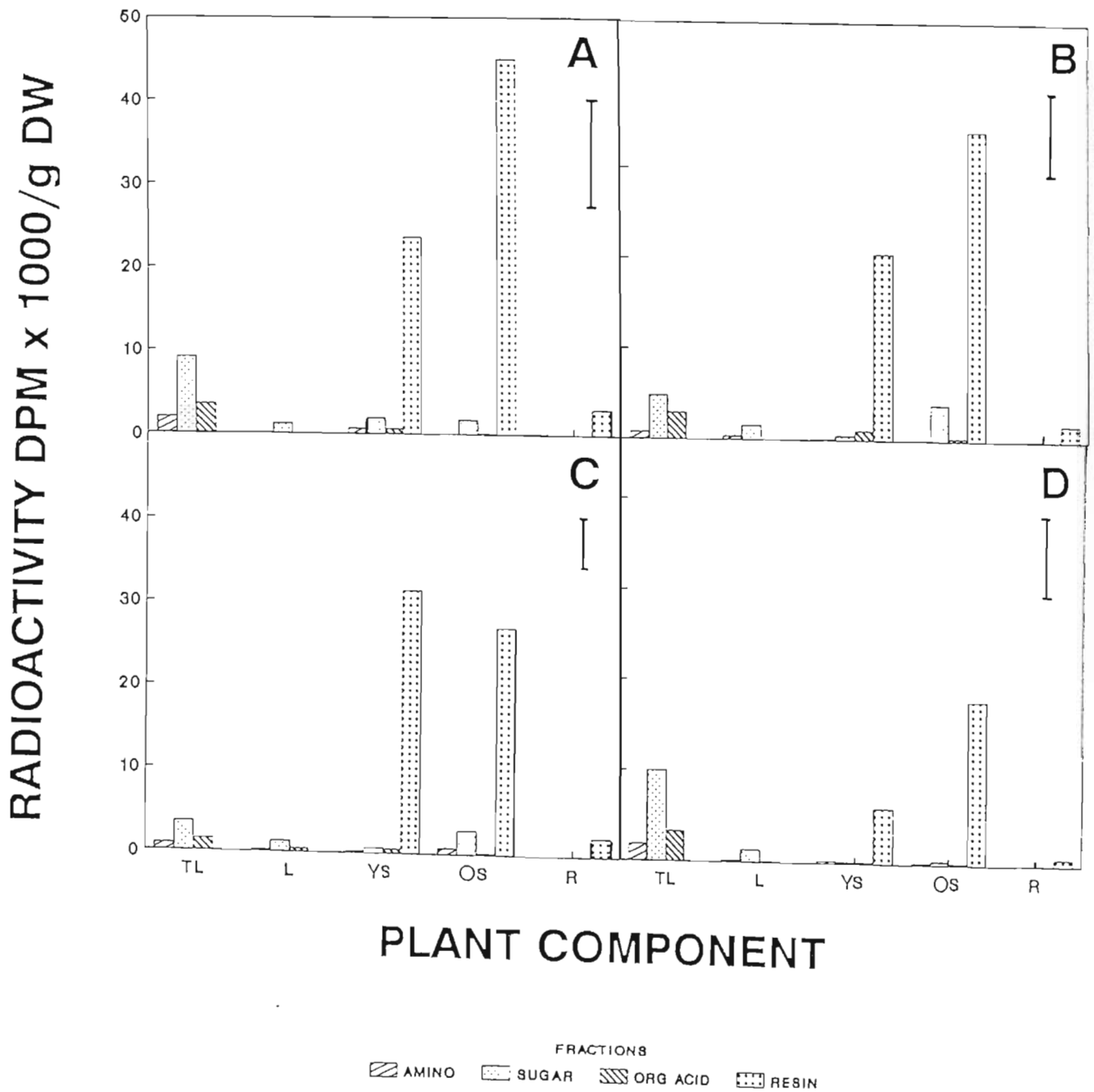


Figure 16.

The radioactivity recovered in the amino acid (▨), carbohydrate (▤), organic acid (▩), acetone (▧) and petroleum ether (□) fractions of the treated leaves (TL), untreated leaves (L), young stems (YS), mature stems (OS) and roots (R) of plants where the substrate was applied as [^{14}C] acetate, [^{14}C] fructose, [^{14}C] glucose and [^{14}C] sucrose, in the Summer. The bars represent the least significant difference (L.S.D.).

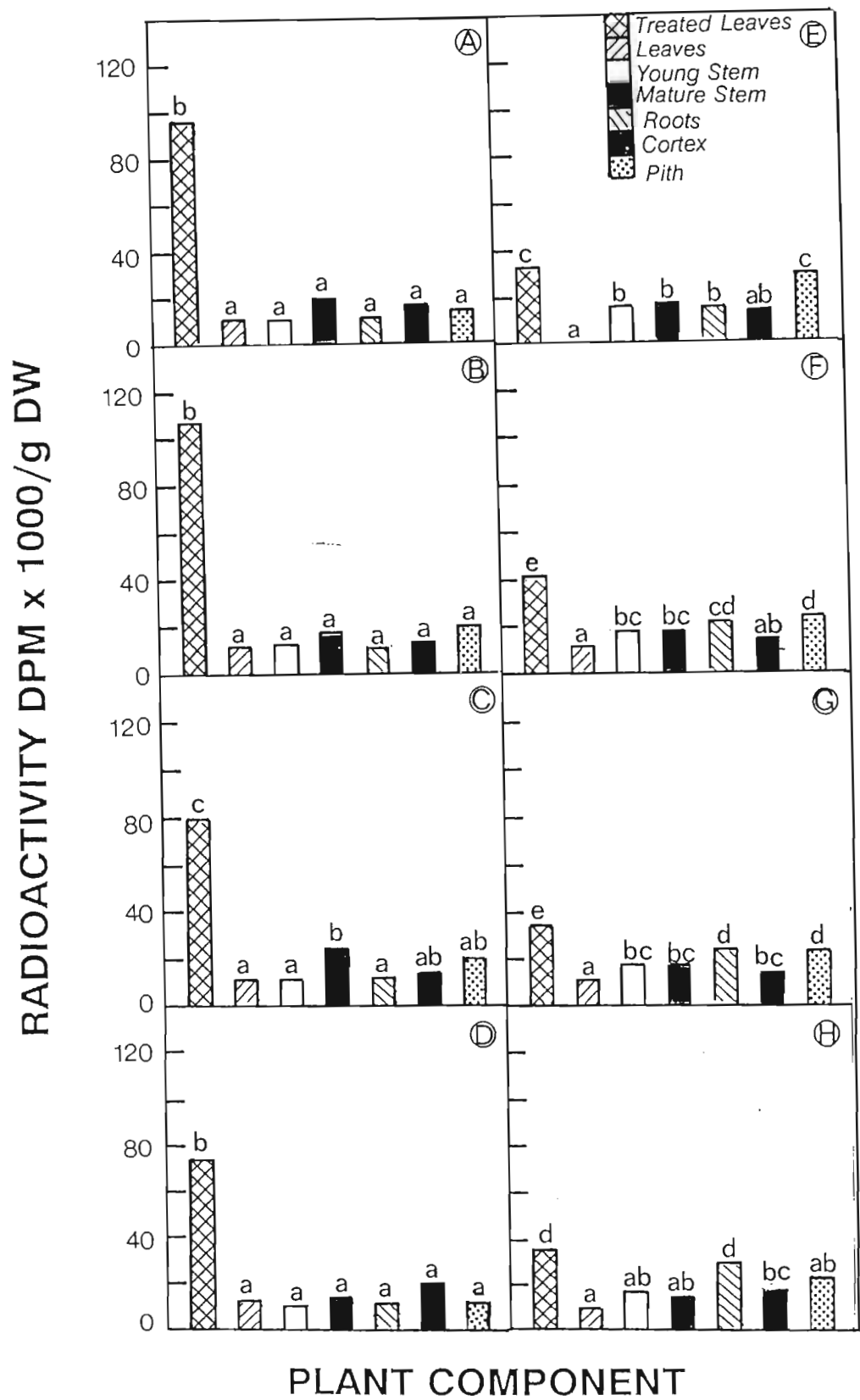


Figure 17. The distribution of radioactivity from [^{14}C] sucrose in Winter (A to D) and Summer (E to H) plants, 6 (A,E), 12 (B,F), 24 (C,G) and 48 (D,H) hours after application of the radioactivity. Plant components analyzed included treated leaves, leaves, young stems, mature stems, roots, cortex and pith. A range analysis at the 95 % confidence level was performed on the data and is represented as letters above the bars.

between the leaves, stems and roots and there appeared to be no evidence of a major sink operative in these plants.

In the Summer, the amount of radioactivity retained by the treated leaves was significantly less than that recovered from the Winter leaves (**Figures 17E-H**). The treated Winter leaves retained between 80 000 to 110 000 dpm compared to 40 000 dpm retained by the treated Summer leaves. The remaining radioactivity was distributed throughout the components of the Summer plants.

Levels of radioactivity recovered from the leaves was low when compared with the stems and roots (**Figure 17F-H**). The roots received a significantly higher amount of radioactivity when compared with the other plant components (**Figure 17H**). This would suggest that the roots were stronger sinks than the stems in the Summer plants.

Analysis of the radioactivity in the cortex and pith of the stem crown during the Summer and Winter, demonstrated a significant movement of the radioactivity into the pith region of Summer plants at all times, indicating the general strength of this sink (**Figure 17E-H**). This was not observed in the Winter plants, where the radioactivity was partitioned evenly throughout the plant with no preferential partitioning into the pith.

In the leaves of the Summer plants, the radioactivity was ethanol-soluble for all harvest times (**Figure 18A**). There was also an increase in radioactivity of the acetone-soluble fraction within six hours. In the young stems, the radioactivity was significantly higher in the ethanolic fractions at all harvest times, including the 48 hour harvest (**Figure 18B**). The mature stems, when compared with the other plant components, showed preferential incorporation primarily into the acetone-soluble fraction and less so into the ethanolic fraction (**Figure 18C**). By 48 hours, levels of radioactivity in the ethanolic and acetone-soluble fractions were equivalent. The radioactivity was highest in the ethanolic fraction of the roots of the Summer plants, but these levels decreased after 48

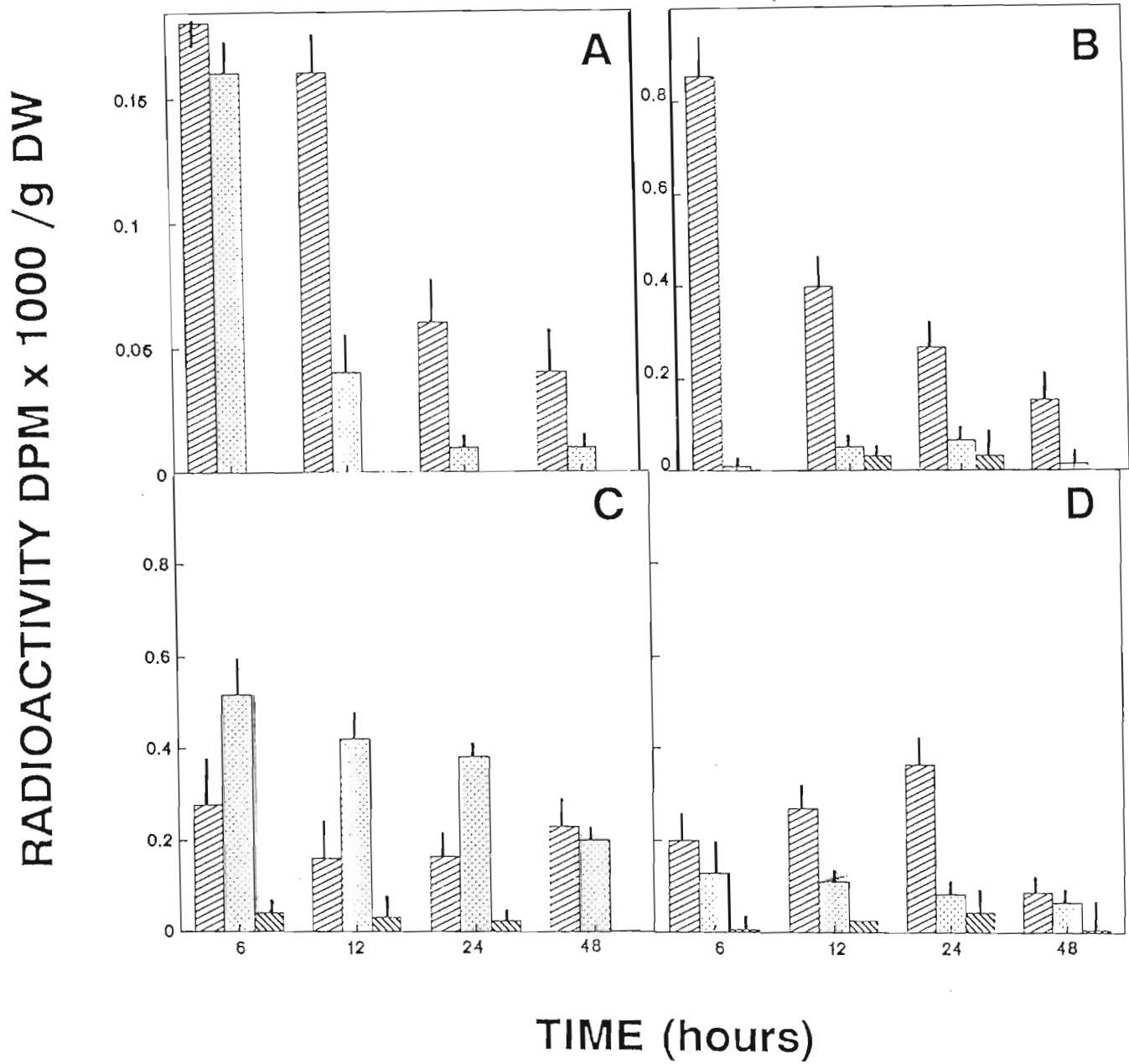


Figure 18. Radioactivity recovered in the ethanolic (▨), acetone (▤) and petroleum ether (▧) fractions of the leaves (A), young stems (B), mature stems (C) and roots (D), 6, 12, 24 and 48 hours after application of [^{14}C] sucrose to Summer plants. Bars represent the standard error.

hours (**Figure 18D**). Incorporation of the isotope into the petroleum ether fraction was observed in the stem and roots (**Figure 18 B-D**).

During the Winter, the radioactivity was primarily found in the ethanolic fraction of the leaves (**Figure 19A**). The young Winter stems differed from the Summer stems in that the radioactivity incorporated into the petroleum ether fraction was significantly higher than the radioactivity extracted in the other fractions (**Figure 19B**). After 48 hours the amount of radioactivity extracted was reduced in all three fractions. The amount of radioactivity in the petroleum ether fraction of the mature Winter stems and roots was less than that extracted from the younger stems (**Figure 19B-D**).

2.4.7 The distribution and metabolism of [^{14}C] fructose in guayule

The radioactivity remaining on the treated leaves was greater in the Winter than in the Summer plants (**Figure 20**). Again, the level of translocated radioactivity remained low with higher levels recorded in the stems rather than in the leaves and roots (**Figure 21A-D**).

The radioactivity extracted from the Summer treated leaves was significantly lower than the radioactivity in the Winter treated leaves (**Figure 21**). The carbon isotope was translocated to all the other plant components with higher levels recorded in the mature stems and roots (**Figure 21 E,G**). After 48 hours, the radioactivity was concentrated in the young and mature stems (**Figure 21H**).

For this experiment, the ethanolic fraction was further divided into the amino acid, carbohydrate and organic acid components. In Winter, the isotope was partitioned between the three components of the ethanolic fraction in all the leaves (**Figure 22A,B**). The primary stem showed preferential incorporation into the acetone fraction (**Figure 22C**), while the radioactivity in the mature stem was incorporated into the amino acid component of the ethanolic fraction

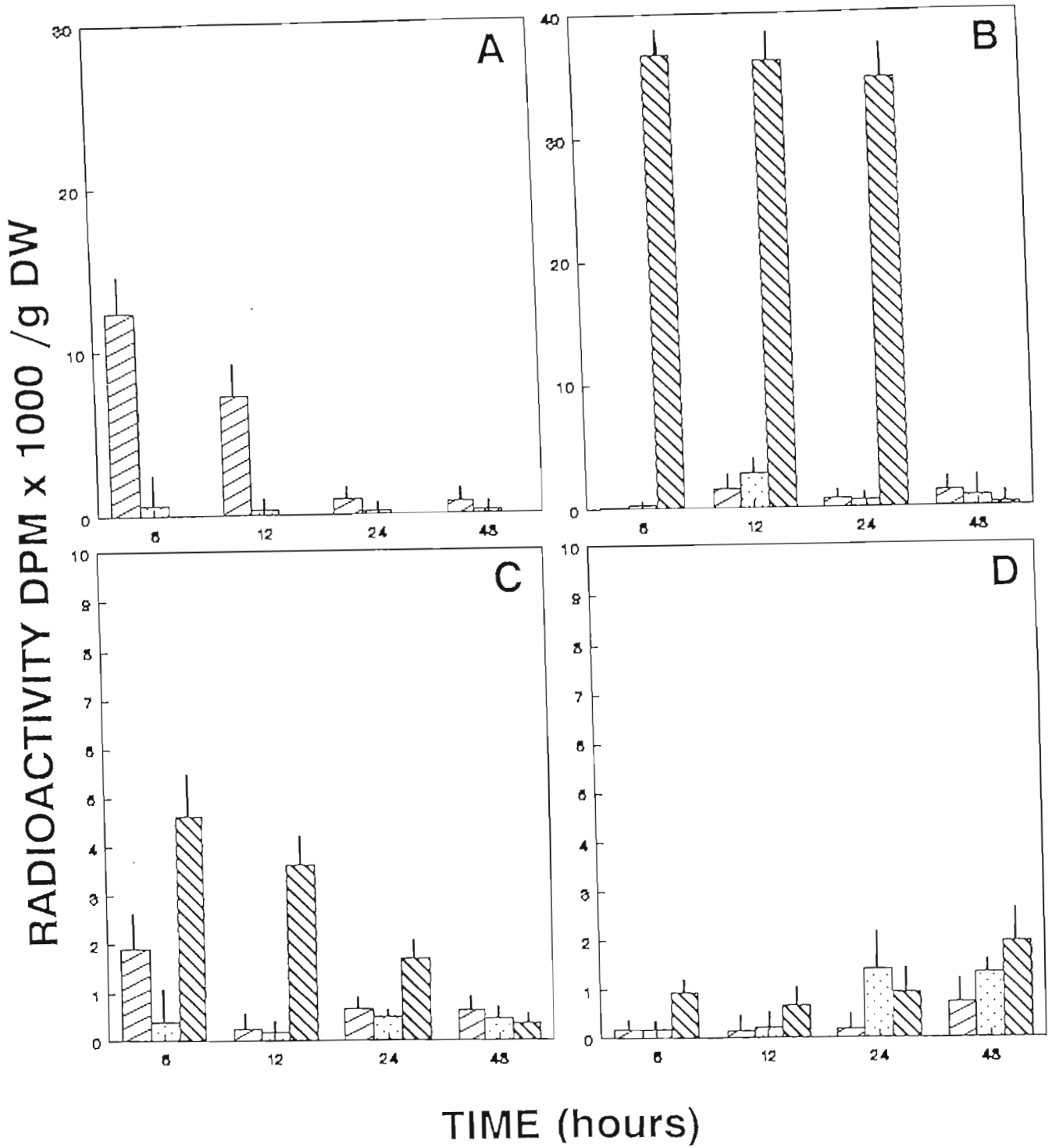


Figure 19.

Radioactivity recovered in the ethanolic (▨), acetone (▤) and petroleum ether (▧) fractions of the leaves (A), young stems (B), mature stems (C) and roots (D), 6, 12, 24 and 48 hours after application of [¹⁴C] sucrose to Winter plants. Bars represent the standard error.

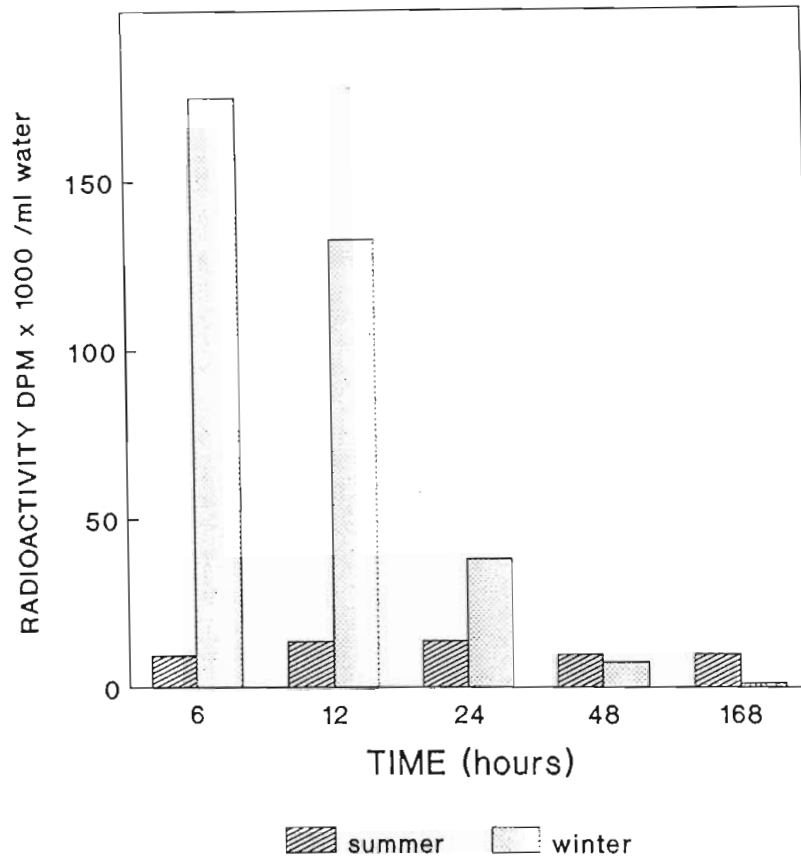


Figure 20. The radioactivity remaining on the treated leaves of Winter and Summer plants following the application of [^{14}C] fructose was washed off after each harvest. Bars represent the standard error.

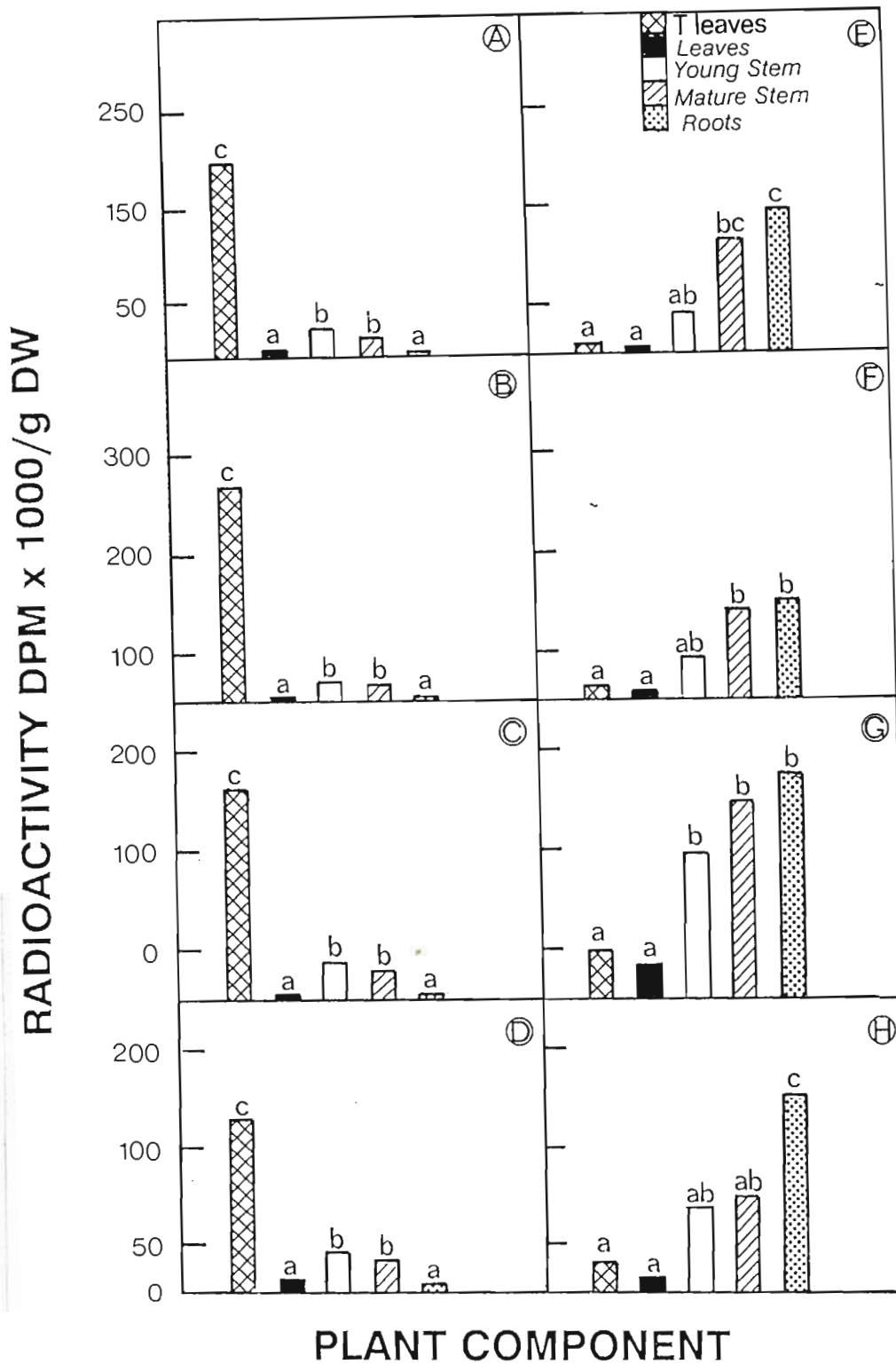


Figure 21.

The distribution of radioactivity applied as [^{14}C] fructose to four apical leaves. Plants were harvested after 6 (A,E), 12 (B,F), 24 (C,G) and 48 (D,H) hours in Winter (A to D) and Summer (E to H) and divided into treated leaves (TL), untreated leaves (L), young stems (YS), mature stems (OS) and roots (R). A multiple range analysis at the 95 % confidence level was performed on the data and is represented as letters above the bars.

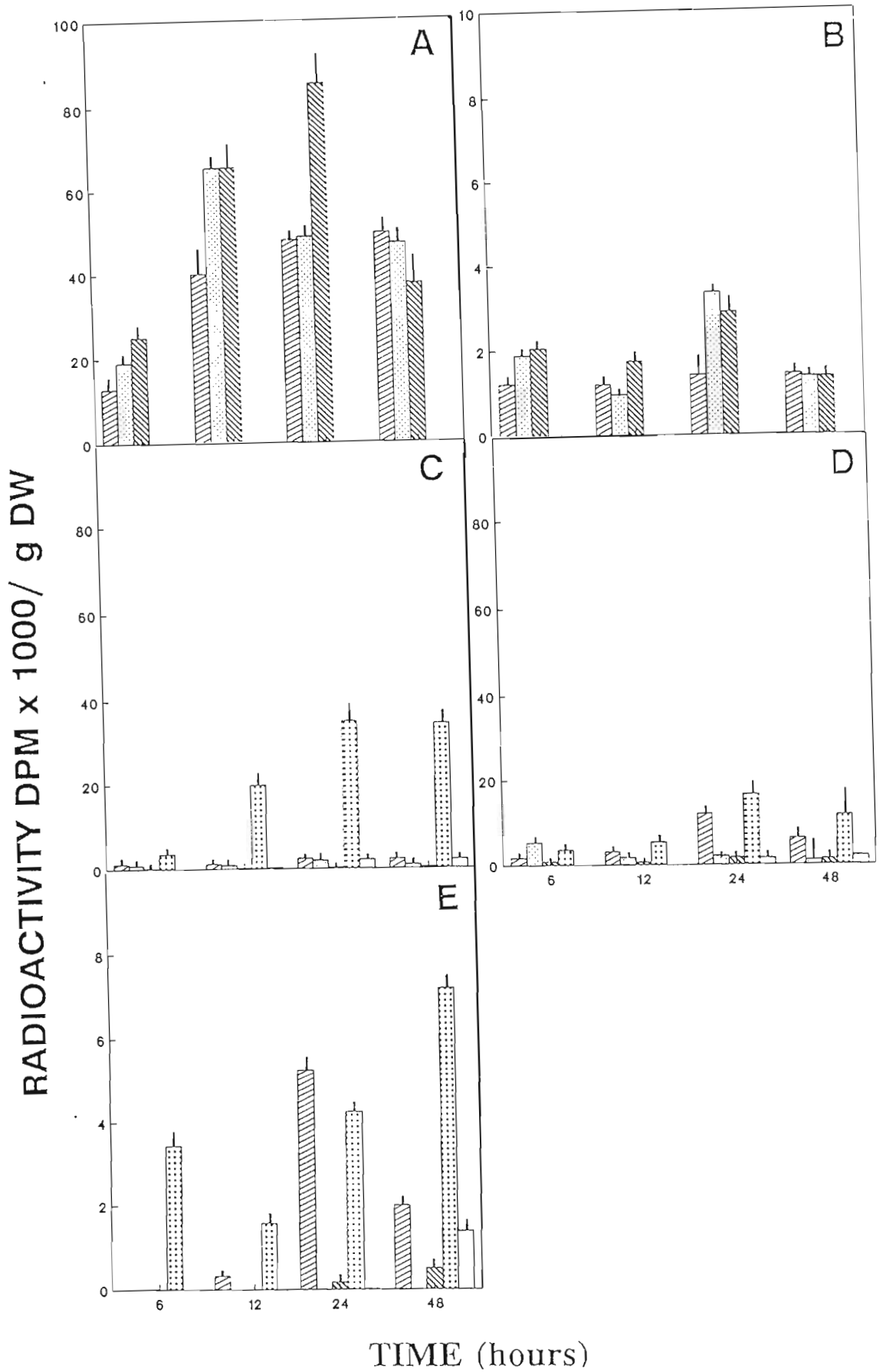


Figure 22.

Incorporation of radioactivity 6,12,24 and 48 hours after application of [^{14}C] fructose into the amino acid (▨), carbohydrate (▤), organic acid (▥), acetone (▧) and petroleum ether (□) fractions of the treated leaves (A), untreated leaves (B), young stems (C), mature stems (D) and roots (E) of Winter plants. Bars represent the standard error.

within 24 hours and steadily increased in the resin component (**Figure 22D**). In the roots, the radioactivity was highest in the amino acid and resin fractions (**Figure 22E**). This experiment differed markedly from the [^{14}C] sucrose experiment in that there was very little activity observed in the petroleum ether (rubber) fraction.

In the Summer, there was a marked decrease in the total amount of radioactivity extracted from the plants (40 000 dpm compared with 10 000 dpm), and this was incorporated predominantly into the carbohydrate fraction of the treated leaves, untreated leaves and the young stem (**Figure 23A-C**). The young, mature stem and roots also showed significant incorporation of the isotope into the resin fraction (**Figure 23C-E**). The radioactivity of the petroleum ether fraction of the roots was much lower than that of the acetone fraction (**Figure 23E**).

The isotope was apparently metabolized along the isoprenoid pathway as it was incorporated into the resins and *cis*-polyisoprene in the Winter plants (**Figure 22B-D**). The Summer plants incorporated the isotope specifically into resins in the mature stem and roots (**Figure 23C-E**).

2.4.8 The role of serine and glycine as precursors in the isoprenoid biosynthetic pathway

The isotopes from acetate, serine and glycine were incorporated into the aqueous fraction of the incubated stem slices (**Figure 24**). The acetone (resin) fraction showed significantly greater incorporation with acetate as substrate. The amino acids were incorporated into resins to a lesser extent. Only acetate was incorporated into the petroleum ether fraction (**Figure 24**).

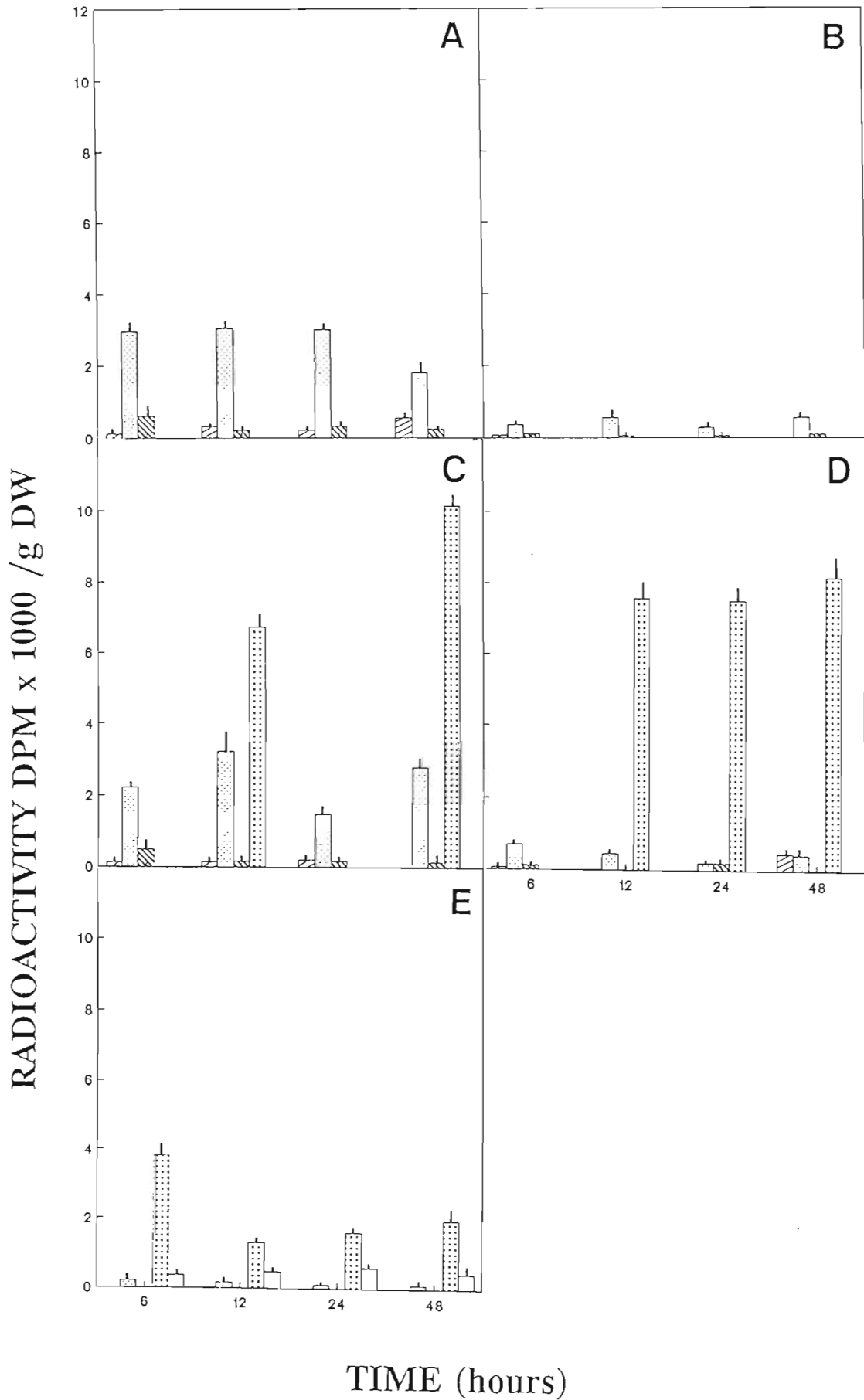


Figure 23.

Incorporation of radioactivity 6,12,24 and 48 hours after application of $[^{14}\text{C}]$ fructose into the amino acid (▨), carbohydrate (▤), organic acid (▧), acetone (▩) and petroleum ether (□) fractions of the treated leaves (A), untreated leaves (B), young stems (C), mature stems (D) and roots (E) of Summer plants. Bars represent the standard error.

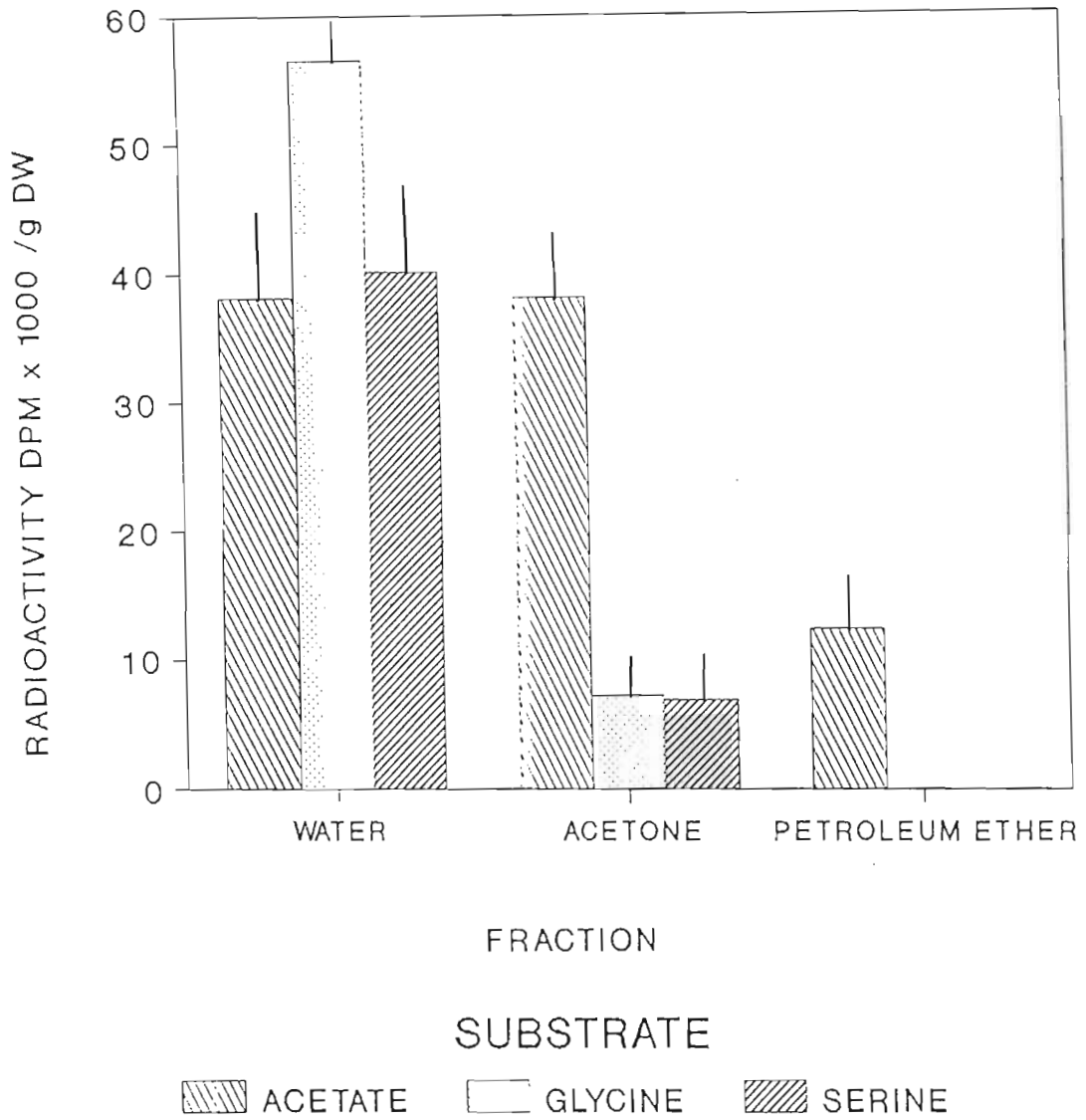


Figure 24.

The incorporation of radioactivity into the aqueous, acetone and petroleum ether fractions of guayule stem slices following incubation in [^{14}C] serine (▨), [^{14}C] glycine (▩) and [^{14}C] acetate (▧). Bars represent the standard error.

2.5 DISCUSSION

In Pietermaritzburg, the dry months coincide with Winter and the rains fall during the Spring and Summer. Rubber production occurs from May to September, with the highest levels recorded after Winter. These experiments were all conducted in June or July (Winter) when rubber synthesis was maximal.

Guayule plants display seasonal growth pattern with a definite period of rest occurring in the Winter. This contrasts with plants growing in the Chihuahuan desert which display no seasonal rhythm with respect to growth, flowering and fruiting. These phenomena take place as long as favourable conditions prevail. This growth pattern is advantageous in an environment where rainfall is not regular (mean rainfall may be 50.8 mm or less in an extreme dry year and 635 mm in an extreme wet year), and temperatures range from -9.4°C to 46°C , with no distinct Winter. In Chapter 4, these seasonal growth patterns will be shown to be associated with biochemical changes in the tissues.

With the onset of colder conditions, guayule plants lose a large percentage of their leaves, retaining only the terminal smaller leaf clusters.

In Spring, the plants produce new leaves and reproductive bud break takes place about 10 September, every year. At the same time, the xylem is differentiated, thus ensuring a fully developed vasculature in the new stems for effective translocation. Phloem differentiates later in the season, in October, at the time of peduncle production. The translocation of assimilates is therefore enhanced. The bark is easily separated from the wood in early Spring, possibly as a result of the division of the cambial cells. The frequency of periclinal division can be indicative of cambial reactivation (AJMAL AND IQBAL, 1987).

This chapter is concerned with the proportioning of the substrate into rubber. Rubber deposition begins in the resin canal epithelial cells and subsequently in

the parenchyma cells of the pith, cortex and vascular rays of the stems and roots. This tissue is non-photosynthetic and thus the productivity of the sink will depend on the supply of assimilates from the source leaves, the long-distance translocation and the allocation and efficiency of the utilization of the mobile sugars.

The reduction of foliage in the Winter months does not reduce the photosynthetic rate, although maximum values are recorded in the Summer months (NAKAYAMA, MITCHELL AND ALLEN, 1985). This must, however, reduce the total assimilate available for metabolism. The net photosynthetic rate of guayule is one third or one fifth that of other economically important plants (NAKAYAMA, ALLEN AND MITCHELL, 1986). Very similar levels have been reported for other arid zone species (ALLEN, NAKAYAMA, DIERIG AND RASNICK, 1987).

The stems are also able to fix [^{14}C] carbon dioxide (GILLILAND, VAN STADEN AND MITCHELL, 1985). The stems of guayule are entirely green in colour from the phelloderm to the central stele region, although chloroplasts are only located in the parenchyma of the cortex (GILLILAND AND VAN STADEN, 1986). The contribution of the chlorophyll-containing organs such as stems, inflorescence parts and fruits can be significant in terms of overall photosynthetic supply, and can influence carbon partitioning (WARDLAW, 1990).

Sucrose is the translocatable form of assimilate in guayule as demonstrated by the short term studies, where the three carbohydrate substrates were translocated as [^{14}C] sucrose in most instances. [^{14}C] Acetate, on the other hand, was first converted into a translocatable form (sucrose) which was then used as a substrate for isoprenoid synthesis. In *Hevea*, it has been observed that radioactive carbohydrates supplied to the bark were converted to sucrose and observed twelve hours later, in the latex vessels, where the sucrose was readily utilized for rubber synthesis (TUPY, 1969). It was decided to use [^{14}C] sucrose and [^{14}C] fructose in two long term experiments. Sucrose was chosen because it was shown to be the translocatable carbohydrate and fructose

because it was evenly distributed throughout the plant. Acetate has already been investigated (KELLY AND VAN STADEN,1987).

Application of the carbohydrates to the leaves resulted in different translocation patterns for the Summer and Winter leaves. In Winter, the uptake and translocation of the radioactivity from the treated leaves in both long term experiments was very poor. This contrasts with the findings of BENZONI AND MILLS (1991), who found that the best translocation of radioactivity occurred in Winter and was attributed to reduced sink activities associated with dormancy. The reduction or possible absence of sinks in Winter plants was noted for both the long term substrate experiments, although the amount of radioactivity translocated was higher in all the Winter plant components when compared with the Summer plant components.

In Winter, although the translocation appears to be limited, the amount of radioactivity reaching the various plant components was greater than that found in the Summer plants. The Summer plants showed a consistent loss of radioactivity from the treated leaves although the remainder was translocated to all other plant components. The radioactivity on and in the leaves is important as conditions which favour a relative enhancement of photorespiratory losses of carbon will appear superficially as an enhancement of translocation based on the loss of carbon from the leaf (WARDLAW,1982). A great deal of the radioactivity retained on the Winter leaves appeared in the wash, while in the Summer leaves the radioactivity recovered in the wash was very low. The retention of radioactivity in the leaves may be due to the poor symplastic connections in the leaves, which appear to transport sucrose apoplastically, leaving a great deal of sucrose in the mesophyll cells (GILLILAND,pers.com.).

The loss of radioactivity from the Summer leaves is due either to hydrocarbon emission or respiration. Hydrocarbon emission from guayule amounts to a loss of 0.23 metric tons per hectare per year, while the carbon equivalent for rubber

is 0.53 per metric ton per hectare per year (NAKAYAMA,1984). Guayule has a compensation point of $72 \mu\text{l.l}^{-1}$ (KEITHLY AND BENEDICT,1985), while *Parthenium hysterophorus* exhibits reduced photorespiration as indicated by the low compensation point of 20 to $25 \mu\text{l.l}^{-1}$ (MOORE,FRANCESCHI,CHENG,WU AND KU,1987). In addition, the numerous peroxisomes present in the Summer leaves of guayule may be used as ultrastructural evidence of the possible involvement of photorespiration (COOMBS,1985).

Inhibition of photorespiration resulted in decreased incorporation of radioactivity into rubber (REDDY,SUHASINI AND DAS,1987). Photorespiration, however, cannot be regarded only as a process whereby carbon is lost from the plants, as the pathway constitutes the major flux of nitrogen within the leaf which increases the rate of primary nitrogen metabolism (WALLSGROVE,KEYS,LEA AND MIFLIN,1983). These reactions take place in the chloroplasts and consequently the rates of nitrogen flux must be attuned to those of carbon flux from photorespiration.

The warm Summer nights stimulate growth and cause depletion of the respiratory substrates, while the cool Winter nights preserve high levels of respiratory substrate. The ideal situation would be a balance between daytime photosynthesis and night time utilization of the assimilate. This temperature effect has been observed in plants which produce monoterpenes (BURBOTT AND LOOMIS,1967). Thus in guayule, it is possible that the cooler night temperatures allow for accumulation of additional substrate by plants since respiratory requirements do not deplete the assimilate.

If both hydrocarbon emission and photorespiration are active then a great deal of carbon may be lost by guayule during the Summer months. However, the results from the metabolic studies of the four substrates suggest that if this carbon was retained it would be partitioned into carbohydrates and then into the isoprenoid pathway. Resins rather than rubber would be produced. Optimum rubber production appears therefore to be confined to the Winter months when peroxisomes are no longer visible in the leaves although

BACKHAUS (1985) reported that peroxisomes were present in tissue during active rubber formation.

REDDY, SUHASINI AND DAS (1987) found that inhibition of photorespiration resulted in a reduction in radioactivity incorporated into rubber which would suggest that serine or glycine are utilised as substrates for rubber synthesis. Serine was proposed as an effective substrate for rubber synthesis by KEITHLY AND BENEDICT (1985), where serine synthesis proceeds via the glycerate and glycolate pathways in guayule leaves. In both these cases it is equally plausible that 3-phosphoglycerate may be acting as the substrate via glycolate and acetyl CoA rather than serine.

Radioactive pyruvate and serine were both incorporated into rubber in excised shoots of guayule (REDDY, SUHASINI AND DAS, 1987), which conflicts with the radiochemical assay in this study where serine and glycine were not incorporated into rubber in stem slices incubated in acetate, serine and glycine. It was expected that the incubated stem slices would be a more effective system with respect to translocation and sub-cellular localization of the pathway than the excised shoot system, thus improving the access of serine and glycine.

It is not unusual for amino acids to be a source of carbohydrates for glycolysis (BOLAND, GARNER, NELSON AND ASAY, 1977). The fate of the radioactivity in the twelve hour experiment suggests that serine and glycine and probably other amino acids can be used as additional carbon skeletons for rubber synthesis. The amino acid and carbohydrate fractions, in the short term Winter experiment, showed the highest levels of radioactivity incorporated. Alanine, threonine, glycine, serine and cysteine can be metabolised to pyruvate (LEHNINGER, 1975), acetyl CoA and the rest of the isoprenoid pathway.

However, serine is also involved as an indirect or direct precursor for a number of other amino acids and other molecules. The level of free amino acids in the

leaves and stem changed after wintering and with the onset of growth. This is not unexpected as the amino acids are the mobile stages of stored nitrogen, and contribute substantially to growth at the beginning of Spring (SAGISAKA,1974).

There appears to be a division of labour in the stem, with the young stem responsible for rubber synthesis in the Winter. In the Summer, assimilate in the younger stems may be used preferentially for growth rather than for secondary metabolism, while the mature stem is involved in the synthesis of isoprenoids. The role of the young stems in rubber synthesis is not unexpected as thin branches were found to store nearly 80% of the detectable rubber in the bark (CURTIS,1947). As the diameter of the stem increases, so the number of cells with the potential to store rubber decreases. In the stem, a large phloem : xylem ratio is favoured as this allows for greater rubber storage (ARTSCHWAGER,1943). The molecular weight of bark rubber is twice that of the wood rubber (ESTILAI,1987).

The radiolabelling studies indicated that in the Summer, movement of the radioactivity is basipetal when the radioactivity was supplied as sucrose, and acropetal when supplied as [^{14}C] fructose (**Figure 17;21**). The radioactivity also moved in a lateral direction towards the pith, an area which was acting as an important sink during the Summer months (**Figure 17**).

Once the substrate had been taken up by the leaves, it was probably loaded via the transfer cells into the minor veins of the phloem as transfer cells are specialized parenchyma cells with wall ingrowths (GUNNING AND PATE,1974) which increase the surface area of membranes available for exchange with the apoplast, where symplastic connections are poor or absent (DELROT,1989). The transfer cells occur where high rates of solute flow over short distances are required (CRONSHAW,1981).

The companion cells are intimately connected with the sieve elements. The sieve elements are the most highly specialized cells of the phloem (BEHNKE,1989) and translocate the assimilate to the rest of the plant. Translocation through sieve elements occur as long as the sieve elements and companion cells are living. In the leaves, the companion cells are usually larger than the sieve elements, probably reflecting their role in assimilate loading, whereas in the stem the diameter of the sieve elements may be up to one hundred times greater than that of the companion cells.

Nacreous thickening covers the wall areas which do not have sieve elements. The function of this layer is unknown although a number of proposals have been made. The wall may serve for storage of wall material to be used during elongation growth. It is seen as a primitive characteristic as it is predominant in the more primitive woody taxa of the angiosperms (BEHNKE,1989). The glistening of the wall observed at the light microscope level would suggest a high level of hydration of the constituents and that the cellulose polymers are widely spaced allowing for a rapid solution flow (BAKER,1978). BUVAT (1989), on the other hand, views the nacreously thickened cells as non-functional cells.

In guayule the nacreously thickened walls of the sieve tube cells probably increase the area for apoplastic transport allowing greater uptake of assimilate in the leaves and unloading of assimilate in the stem. Once the assimilate has reached the stem, it is unloaded apoplastically and then moves symplastically via the parenchyma to the resin canal epithelial cells and parenchyma cells. In *Hevea*, the sugars are transported symplastically by numerous plasmodesmata especially in the parenchyma cells adjacent to the laticifers.

An important factor in the control of carbon partitioning is the timing of organ initiation and growth. Competitive advantage in terms of a successful sink is achieved by the high relative growth rate of the organs, the efficiency of transfer from the vascular system to the sink, and the spatial or biochemical isolation of assimilates in the growing or storage organs. This will lower the

concentration of photosynthates in the sieve elements servicing the sinks and establish a favourable concentration gradient between sink and source. In guayule, the onset of growth results in a reduction in the rubber producing potential of the enzymes although rubber synthesis continues until September utilizing excess IPP (APPLETON,1990) (**Figure 6**).

Storage in the leaf and in tissues along the path of transport between source and sink may provide an important control in relation to partitioning of carbon in plants. Fructans are an alternative form of carbohydrate storage, having a lower osmotic concentration per unit carbon and would appear to have an advantage over sucrose (HENDRIX,1985). Guayule stores its reserve carbohydrate as fructans and starch (KELLY AND VAN STADEN,1991). The phenomenon of carbohydrate storage in guayule will be considered in greater detail in Chapters 4 and 5.

cis-Polyisoprene production in guayule relies therefore upon three factors : cellular maturity (GOSS,1991), rubber transferase activation (APPLETON,1990) and the availability of substrate. The cellular location of the isoprenoid pathway has been reviewed in Chapter 1, where it was shown that there is still no consensus regarding the location and fate of the IPP synthesised by the plastid. As the factors controlling isoprenoid and *cis*-polyisoprene synthesis are different, it could be possible that their sub-cellular localizations differ. The penultimate stage of the pathway is probably cytoplasmic (GOSS,1991) while isoprenoid (*trans*-terpenes) production may require IPP processed by the plastids of the resin canal epithelial cells. If IPP in the cytoplasm can be utilized for rubber synthesis, then the fact that the plastid acts autonomously in the synthesis of isoprenoids, allows the two processes to occur simultaneously and independently of one another. In a plant, which exhibits a great deal of division of labour, the two processes with a high demand for sucrose are unlikely to rely on processing by the same organelle.

The nature of the applied substrate is also important as [^{14}C] sucrose was efficiently incorporated into rubber whereas the [^{14}C] fructose was incorporated into resin in the Winter plants. This could indicate that sucrose is the more effective substrate because it does not have to be processed by the plastids and can be utilized, once metabolised to IPP, in the cytoplasm. Fructose, on the other hand, can enter the plastid and be utilized for the synthesis of resins thus backing up the model proposed by KREUZ AND KLEINIG (1984).

The early appearance of rubber particles in the resin canal epithelial cells is probably due to the close proximity of these cells to the phloem, ensuring a regular supply of sucrose prior to further translocation.

cis-Polyisoprene is synthesized from either FPP or GGPP. The initial steps of the isoprenoid pathway leading to the isoprenoids are not limited by temperature variables, although a correlation exists between minimum temperature and the penultimate stage of the pathway leading to the formation of *cis*-polyisoprene in guayule (APPLETON AND VAN STADEN, 1989). Guayule uses the reliable environmental cue of temperature to ascertain whether Winter is approaching. This does not result in the immediate synthesis of rubber; instead the plant requires a fixed number of days during which time the difference between the maximum and minimum temperatures should be seven to nine degrees, before rubber synthesis begins (APPLETON AND VAN STADEN, 1989). During this time, a genetic induction could occur for the activation of the rubber transferase enzyme. As a particular period of time occurs before rubber synthesis takes place it may be possible that the activity of the rubber transferase enzyme requires promoter action which may be in the form of precursor availability.

Once the enzyme has been activated it requires substrate (IPP) for rubber to be synthesized. There has been a dispute as to the role of the substrate in the synthesis of *cis*-polyisoprene. GILLILAND AND VAN STADEN (1990) proposed that it is the increased levels of substrate which act on the genes which in turn activates the enzymes. However, in this study it has been shown that there are

a number of factors which influence the partitioning and fate of the assimilate and that the level of substrate is not a reliable cue for the activation of the rubber transferase enzyme. Although the loss of radioactivity from the treated leaves decreases during the Winter months and the growth requirements are minimal, thus ensuring excess substrate, there is no preferential partitioning of this substrate to the site of rubber deposition. However, once the substrate reaches the site of rubber deposition the process of rubber production has priority on the substrate.

In the Spring, longer daylengths favour growth, budbreak overrides the temperature effect, and the potential to produce rubber decreases (APPLETON AND VAN STADEN,1989). For two months (August and September), the processes of growth and rubber production continue simultaneously. Experiments with Spring plants have shown that applied substrate is incorporated into rubber (KELLY AND VAN STADEN,1987). The amount of available substrate increases during the Spring, as the cortical parenchyma cells become filled with starch grains, indicative of excess sucrose (KELLY AND VAN STADEN,1991). By October, when growth is significantly higher, and the plants possess new Summer leaves containing peroxisomes, the level of starch decreases (KELLY AND VAN STADEN,1991), and the incorporation of carbon into rubber decreases substantially. If the loss of carbon by photorespiration during the Summer months was to be circumvented it would not bring about increased rubber production as the rubber transferase activity decreases in the Spring, once growth begins (APPLETON,1990). This enzyme is of paramount importance for rubber production.

In some instances, all three factors may be involved and still no rubber will be produced. For example, the pith parenchyma receives an adequate supply of substrate in the Summer (**Figure 17**), the pith also possesses the greatest enzymatic potential to incorporate acetate into rubber (APPLETON,1990), and there are resin canal epithelial cells present, albeit in smaller numbers. However, the actual rubber content of the pith is low (KELLY AND VAN

STADEN,1991). This area, as will be seen in Chapter 4 has other priorities with respect to the fate of the translocated sucrose. Once again, guayule exhibits a division of labour with respect to the predominant metabolism of the plant components.

The partitioning of substrate in guayule is not a simple process and as WARDLAW (1990) says it is a series of factors rather than a limiting event which controls carbon partitioning. Improving the assimilate supply in the Winter months will not ensure that this assimilate is partitioned into rubber. The precursor, sucrose, will be utilized for IPP synthesis and ultimately rubber synthesis, in the Winter months. Continuation of this process in Summer would depend on altering the nature of the rubber transferase enzyme and preventing the loss of carbon via photorespiration and the provision of adequate storage space for rubber deposition.

CHAPTER 3

PLANT CARBOHYDRATE RESERVES

3.1 A PERSPECTIVE ON THE FRUCTANS

3.1.1 INTRODUCTION

Fructans have been largely ignored in the field of plant oligosaccharide research and assimilate partitioning. Fructans are classified as primary oligosaccharides, constituting a series of homologous oligosaccharides, considered to be derivatives of sucrose (HIRST,1957). There are many other oligosaccharides based on sucrose where galactopyranosyl, glucopyranosyl or fructofuranosyl is transferred to either the glucosyl (for example, raffinose, stachyose, verbascose, gentianose) or fructosyl (for example, kestose and isokestose) moieties of sucrose (KANDLER AND HOPF,1982). An oligosaccharide is a compound that on complete hydrolysis, gives only monosaccharide units, in relatively small numbers per molecule (WOLFROM,VERCELLOTTI AND HORTON,1963). Oligosaccharides, in turn are classified as disaccharides, trisaccharides, tetrasaccharides and so forth, with no sharp distinction between oligosaccharides and polysaccharides. It is generally agreed that a carbohydrate consisting of two to ten monomeric residues linked by an O-glycosidic bond is an oligosaccharide. Primary oligosaccharides occur in significant amounts in plants and are of metabolic relevance, while secondary oligosaccharides arise by hydrolysis of higher oligosaccharides, polysaccharides, glycoproteins and glycolipids.

A confusing situation has arisen in the literature as far as the nomenclature of these fructans is concerned. The polymers which occur in microorganisms are called levans while those present in plants are called polyfructosans, fructosans and fructans. Neither of the first two terms should be used as the former refers to polymerized fructosan and the latter corresponds to a defined type of

fructose dianhydride that is produced on acid hydrolysis of fructose polymers. Therefore during this dissertation, the polymers of D-fructose will be referred to as fructans.

3.1.2 DISTRIBUTION AND OCCURRENCE OF FRUCTANS

Fructans occur primarily in the Gramineae (1200 species)(SMITH,1986) and Compositae (24000 species) and the Liliales (6300 species) (SMOUTER AND SIMPSON,1989). The first two families are considered to be fairly advanced (LEWIS,1984), while the Liliales is not considered to be highly evolved (HENDRY,1987). It is thought that fructans are probably more widespread than established to date (KANDLER AND HOPF,1980).

The geographical distribution of these families is diverse and as such fructan accumulators are no longer considered to be only temperate in occurrence (SMITH AND GROTELUESCHEN,1966; SMITH,1968). The diversity of occurrence of fructan metabolism among the highly evolved families of the vascular plants indicates that fructan accumulation may have developed recently, perhaps in response to one or two selective pressures. Fructan accumulation may confer a selective advantage and should not be considered as a minor pathway of declining evolutionary significance (LEWIS,1984).

Fructans are distributed throughout the organs of the plants in which they occur, although levels may differ significantly in the different plant components (DARBYSHIRE AND HENRY,1978). Leaves usually accumulate low levels while roots, tubers, bulbs, rhizomes, sometimes immature fruit (MEIER AND REID,1982) for example wheat kernels (SCHNYDER, EHSES, BESTAJOVSKY, MEHRHOFF AND KÜHBAUCH,1988), barley kernels (BÜTTNER,DE FEKETE AND VIEWEG,1985), and seeds (HOUSLEY AND DAUGHTRY,1987) accumulate high levels. Fructan composition has been found to be affected by plant developmental changes (ARCHBOLD,1940; WHISTLER AND SMART,1953; SOJA, HAUNOLD AND PRAZNIK,1989), by the age of the plant component (WAGNER AND WIEMKEN,1989) and by environmental factors. For

example, high irradiance or low temperatures favour fructan accumulation (LABHART, NÖSBERGER AND NELSON,1983) while high nitrogen or low potassium decrease fructan accumulation (ARCHBOLD,1940). There are also large differences in the fructan content within species in a genus (DARBYSHIRE AND HENRY,1981). The ability to synthesize fructans may be passed on in culture as found in *Symphytum officinale* L.. Leaf callus synthesized fructan while calli from seeds, anthers and roots did not. The regenerated plant only produced fructan if the parent callus had done so (HAAB, STADLER, FRANZ, ABOU-MANDOUR AND CZYGAN,1986).

3.1.3 THE CHEMICAL STRUCTURE OF FRUCTANS

Fructans are polymers of D-fructose carrying a D-glucosyl residue at the end of the chain attached via a (2→1) linkage as in sucrose. This glucose is derived from the parent sucrose molecule. No bond of the fructose furanose ring is part of the macromolecular backbone and the carbohydrate exists in the furanose form, adding flexibility to the whole fructan molecule (MARCHESSAULT,BLEHA,DESLANDES AND REVOL,1980).

The simplest fructan, monofructosyl sucrose can be considered a trisaccharide (POLLOCK AND CHATTERTON,1988). Three distinct forms of monofructosyl sucrose have been isolated (POLLOCK,1982a) and these form the basis of three fructan series with different linkage patterns. There are a number of synonyms of fructans and so all the variations will be included in the following paragraph.

The first series is based on the trisaccharide isokestose (1-kestose, 1^F-fructosylsucrose) (EDELMAN AND BACON,1951), consisting of linear nonreducing chains of β -(2→1)-D-fructofuranosyl units (**Figure 25A**). These fructans are commonly known as inulins, and are typical of the Compositae (AKAZAWA,1976), Boraginaceae and Iridaceae (KANDLER AND HOPF,1980), and are used commercially for food and medicine.

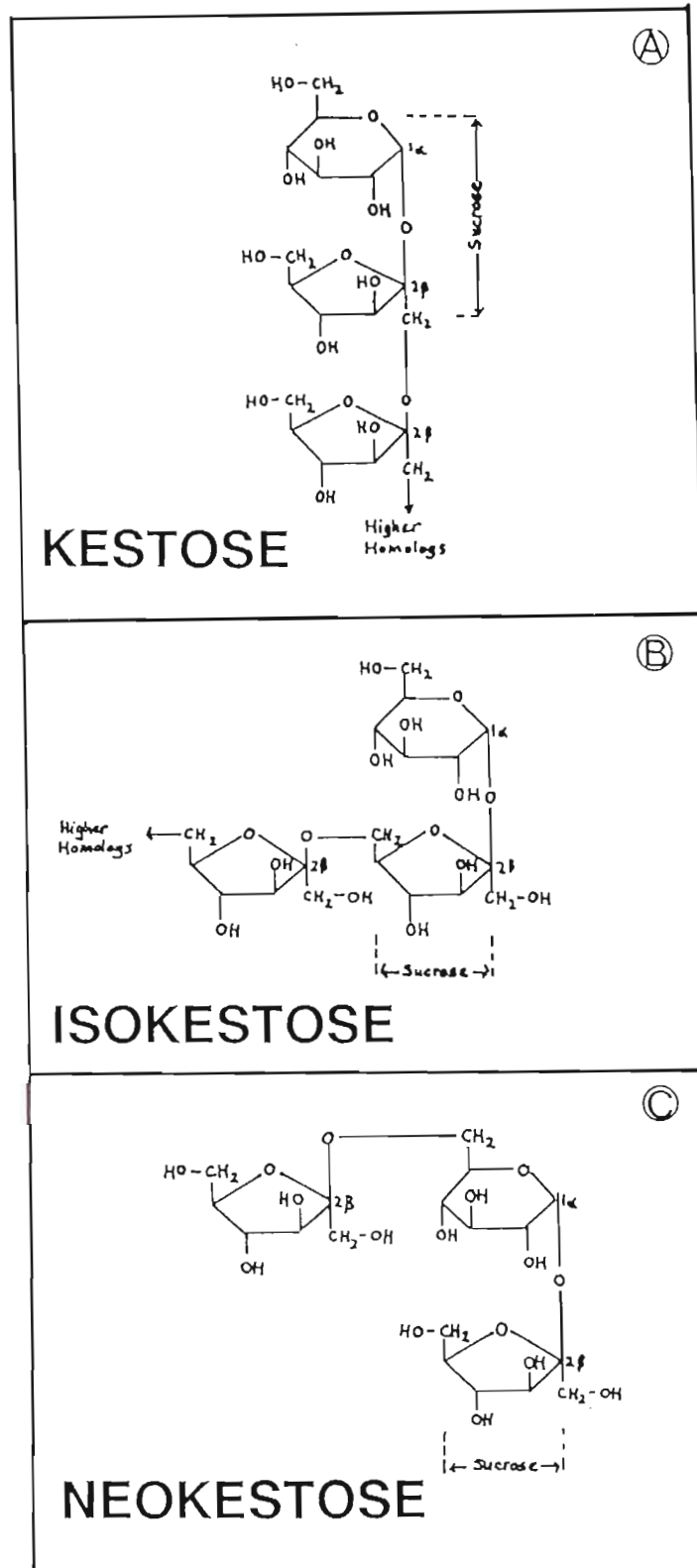


Figure 25.

The structure of the trisaccharides, kestose (A), isokestose (B) and neokestose (C).

The kestose (6-kestose, 6^F-fructosylsucrose) series, where adjacent residues are linked by β -(6 \rightarrow 2) bonds, are based on the trisaccharide kestose (**Figure 25B**). These fructans are commonly known as levans or phlein, and are typical in the Gramineae (FREHNER, KELLER AND WIEMKEN, 1984; HOUSLEY AND DAUGHTRY, 1987).

The last series and a minor group is formed from the trisaccharide neokestose (6^G-fructosylsucrose). It differs from the other two trisaccharides in that the glucose residue is linked directly to the fructose moieties through carbon 1 and 6 and has β -(2 \rightarrow 1)-D-fructofuranosyl linkages (KANDLER AND HOPF, 1980) (**Figure 25C**). Chain elongation can occur on both fructose residues.

In addition to these three groups there are branched fructans and fructans which have different isomers and linkages within the same chain. In some plants, mixtures may exist of isokestose and neokestose fructans (HAMMER, 1969; SHIOMI, 1981; HENRY AND DARBYSHIRE, 1981), and all three types of trisaccharides have been identified in *Lolium temulentum* L. (CAIRNS AND POLLOCK, 1988ab), *Triticum aestivum* L. cv Caldwell and in *Festuca arundinacea* Schreb. (CARPITA, KANABUS AND HOUSLEY, 1989). With the advent of more sensitive analytical procedures these will be identified more accurately (POLLOCK, 1986). Once the range of forms has been established, hypotheses concerning synthesis and degradation may be proposed.

Fructans occur in two forms (α and β). They have the same optical rotation, although the α form is not readily soluble in cold water and is crystalline while the β form is amorphous, easily soluble in cold water, and is usually extracted in alcohol. Once precipitation takes place, it reverts to the α -form which is partially water soluble (BECK AND PRAZNIK, 1986).

All fructans are non-reducing, not hydrolysable by yeast invertase (although this may not be true of the low molecular weight fructans), resistant to

amylase action, but very susceptible to acid hydrolysis (ARCHBOLD,1940). The degree of polymerization varies with plant species but all fructans have a low degree of polymerization (DP) when compared with starch. The DP may range from 10 to 260.

3.1.4 EXTRACTION, PURIFICATION AND QUANTIFICATION OF FRUCTANS

Current methodology requires that the tissue first be freeze dried to halt all enzymic reactions and then boiled in 80% ethanol. All soluble carbohydrates are then extracted using warm water and dilute ethanol, and the neutral components separated from the charged species using ion exchange resins (POLLOCK,1982a). Low molecular weight sugars can be removed by dialysis or gel filtration.

As two consecutive members of any of the homologous fructan series differ in only a single fructosyl residue, this makes resolution very difficult and separation occurs on the basis of size or solubility. Individual fructo-oligosaccharides were originally resolved using paper chromatography with the associated problems of chromatographic times of five days to two weeks (TREVELYAN,PROCTER AND HARRISON,1950; BACON AND EDELMAN,1951; BACON,1959; ERVIN AND SYPERDA,1971; BHATIA,MANN AND SINGH,1974). Later, thin layer chromatography improved on the separation times, ranging from 90 minutes to eight hours (COLLINS AND CHANDORKAR,1971; NELSON AND DICKSON,1981). Gas liquid chromatography (POLLOCK,HALL AND ROBERTS,1979) or exclusion chromatography using gel filtration (PONTIS,1968; DARBYSHIRE AND HENRY,1978; FREHNER,KELLERAND WIEMKEN,1984; JOHN,SCHMIDT,WANDREY AND SAHM,1982; PRAZNIK AND BECK,1985; BUCHER,MACHLER AND NÖSBERGER,1987; LECACHEUX AND BRIGAND,1988; CHATTERTON, HARRISON, THORNLEY AND BENNETT,1989) improved on the resolution of the thin layer chromatography. High performance liquid chromatography has been shown to improve the resolution and involves much shorter separation times (MELLIS AND BAENZIGER,1981; PRAZNIK AND BECK,1984; BECK AND PRAZNIK,1986; POLLOCK AND CHATTERTON,1988; BANCAL AND GAUDILLÉRE,1989;

GONZALEZ,BOUCAUD AND LANGLOIS,1989). However, most of the analytical research has been hampered by a lack of fructan standards.

Determination of fructans involves measuring the reducing power using ketose-specific colorimetric assays (ROE,1934; FORSYTH,1948; ROE,EPSTEIN AND GOLDSTEIN,1949; JERMYN,1956).

3.1.5 THE SYNTHESIS OF FRUCTANS

INITIATION OF FRUCTAN SYNTHESIS

Transcription, polyadenylation of messenger RNA and translation on 80S ribosomes appear to be involved in the "induction" process of fructans (CAIRNS AND POLLOCK,1988a). Photosynthates appear to be the effectors for induction of sucrose-sucrose-1F-fructosyltransferase (SST:EC 2.4.1.99) activity, where not only sucrose (CHANDORKAR AND COLLINS,1974a), but maltose, trehalose and melezitose were inducers of SST activity (WAGNER, WIEMKEN AND MATILE,1986). Yet, the mechanisms by which induction stimuli are sensed by the plants and subsequently manifested as alterations in carbon allocation, remain a mystery.

THE SYNTHESIS AND DEGRADATION OF FRUCTANS

The first model for the synthesis of fructans was elucidated by EDELMAN AND JEFFORD (1968), using *Helianthus tuberosus* (Figure 26A). There were two enzymes involved, sucrose-sucrose-1F-fructosyltransferase (SST:EC 2.4.1.99) which catalyses the synthesis of the trisaccharide intermediate, isokestose (*) from donor to acceptor sucrose, and β -(2 \rightarrow 1')-fructan-1F-fructosyltransferase (FFT:EC 2.4.1.100) which catalyses the transfer of a fructose moiety to sucrose or a higher homologue of fructan and is therefore responsible for chain elongation (CHANDORKAR AND COLLINS,1974a). SST and the starter molecule were said to be located in the cytosol, while FFT transferred fructosyl residues from the trisaccharide across the vacuole.

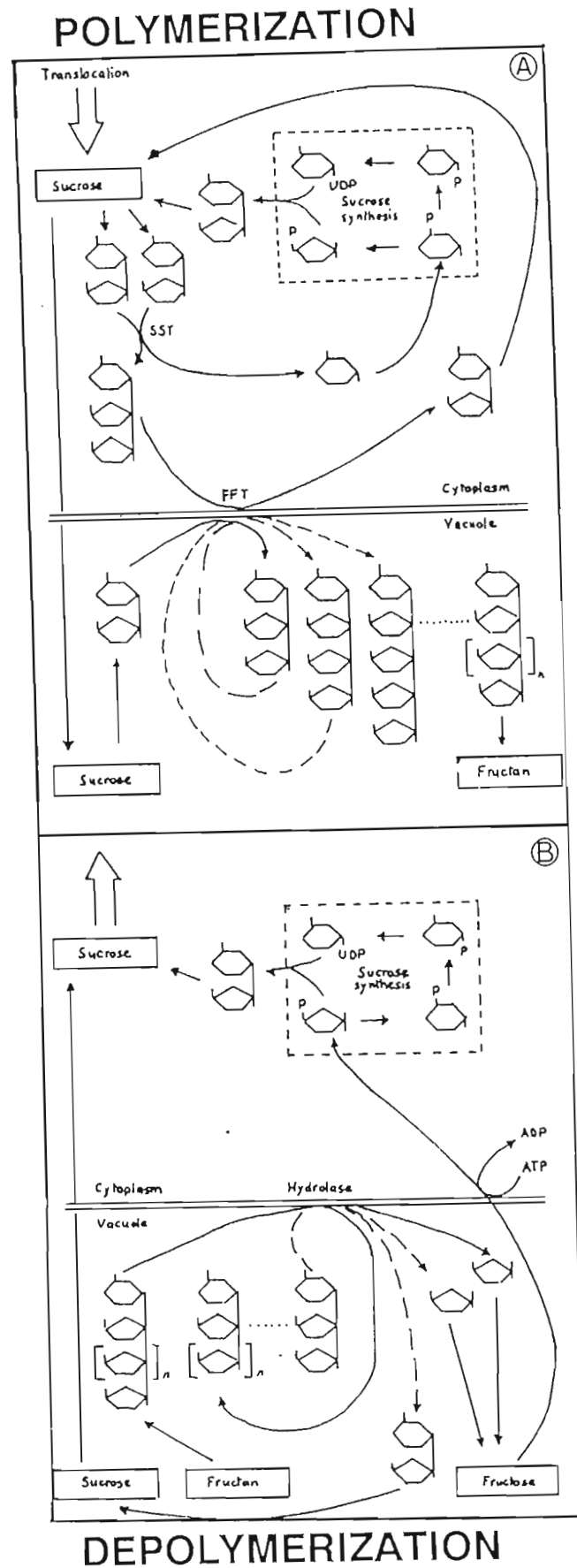


Figure 26.

The polymerization of sucrose to fructan in the cells of developing tubers of *Helianthus tuberosus* (A) and the depolymerization of fructan to sucrose and fructose in sprouting tubers of *Helianthus tuberosus* (B) (from EDELMAN AND JEFFORD, 1968).

Experimental evidence for this model was based on the premise that sucrose accumulation precedes fructan synthesis and that the subsequent patterns of carbon partitioning implicate sucrose as the precursor of fructan (POLLOCK,1982a; LABHART, NÖSBERGER AND NELSON,1983; WAGNER,KELLER AND WIEMKEN,1983; POLLOCK,1984a; HOUSLEY AND POLLOCK,1985; WAGNER,WIEMKEN AND MATILE,1986; CAIRNS AND POLLOCK,1988a). Prevention of fructan synthesis by inhibiting gene expression in leaves caused elevated sucrose accumulation (CAIRNS AND POLLOCK,1988).

The activity of FFT is low in the leaves depleted of carbohydrate and in which fructan synthesis does not take place. Increases in the extractable activity of FFT are concomitant with increases in tissue sucrose concentration and the onset of fructan biosynthesis. However, the existence of these fructosyl transferases was disputed, at least in *Lolium temulentum* L., where some or all of the fructosyl transferase activities measured were found to be artifacts of the extraction and/or assay conditions (CAIRNS, WINTERS AND POLLOCK,1989). More recently, CAIRNS (1989) confirmed that fructosyl transferases existed at low and physiologically relevant concentrations. A major problem with the enzymatic work was due to the use of abnormally high substrate concentrations which produced intermediates which were not representative of *in vivo* leaf fructan.

EDELMAN AND JEFFORD (1964) found two fructan hydrolases in the tubers of *Helianthus tuberosus*. These hydrolytic enzymes $\beta(2\rightarrow1')$ fructan 1-fructanohydrolase (FH: EC 3.2.1.26), cleave terminal β -2,1-and/or β -2,6 fructofuranoside linkages such as those found in inulin, phleins and sucrose (EDELMAN AND JEFFORD,1968). Hydrolysis of the polysaccharide occurs by the step-wise removal of a single fructosyl residue, progressing until only sucrose remains (RUTHERFORD AND DEACON,1972ab) (Figure 26B). The rate of depolymerization depends on the DP and the nature of the group at the non-reactive end of the molecule. A polymer is then converted to a mixture of sucrose and fructose.

In *Helianthus tuberosus*, SST is located in the cytosol while FFT and β -(2 \rightarrow 1')-fructan-1-fructan-exohydrolase (FEH: EC 2.4.1x) are located on the tonoplast and in the vacuole. FEH becomes active once SST activity disappears. This enzyme removes fructosyl residues from the ends of the fructan chains, thus initiating the depolymerization and remobilization of stored fructan. The fructose so formed is released into the vacuolar sap and exported via a sugar carrier to the cytosol (DARWEN AND JOHN,1989). FFT is active for the entire life of the plant, FEH during sprouting and SST during tuber development.

FREHNER, KELLER AND WIEMKEN (1984) concluded that SST and FFT were located in the vacuole and the activity of SST and FFT was associated with the vacuolar sap and not the tonoplast (DARWEN AND JOHN,1989). In dormant tubers, FEH activity was found in the tonoplast, and FFT in the vacuolar sap. However, so little is known about the vacuolar ultrastructural changes associated with fructan synthesis (KAESER,1983). There are problems associated with the technique for vacuole isolation which may isolate the larger storage vacuoles rather than developing vacuoles which may have lead to spurious reports of the presence of enzymes.

Great variation has been found in the patterns of accumulation of fructans in the Graminae. Some species accumulate oligosaccharides and high molecular weight fructans (*Lolium temulentum*) whereas *Dactylis glomerata* accumulate fructans of only a high molecular weight (POLLOCK,1982b,1986). All three trisaccharides have been observed in *Lolium temulentum* (BACON,1959), and two in barley (BANCAL AND GAUDILLÉRE, 1989). This suggests that fructan biosynthesis in grasses must be more complex than the process in *Helianthus tuberosus*. The biosynthetic pathway would appear to proceed from sucrose, through isokestose to higher polymers of fructan mediated by the action of fructosyl transferases. However evidence is not complete. Isokestose occurs ubiquitously in all fructan accumulators (WAGNER AND WIEMKEN,1989). It has been suggested that there are multiple pathways of synthesis in *Lolium temulentum*

(CAIRNS AND POLLOCK,1988a) similar to those found in *Asparagus officinalis* (SHIOMI,1981,1982).

BANCAL AND GAUDILLÉRE (1989) suggested that the kestose found in barley may be formed by an alternative pathway, independent of isokestose formation. Purified SST from barley produced isokestose exclusively, whereas invertase yielded kestose as a byproduct. However, kestose may be an artifact, the result of a high concentration of sucrose in the incubation media (CAIRNS,1989).

Many of the earlier theories on fructan synthesis were based on evidence using insensitive, low-resolution analytical techniques. The cleared homogenate system that has been used by CAIRNS (1989) may provide a more convincing starting point for further *in vitro* studies on fructan synthesis. Furthermore, there is no evidence at present, as to how the cell controls the cycles of fructan synthesis and degradation.

3.1.6 THE SITE OF FRUCTAN STORAGE

The vacuole occupies as much as 80 to 90% of the total cell volume. The totality of vacuolar solutes determines water potential, the cell shape is maintained by the turgor pressure of the vacuole, it provides a repository for metabolites such as amino acids, organic acids, saccharides, and inorganic nitrogen and phosphate (MATILE,1990). In mesophyll cells, starch storage may be limited and the vacuole then acts as a site of storage for sucrose, which can be withdrawn and transported as the plant demands. If the accumulated saccharide comprises a large percentage of the dry weight, the resulting low water potentials are avoided by replacing sucrose by a polysaccharide, usually fructan (WAGNER,KELLER AND WIEMKEN,1983). The accumulation of fructan in the vacuole removes this carbohydrate from the site of carbon fixation in the chloroplast.

Fructans were originally thought to accumulate in the vacuole (MOLISCH,1921; SACHS,1964). This was later confirmed (WAGNER,KELLER AND WIEMKEN,1983; WIEMKEN,FREHNER,KELLER AND WAGNER,1986; FREHNER, KELLER AND WIEMKEN,1984). It is believed that all the major events of fructan synthesis occur at or within the tonoplast. SST and FEH have been located in the barley mesophyll cell vacuoles (WIEMKEN,FREHNER, KELLER AND WAGNER,1986; WAGNER AND WIEMKEN,1986), with both enzymes having optimum activity at a pH of 5 to 6, and both appear to be glycoproteins (WAGNER AND WIEMKEN,1986).

Storage may be promoted by auxins (WAIN,RUTHERFORD,WESTON AND GRIFFITHS,1964; RUTHERFORD AND DEACON,1974) and cytokinins (PONTIS,1966). Cytokinins have been shown to improve assimilate supply (HAYES,OFFLER AND PATRICK,1985), which may in turn promote fructan synthesis.

3.1.7 FACTORS AFFECTING THE SYNTHESIS OF FRUCTANS

Sucrose as substrate

Sucrose is the primary precursor for the synthesis of fructan (EDELMAN AND DICKERSON,1966; WOLOSUIK AND PONTIS,1974; CAIRNS,1989) although the hydrolysis of assimilatory starch may provide endogenous substrate for sucrose and thereby for fructan synthesis (CHANDORKAR AND COLLINS,1974b). Sucrose accumulation appears to be more important than active carbon fixation for the initiation of fructan synthesis, with different threshold concentrations of endogenous sucrose necessary for initiating fructan synthesis (LABHART, NÖSBERGER AND NELSON,1983; SICHER,KREMER AND HARRIS,1984). The sucrose that is utilized for fructan synthesis appears to be the product of current photosynthetic carbon production (HOUSLEY AND POLLOCK,1985).

Sucrose is divided between the cytoplasm (60%) and the vacuole (40%) (THORNE AND GIAQUINTA,1984), with the cytoplasmic sucrose being used for translocation and the vacuolar sucrose for storage (FARRAR AND FARRAR,1985).

The percentages of sucrose in the cytoplasm and vacuole may alter, for example in *Hordeum vulgare* L. 80% of the sucrose is found in the vacuole (FARRAR AND FARRAR,1986).

SST activity increases prior to fructan synthesis (WAGNER,WIEMKEN AND MATILE,1986). Only a portion of the total pool of sucrose present in the cells, most probably the cytosolic could be acting as an effector for the induction of SST (WAGNER AND WIEMKEN,1989).

Sucrose is also stored in the vacuole, although it is synthesized in the cytoplasm (KRUGER,1990). Sucrose synthase, the enzyme associated with the breakdown of sucrose was found to be extravacuolar in celery (*Apium graveolens* L. var. *dulce*) (KELLER AND WIEMKEN,1982) and in *Helianthus tuberosus* L.(FREHNER,KELLER,MATILE AND WIEMKEN,1987). No evidence exists for a sucrose uptake mechanism at the tonoplast, therefore a precursor must be converted to sucrose either during or after transport through the tonoplast (KRUGER,1990). In the vacuole, vacuolar acid invertase regulates sucrose storage. When the activity of this enzyme is high then sucrose in the vacuole is rapidly converted to hexoses which are rapidly transported into the cytoplasm (REES,1984).

This short-term fructan accumulation in the leaves does not take place at the expense of the starch. The factors which predispose the leaves to utilize a large proportion of fixed carbon for the synthesis of sucrose and then to modulate the export of the sucrose in order to synthesize fructans, have yet to be elucidated.

Low temperature effects on fructan synthesis

Low temperatures promoted depolymerization of fructans in *Helianthus tuberosus* L. (JEFFORD AND EDELMAN,1963; EDELMAN AND JEFFORD,1964), *Taraxacum officinale* Weber (RUTHERFORD AND DEACON,1974) and in four wheat cultivars (TOGNETTI,SALERNO,CRESPI AND PONTIS,1990). Associated with low temperatures

are increases in sucrose synthase (TOGNETTI,CALDERÒN AND PONTIS,1989), sucrose phosphate synthase activity (SALERNO,IANIRO,TOGNETTI,CRESPI AND PONTIS,1989) and sucrose concentration (POLLOCK,1984b). SST increases (WAGNER, KELLER AND WIEMKEN,1983) and FEH decreases (TOGNETTI,CALDERÒN AND PONTIS,1989). Reversal of cold acclimation was associated with a reversal in the enzyme activities. POLLOCK, CAIRNS, COLLIS AND WALKER (1986) proposed that low temperatures stimulated fructan synthesis by altering metabolic interactions and the catalytic specificity of the enzymes involved in fructan synthesis. The relationship between temperature and enzymes may not be as simple, because FEH in *Hordeum vulgare* (L.) Morx. has an Arrhenius E_a of 8.8 kcal mol⁻¹, and this value remained linear between 5 and 40°C, which suggests that fructan content is not regulated primarily by a direct temperature effect on FEH activity (HENSON,1989). In the case of *Agropyron cristatum x desertorum*, the leaves remained green during the colder period and the fructan level only increased in the spring and autumn (CHATTERTON,THORNLEY,HARRISON AND BENNETT,1988) which would not be expected if temperature was acting directly on enzyme activity.

3.1.8 THE REGULATION AND CONTROL OF FRUCTAN SYNTHESIS

Apart from the above correlations between temperature and fructan levels, very little information is available on how the accumulation of fructans is controlled. It has been suggested that the initiation of fructan synthesis is controlled at the level of gene expression (CAIRNS AND POLLOCK,1988a). If the increase in SST, which usually precedes fructan synthesis, was inhibited by cycloheximide no fructan was synthesized, suggesting that protein synthesis is also involved.

3.1.9 THE FUNCTION OF FRUCTANS IN THE PLANT

Short term carbohydrate storage

The diurnal differences between production, export and utilization of photosynthates in the leaves are largely balanced by the storage and

remobilization of sucrose and starch (HALMER AND BEWLEY,1982). Fructan on the other hand, appears to be used for balancing seasonal differences associated with plant development and environmental factors (WAGNER, WIEMKEN AND MATILE,1986). This is the more dominant role. Fructan accumulation is limited by the plant's capacity to produce photosynthate in excess of that required for growth (a changed ratio of carbon utilization to carbon fixation) (POLLOCK,1984b). Defoliation (SUZUKI,1971), and the low availability of nitrogen or phosphate (ARCHBOLD,1940), modify this ratio and thereby induce fructan synthesis.

Ratio modification is typically observed in early spring when demand for assimilate exceeds photosynthate supply prior to renewed growth (WAGNER,KELLER and WIEMKEN,1983). At this time, fructans are readily mobilized, availing carbohydrates to a growing plant in a short time (EDELMAN AND JEFFORD,1968). This would be important if the photosynthetic apparatus was not yet fully developed.

Osmoregulation of cellular water potential

Fructans function as reserve carbohydrates and act as osmotic buffers as their osmotic potential is easily altered by polymerization and depolymerization. The polymerization and depolymerization of fructans redistributes fructose without altering the net carbohydrate content (BACON AND LOXLEY,1952; EDELMAN AND JEFFORD,1968; PONTIS,1989). An increase in osmotic potential is brought about by decreasing the DP of the fructan, which may also assist in leaf expansion in the early spring (POLLOCK AND JONES,1979).

Adaptation of plants to stress

The synthesis and accumulation of fructans appears to be associated with adaptation of the plants to stress factors such as low temperatures (RUTHERFORD AND WESTON,1968; POLLOCK AND RUGGLES,1976; PONTIS AND DEL

CAMPILLO,1985), cryoprotection (KANDLER AND HOPF,1982; SUZUKI AND NASS,1988) and water stress (TRAUB,SLATTERY AND McRARY,1946; BENEDICT,McRARY AND SLATTERY,1947;BLACKLOW,DARBYSHIRE AND PHELOUNG,1984).

The characteristics of frost, drought and heat hardy plants are closely connected to the ability of the plant tissues to endure desiccation. Excessive dehydration and shrinkage of tissues may be prevented by the accumulation of a high solute concentration in small vacuoles. Growth slows down, and the insoluble carbohydrate reserves become converted to solutes which accumulate in the vacuole leading to an increase in osmotic potential. The plasma membrane has been found to be a critical structural component in the cell and changes in the protein conformation may contribute to frost hardness (SAKAI AND YOSHIDA,1968).

Additional carbon source during the CAM cycle

In *Fourcroya humboldtiana* Treal, a rosette CAM plant, the DP of fructans changes during the CAM cycle. Fructose molecules are hydrolyzed from fructans and used for PEP synthesis. The DP increases during the day as the CO₂ from the decarboxylation of malate is incorporated into photosynthate (OLIVARES AND MEDINA,1990).

Sucrose unloading at the phloem

Presently, fructan storage can reach up to 70% of the dry weight in leaf tissue without affecting photosynthesis. Sink strength can be altered by changing polymerization of fructans which will effect cell turgor which is thought to mediate unloading (SCHNYDER,NELSON AND SPOLLEN,1988). The osmolality of cell sap remains the same, despite the changes in sucrose concentration.

Fructan concentration increases in the zone of elongation of grasses where fructans are thought to sequester sucrose thus facilitating sucrose transport at the base of the leaves (SPOLLEN AND NELSON,1988).

3.2 STARCH

3.2.1 The chemical structure of starch

Starch is composed of amylose and amylopectin which are not represented by uniform molecular structures; instead different chain lengths and branching characteristics exist. Amylose consists of (1→4)- α -linked D-glycosyl residues; amylopectin contains, in addition, branching via (1→6)- α -bindings (STITT AND STEUP,1985).

3.2.2 The site of storage of starch

Starch is found in chloroplasts during active photosynthesis and in amyloplasts which synthesize and store starch in grains as their sole function.

3.2.3 The synthesis of starch

Accumulation of starch may be transitory in the leaves, where the starch is regularly metabolized. In this case, starch builds up during the day, reaches a peak at the end of the light period, and breaks down during the night, although synthesis and degradation can occur simultaneously (FOYER,1984). Not all the starch synthesized during the day will be degraded during the night and there is another pattern of a longer duration superimposed on the diurnal pattern (JENNER,1982).

Accumulation in non-photosynthetic tissues, for example storage organs such as seeds, rhizomes and tubers, may occur over an extended period of time,

ending with the degradation and disappearance of the starch. These levels of starch may change in response to environmental stress. For example, *Robinia pseudo-acacia* L. accumulates starch in the bark during the late summer, reaching its maximum as plant growth ceases while spring is associated with a temporary increase in starch (SAKAI AND YOSHIDA,1968). A similar pattern was observed in *Populus canadensis* "robusta" (SAUTER,1988) and in *Pinus resinosa* (POMEROY, SIMINOVITCH AND WIGHTMAN,1970). The temperature-related changes in the quantity of starch synthesized in woody species has lead to suggestions that starch accumulation imparts a frost-hardiness to the plant, hardening the plants for winter. Changes in temperature may shift the flow of carbon between starch and sucrose reversibly in either direction and any deprivation of the sucrose source results in the breakdown of starch until a balance is reached.

Starch synthesis may (PREISS,1984) or may not be (SICHER AND KREMER,1986) related to the sucrose levels in the leaves. Sucrose accumulation is initiated after a dark to light transition, whereas leaf starch synthesis usually follows a period of induction (FONDY AND GEIGER,1982). The rate of sucrose storage in leaves decreases or even stops when starch accumulation occurs in the chloroplast (FONDY AND GEIGER,1982). Triose phosphates are transported across the membrane from the chloroplast to the cytosol by the phosphate translocator. If the concentration of triose phosphate is greater than that required by the cell, then it is converted to hexose-phosphate and the concentration of the hexose-phosphate determines the synthesis of sucrose (DENNIS AND EMES,1990). Starch synthesis occurs predominantly via the ADP-glucose pathway and also draws directly from the hexose-phosphate pool (PREISS AND LEVI,1980). That is why starch metabolism is regarded as a buffer for the metabolism of sucrose (STITT,1984).

Most references combine starch with inulin as storage polysaccharides. This storage function has been challenged as the quantities of starch that are produced are in excess of the requirements for growth and maintenance. The

amount of starch produced is not simply related to the magnitude of the assimilate produced since other factors are involved. For example, if the demand for sucrose from the sink(s) is great, then there will be greater partitioning of carbon into sucrose than into starch. Rapid export of sucrose results in reduced starch accumulation, while decreased sucrose export results in reduced sucrose biosynthesis and carbon then moves into starch (FOYER,1984).

Heterogeneous enzymes are involved in the synthesis of starch. The multiple forms of starch synthase and branching enzymes may participate in the formation of different sections of the amylopectin structure (PREISS AND LEVI,1980). There is no information on whether the amylase and phosphorylase levels also fluctuate.

3.2.4 The degradation of starch

Starch degradation involves three phases: reduction of the granule to a soluble form (maltodextrins), debranching and degradation of these large polymers to glucose and glucose-1-phosphate, and finally further metabolism of glucose and glucose-1-phosphate and the export of these products from the site of polysaccharide storage (PREISS AND LEVI,1980). Degradation of starch is slower in the reserve tissues than in leaf starch granules. In leaves, the products of starch breakdown account for only part of the sugar, sugar phosphate and organic acid transformations in the dark, and it is difficult to assign the appearance of intermediates to starch breakdown. In germinating seeds or other reserve tissues, however it is relatively easy to determine the major products of starch degradation (PREISS AND LEVI,1980).

3.2.5 Concluding remarks

Many questions still need to be answered about the synthesis and degradation of starch. There has been no *in vitro* demonstration of starch synthesis; the

functions and interactions of the multiple forms of starch synthase and the branching enzymes need to be characterized. It is not known how the plant controls the amount of amylose and amylopectin nor how starch synthesis is initiated in the absence of a primer.

3.3 THE SIGNIFICANCE OF FRUCTAN RATHER THAN STARCH ACCUMULATION

The accumulation of fructan does confer advantages on the plant. Fructans and starch differ in their physical properties, site of storage and biosynthetic pathway. Fructans are water-soluble, have a unique molecular structure with a flexible furanose configuration (MARCHESSAULT, BLEHA, DESLANDES AND REVOL, 1980), which allows for rapid polymerization and depolymerization. Sucrose, fructose and glucose can therefore be produced on demand, which will influence the osmotic properties of the cell.

Starch, on the other hand, has a rigid pyranose configuration and starch accumulation is more sensitive to low temperatures (POLLOCK AND LLOYD, 1987). SST and FFT have Q_{10} s of 1.4, 1.6 and 1.5 respectively (2 to 10°C) and these enzymes continue to operate at low temperatures, while ADPG starch synthase has a Q_{10} of 3.9, making starch synthesis more sensitive to low temperatures (FARRAR, 1988).

Both starch and fructan require sucrose as substrate although the enzymes involved in fructan synthesis are soluble and less sensitive to changes in the supply of sucrose than the enzymes involved in the synthesis of starch (POLLOCK AND JONES, 1979). Thus fructan synthesis can continue at temperatures where growth is severely reduced.

The conversion of sucrose to fructan by SST conserves much of the energy of the glycosidic bond of sucrose (REES,1984), while the synthesis of starch is an energy-consuming process (STITT,1984).

Although it appears that fructan storage offers a greater advantage than starch storage, it is still disputed whether one is stored as an alternative or in addition to, the other (BROCKLEBANK AND HENDRY,1989). The presence of fructans in plants shows no obvious correlation with the presence or absence of starch (PONTIS AND DEL CAMPILLO,1985; CHATTERTON,HARRISON,BENNETT AND ASAY,1989). Instead, fructans may be seen as an extension of the sucrose pool, complementing the storage of starch as a reserve substance (NELSON AND SPOLLEN,1987).

CHAPTER 4

THE SEASONALITY OF CARBOHYDRATE PRODUCTION IN GUAYULE

4.1 THE STATUS OF CARBOHYDRATE RESEARCH IN GUAYULE

4.1.1 INTRODUCTION

The application of radioactive substrate showed that partitioning of carbon in guayule differed between Summer and Winter. Firstly, a limited amount of the total applied radioactivity was translocated from the leaves of the Winter plants. However, the total radioactivity translocated from the treated leaves during Winter exceeds that translocated in the Summer. The loss of applied carbon from the Summer leaves limits the amount of carbon that can be translocated. Secondly, the carbon translocated to the young stems is utilized for rubber in the Winter and for growth in the Spring and Summer. The mature stems, however utilized the translocated carbon for resin synthesis in the Summer and Autumn. Thirdly, there is no preferential partitioning of the translocated radioactivity to the site of rubber synthesis, although once there, the carbon is utilised for rubber synthesis. Fourthly, the nature of the applied substrate was found to be important with respect to its fate. Glucose and fructose were converted to sucrose and then translocated, with radioactive sucrose utilized for rubber synthesis and radioactive fructose for resin synthesis. As the applied radioactive carbohydrates are incorporated into rubber, it was necessary to establish the nature and interaction of the endogenous carbohydrates with respect to isoprenoid biosynthesis.

4.1.2 FRUCTANS IN GUAYULE

McRARY AND TRAUB (1944) were the first to provide evidence for the existence of a polysaccharide, probably a fructan, in guayule. As guayule is a temperate

plant this was not unexpected. Fructan synthesis is thought to be a refinement of the existing carbohydrate pathways in species evolved in temperate climates. The fructan identified by McRARY AND TRAUB (1944) was comparable with inulin which had been isolated and identified by HAWORTH, HIRST AND PERCIVAL (1932), and was similar to that found in other members of the Compositae such as dahlia, Jerusalem artichoke, chicory, dandelion and the Russian dandelion (HASSID, McRARY, DORE AND McREADY, 1944). McRARY AND SLATTERY (1945) established a rapid method for the determination of fructosan using a modified method of ROE (1934). By using hot and cold water extractions, TRAUB AND SLATTERY (1946a) were able to identify another component of the polysaccharide fraction which was referred to as levulin. The levulins were later separated into two fractions, a 89% ethanol soluble and a 89% ethanol-insoluble component. Levulins were referred to as a mixture of non-reducing polysaccharides in Compositae, containing mainly fructose and lesser proportions of glucose (TRAUB AND SLATTERY, 1946a).

4.1.3 THE ROLE OF FRUCTANS IN GUAYULE

Levulins were regarded as the chief reserve carbohydrate in guayule. In the stems and roots of guayule the soluble levulins increased during the Summer months and decreased during the Winter, while the insoluble levulins increased during the Winter months and were very low during the Summer months (TRAUB AND SLATTERY, 1946b). Temperature had a marked effect on levulin content (BENEDICT, 1950). Very little information exists on levulins today and the terminology may no longer be applicable.

Plants grown under conditions of low carbon utilization utilized their free sugar, levulin, inulin and pentosan fractions while still accumulating rubber (TRAUB, 1946). As this experiment was carried out in the Autumn and Winter months, BENEDICT (1949) repeated it in the Spring months. The results differed in that the defoliated plants used in the former experiment accumulated rubber while the defoliated plants in the latter experiment did not. Although Spring

plants synthesize rubber, by October, the potential of the rubber transferase enzyme has decreased (APPLETON AND VAN STADEN,1989), which would explain why the plants that Benedict used failed to accumulate rubber.

A debate arose as to the relationship between growth, assimilates and rubber. Growth and rubber were said to be inversely related (LLOYD,1911),and rubber was either formed at the expense of the assimilates (RITTER,1954), or the two processes were independent of one another. As there was no rubber formation without growth, and rubber and inulin accumulated simultaneously, carbohydrate and rubber synthesis were regarded as separate mechanisms independent of one another (BONNER AND GALSTON,1947).

4.1.4 FREE SUGARS IN GUAYULE

The amount of free non-fructose sugars appeared to be greater than the free fructose, in spite of the fact that the chief reserve carbohydrates were fructose polymers (TRAUB AND SLATTERY,1946b). Levulins were thought to be the chief translocatory carbohydrates in guayule as they were present in small amounts in the stems and roots but predominated in the leaves and inflorescences (TRAUB AND SLATTERY,1946b). This is not a new idea as JEFFORD AND EDELMAN (1961) suggested that fructans are translocated in *Helianthus tuberosus* L. tubers.

Fructose comprised about 10% of the total free sugars in the stem and roots, and 25% in the inflorescences in guayule. Free sugars were thought to occur in minimal amounts due to their utilization and conversion to polysaccharides (TRAUB,SLATTERY AND WALTER,1946). Little variation was noted in the free sugars and inulin levels on a seasonal basis (TRAUB AND SLATTERY,1946b).

4.1.5 STARCH IN GUAYULE

The seasonality of starch production has not been investigated in guayule although starch has been observed in leaves during Autumn (KELLY,GILLILAND AND VAN STADEN,1985), in the parenchyma cells immediately inside the endodermis, between sclerenchyma fibres, and in the layer of parenchyma cells sheathing the resin canal epithelial cells (ARTSCHWAGER,1943).

4.1.6 OBJECTIVES OF THIS STUDY

Sucrose is usually not stored for any length of time and is either complemented or replaced by other forms of carbohydrates (KELLER,1989). Guayule is no exception, and accumulates starch and fructans. Fructans and rubber accumulate simultaneously in the Winter, while starch accumulates from Spring to Autumn in the leaves, and sometimes in the young stem (KELLY AND VAN STADEN,1991). The function of these reserve carbohydrates in the carbon economy of guayule and the contribution of these reserves to the isoprenoid pathway, has not been addressed.

The objectives of this study were to :

- 1) investigate and update the methods used to extract, separate and identify fructans in guayule;
- 2) establish whether reserve carbohydrate accumulation is seasonal ; and to
- 3) determine the relationship between reserve carbohydrate and isoprenoid production;
- 4) assess the contribution of fructose, glucose and sucrose, to reserve carbohydrate as well as isoprenoid production.

4.2 MATERIALS AND METHODS

4.2.1 Climatic parameters

The experimental period spanned from January 1988 to December 1989. The daily maximum and minimum temperature recordings were made on a thermohygrograph alongside the experimental site (**Figure 4**, section 1.1.2).

4.2.2 Plant material

Uniform, intact two-year-old W10 (R.S.A.) (A48118; U.S.A.) guayule plants were maintained in 30 cm plastic pots in a bark medium. Plants were watered on a daily basis and fertilized at regular intervals. Plant material was collected between 09 00 and 10 00 hours to minimise diurnal variation in the carbohydrate content. Upon harvest, plant components were divided into leaves, stems and roots and the fresh weight of all components was established. All plant components were frozen in liquid nitrogen and lyophilized in a freeze drier, thus alleviating the possibility of artifacts arising during sugar analysis (HENDRIX AND PEELIN,1987). Once dried, samples were weighed to determine dry weight and then ground into homogeneous powders on a Wiley Mill, and stored in glass vials at -10°C until required.

4.2.3 Extraction, separation, purification and determination of fructans

Extraction Procedures

Graded ethanolic extraction

A graded ethanol series was used to determine whether fructan polymers of one or mixed sizes exist (NELSON AND SMITH,1986). The free sugars and short chain polymers can be extracted with high concentrations of ethanol, while the

long chain fructans can be extracted with water and 25% ethanol, at room temperature (SMITH,1968).

One g dried leaf, stem and root material was harvested in September and March and was extracted in 50 ml 0, 25, 50, 75 and 90% ethanol (v/v) respectively, overnight. Samples were then filtered through Whatmans No.1 filter paper.

The filtrates were clarified as follows: a saturated lead acetate solution was added to the water or ethanolic filtrates to precipitate the proteins. The resulting solution was filtered through double Whatmans No. 1 filter paper into flasks containing sodium oxalate which precipitated the excess lead (TRAUB AND SLATTERY,1946a). This filtrate was filtered through double Whatmans filter paper, reduced to dryness under vacuum at 45 °C, and then redissolved in 10 ml *iso*-propanol (10%). Samples were centrifuged at 10 000 x g for 20 minutes and the supernatant was retained and filtered through a 0.22 µm Millipore filter.

Polymer length was determined by paper and thin layer chromatography. A 500 µl aliquot of the *iso*-propanol solution was applied to the marked origin of Whatman No. 1 chromatogram paper. Known standards (50 µl) of sucrose, fructose and glucose (in 80 % ethanol (v/v) were spotted on each chromatogram. The chromatogram was then dried. The tank followed by the chromatogram was equilibrated for three hours with the solvent, n-butanol: acetic acid: water (4:1:5;v/v upper phase) (PARTRIDGE AND WESTALL,1948; GUPTA,KAUR AND SINGH,1986). The chromatogram was run for 3 days after which time it was removed and dried. Individual carbohydrates were visualised by spraying (not dipping) the chromatograms with aniline-diphenylamine phosphate (Section 2.8.9). Spots were marked and those co-eluting with the standards were identified and a diagrammatic record was kept.

For thin layer chromatography, 100 µl aliquots of the *iso*-propanol solutions from all the fractions were spotted onto Kieselgel 60F₂₅₄ ready foils. These

were run in butan-1-ol:propan-2-ol:water (3:12:4; v/v upper phase) for 10 hours, dried and developed again. Once dry, the foil was sprayed with a ketose-specific urea-phosphoric acid spray^{***}.

UREA PHOSPHORIC SPRAY^{***} (ZWEIG AND SHERMA, 1972)

REAGENTS

1 g urea

4.5 ml H_3PO_4

48 ml n-butanol saturated with water

PROCEDURE

Mix together above reagents

Spray

Heat at 90°C until spots are visualised (10 to 15 minutes)

A comparison of hot and cold water extractions

One g dried young stem, mature stem and root material collected from plants during September, was extracted in either water or 80 % ethanol (v/v). One water sample was boiled for 20 minutes, the second water sample was extracted at room temperature and the 80% ethanol (v/v) sample was extracted at room temperature. Samples were then filtered through Whatmans No.1 filter paper, purified and reduced and 100 μl aliquots of the reduced samples were spotted onto TLC plates as outlined above.

4.2.4 Routine Extraction Procedure Adopted for all Analysis

Samples were extracted in 50 ml water or in 50 ml 80% ethanol (v/v) at room temperature overnight, filtered through Whatmans No. 1 filter paper and the residue retained for starch analysis. The filtrate was retained and clarified as follows: a saturated lead acetate solution was added to the water or ethanolic

filtrates to precipitate the proteins. The resulting solution was filtered through double layers of Whatmans No. 1 filter paper into flasks containing 2 g sodium oxalate which precipitated the excess lead (TRAUB AND SLATTERY,1946a). This filtrate was filtered through double layers Whatmans No. 1 filter paper, reduced to dryness under vacuum at 45 °C, and then redissolved in 10 ml *iso*-propanol (10%). Samples were centrifuged at 10 000 x g for 20 minutes and the supernatant was retained and filtered through a 0.22 μ m Millipore filter.

4.2.5 Colorimetric Determination of Fructans

The first part (a) of this procedure hydrolyses all fructose (free and combined) including sucrose, thus providing a value for total fructose (FORSYTH,1948). The second part (b) hydrolyses sucrose, and the resulting free fructose and glucose is destroyed by boiling in NaOH, as the monosaccharides are unstable under alkaline conditions (PONTIS,1966). The fructan polymers are then hydrolysed using resorcinol.

- a) *Mild acid hydrolysis (20 minutes in 30% HCl at 80°C), followed by a ketose-specific resorcinol-HCl colorimetric test*.*

RESORCINOL TEST* (ROE,1934; McRARY AND SLATTERY,1945)

REAGENTS

Alcoholic resorcinol: 1 g resorcinol dissolved in 1 l 95% ethanol (v/v)

30% HCl: 1 volume distilled water in 5 volumes concentrated HCl

Fructose standard: Dissolve 100 mg fructose in 100 ml saturated benzoic acid solution. Two working standards were prepared by diluting 6 ml and 10 ml fructose solution in 100 ml of a saturated benzoic acid solution.

METHOD

0.5 ml extract (make up three replicates)

Add 0.5 ml alcoholic reagent

Add 1.5 ml 30% HCl

Vortex sample

Place tubes in 80°C water bath for 20 minutes

Cool to room temperature

Determine absorbance at 520 nm

The spectrometer (Beckman DU 60 series) was calibrated with a 0.5 ml water sample and the following known standards were used to obtain a calibration curve: 50; 40; 30; 24; 18; 12; 10; 6; 5; 3 μ g fructose. In this range a straight line which fitted the formula $y = mx + c$ was calculated at the beginning of each batch of samples analyzed or when fresh reagents were made up. Five replicates for each concentration were used. The standard calibration curve was then used to calculate the concentration of fructose present in each sample.

b) *Hydrolysis of the sucrose by yeast invertase** followed by boiling in NaOH.*

YEAST INVERTASE HYDROLYSIS**

REAGENTS

Yeast invertase: Although this step is included in all fructan determinations, only BROCKLEBANK AND HENDRY (1989) included details on the preparation of invertase. Sigma specify that one unit yeast invertase hydrolyses 1.0 μ mole sucrose per minute at pH 4.5 at 55°C. For these experiments 50 mg invertase was added to 100 ml sodium acetate buffer, pH 4.55.

Sodium acetate buffer (GOMORI,1955):

Acetic acid 1.155 ml / 100 ml

Sodium acetate 1.64 g / 100 ml

Take 5 ml acetic acid and 8 ml sodium acetate to yield a pH of 4.55

PROCEDURE

Take 0.5 ml clarified, centrifuged sample

Add 0.5 ml invertase in sodium acetate buffer pH 4.55

Leave in water bath at 55°C for 20 minutes

Add 0.5 ml 0.25M NaOH (PONTIS,1966)

Vortex

Heat for 10 minutes at 100°C

Cool

Determine fructose content using the resorcinol-HCl test*

4.2.6 Chromatographic Determination

Paper Chromatography

Leaf and stem samples were collected at the end of each month at 9 00 hours from January 1987 to December 1987. Fructans were extracted in water and 80% ethanol (v/v) and fructan was determined colorimetrically (section 4 and 5). A 500 μ l aliquot of the *iso*-propanol solution was applied to the marked origin of Whatman No. 1 chromatogram paper. Known standards (50 μ l) of sucrose, fructose and glucose (in 80 % ethanol (v/v)) were spotted on each chromatogram. The chromatogram was then dried. The tank holding the chromatogram was equilibrated for three hours with the solvent, n-butanol: acetic acid: water (4:1:5;v/v upper phase) (PARTRIDGE AND WESTALL,1948; GUPTA,KAUR AND SINGH,1986). The chromatogram was run for 3 days after which time it was removed and dried. Individual carbohydrates were visualised by spraying (not dipping) the chromatograms with aniline-diphenylamine phosphate (Chapter 2, section 2.3.9). Spots were marked and those co-eluting with the standards were identified and a diagrammatic record was kept.

Thin layer chromatography

A number of techniques were attempted but the technique of CAIRNS AND POLLOCK (1988b) was adapted and utilised as it proved the most rapid and successfully separated the fructan polymers. Following extraction, 100 μ l aliquots of the *iso*-propanol solutions from the 80% ethanolic fractions were spotted onto Kieselgel 60F₂₅₄ ready foils. These were run in butan-1-ol:propan-2-ol:water (3:12:4; v/v upper phase) for 10 hours, dried and developed for a second time. Once dry, the foil was sprayed with a ketose-specific urea-phosphoric acid spray^{***}.

UREA PHOSPHORIC SPRAY^{***} (ZWEIG AND SHERMA, 1972)

REAGENTS

1 g urea

4.5 ml H₃PO₄

48 ml n-butanol saturated with water

PROCEDURE

Mix above reagents

Spray

Heat at 90°C until spots are visualised (10 to 15 minutes)

4.2.7 EXTRACTION AND DETERMINATION OF STARCH IN GUAYULE

The residue from the water/ethanol extractions were dried at 50°C and stored at -10°C until required. Two ml HCl (1N) was added to 0.1 g of the residue sample. This was vortexed and hydrolysed at 94-98°C in a water bath for 2 hours. Ten ml distilled water was added and the sample was vortexed, then centrifuged at 8 000 x g for 15 minutes. The supernatant was filtered through a 0.45 μ m Millipore filter and then made up to 20 ml with distilled water. Starch was determined using the glucose oxidase^{****} test.

GLUCOSE OXIDASE TEST FOR STARCH**** (EBELL,1969ab)

REAGENTS

A:41 g KH_2PO_4 ; 21 g K_2HPO_4 ; 6.5 g $(\text{NH}_4)_6\text{MO}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ up to 1 litre

B:2 g KI in 100 ml H_2O , use fresh

C:1000 units glucose oxidase, use fresh

Dissolve C in 100 ml A and 100 ml B

Dilute to 500 ml with water

Adjust pH to 6.0

Keep cold in the dark

PROCEDURE

0.5 ml taken from the 20 ml filtrate

Add 4 ml reagent

Vortex

Leave in the dark for 40 minutes at 25°C

Measure absorbance at 353 nm

4.2.8 SEPARATION AND IDENTIFICATION OF FREE SUGARS BY GAS LIQUID CHROMATOGRAPHY

Plant components were dried and extracted in 80% ethanol (v/v), as described in section 4.2.2.

Sample preparation

Aliquots of 100 μl were withdrawn from each *iso*-propanol solution.

Conversion to oximes

In order to convert the sugars to oximes, the 100 μl aliquots were dried under nitrogen at 40°C and a 0.5 ml hydroxylamine / pyridine **** was added, the vial was sealed and heated at 40°C for 20 minutes. A 100 μl aliquot was then

removed from the hydroxylamine / pyridine solution, reduced to dryness under nitrogen at 40°C, and cooled to room temperature.

Hydroxylamine / pyridine solution ****(HOLLIGAN,1971)

0.5 ml of a solution of 25 mg/ml hydroxylamine hydrochloride dissolved in pyridine.

Sililylation

The sugars were sililylated by adding 50 µl Sylon BTZ (Supelco) or Trisil (Pierce,U.S.A.). The Sylon BTZ required 15 minutes for the reaction to be completed while the Trisil required one hour standing time at room temperature before the reaction was completed and an injection was possible.

Injection

One µl of the sililylated sample was injected into a Varian Gas Chromatograph, model 3700. Sugars were detected using a flame ionising detector. The sugars were separated using an OV-17 glass column (1.8 m x 6 mm I.D.), packed with Chromosorb HP 80-100. Nitrogen was the carrier gas. Peaks were integrated electronically.

Programme

140°C for three minutes

2°C per minute increase to 250°C

Temperature held at 250°C for 10 minutes

Injector temperature 200°C

Ion detector 350°C

Separation of Standards

Separation times

One mg solutions of the following solutions were prepared to determine their separation times: erythritol, deoxyribose, xylose, arabinose, ribose, rhamnose, mannitol, sorbitol, fructose, galactose, glucose, inositol, sucrose, trehalose, maltose and cellibiose. These were run separately and then combined in the order erythritol and deoxyribose, and then erythritol, deoxyribose and xylose and so forth until all the standards were run concurrently. It was then possible to tentatively identify the sugars in each plant sample. The standards were prepared for G.L.C. as outlined above.

Calibration curve for fructose, glucose and sucrose

One mg solutions of glucose, fructose and sucrose were prepared in one ml 80% ethanol (v/v). These standards were used to make a dilution series of 2, 1.6, 1.2, 0.8, 0.4 and 0.2 μg sugar. Each of these were prepared for G.L.C. as previously described. These samples were run to ascertain the area of the peak corresponding with the known concentration of the sugar. In this range these values were fitted into the equation $y = mx + c$, and the correlation coefficient was calculated thus enabling the concentration of fructose, glucose and sucrose in each plant component to be calculated.

4.2.9 APPLICATION OF THE TECHNIQUES

The effect of season on the accumulation of carbohydrates in guayule

Five plants were harvested from January 1988 to December 1989 on a monthly basis. Plants were divided into leaves, young stems (1 to 5 cm from stem apex), mature stem (rest of the stem) and roots (including the crown), and the fresh mass and dry mass determined (Section 4.2.1). Using the

techniques that had been established the level of fructan, starch and the free sugars were extracted from the leaves, stems and roots of guayule plants from January 1988 to December 1989. The amount of fructose, glucose and sucrose was quantified using G.L.C.. In this case, 100 μl aliquots from the five replicates were combined and the resulting 500 μl was dried under nitrogen and the conversion to oximes performed as described. As the programme required 100 minutes of running time, samples were only run once. The procedure followed is outlined in **Figure 27**.

4.2.10 RADIOCHEMICAL EXPERIMENT

A three-year-old plant was placed in a sealed bell jar in June. It was exposed to $^{14}\text{CO}_2$ liberated from $\text{Ba}^{14}\text{CO}_3$ reacting with sulphuric acid. Plants were exposed to the $^{14}\text{CO}_2$ for 3 hours (3 hours light; 09 00 to 12 00 hours) and 12 hours (9 hours light and 3 hours dark; 09 00 to 21 00 hours), at ambient environmental conditions. Air was circulated through the bell jar for the entire experimental period with an ethanolamine trap for the recovery of excess $^{14}\text{CO}_2$. Minimum and maximum temperatures were 21-29°C and 9-22°C; light intensity was from 1549 to 2290 $6.37 \mu\text{mol m}^{-2} \text{s}^{-1}$ for the three hour experiment and from 1750 to 2440 to 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for the 12 hour experiment. Each experiment was repeated three times.

Plants were harvested immediately after the radiolabelling, weighed and then dried at 60°C for 24 hours, separated into leaves, stems and roots, and ground into homogeneous powders. The dried ground material was placed in cellulose thimbles for Soxhlet extraction, and the weight of the material to be extracted was recorded. This material was then extracted with 80% ethanol (v/v), acetone and petroleum ether (40 to 60°C) in succession. The three fractions thus obtained were brought to constant volume (10 ml) by flash evaporation. Aliquots (1 ml) of the three fractions were placed in 5 ml plastic scintillation vials (Beckman) and 4 ml Ready Solve EP (Beckman Instruments) was added to each. The vials were left in the dark for 12 hours, whereafter the

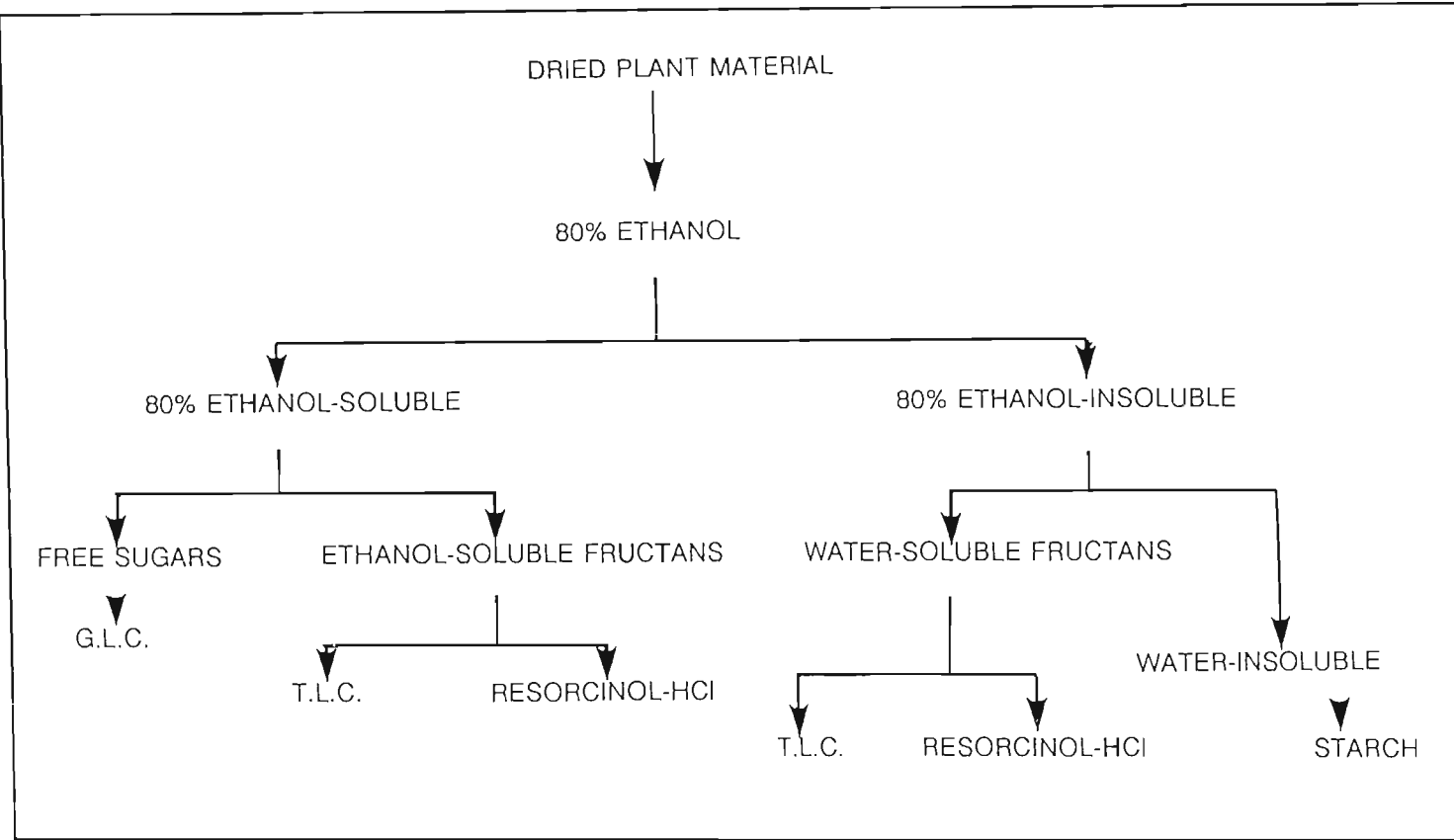


Figure 27.

A flow diagramme of the experimental procedure followed to extract starch, water-soluble and ethanol-soluble fructans in guayule. Bars represent the standard error.

radioactivity (DPM) was recorded. Fructans and starch were extracted as described. The radioactivity in these two fractions was determined by drying the 1 ml aliquots used for the colorimetric analysis. Four ml Ready Solve was added (Beckman Instruments) and radioactivity determined using a Beckman LS 3800 scintillation counter.

4.2.11 STATISTICAL ANALYSIS

A multiple regression analysis (STATISTICAL GRAPHICS CORPORATION,1987) was used to establish the nature of the relationship between the level of fructans, free fructose and starch and to assess the influence of growth and temperature on these levels.

4.3 RESULTS

4.3.1 Establishment of the routine procedures for the extraction, separation and purification of carbohydrates in guayule

In most fructan experimental procedures an ethanolic extraction is followed by a water extraction. In some cases the ethanol is boiled (CARPITA,KANABUS AND HOUSLEY,1989; GONZALEZ, BOUCAUD AND LANGLOIS,1989; PRESSMAN,SCHAFFER,COMPTON AND ZAMSKI,1989) while in other cases it is not (HOUSELY,KANABUS AND CARPITA,1989). The same situation applies to the second extraction where the water may be boiled (GONZALEZ,BOUCAUD AND LANGLOIS,1989),in some cases it is not stipulated (CARPITA,KANABUS AND HOUSLEY,1989; HOUSLEY,KANABUS AND CARPITA,1989), or not included (PRESSMAN, SCHAFFER,COMPTON AND ZAMSKI,1989; RUTHERFORD AND DEACON,1974). These examples have been selected at random to show that there are a variety of ways in which fructans can be extracted.

The general principal in these extractions is that the ethanolic extraction, usually 80%, removes the free sugars and low DP fructans, followed by the water extraction of the residue from the ethanolic extraction, in which the high DP fructans are soluble.

Early research on guayule carbohydrates adopted another approach which involved boiling the plant material in 80% ethanol (v/v), followed by a cold water extraction of the ethanolic-insoluble fraction and a boiling water extraction. This would extract the free sugars, levulins and inulin respectively (TRAUB AND SLATTERY, 1946b).

The graded ethanol series indicated that free sugars were extracted in water and ethanol, although the amount of fructose was greatest in the dilute ethanol concentrations (**Figure 28**). The greatest amount of fructose was extracted in the water and 25% ethanol fractions, (**Figure 28A**), specifically in the root samples. The amount of fructose was estimated using a modification of the technique of ROE (1934) and McRARY AND SLATTERY (1945), by decreasing the reagents by one twentieth.

Separation of the extracts from the ethanolic series by paper chromatography showed that free sugars were present in the leaves and young stem in September while longer polymers were present in the mature stem and roots (**Figure 28B**).

As separation of the fructan polymers by paper chromatography was well documented this method was used initially. Thin layer chromatography (TLC) techniques were under investigation and the experiment was repeated in March when a rapid, successful TLC technique was adopted (CAIRNS AND POLLOCK, 1988b).

The amount of fructose was high in the mature stem and roots in March which were extracted using water, 25% and 50% ethanol (**Figure 28C**). Separation

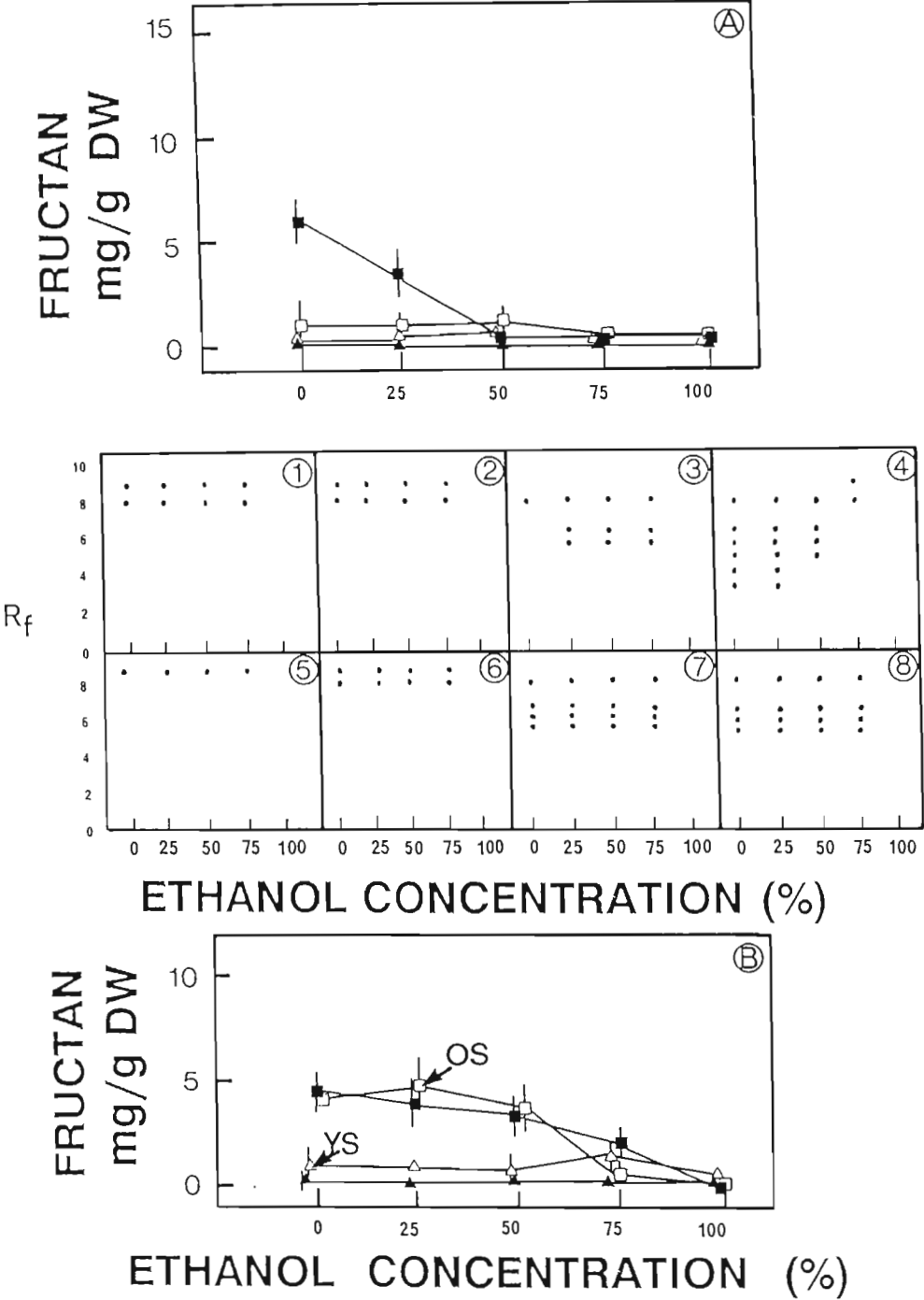


Figure 28. A serial ethanolic extraction of guayule leaves (▲), stems (△) and roots (■) in September and March followed by separation of the polymers using paper (C, 1-4) and thin layer (C, 5-8) chromatography.

of these extracts by TLC indicated that the leaves and younger stem contained only free sugars, while the larger polymers were present in the mature stem and roots (**Figure 28D**). There does not appear to be any separation of chain length with changing concentration of ethanol as suggested by SMITH AND GROTELUESCHEN (1966), except for the roots of the September plants (**Figure 28B,4**).

A comparison between extracting in boiling water or leaving samples at room temperature showed no difference in polymer length separated (**Figure 29**). Both water extracts of the mature stem had a crystalline appearance. This could mean the although the levulins had a greater solubility than inulin in water with a lower specific rotation (TRAUB AND SLATTERY,1946a), they were only low molecular weight fructans. TRAUB AND SLATTERY (1946b) identified this as a DP less than 30. These shorter polymers should have been extracted in 80% ethanol with the free sugars while the larger polymers were extracted in water.

4.3.2 Application of techniques to establish the effect of season on carbohydrate production in guayule

Guayule exhibits three cycles with respect to fructan synthesis. These occur at the end of Summer (March), at the end of Winter (July), and at the beginning of Summer (September/October) (**Figure 30**). The appearance of this pattern of accumulation when compared with that of TRAUB AND SLATTERY (1946b) was due to sampling for two years. The inverse relationship shown between 80% soluble and 80% insoluble levulins by TRAUB AND SLATTERY (1946b) was not identified in this study. Instead, the amount of water-soluble fructans increased in March, June/July and October in the young and mature stem and roots (**Figure 31**). The maximum amount of water-soluble fructans extracted from the roots was 10 mg, for mature stems 4 mg, for young stems 4 mg and leaves 0.2 mg g⁻¹ dry weight (**Figure 31**).

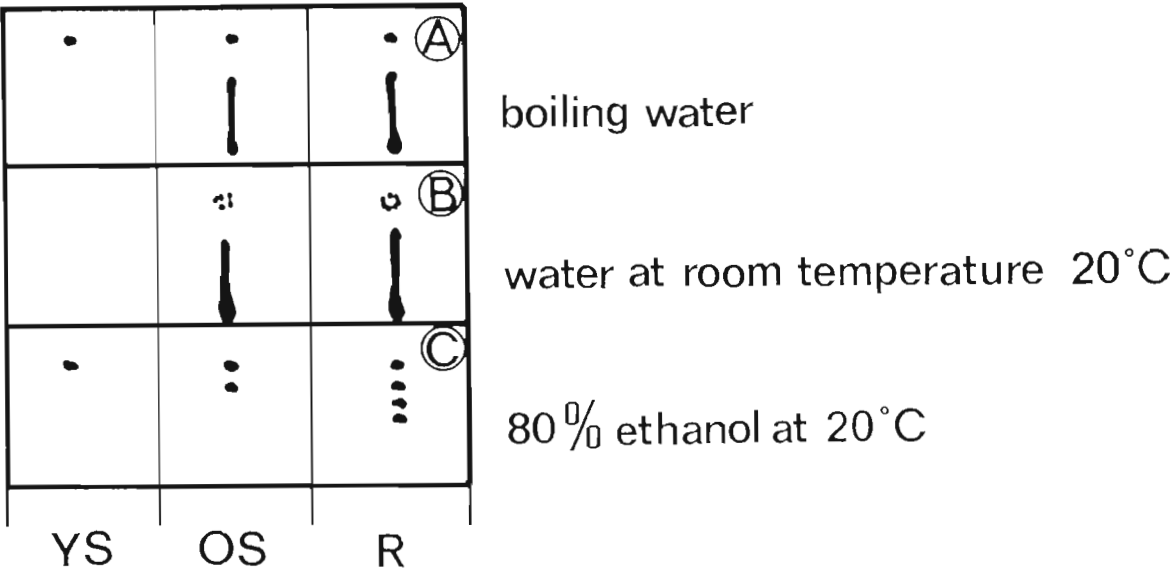


Figure 29. A comparison of polymer length separated when guayule young stems (YS), mature stems (OS) and roots (R) were extracted in water and ethanol.

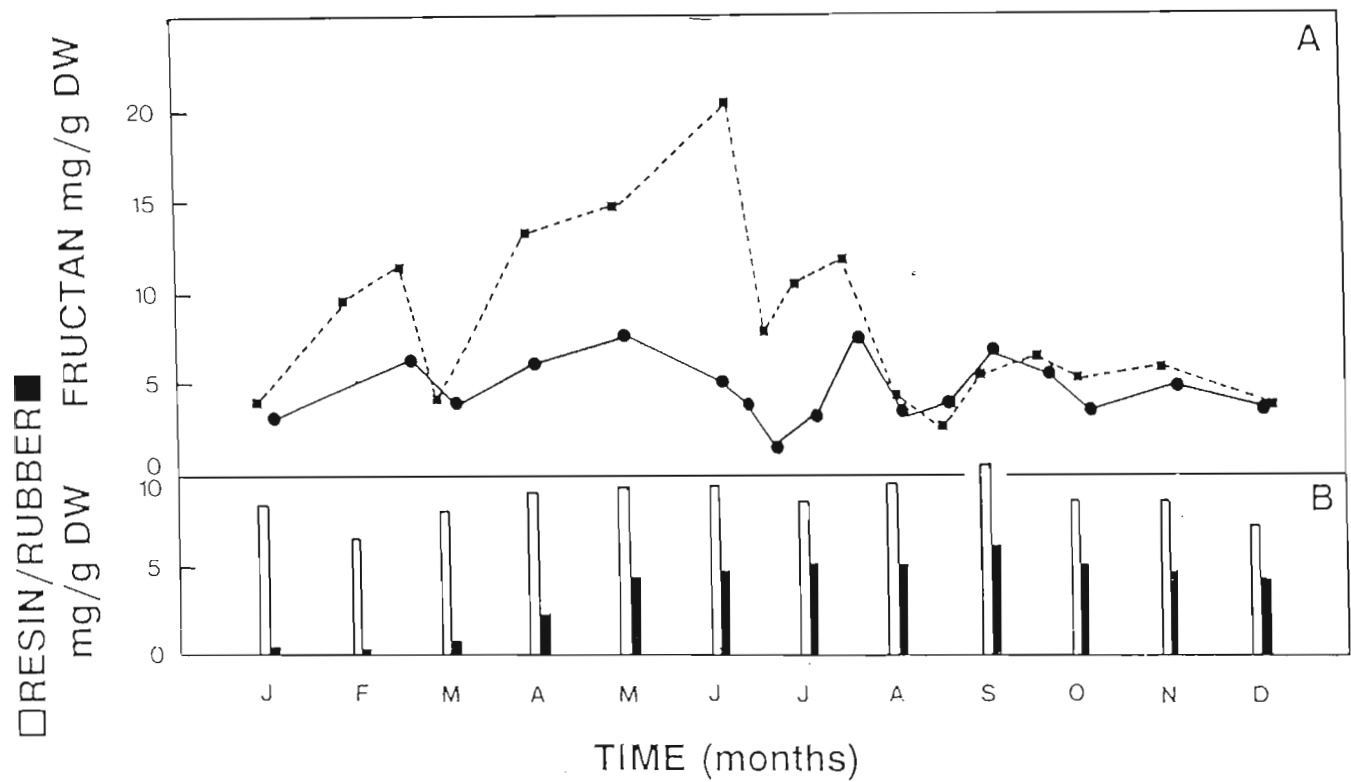


Figure 30. Total fructans, resin and rubber extracted from the leaves (●) and stem (■) of guayule from January to December 1988.

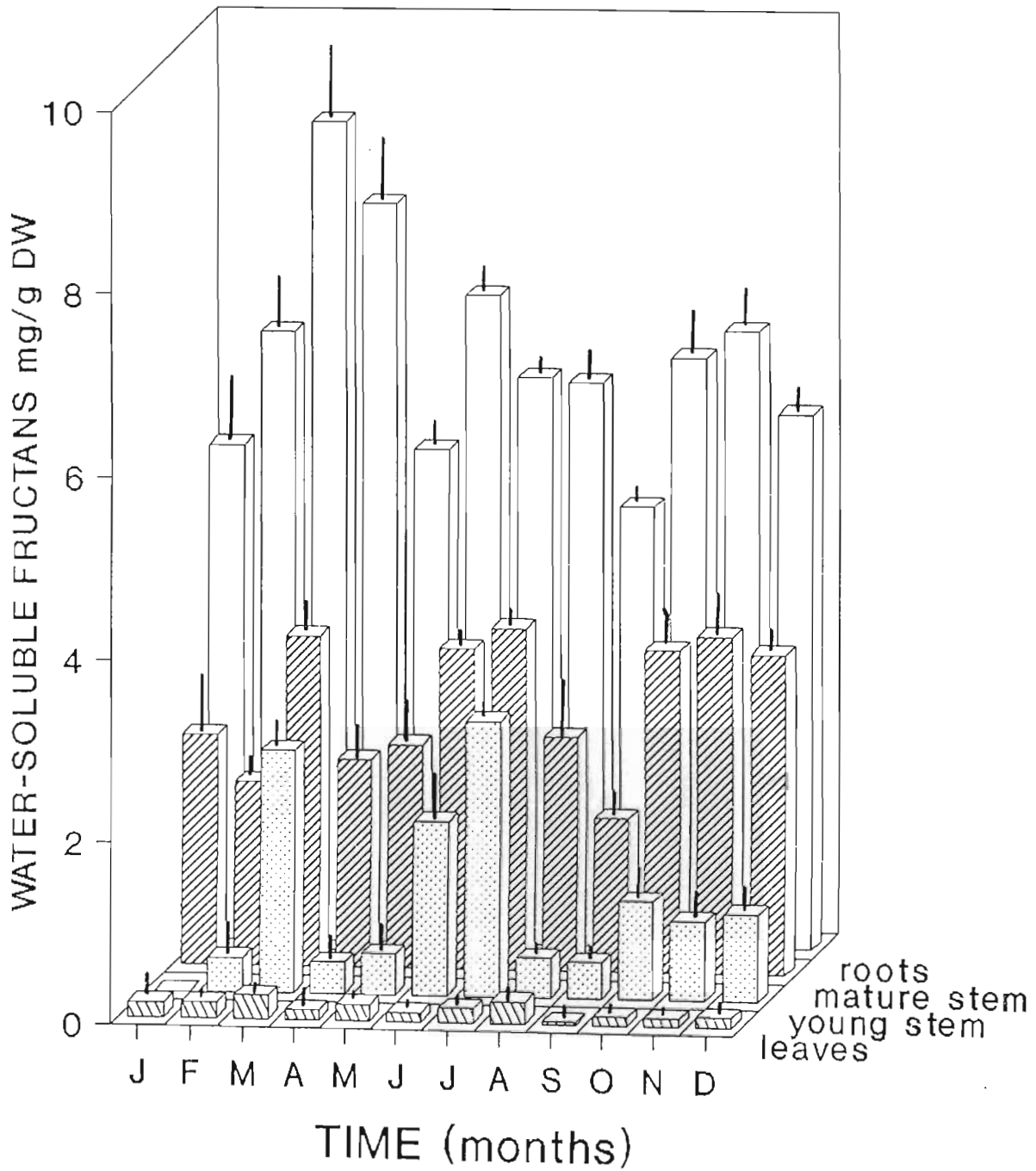


Figure 31.

The pattern of accumulation of water-soluble fructans in the leaves (▨), young stems (▤), mature stems (▧) and roots (□) of guayule from January to December 1989. Bars represent the standard error.

The same trend was observed in the ethanol-soluble fructans (**Figure 32**). The mature stem contained the highest level of ethanol-soluble fructans followed by the roots, young stem and leaves (**Figure 32**). Initially it was thought that the same molecules which were being extracted in the water and 80% ethanol.

However, TLC separation showed that polymer length varied throughout the year for both water-soluble and ethanol-soluble fructans. In the young stem polymer length increased in May, June and July and depolymerization occurred in August (**Figure 33A**). The size of the fructan polymer remained approximately the same throughout the year in the mature stem (**Figure 33B**). The largest water-soluble polymers occurred in the roots from April to August (during the colder months), depolymerization occurred after budbreak, coinciding with flowering followed by polymerization in November/December and depolymerization in January/February coinciding with the second flowering flush (**Figure 33C**).

In the roots, depolymerization of the ethanol-soluble fructans occurred from May to June and again from August to October, polymerization took place from November to February (**Figure 34C**). Depolymerization of fructans in the mature stem occurred from May to August. Polymer length increased in September and was the longest in November/ December (**Figure 34B**). The opposite occurred in the young stem where polymer length increased in June/July, decreased in August and increased from September to January (**Figure 34A**).

Associated with the carbon requirement for growth in August and September was a depolymerization of all fructans (**Figures 33,34**) and a reduction in the amount of fructans in all plant components (**Figures 31,32**). The free fructose present in the young stem (**Figure 35B**) and roots (**Figure 35D**) decreased dramatically in August. The appearance of new leaves resulted in higher

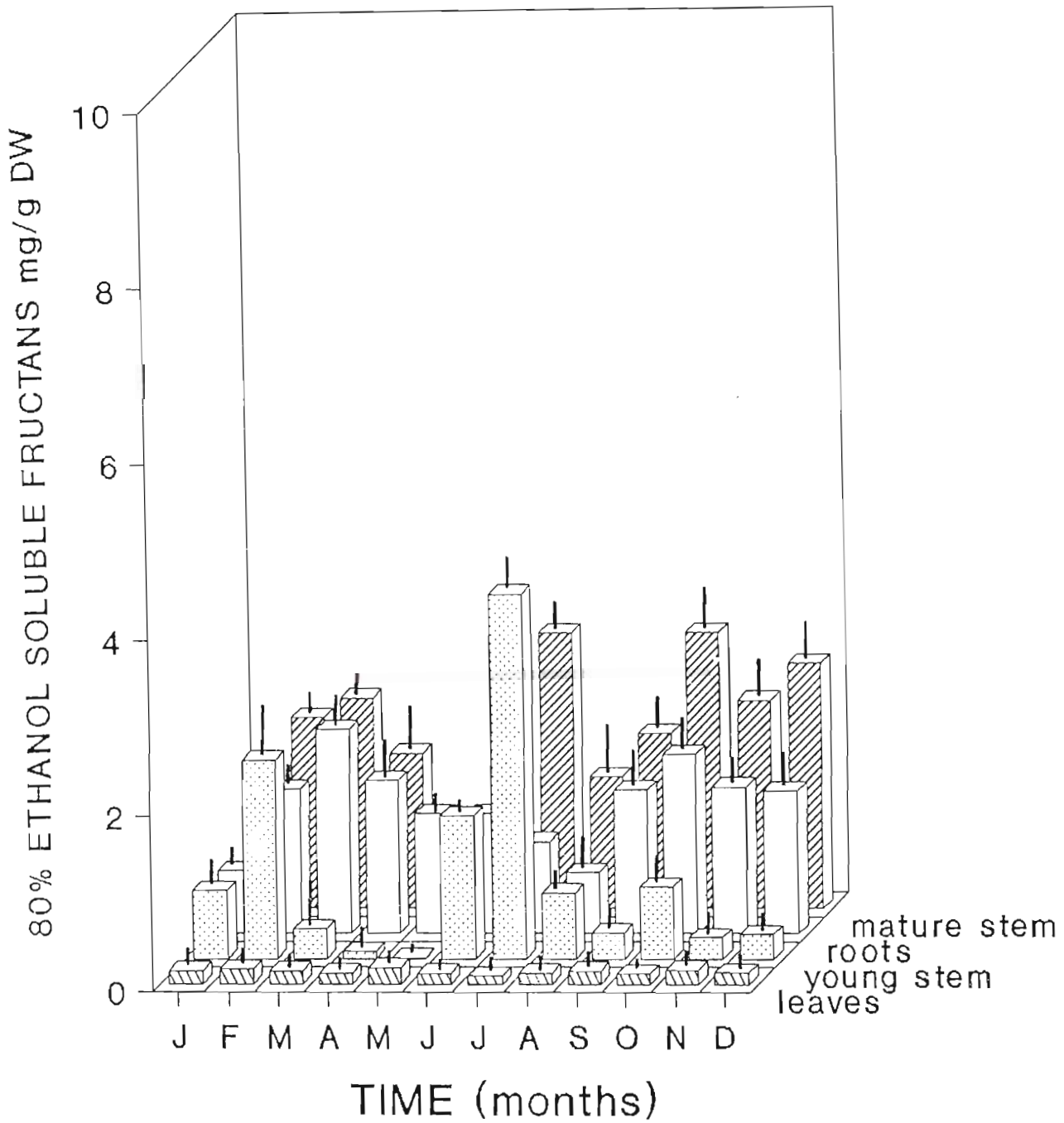


Figure 32.

The pattern of accumulation of ethanol-soluble fructans in the leaves (▨), young stems (▤), mature stems (▧) and roots (□) of guayule from January to December 1989. Bars represent the standard error.

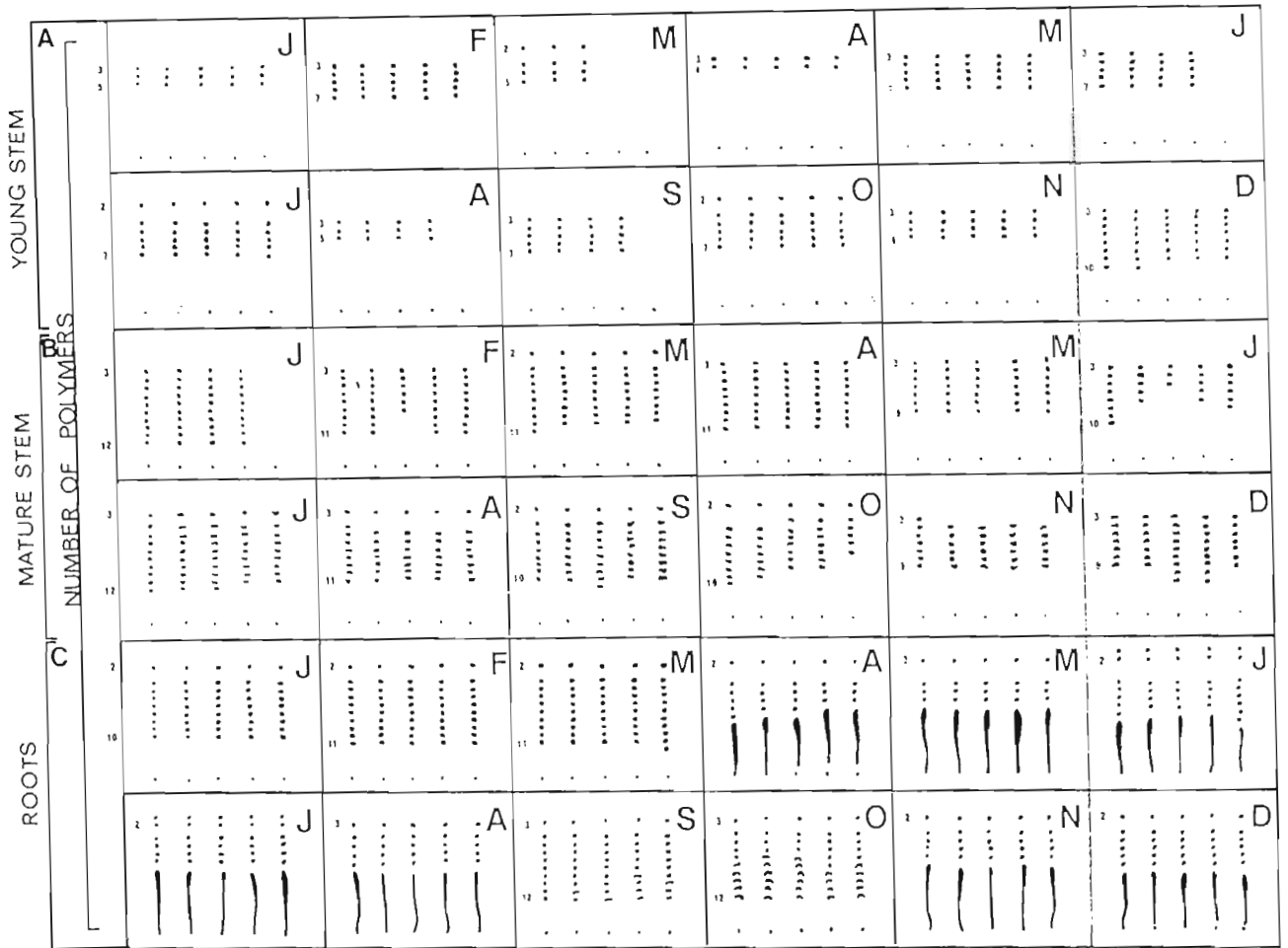


Figure 33. A diagrammatic representation of the TLC separation of water-soluble fructans in the young stems, mature stems and roots of guayule from January (J) to December (D).

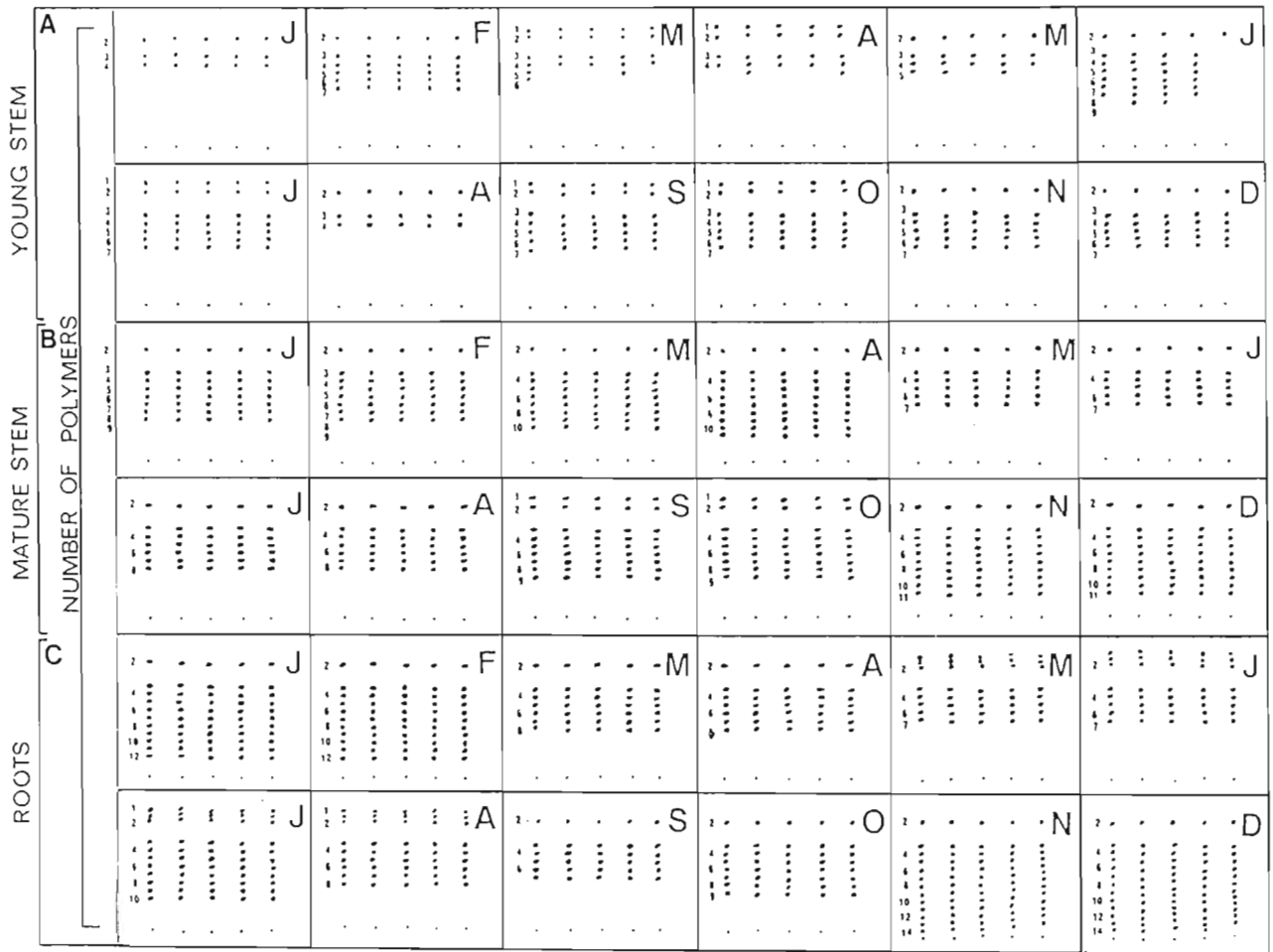


Figure 34. A diagrammatic representation of the TLC separation of water-soluble fructans in the young stems, mature stems and roots of guayule from January (J) to December (D).

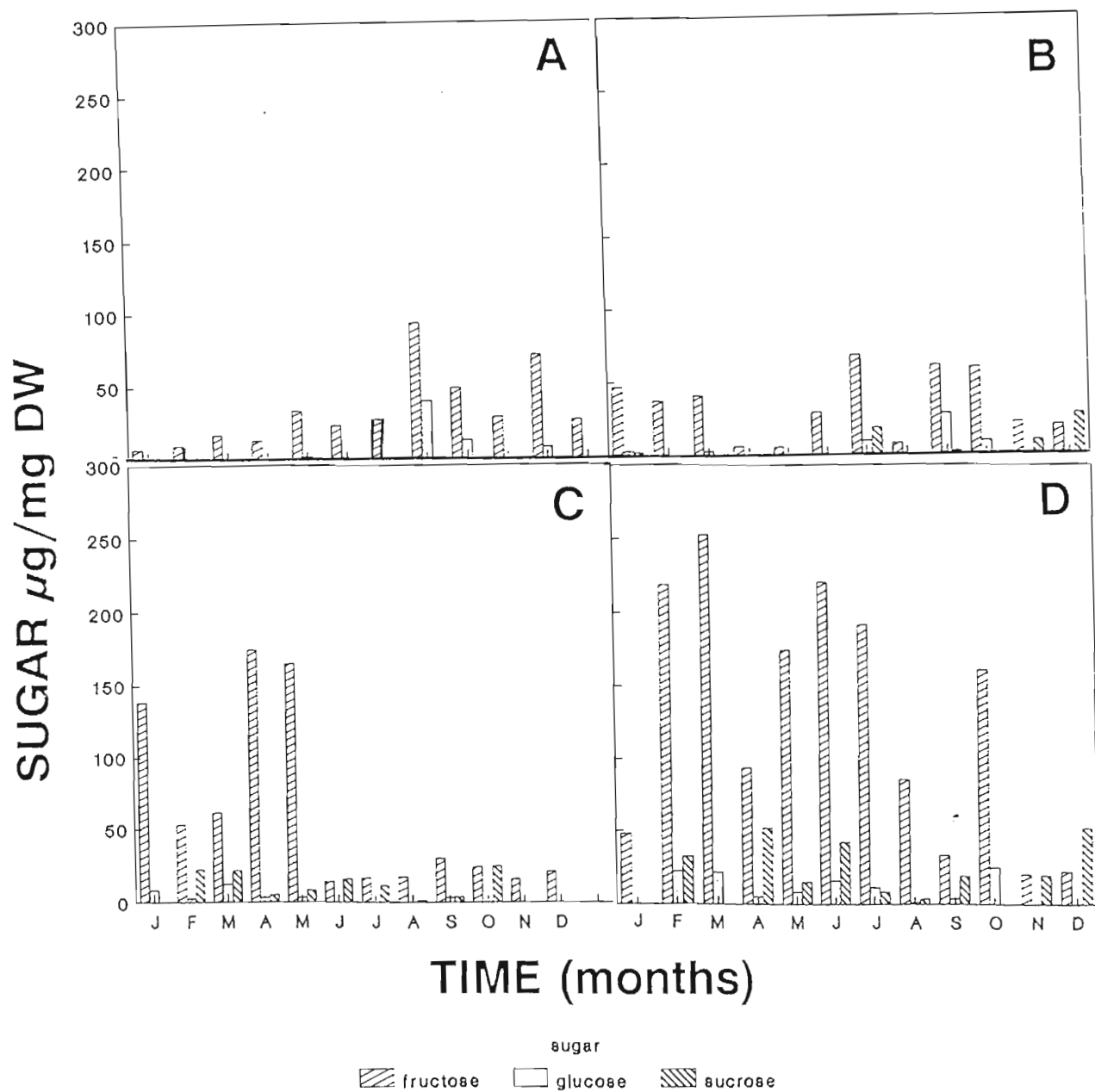


Figure 35. The level of fructose (▨), glucose (□) and sucrose (▩) extracted from the leaves (A), young stems (B), mature stems (C) and roots (D) of guayule.

fructose levels in the leaves (**Figure 35A**), although the level of fructan extracted from the leaves remained low (**Figure 31,32**).

Fructose was the major free sugar in the leaves (**Figure 35A**). The highest level of fructose was extracted in August, this was followed by a drastic reduction in September, and another increase in November. Glucose was highest in August when new leaves were produced and starch synthesis restarted (**Figure 37**). Fructose was dominant in the young stem with the highest levels recorded in July, September and October (**Figure 35B**). The level of sucrose was high in July. All free sugar levels decreased in August coinciding with budbreak. Glucose was highest in September and sucrose in December. In the mature stem, fructose was highest in April/May and from June to November, the levels of sucrose and fructose were similar (**Figure 35C**). In the roots, fructose was the dominant free sugar with increases occurring in February/March, June and October, resembling the patterns observed in the water soluble fructans (**Figure 31**). Sucrose levels were highest in April, June and December.

The G.L.C. programme used separated glucose, fructose and sucrose with elution times of 26, 23 and 52 minutes respectively (**Figure 36**). Increasing the length of the programme and holding the temperature at 250°C for 20 minutes did not separate the trisaccharides (HOLLIGAN AND DREW,1971). Separation of water samples by G.L.C. was unsuccessful and no peaks were detected.

These equations were used for the calibration curves :

fructose: $y = 51.52x + 8915$ correlation coefficient 97.01%

glucose: $y = 95.13x + 56118$ correlation coefficient 98.63%

sucrose: $y = 68.16x + 30768$ correlation coefficient 97.49%.

Growth exhibited a seasonality. Leaf production was renewed in August and increased from September to February (**Figure 37A**). This was visible from the higher ratio of upper to lower plant components (**Figure 37B**). Stem growth showed a similar trend although maximum growth occurred in November

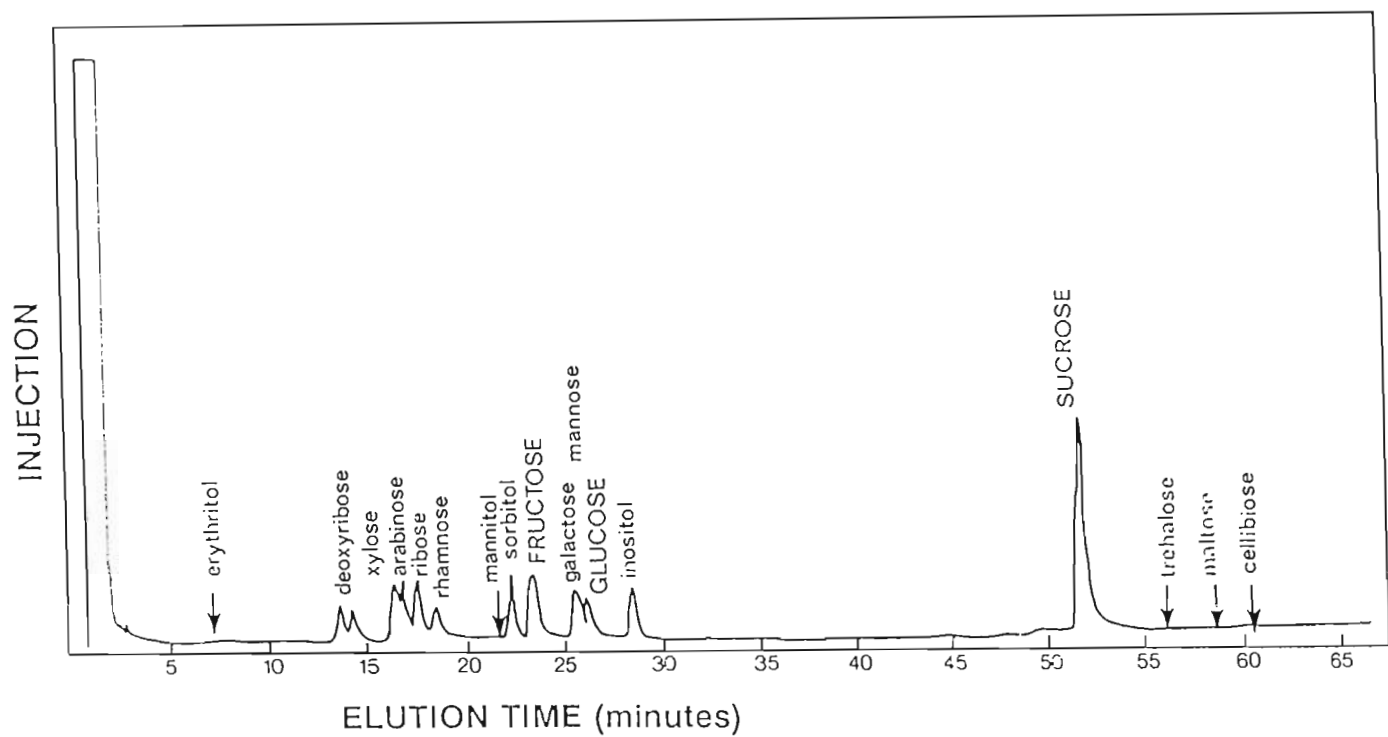


Figure 36. The elution times at which the standards erythritol , deoxyribose, xylose, ribose, arabinose, rhamnose, sorbitol, mannitol, fructose, galactose, glucose, inositol, sucrose, trehalose, maltose and cellibiose were separated by G.L.C.

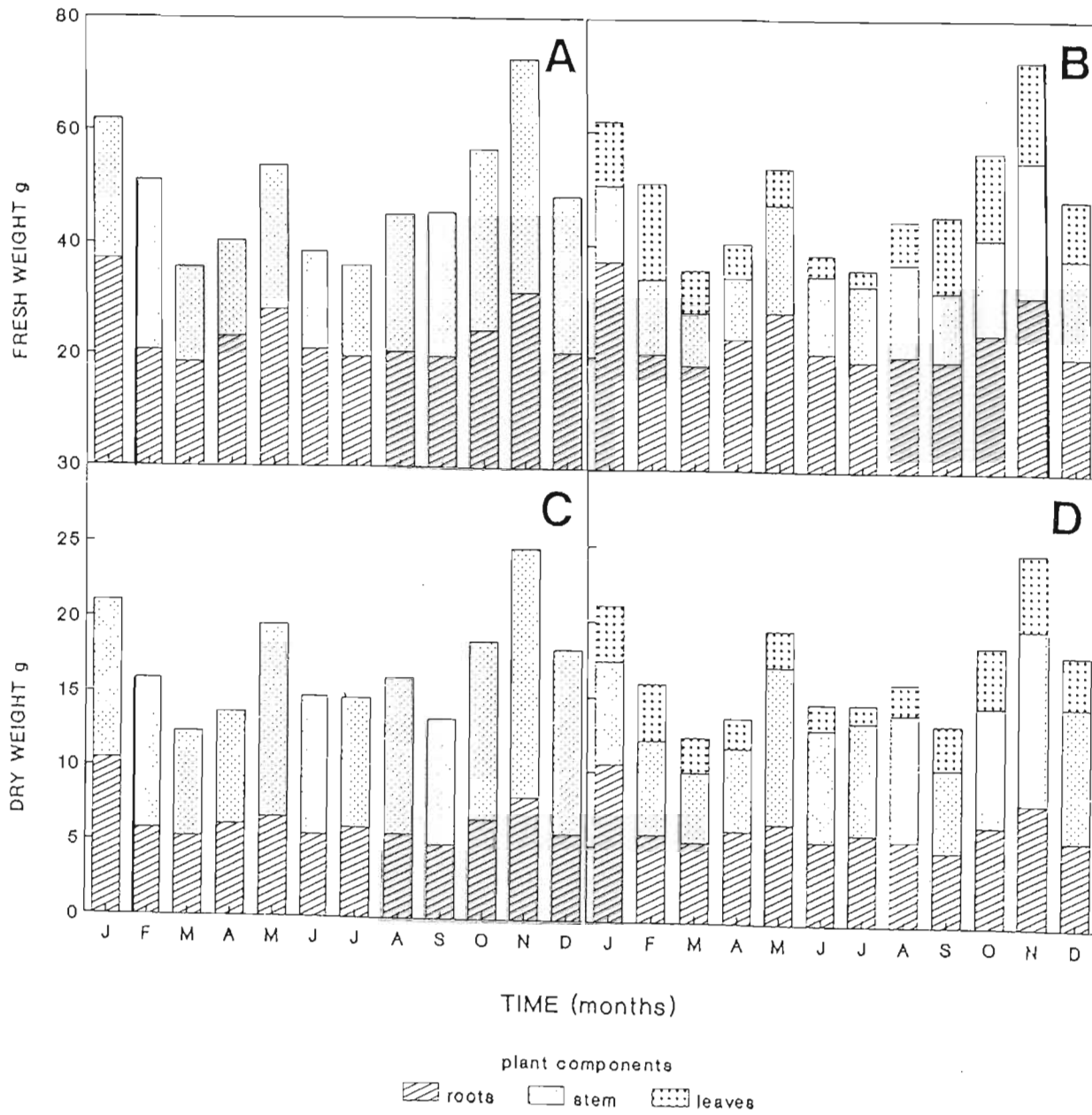


Figure 37.

The fresh (A) and dry (C) mass of the leaves and stems were combined and represented the above ground growth (□), the roots represented below ground mass (▨). The exact fresh (B) and dry (D) mass of the leaves (▤), stems (▥) and roots (▨) is also represented.

(**Figure 37A**). Root growth was not as marked and it was greatest in January (**Figure 37A**). The dry weight of these plants showed that there was a maximal carbon investment in November in all plant components (**Figure 37C**).

Total fructose (combined fructose, free fructose and sucrosyl fructose) extracted in water and 80% ethanol displayed similar levels (**Figures 38,39**). Total fructose in the water fraction increased in March, July and October in the young stems and roots (**Figure 38**). The level of free fructose extracted from the leaves was low when compared with the mature stem and roots (**Figure 38**). Total fructose in the mature stems was constant throughout the year (**Figure 38**). Polymers of fructose (fructans), made up half the total fructose in the water fraction (**Figure 31**).

Total fructose was greatest in February in the ethanol fraction of the leaves and young stem (**Figure 39**). The second increase in the leaves in total fructose was observed in August and September. In June and July and again in September and October increases were observed in the young stems (**Figure 39**). Total fructose in the ethanolic fraction of the mature stem and roots remained constant. The amount of fructose in the roots had decreased substantially when compared with the water fraction (**Figure 39**).

During the first year of analysis, starch was extracted from the leaves and stems (**Figure 40**). During the second year, starch was extracted from only the leaves and not the young stems (**Figure 41A**). For both years, elevated levels of starch occurred at the end of Summer (January to March) while the plants were losing their leaves. The second peak observed during the second year of harvest at budbreak (August) was more marked than the first (**Figure 41A**). When the levels of chlorophyll, which were extracted over the same year, were superimposed on the levels of starch (**Figure 41B**), there was a decrease in the chlorophyll content as starch increased in the Spring. In Summer, chlorophyll and starch accumulated simultaneously (**Figure 41B**).

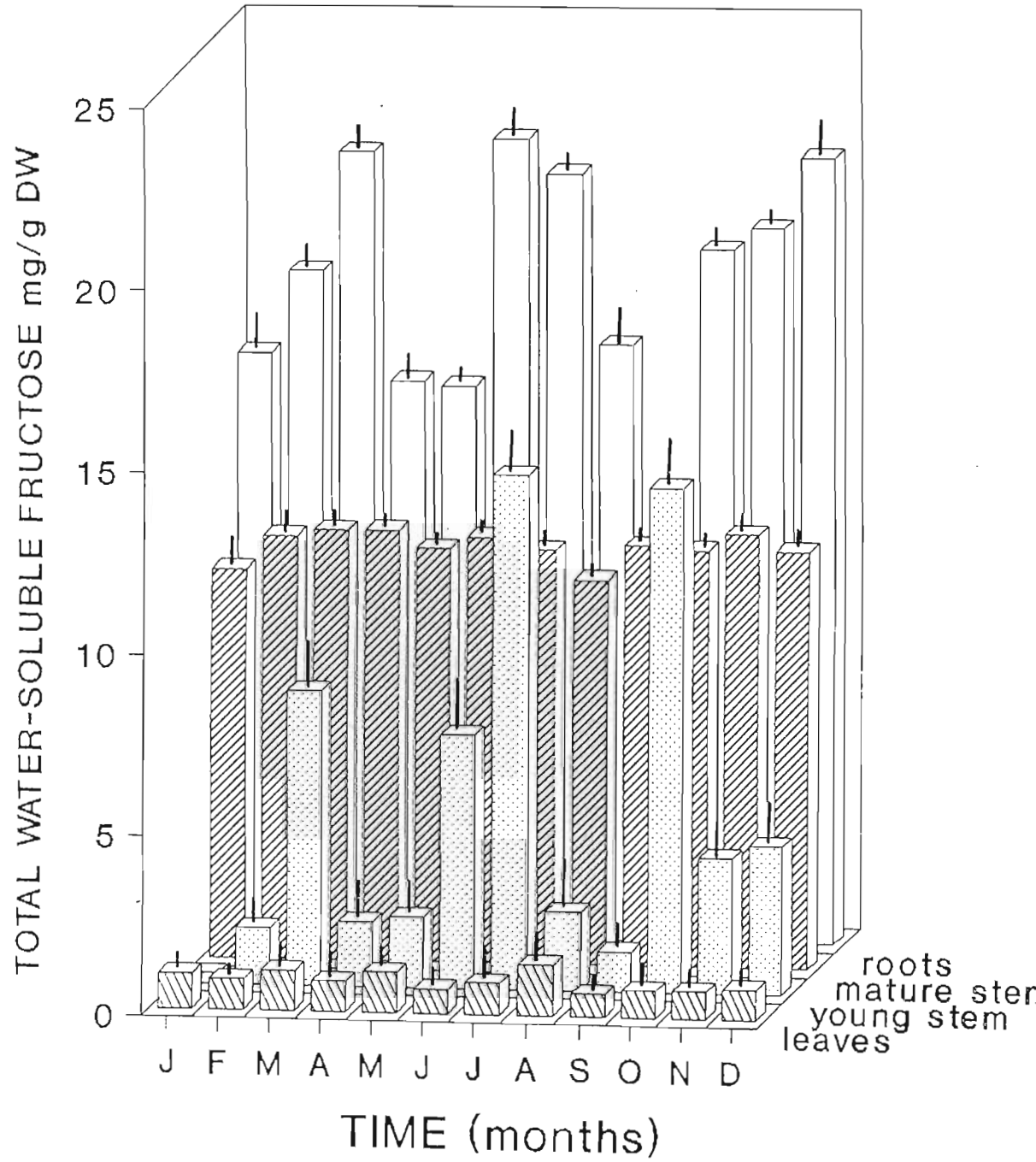


Figure 38. Total fructose in the aqueous fraction was extracted from the leaves (▨), young stems (▤), mature stems (▩), and roots (□) from January to December 1989. Bars represent the standard error.

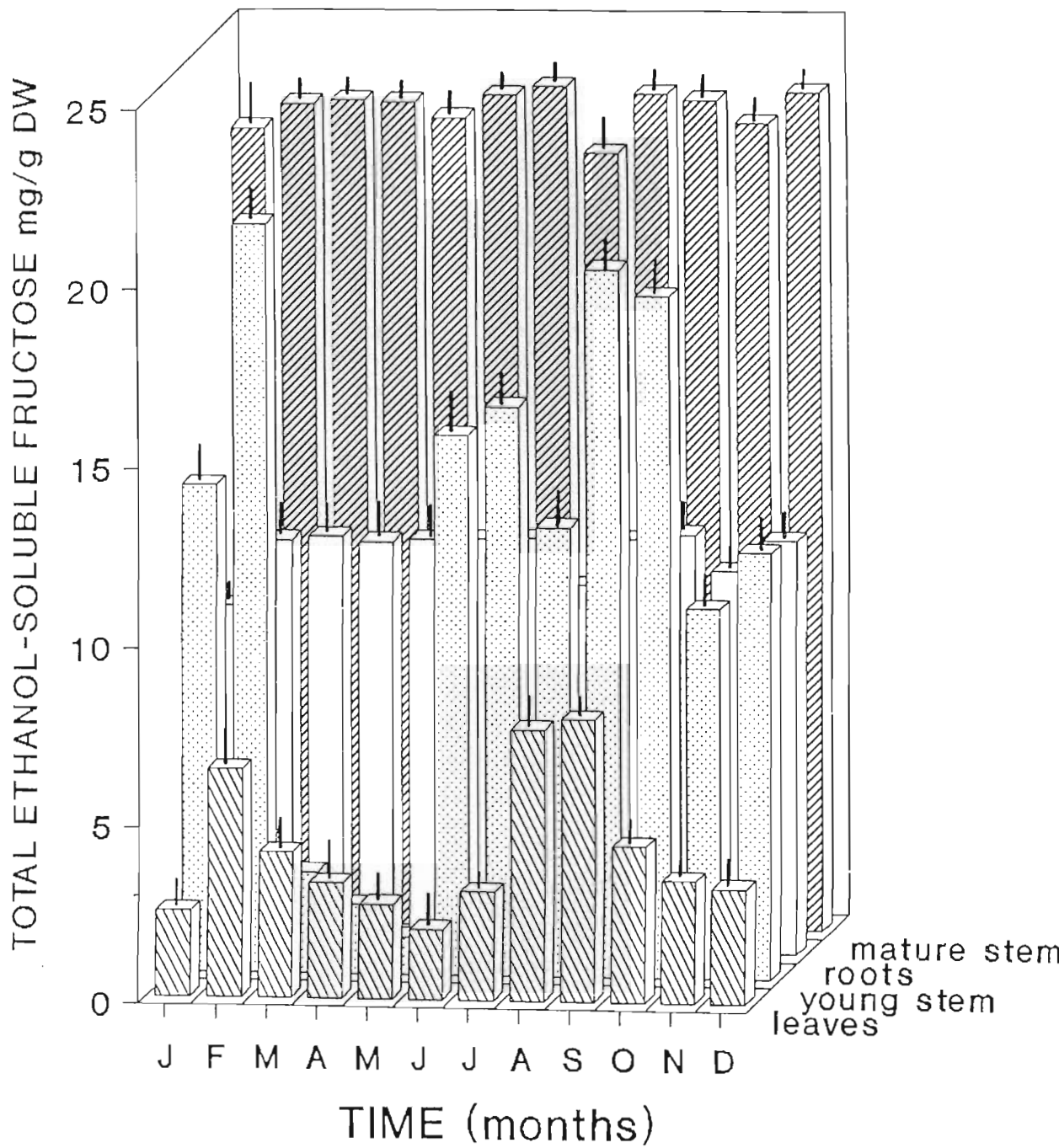


Figure 39. Total fructose (free and combined) in the 80% ethanol fraction was extracted from the leaves (▨), young stems (▤), mature stems (□) and roots (▩) from January to December 1989. Bars represent the standard error.

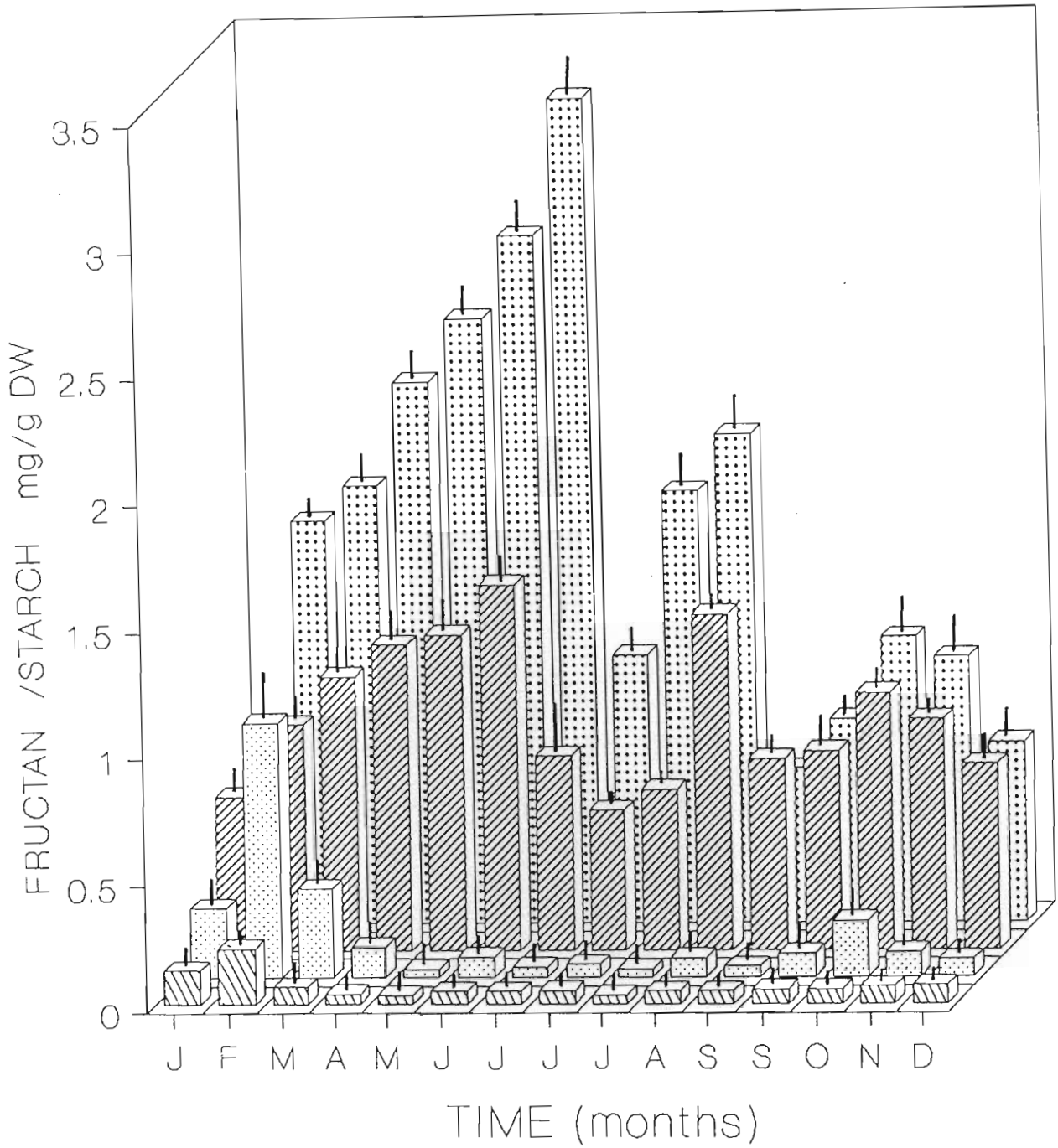


Figure 40. A comparison of the level of fructan extracted from the leaves (▨) and young stems (▤) with that of starch extracted from the leaves (▧) and young stems (▩) of guayule from January to December 1988. Bars represent the standard error.

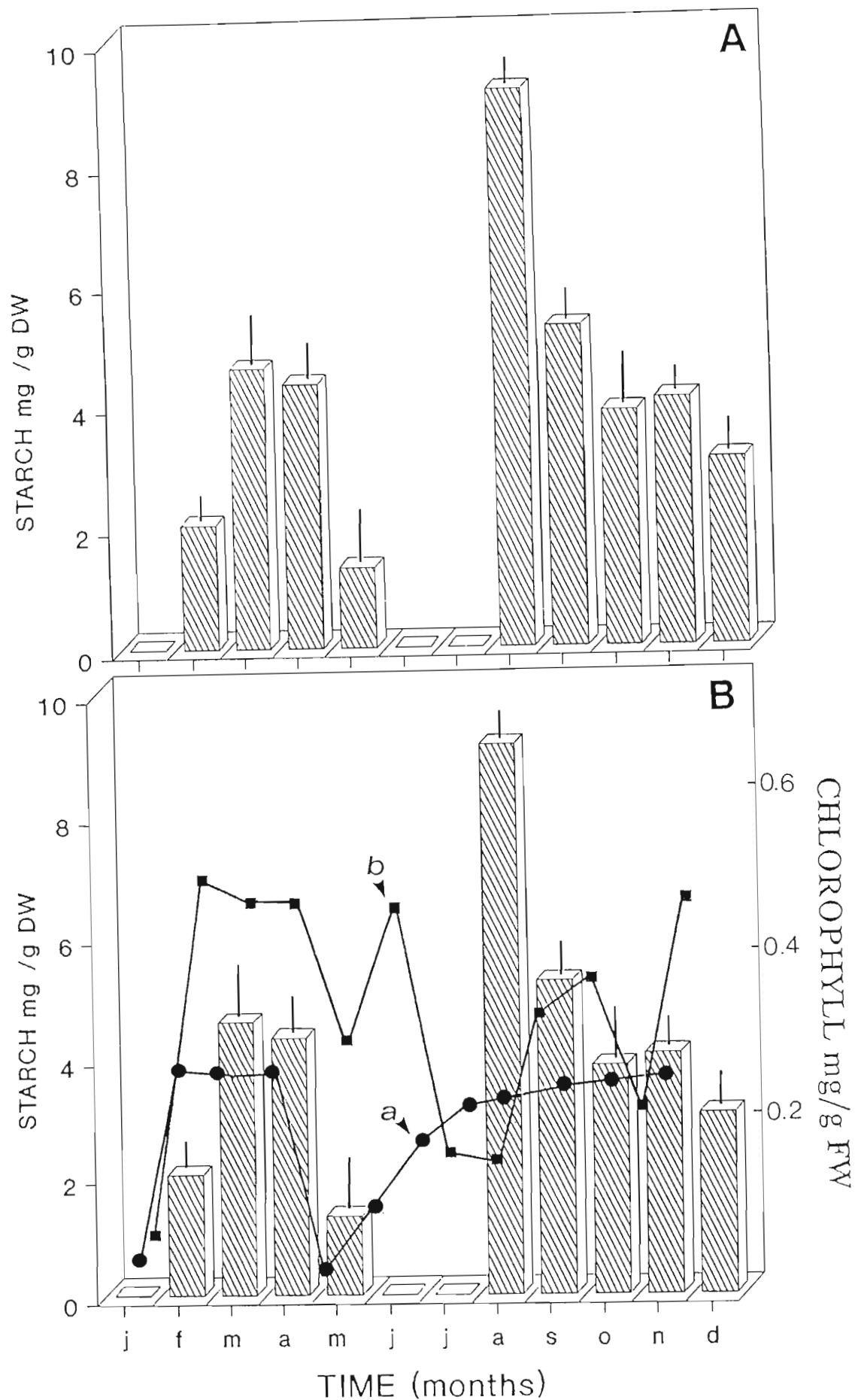


Figure 41. The level of starch from guayule leaves of the 1989 harvest (A). Levels in the stems and roots were negligible. The amount of chlorophyll was superimposed on the amount of starch extracted from the leaves (B). Bars represent the standard error.

Fructans were extracted from the leaves, stems, roots and cortex and pith of the crown (**Figure 43**). At the same time the potential of the enzymes of the above plant components to incorporate acetate into resin and rubber during flowering, budbreak and Spring growth was established. Most radioactivity was incorporated into the resin and rubber in the pith at the end of Winter (**Figure 42AB**). The pith of the crown, followed by the roots appeared to be the major storage area for fructans (**Figure 43A,44**). During the 1988/1989 harvests the crown was not analyzed separately and was included with the roots (**Figure 44**). Starch was measured in the leaves, stems and roots although starch was not measured in the mature stem and roots during the 1988/1989 harvests (**Figure 43B**).

Temperatures and light intensity were high during the three hour $^{14}\text{CO}_2$ experiment. The carbon dioxide was preferentially incorporated into the ethanolic fraction of the leaves and stems (**Figure 45A**). Levels of radioactivity incorporated into rubber were low, as indicated by the low recovery in the petroleum ether fraction (**Figure 46A**). This result may indicate that three hours was too short a time period for the incorporation of the isotope into rubber, or that conditions for either incorporation or translocation were limiting. It was noticeable that after the 12 hour experiment little radioactivity was found in the ethanolic fraction of the leaves (**Figure 46B**).

Much of the newly fixed carbon was partitioned into ethanol-soluble fructans (**Figure 45A**) and starch (**Figure 45C**) within 3 hours. No radioactivity was incorporated into the water-soluble fructans after 3 or 12 hours (**Figure 45B**). After 12 hours, the radioactivity was incorporated into rubber in all plant components (**Figure 46B**). The endogenous level of ethanol-soluble fructans declined after 12 hours (**Figure 45A**) while the endogenous level of water-soluble fructans increased (**Figure 45B**). It would appear that the water-soluble fructans are utilizing the endogenous ethanol-soluble fructans and not the current photosynthetic pool as a carbon source (**Figure 45A,B**).

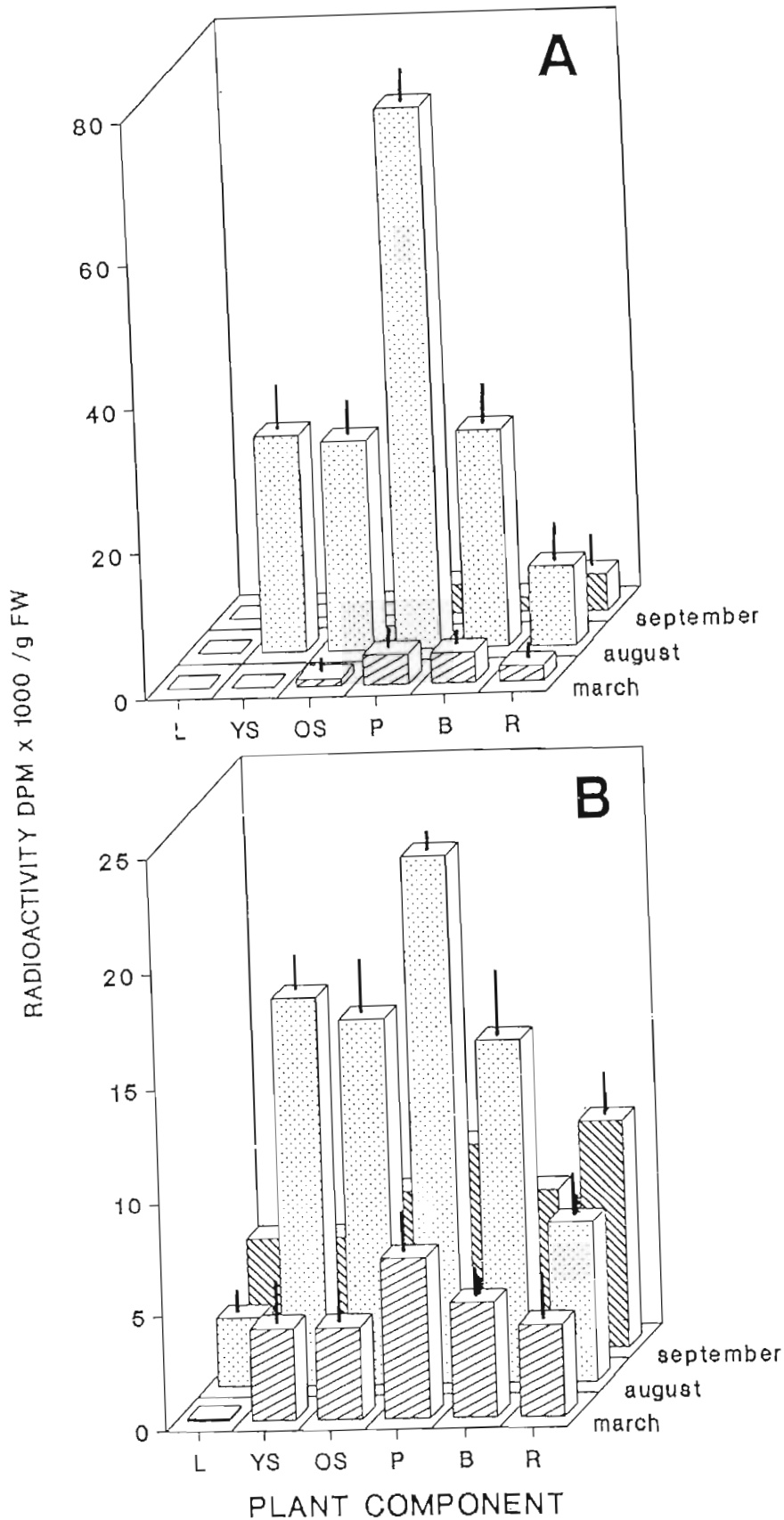


Figure 42.

The potential of the enzymes to synthesize rubber (A) or resin (B) was measured by the incorporation of radioactivity from $[^{14}\text{C}]$ acetate into resin or rubber. This was measured in the leaves (L), young stem (YS), mature stem (OS), pith (P) and cortex (B) of the crown, and roots (R) in March (flowering), August (at budbreak) and in September (Spring). Bars represent the standard

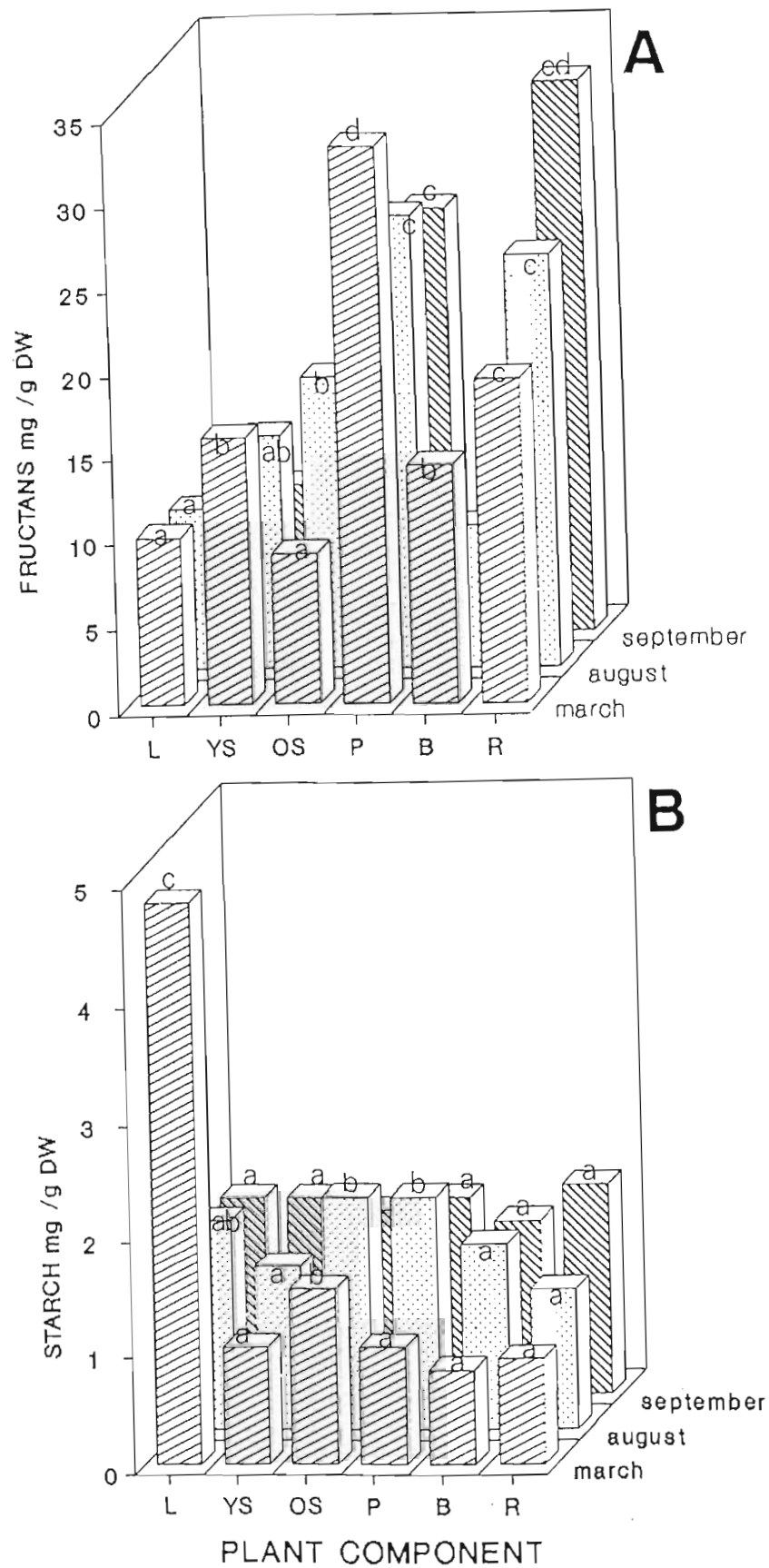


Figure 43.

Fructan (A) and starch (B) were measured in the leaves (L), young stem (YS), mature stem (OS), pith (P) and cortex (B) of the crown, and roots (R) in March (flowering), August (at budbreak) and in September (Spring). A range analysis at the 95 % confidence level was performed on the data and is represented as figures above the

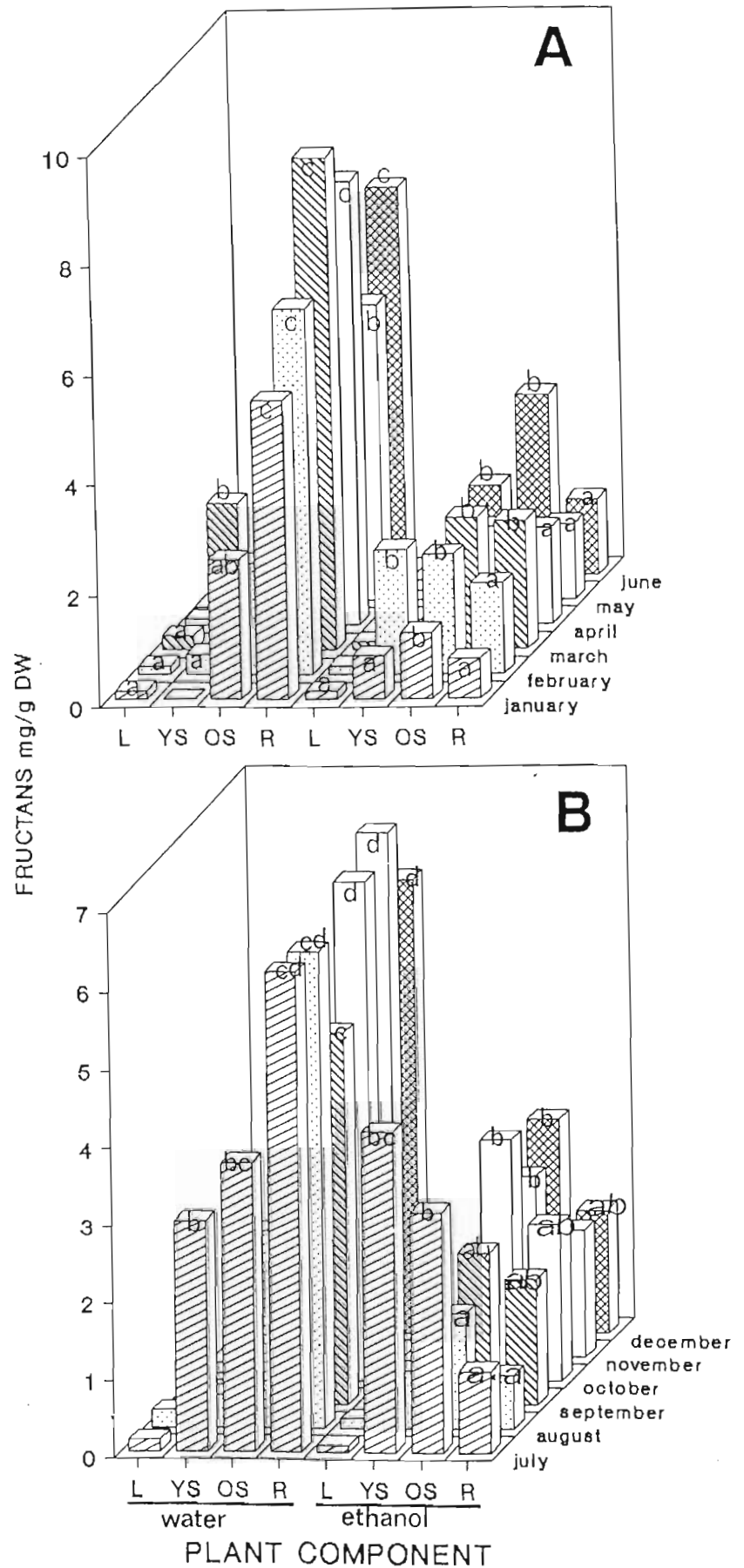


Figure 44.

A comparison of the distribution of water and ethanol-soluble fructans in the leaves (L), Young stem (YS), mature stem (OS) and roots (R) from January to December 1989. A range analysis at the 95 % confidence level was performed on the data and is represented as figures above the columns

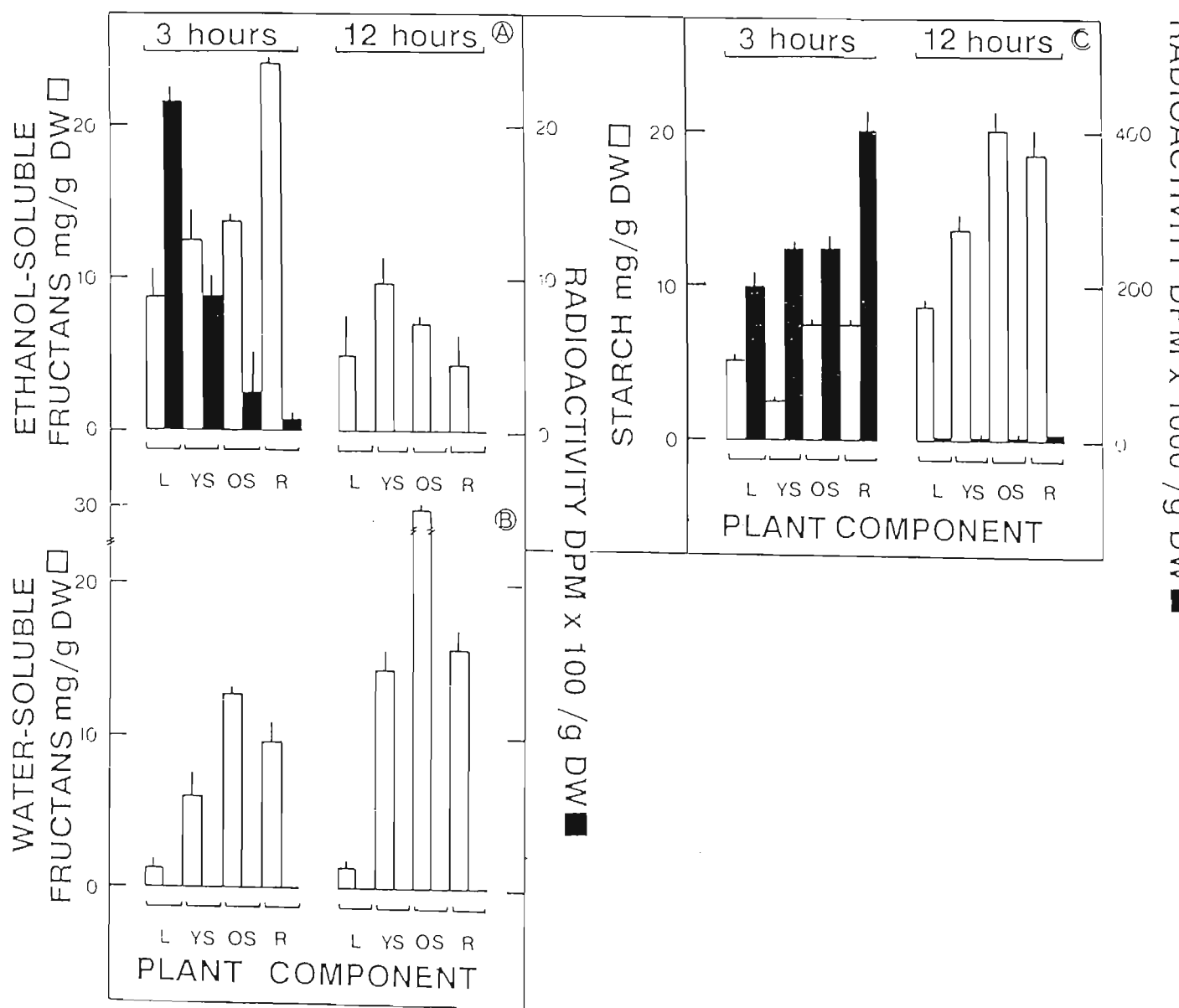


Figure 45. The endogenous ethanol-soluble fructans and the level of ^{14}C -labelled ethanol-soluble fructan (A), the endogenous water-soluble fructans and the level of ^{14}C -labelled water-soluble fructans (B), total starch and the level of ^{14}C -labelled starch (C) in guayule plants which had been exposed to $^{14}\text{CO}_2$ for 3 and 12 hours. Bars represent the standard error.

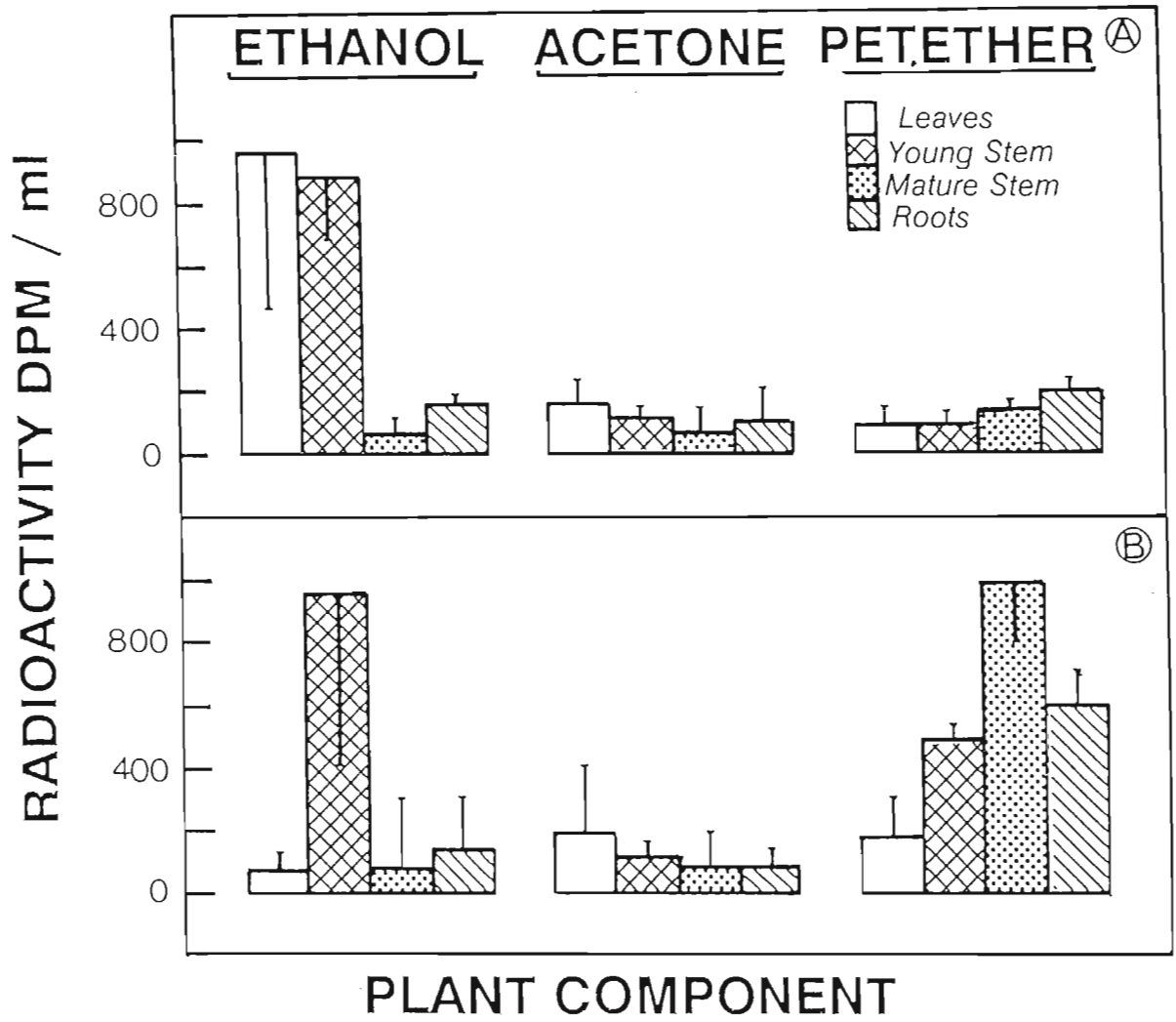


Figure 46.

The level of ^{14}C in the ethanolic, acetone and petroleum ether fractions of leaves, stems and roots following exposure of the plants to $^{14}\text{CO}_2$ for 3 (A) and 12 (B) hours. Bars represent the standard error.

As fructans appear to be stored in the roots of guayule, a regression analysis was carried out to determine the relationship between the level of fructans, total free fructose and temperature.

The results indicated that 88.53 % of the variability in root fructan levels was due the level of total fructose in the young stems, mature stems and roots, and the maximum and minimum temperatures. The significant contribution came from the total fructose fraction of the roots (86.39%). Therefore the effect of temperature, and free fructose in the stems is minimal.

When the variability in total fructose levels in the roots was analyzed, the variation was significantly influenced by fructans in the roots (86.39%). The level of free fructose in the young and mature stems was only responsible for 8.9% of the variability.

Starch levels on the other hand appear to be influenced by temperature. Variability was found to be due to growth and temperature (48.12%). Fructans, total fructose, growth and temperature accounted for 52.29% of the variability. Starch levels are being influenced by other factors.

4.4 DISCUSSION

Storage of photosynthates between the source and sinks provides an important control in the partitioning of carbon in plants. It was originally thought that mobilization of these reserves occurred when current photosynthates were low or when the demand for photosynthates increased (WARDLAW,1990). However, the variable forms of carbohydrate stored, the different degrees of storage, sites of storage and functions of these carbohydrates indicate that the reserve carbohydrates play a major role in the carbon economy of the plant.

Guayule stores fructans and starch. Starch is thought to be responsible for ensuring that short-term diurnal changes in carbohydrate availability are minimal. The simultaneous increase in chlorophyll and starch in Summer could be due to the production of assimilation starch, while in March reserve starch was synthesized. The occurrence of starch grains in Autumn, reinforces the role of the plastids as amyloplasts in the Autumn and chloroplasts in the Summer. The ratio of chlorophyll a to chlorophyll b changes throughout the year. In Winter, the ratio of chlorophyll a to chlorophyll b is high, indicating that photosystem I is operative (BOARDMAN,1971). This is followed by the restoration of chloroplasts when the ratio of chlorophyll a to chlorophyll b is low and photosystem II is functional and the activity of both photosystems is restored (SENDER, SCHÖTZ AND BECK, 1975).

Fructans function to alleviate differences due to environmental/ seasonal factors (WAGNER, WIEMKEN AND MATILE, 1986). Fructans are the dominant storage carbohydrate in guayule. Low levels of fructans occur in the leaves, the site of starch synthesis. The synthesis and storage of fructans in heterotrophic sinks like the stems and roots relies on imported carbon and is less affected by short term environmental fluctuations (POLLOCK AND CHATTERTON, 1988). The synthesis of fructans is unaffected by colder temperatures while starch is resynthesized once the temperatures increase and new leaves are formed.

In guayule, these two reserve carbohydrates fulfil different roles, starch is responsible for immediate sucrose storage as shown by the radiochemical experiment, while fructans are reserve carbohydrates. The storage of starch in chloroplasts of the leaves confines the role of starch to immediate carbon metabolism. The fructans are synthesized on the tonoplast and stored in the vacuole of parenchyma cells allowing fructan a greater distribution and therefore a role in immediate cellular metabolism in all tissues.

Fructans have an important role as the source of assimilates for new Spring growth, with growing buds acting as sinks for reserves (WARDLAW, 1990). Free

fructose increases in the young stems prior to budbreak, and in the leaves after budbreak, suggesting that fructans provide a readily available source of carbon especially important when the photosynthetic apparatus is undeveloped. Metabolic activity at the end of Winter, Autumn and early Spring occurs at a time when small temperature shifts can have large effects on sink demand and readily accessible reserves will be an advantage (WARDLAW,1990). As growth is more sensitive to temperature changes than photosynthesis, photosynthesis would continue through Winter (POLLOCK,LLOYD,STODDART AND THOMAS,1983).

The synthesis of fructans is limited by the plants capacity to produce photosynthate in excess of that required for growth (POLLOCK,1984b). Increases in fructan levels will therefore be indicative of excess photosynthate, and low levels of fructan will occur when other processes require carbon or when photosynthesis is limited. Thus fructans (both ethanol and water-soluble) decreased in all plant components during bud growth and new leaf development in August and September. The decrease observed in May occurred during defoliation at a time when the plant was entering dormancy and the ratio of carbon utilisation to production decreased. Theoretically, defoliation should provide excess carbon thereby inducing fructan synthesis and the increased translocation of assimilates from leaves (SUZUKI,1971).

Once rubber production begins, carbon is required for the synthesis of IPP the precursor of *cis*-polyisoprene. Fructans may be providing carbohydrates for this process as the amount of free fructose increases. However, the hypothesis that excess substrate induces the rubber transferase gene (GILLILAND AND VAN STADEN,1990), cannot be substantiated as both free and reserve carbohydrates are low prior to Winter. Rubber synthesis is not a device for consuming excess photosynthate (GILLILAND AND VAN STADEN,1990) as fructan levels increased during June and July indicative of excess substrate.

Both rubber and fructan synthesis occur simultaneously in the Winter, suggesting competition for the same substrate or utilization of different carbon

sources. The ultrastructural location of the two pathways provides a solution for substrate utilization: fructans are probably stored in the pith parenchyma vacuoles while rubber occurs in the vacuoles of the cortical cells. Secondly, rubber synthesis is using the currently photosynthesized sucrose pool while the storage sucrose pool is converted to fructans. During growth, fructans are utilized as an additional carbon source, indicative of the demand for carbon at a time when there is insufficient carbon to maintain rubber production.

In August and September, despite the introduction of growth, rubber levels increase. By November, when maximum growth is observed, rubber synthesis is completed. The reappearance of starch coincides with the production of new leaves which would increase the amount of carbon available for the plant. Rubber synthesis may use this source to continue production. Once starch synthesis is re-established as a major carbon-consuming process in the leaves and young stem, fructan levels remain low until the period of defoliation in the new year.

The depolymerization of fructans does not occur when the temperatures are low. Instead, depolymerization of the ethanol-soluble fructans is accompanied by polymerization of the water-soluble fructans during Autumn. At no time are the polymers completely depolymerized to sucrose, fructose and glucose. The presence of high levels of fructose can be used as an indication of depolymerization. Large amounts of free glucose rarely accumulate during fructan synthesis, glucose is probably phosphorylated and resynthesized into sucrose (POLLOCK AND CHATTERTON, 1988).

In the young stem, sucrose and the trisaccharide (presumably isokestose), are present with the polymer length increasing to five. The level of free sugars in this region is low making it difficult to assess whether this is the region of fructan synthesis with translocation of the smaller trisaccharides to the mature stem and roots where the larger polymers are stored.

The tonoplast and vacuole have been implicated as the site of fructan synthesis and storage (WAGNER, KELLER AND WIEMKEN, 1983). GOSS, FOSTER AND BENEDICT (1986) suggested that in non-rubber producing cells, the tissue progresses towards the univacuolate state which would provide a great deal of storage space for fructans. Storage in the vacuole would minimize feedback inhibition associated with sucrose synthesis.

The seasonality exhibited by guayule is not unusual as temperate plants have an annual rhythm associated with the seasons, while tropical plants like *Hevea* base their activity on the availability of water. *Helianthus tuberosus* L. accumulates fructans in the Autumn followed by depolymerization upon exposure to low temperatures. Sprouting of the tubers then causes extensive mobilization of fructan and resynthesis of sucrose (EDELMAN AND JEFFORD, 1968). In temperate grasses, maximum levels of high molecular weight are measured in Winter followed by large scale mobilization prior to rapid Spring growth (POLLOCK AND JONES, 1979). In guayule there is depolymerization of ethanol-soluble fructans and polymerization of water-soluble fructans during the Winter months. From August (budbreak) depolymerization occurs in both fractions providing the plant with extra carbon. Depolymerization requires hydrolase and transferase activity which causes the fructose residues to be redistributed (BACON AND LOXLEY, 1952).

Guayule accumulates fructans in the crown region of the stem and in the roots. Fructans also accumulate in the roots of *Taraxacum officinale* (RUTHERFORD AND DEACON, 1972a) and *Cichorium intybus* (BHATIA, MANN AND SINGH, 1974) and tubers of *Helianthus tuberosus* (BACON AND LOXLEY, 1952).

4.5 CONCLUSION

Fructan synthesis retains an independence from starch synthesis and can be seen as an extension of the sucrose pool which has the advantage of being independent from starch. Unlike starch, fructan synthesis and degradation requires no UDP-glucose or other phosphorylated sugar (DARBYSHIRE AND HENRY,1978).

The enzymes involved in fructan synthesis are not inhibited by low temperatures (POLLOCK AND LLOYD,1987) whereas those associated with starch synthesis are (SAKAI AND YOSHIDA,1968). The accumulation of fructan may enhance the performance of the photosynthetic carbon reduction cycle at low temperatures by recycling inorganic phosphate during the export and the storage of carbon (POLLOCK AND LLOYD,1987).

The mature stem appears to be the area associated with metabolic activity as it has the highest level of 80% ethanol-soluble fructans with intermediate polymer length throughout the year. If resin production is greatest in this area and fructose is utilized primarily as the precursor for resin synthesis then free fructose and glucose could be provided by the ethanol-soluble fructans. This fructose and glucose would then be able to move into the plastids of the resin canal epithelial cells and be utilized as substrate for *trans*-terpene synthesis. In guayule the pith region of the crown of the mature stem and roots act as the site of storage for the fructans.

The excess carbon supplied during the radioisotope experiment was initially incorporated into starch and ethanol-soluble fructans. In the long term (12 hours), incorporation of the radioactivity into rubber increased, while the ethanol-soluble fructans decreased, suggesting utilization of the same carbon source. However, there was a concurrent increase in the endogenous water-soluble fructans. The ethanol-soluble fructans are probably acting as the carbon source for the water-soluble fructans rather than rubber, as the level of

ethanol-soluble fructans increased during the time of rubber production (June/July). If the ethanol-soluble fructans were acting as the carbon source for rubber synthesis then the level of these fructans would be low in winter. Rubber must be utilizing the current translocated sucrose and is independent of fructan synthesis although carbohydrate and rubber synthesis cannot be regarded as separate mechanisms (BONNER AND GALSTON,1947).

During the Winter, the amount of ethanol-soluble fructans increased and the polymer length decreased. The cells contained many more molecules of a shorter length which must alter the cell solute concentration and maintain cell turgor during these times of stress. This depolymerization would have implications for the partitioning of carbon to the site of rubber and fructan synthesis if the allocation of carbon is favoured by turgor-related membrane transport (DAIE,1988). The altering of cell turgor could provide a signal for additional assimilate transport.

Fructans function as an additional carbon source during growth as there is a decrease in both water and ethanol-soluble fructans in August in the young stems. The ethanol-soluble fructans act as the immediately available source of carbon for growth, as fructan molecules are able to undergo continuous rapid modifications in structure. Upon depletion of this source, water-soluble fructans in the roots undergo depolymerization in September/ October thus replenishing the carbon source for growth and flowering.

Depolymerization of fructans occurs when the carbon supply is low while polymerization occurs when there is excess sucrose. The occurrence of polymerization in November to February in the roots and November/ December in the mature stem would suggest that sucrose is being produced in excess of that required by the plant. This takes place despite the loss of carbon from the plants via the leaves in the Summer (Chapter Two). Carbon loss in Summer, does not therefore disadvantage the plant with respect to its carbon economy.

Growth and flowering are seen as priorities in carbon resource allocation. The high levels of starch observed in the first year of experimentation, in all plant components, occurred during floods in Pietermaritzburg when flowering was very poor. Starch was therefore thought to provide carbon for flowering (KELLY AND VAN STADEN, 1991), however it appears that the fructans provide carbon for growth and flowering. This will be discussed in Chapter Five. Rubber synthesis relies on current photosynthesis for carbon which means that the continuation of rubber synthesis in August and September would remove the current photosynthates from the carbon pool. This indicates a vital role for fructans in supporting new growth and flowering.

CHAPTER 5

MANIPULATION OF GROWTH IN GUAYULE PLANTS

5.1 REPRODUCTIVE BIOLOGY

5.1.1 INTRODUCTION

Reproduction can have profound physiological effects on plant growth and development. Flowering in any plant requires the allocation of carbon to the reproductive sink for inflorescence development (HENDRIX,LINDEN,SMITH,ROSS AND PARK,1986). Developing fruits and seeds are the among the strongest of sinks in plants, while developing flower initials are the weakest (DAIE,1985). A stimulus is usually perceived in order to achieve relocation of the assimilates. This stimulus usually relies on a change in the duration of the light period and/or daylength (CHARLES-EDWARDS,DOLEY AND RIMMINGTON,1986) or temperature as in vernalization (NAPP-ZINN,1987). The occurrence of short day and long day flowering plants has made daylength a critical factor in the induction of flowering. Temperature, on the other hand, provides both information, and is an essential condition for growth.

The length of the photoperiod is a function of the latitude of the experimental site and the time of the year. Tropical plants experience very little seasonal variation as daylengths remain at about twelve hours (SALISBURY,1981). Photoperiodic responses are not limited to flowering but also effect stem elongation, leaf growth, dormancy and leaf abscission (SALISBURY,1981).

5.1.2 FLOWERING IN GUAYULE

In guayule it is thought that flowering occurs during active growth and not as a response to temperature or seasonality (LLOYD,1911; HAMMOND AND

POLHAMOUS,1965). Flowering occurs after rainfall, with large inflorescences forming in times of abundant rainfall and short pedicels with masses of crowded capitula when rains are infrequent (POLHAMOUS,1962).

The flowering response is thought to be under photoperiodic control induced by long days (WHITEHEAD AND MITCHELL,1943), although flowering has never been characterized for this plant. Recently, BACKHAUS,HIGGINS AND DIERIG (1989) established that guayule required a critical daylength of between 9.5 and 11 hours to induce flowering. Rapid flowering occurred when days were 20 hours long. This decreased as daylengths approached 11 hours. With exposure to the correct daylength of as few as three 20 hour photocycles, flowering was achieved although 100% flowering occurred after 10 photocycles (BACKHAUS, HIGGINS AND DIERIG,1989).

Initial flowering in guayule is characterized by a single inflorescence. After branching begins, the number of flowers increase because each branch terminates in an inflorescence. The flowering stalk is a compound cyme with the main peduncle and secondary peduncles bearing heads about one centimetre in width. Each head has five ray florets around the outside that produce seed and numerous disc florets in the centre that produce pollen. The growth habit results in a multi-branched plant with an upper layer of leaves surmounted by the inflorescences on long peduncles.

In the 1988 carbohydrate experiment described in Chapter Four, the fructan levels were low during flowering (**Figure 40**). Flowering in September was accompanied by a decrease in the rubber producing potential of the stem tissue (**Figures 6, 10**). Chapter Four showed that fructan and rubber synthesis appear to be utilizing different sucrose pools.

It was originally proposed by KELLY AND VAN STADEN (1991) that starch was providing carbon for flowering. However, later experiments showed that fructans were utilised at the time of renewed growth and never reached high

levels during flowering. Therefore it was decided to extend the period of growth and flowering artificially and thereby establish whether starch and/or fructans were utilized as the carbon source for flowering, thus reducing the demand on current photosynthesis.

To do this it was necessary to establish

- i) whether growth and flowering can be prolonged or stimulated by removing plants from the ambient conditions and exposing the plants to long days and increased temperatures and
- ii) what effect these manipulations had on the level of fructans, free fructose and starch.

5.2 MATERIALS AND METHODS

5.2.1 Control Plants

The two-year-old W10 guayule plants which were harvested on a monthly basis from January to December for the 1989 harvest in Section 4.2.9 were used as the control plants for this experiment.

5.2.2 Greenhouse Experiment

At each monthly harvest in the garden, thirty plants grown in pots, were randomly selected from the experimental site and moved to the greenhouse. The maximum and minimum temperatures were recorded on a daily basis (Figure 47). Plants received natural radiation during the day. A 12 hour photoperiod (long days) was maintained artificially using Tungsten-Halogen lights from April to September at 80% of the ambient. Light intensity ranged from $1920 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 09 00 hours in the morning to $2300 \mu\text{mol m}^{-2} \text{s}^{-1}$ at midday, and $140 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 17 00 hours in the afternoon. This altered on cloudy days. Light intensity then remained at about $850 \mu\text{mol m}^{-2} \text{s}^{-1}$ for the entire day.

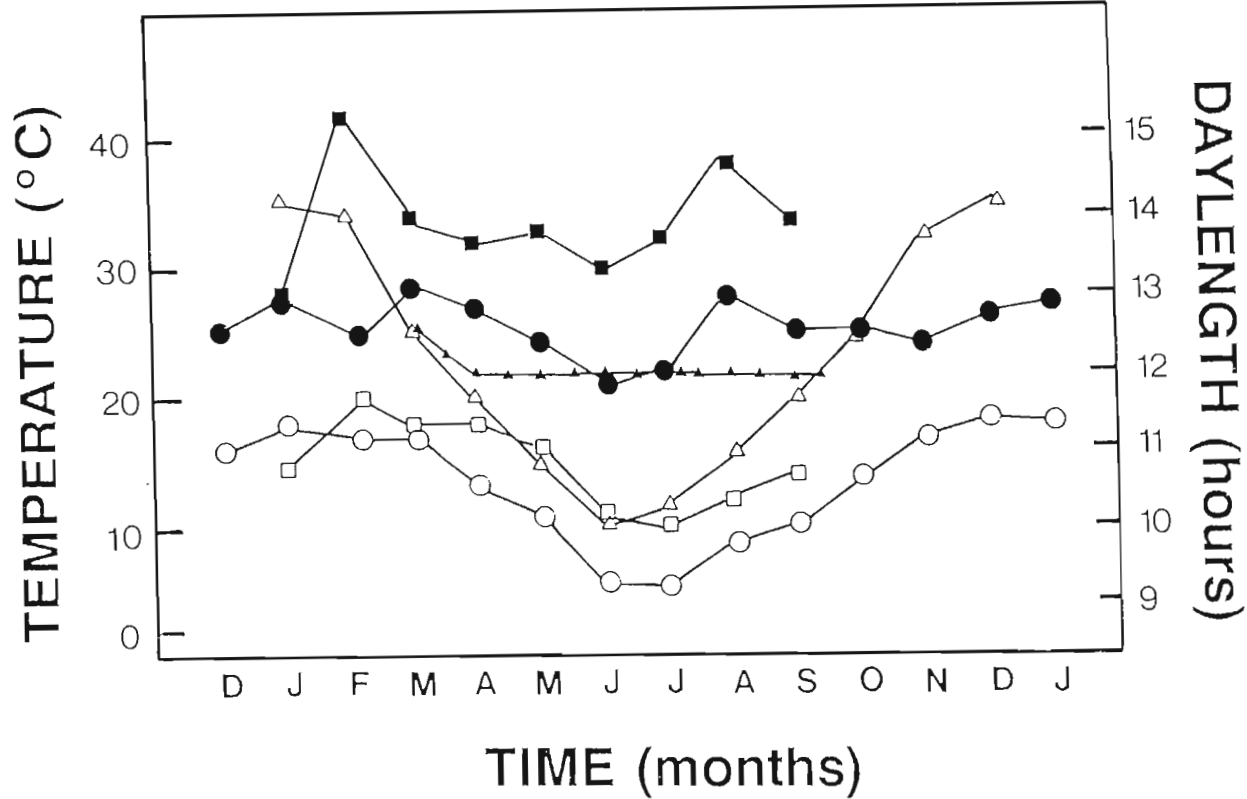


Figure 47. The maximum and minimum temperatures recorded from December 1988 to January 1990 in the garden (●) and phytotron (■). The natural daylength (△) was supplemented in the phytotron (▲).

Once a month, five plants were harvested from each thirty plant block. In an attempt to keep the number of plants harvested to five, a problem arose with total number of plants in the greenhouse. Thus there were months when harvesting was impossible and this was represented by gaps in the graphs. The experiment was terminated after the first flowers appeared in September 1989. Upon harvesting, the fresh mass of the leaves, stems and roots was recorded. These plant components were then frozen in liquid nitrogen and lyophilized on a freeze drier to ensure that no further metabolic activity took place (HENDRIX AND PEELIN,1987). Plant components were weighed to determine dry mass and then ground to homogeneous powders on a Wiley Mill and stored at -10°C, until required. The levels of fructans, free fructose, starch and free sugars were determined as described in the routine analysis in Chapter Four.

5.3 RESULTS

All the guayule plants which were growing in pots, were flowering when the experiment began in January (**Table 2A**). Thirty per cent flowering was observed in the plants in pots. From March until September no flowering occurred. However, plants continued to lose leaves until May when only terminal clusters of leaves and dead flowering stalks remained.

Plants moved to the greenhouse in January, when the maximum temperature was 36°C and minimum temperature 16°C (**Figure 47**), flowered until July although the size of the flowering head was small and there were few capitula per plant (**Figure 48A**). Plants moved in February stopped flowering in March and flowered again in September (**Table 2A, Figure 48B**). Plants were not flowering in the field in March and did not flower when moved to the greenhouse until September (**Figure 48C**). A similar trend was observed for the plants which were moved to the greenhouse in April, May, June and July (**Table 2A, Figure 49A,B,C**).

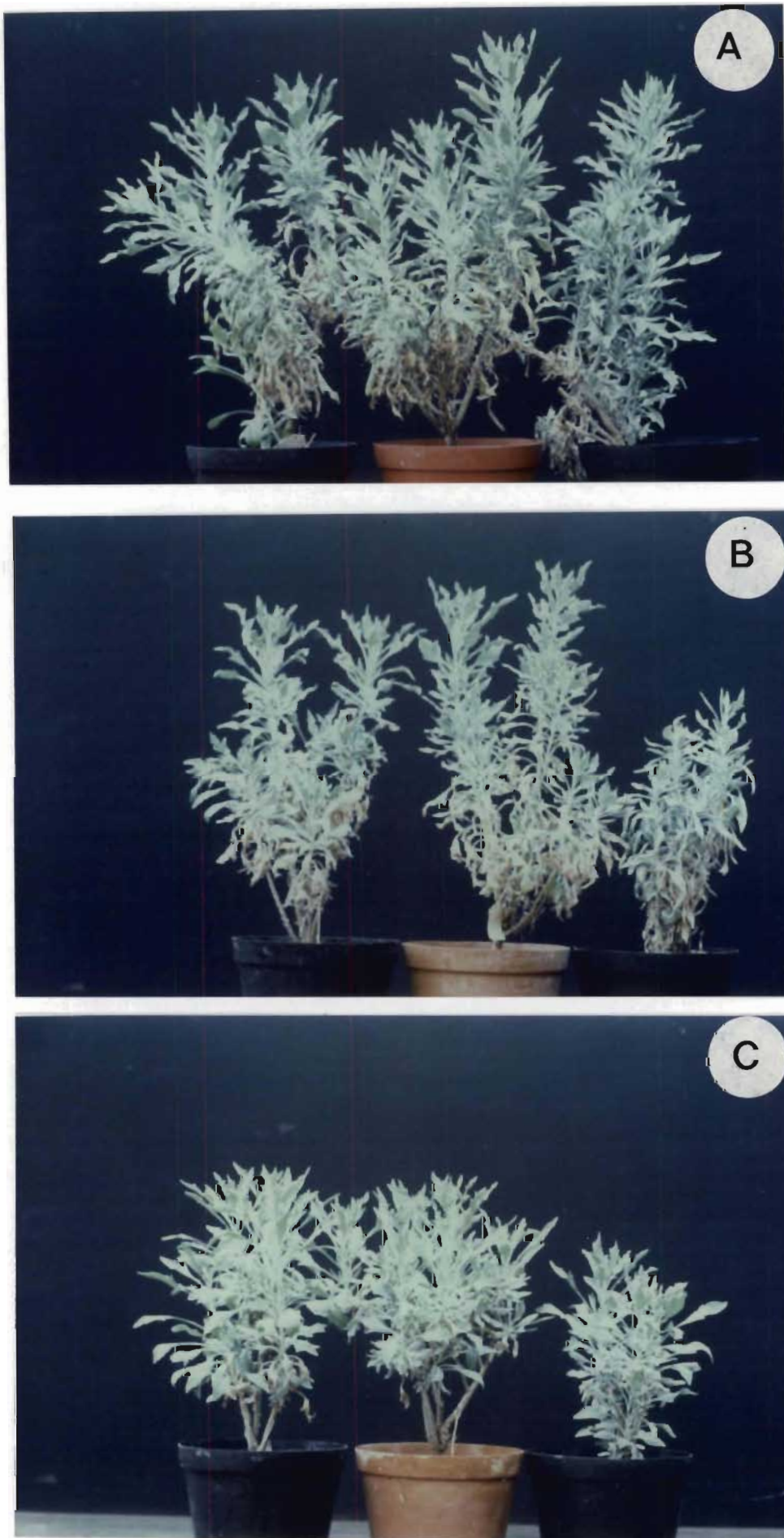


Figure 48.

The morphology of plants which were moved to the greenhouse in January (A), February (B) and March (C) at the end of the experiment.

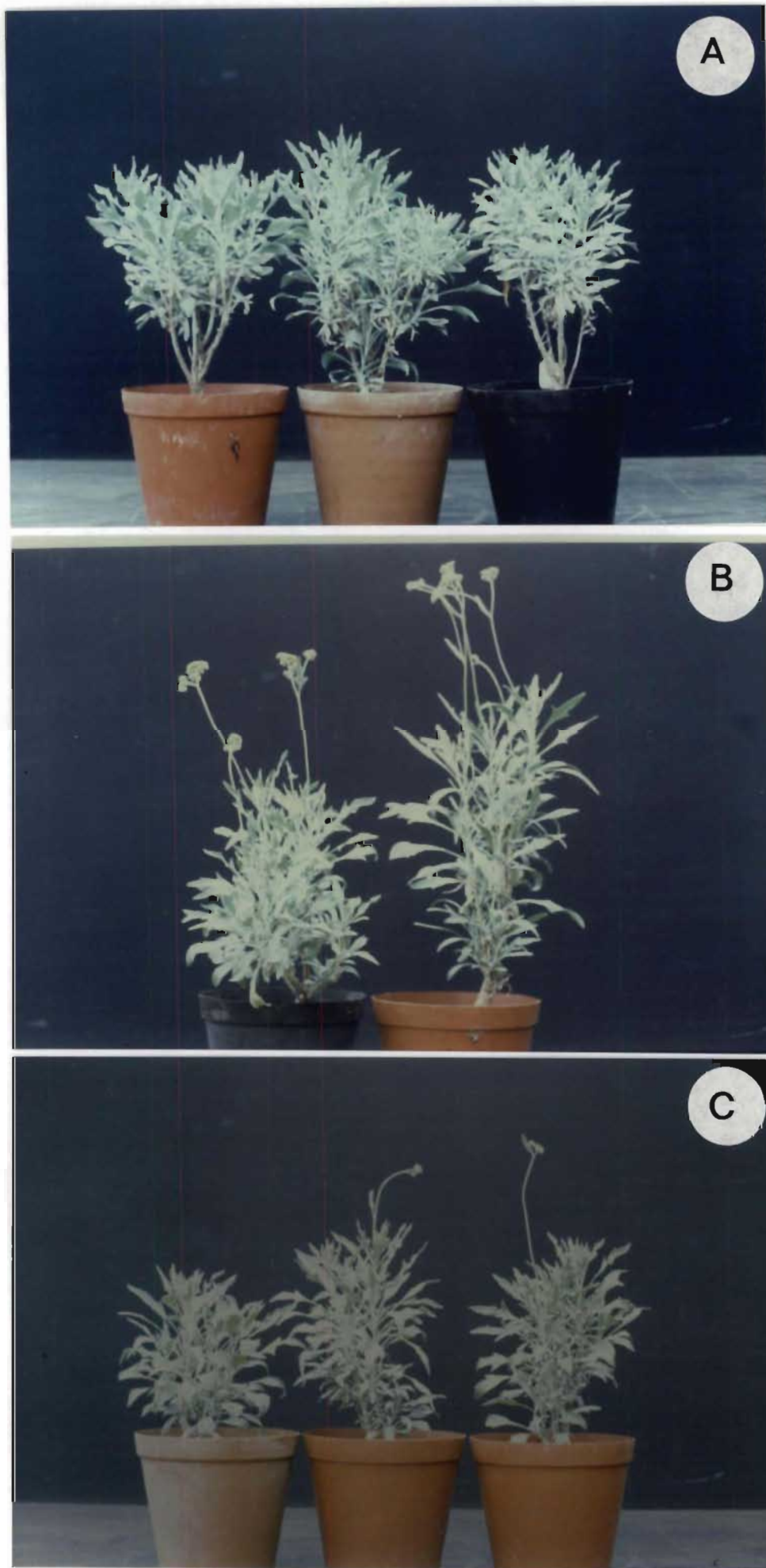


Figure 49.

The morphology of plants which were moved to the greenhouse in April (A), May (B) and June (C) is shown.

Table 2A: The time of flowering (F) or lack of flowering (NF), of guayule plants moved to the greenhouse from January to July (first column) was compared with that of plants grown in the field (FD) or in pots (POT).

	J	F	M	A	M	J	J	A	S	O	N	D
FD	F	F	F	NF	NF	NF	NF	NF	F	F	F	F
POT	F	30	NF	NF	NF	NF	NF	NF	30	F	F	F
JAN	F	F	F	F	F	F	NF	NF	F			
FEB		30	NF	NF	NF	NF	NF	NF	F			
MAR			NF	NF	NF	NF	NF	NF	F			
APR				NF	NF	NF	NF	NF	F			
MAY					NF	NF	NF	NF	F			
JUNE						NF	NF	NF	F			
JULY							NF	NF	F			

Table 2B: The growth (NG) of guayule plants was monitored in the plants which were moved into the greenhouse from January to July. This was compared with plants in the field (F) and in pots (POT). The morphological stages represented are dormancy (D) and vegetative budbreak (BB).

	J	F	M	A	M	J	J	A	S	O	N	D
F	NG	NG	NG	D	D	D	D	BB	NG	NG	NG	NG
POT	NG	NG	D	D	D	D	D	BB	NG	NG	NG	NG
JAN	NG	NG	NG	NG	NG	NG	NG	NG	NG			
FEB		NG	NG	NG	NG	NG	NG	NG	NG			
MAR			NG	NG	NG	NG	NG	NG	NG			
APR				D	D	D	D	BB	NG			
MAY					D	D	D	BB	NG			
JUN						D	D	BB	NG			
JUL							D	BB	NG			

Growth continued in the plants which were moved into the greenhouse in January, February and March for the entire experimental period (**Table 2B**). The plants which were moved into the greenhouse in April, May, June and July did not resume growth although these plants were exposed to growth-promoting temperatures and longer daylengths. These plants remained dormant (**Table 2B**). When harvesting these plants, it was noticed that the bark and cortex were easily separated from the pith.

The plants which were moved to the phytotron in January, February or March, did not enter a dormant period. Growth (fresh mass gain) occurred primarily in the leaves and later in the stem and roots of these plants (**Figure 50A,B,C**). There was a continuous carbon investment in growth in these plants as indicated by the dry mass increase (**Figure 51A-H**). Dormancy was broken in all plants on the 21 August in the greenhouse and at the experimental site even though plants in the greenhouse had been exposed to higher temperatures and longer days than those remaining at the experimental site (**Figure 50,51**). The bark and cortex were not easily separated from the pith in plants which were harvested during this time.

The trends identified, with respect to water-soluble and ethanol-soluble fructan accumulation, in the plants moved into the greenhouse in January (**Figure 52A,53A**) were not observed in any other plants which were moved into the greenhouse in later months (**Figure 52B-G,53B-G**). In February, one month after these plants were moved into the greenhouse, a decrease in water-soluble fructans in all plant components was observed (**Figure 52A**). This was followed by an increase four months later which was the same pattern of accumulation observed in the 1988 and 1989 harvests (**Figures 31,32,40**). A similar trend occurred in the plants which were moved into the greenhouse in March (**Figure 52C**).

The level of water-soluble fructans was high in the mature stems and roots in all plants (**Figure 52A-G**), suggesting a possible area of metabolism and

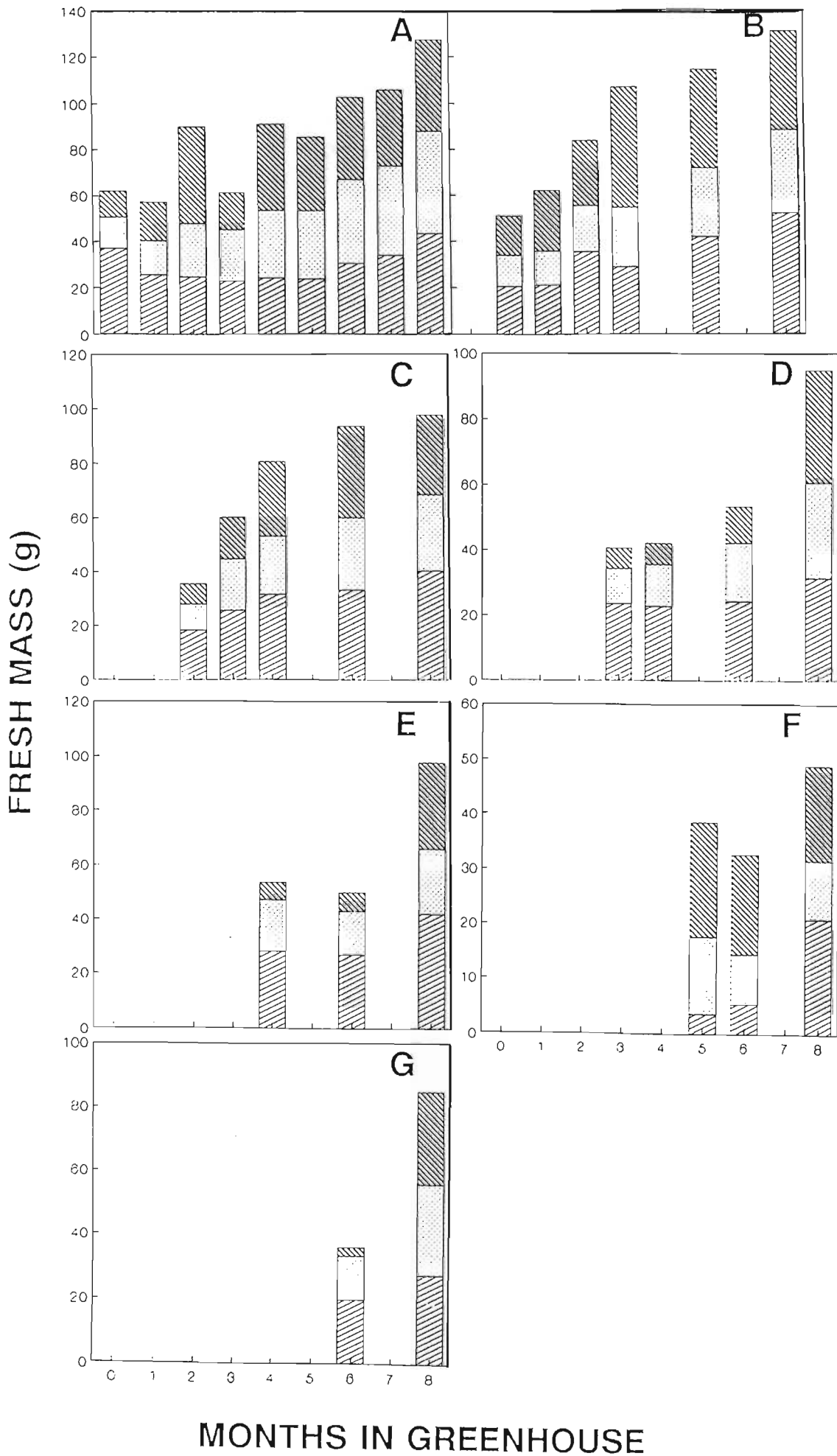


Figure 50.

The growth (fresh mass) of leaves (▨), stems (▤) and roots (▥) after plants were moved to the greenhouse in January (A), February (B), March (C), April (D), May (E), June (F), and July (G).

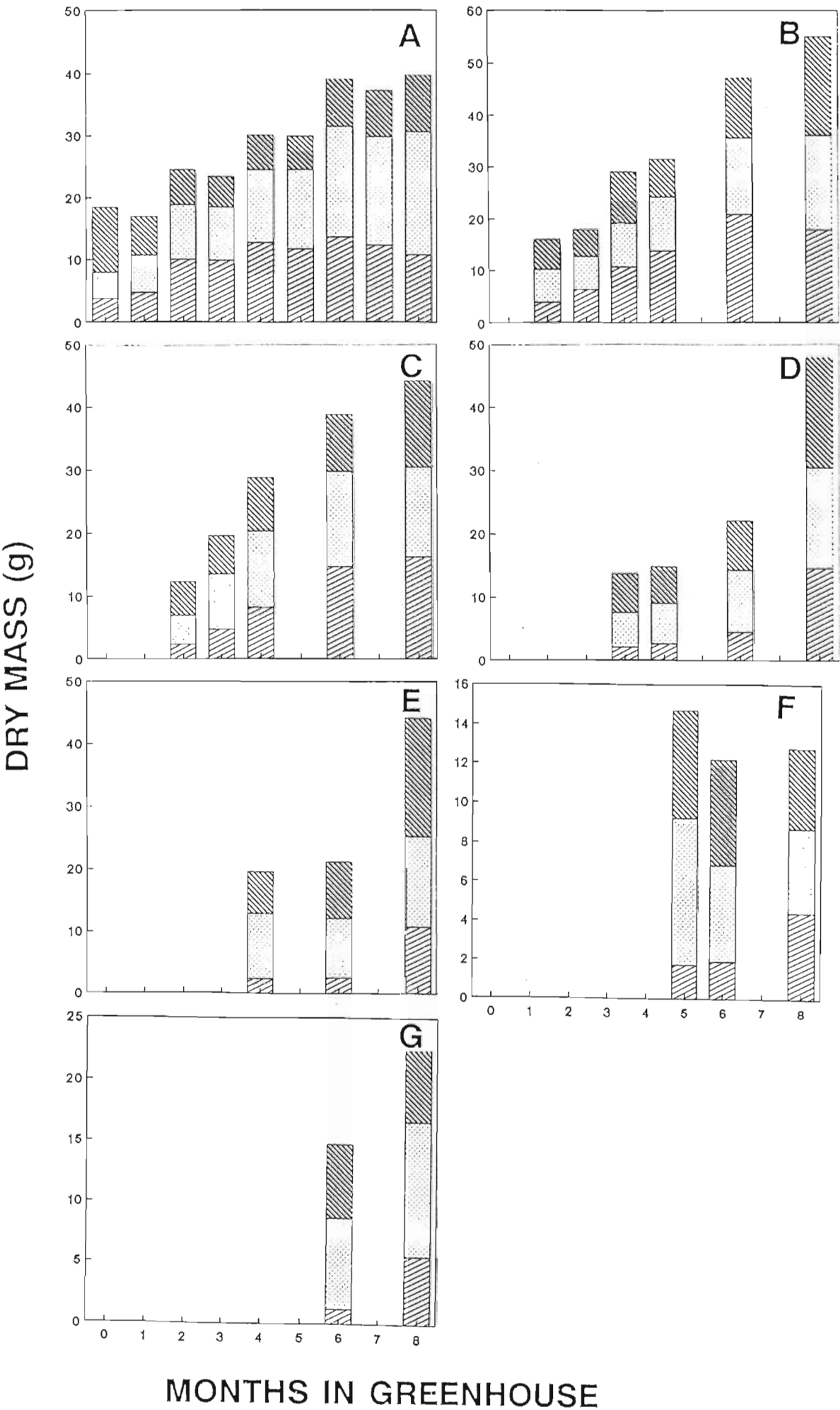
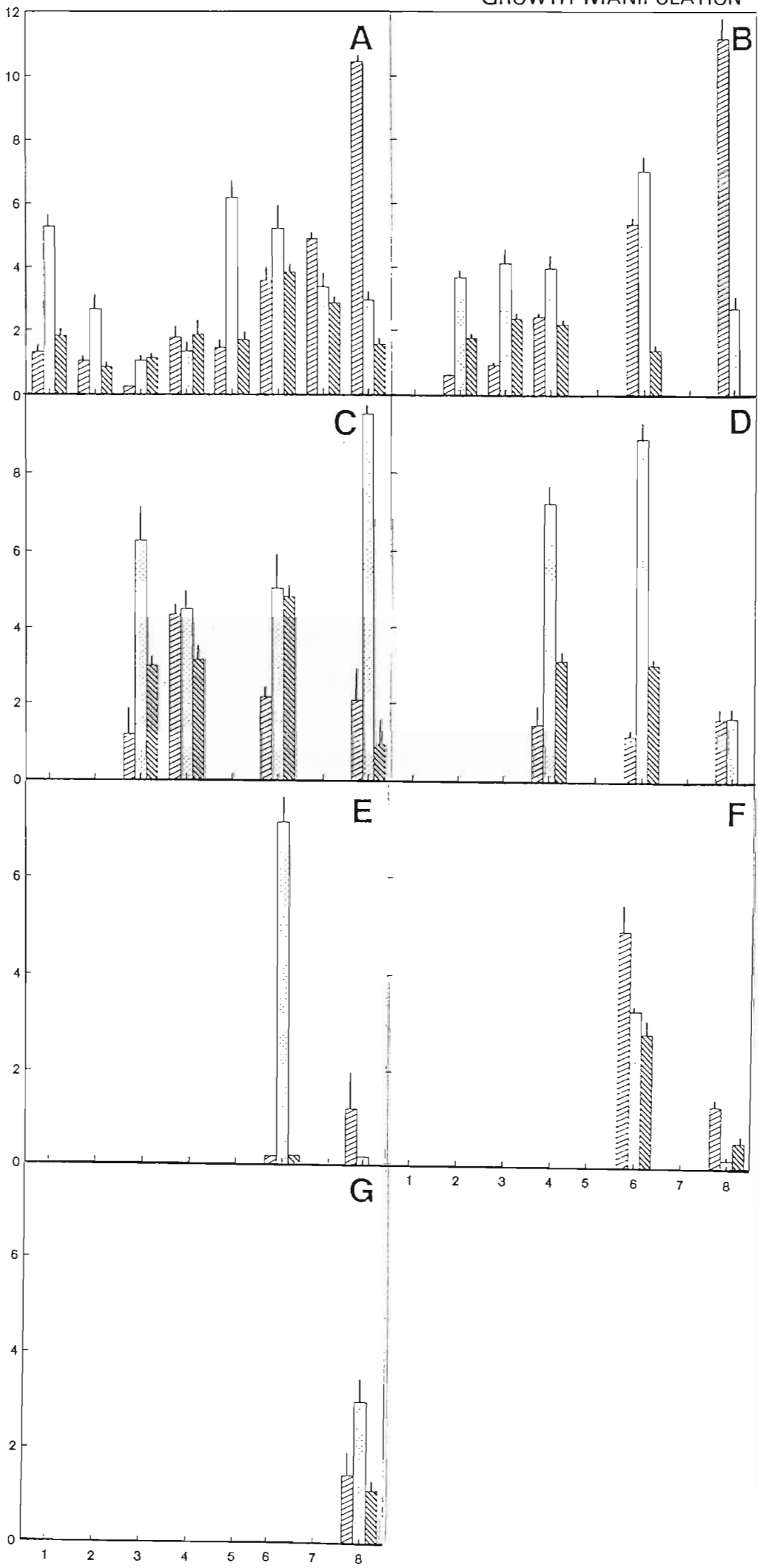
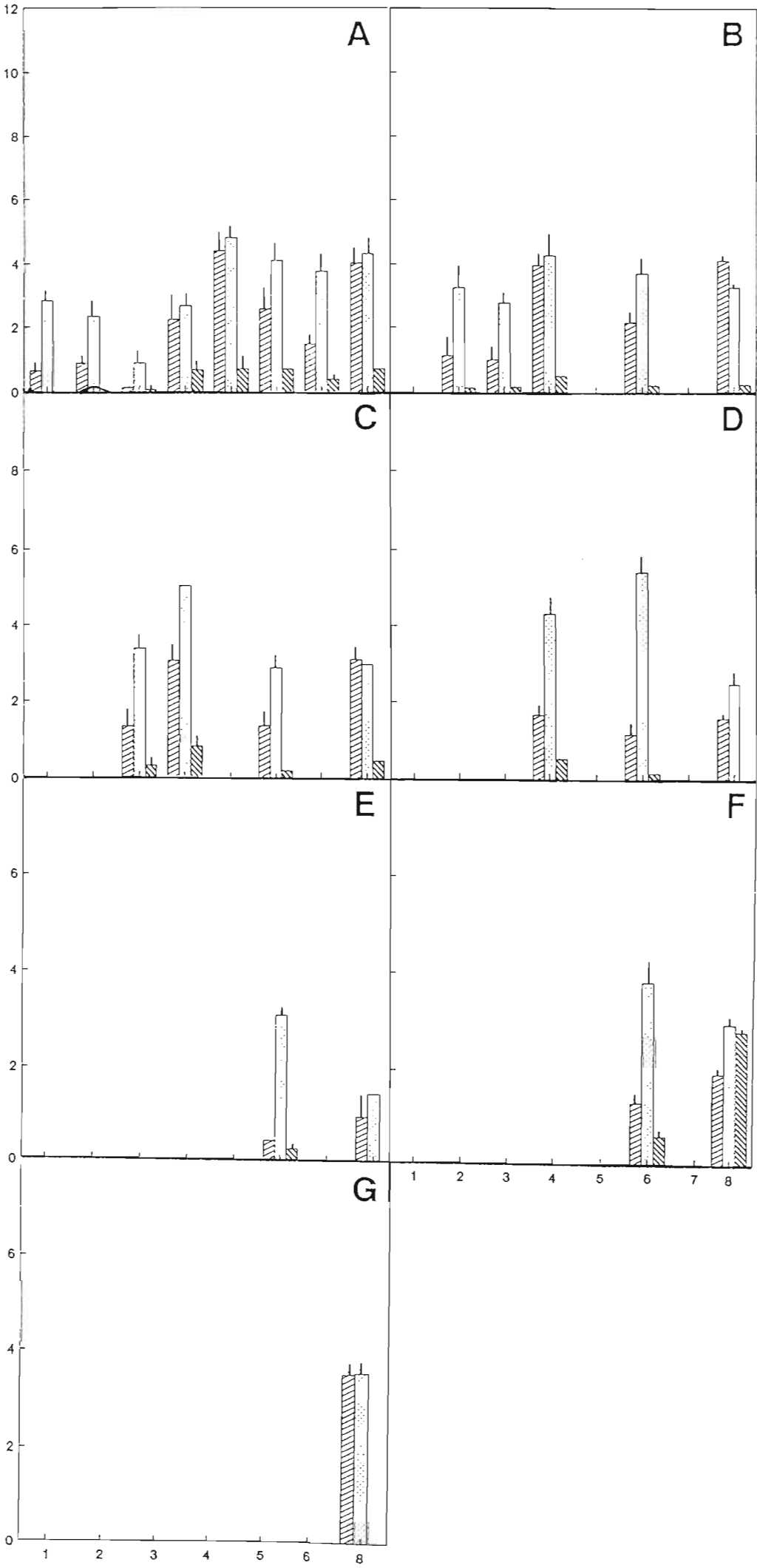


Figure 51. The growth (dry mass) of leaves (▨), stems (▤) and roots (▥) after plants were moved to the greenhouse in January (A), February (B), March (C), April (D), May (E), June (F), and July (G).

WATER-SOLUBLE FRUCTANS mg/g DW



ETHANOL-SOLUBLE FRUCTANS mg/g DW



storage, especially in the dormant plants (**Figure 52D-G**). In all plants, except the January plants there was a significant decrease in the level of water-soluble fructans in all plant components when dormancy was broken (**Figure 52B-G**). This resembled the trend observed for the 1988 (**Figure 40**) and 1989 (**Figure 31**) harvests.

The level of ethanol-soluble fructans extracted from the roots (**Figure 53A-G**), decreased substantially when compared to the water-soluble fructans (**Figure 52A-G**). The mature stem emerged as the principal site of metabolism or storage of the ethanol-soluble fructans (**Figure 53A-G**). This was also observed in the control plants (**Figure 32**).

Total fructose was concentrated in the mature stems of all plants (**Figure 54A-G**) probably due to a major contribution from the ethanol-soluble fructans (**Figure 53**). Once again, a significant decrease in total fructose occurred at budbreak and flowering (**Figure 54A-G**).

A comparison of the free fructose, glucose and sucrose extracted showed that at all times significant levels of fructose were measured prior to flowering (**Table 3**). Once flowering began the level of free fructose decreased (**Table 3**). The level of sucrose and glucose was not significant at any time during the experiment (**Table 3**).

The higher temperatures in the greenhouse affected the level of starch in the leaves (**Figure 55**). The endogenous levels in the plants in pots showed the characteristic increase prior to winter, a decrease during winter and increase at budbreak (**Figure 55A**). Plants moved into the greenhouse in January and February showed similar trends (**Figure 55B,C**). Plants moved into the greenhouse in March produced higher levels of starch for a longer period (**Figure 55D**). Starch increased in plants moved into the greenhouse in April/May after these plants had been in the phytotron a month (**Figure 55E,F**). Starch increased slightly at budbreak in the plants which had been moved into

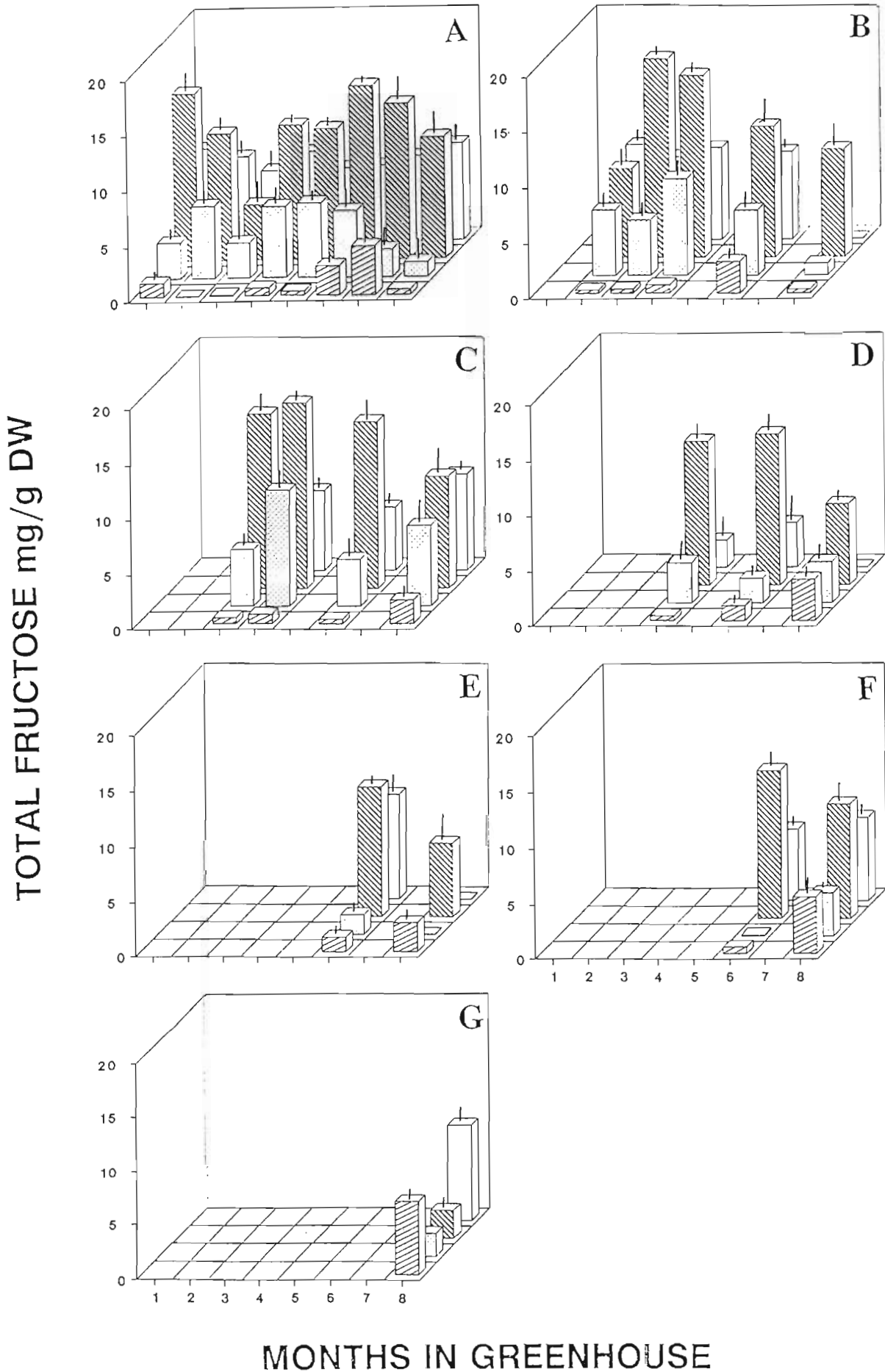


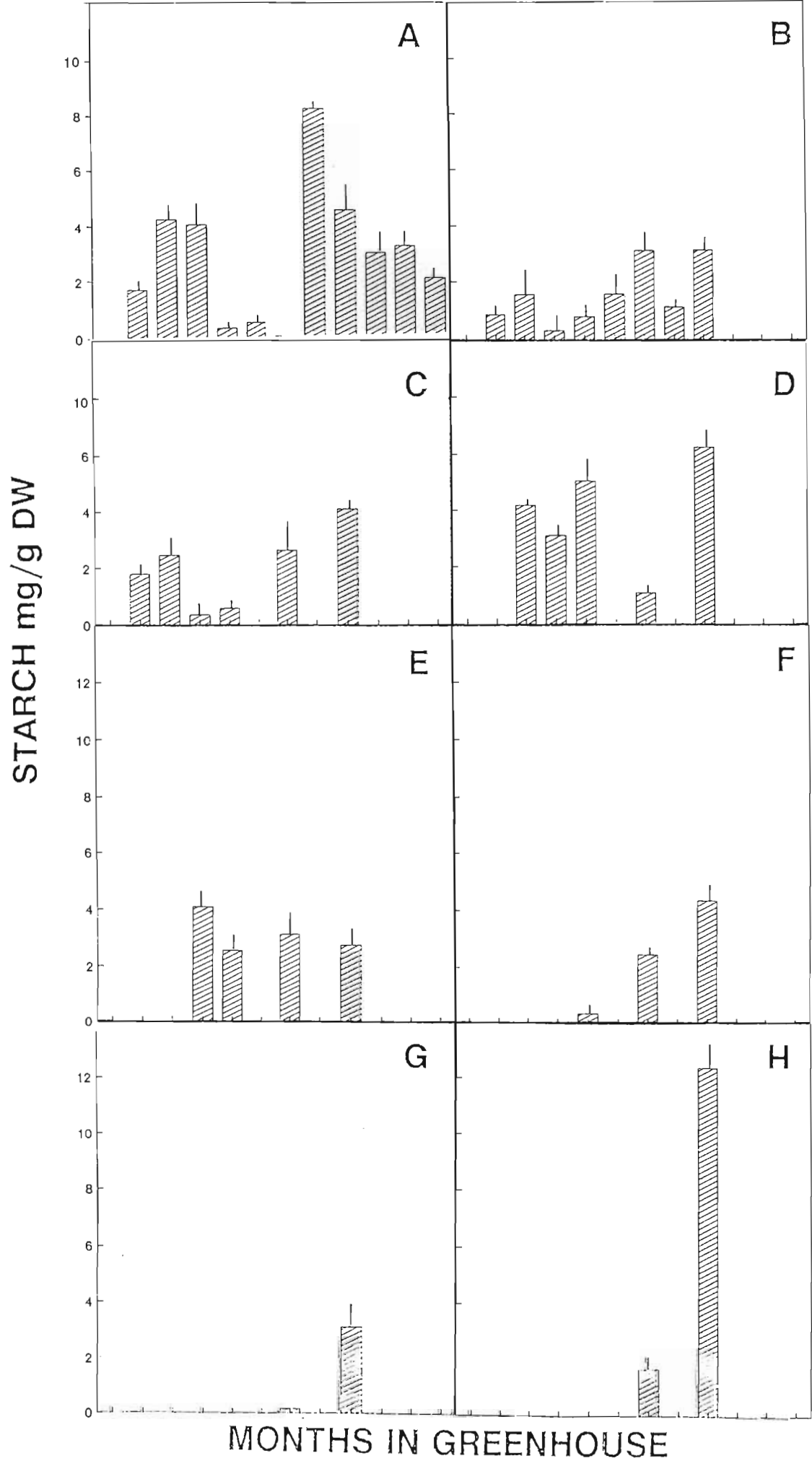
Figure 54.

Total fructose extracted from the leaves (▨), stems (▩) and roots (□) of plants which were moved to the greenhouse in January (A), February (B), March (C), April (D), May (E), June (F) and July (G).

Bars represent the standard error

TABLE 3: The level of fructose, glucose and sucrose was measured in guayule plants before and after flowering occurred in the greenhouse. The first column represents the month in which the plants were moved to the greenhouse and the second column represents the number of months that the plants had been in the greenhouse. Plants were harvested and the sugars separated using G.L.C. Known standards of fructose, glucose and sucrose were run and the areas of these peaks used to determine a straight line. The correlation coefficients were as follows: fructose (97.01%), glucose (98.26%) and sucrose (97.47%). A range analysis at the 95% confidence level was conducted for each month to determine if these differences were significant (**) or not (*).

MONTH		FRUCTOSE	GLUCOSE	SUCROSE
JAN	6	**	*	*
	7	*	*	*
FEB	5	**	*	*
	7	*	*	*
MARCH	4	**	*	*
	6	*	*	*
APRIL	3	**	*	*
	5	*	*	*
MAY	2	**	*	*
	4	**	*	*
JUNE	1	**	*	*
	3	*	*	*



the greenhouse in June (**Figure 55G**). While starch increased significantly in those moved in July (**Figure 55H**).

5.4 DISCUSSION

The natural distribution of guayule spans a latitudinal range from 23°39' to 30°00', with an altitudinal range of 743 m to 2340 m. The natural climatic environment is subtropical to warm temperate and continental in character with extremes of Summer and Winter temperatures and a large annual temperature range. Rainfall occurs in the warmer months while the colder months are dry (NIX,1986).

Pietermaritzburg is situated at 29° 37'S , altitude 762 m. The climate has wet Summers with dry Winters where the temperature range is very large. This location, at an approximate latitude of 30°S, results in more rapid changes in daylength during the Autumn and Spring equinoxes when compared to Winter and Summer (SALISBURY,1981). As the minimum Winter temperatures can reach 0°C in Mexico and in Pietermaritzburg, the plants must become frost-hardy before the cold season begins.

Leaf loss can be used as an indicator of plants entering dormancy. This process was not controlled by photoperiod as plants moved to the greenhouse from January to March continued growing. In this case, the plants responded to the elevated temperatures. However, after March, the plants entered a phase in which dormancy could not be reversed. During the period in the greenhouse, the level of reserve carbohydrates remained the same until budbreak in August. Fructans must be providing carbon for growth and/or flowering. The problem is that the carbon requirements for these two processes are difficult to separate.

The experiment was not successful in terms of initiating flowering. Plants which were flowering in January continued to do so in the greenhouse. The elevated temperatures and increased photoperiod in the greenhouse did not promote flowering in any of the guayule plants moved after January, although WHITEHEAD AND MITCHELL (1943) and DOWNES (1986) have been able to promote flowering under those conditions.

Vegetative budbreak in the plants in the greenhouse occurred within two days of those in the field. The plants in the field had been exposed to lower maximum and minimum temperatures as well as shorter photoperiods. The plants moved to the greenhouse in February and March were exposed to months of 12 hour photoperiods which did not induce flowering.

There are two events occurring in guayule in Spring. One concerns the growth of the determinate axis which precedes flower initiation (SACHS,1987) and the other is flower initiation. It is therefore not unusual that vegetative and reproductive budbreak may have different requirements (REES,1987). Thus it should be possible to identify the initial evocational event which is the formation of the determinate shoot axis.

Prior to vegetative budbreak the plants in the greenhouse had been exposed to longer and warmer days than those in the field. However, budbreak in the greenhouse and field occurred within days of one another. This process appears to be under genetic control. Reproductive budbreak, on the other hand, may utilize a temperature cue like accumulated degrees above a certain temperature. Once the maximum temperatures increased by 6°C in the greenhouse and experimental site, flowering began. Temperatures in the greenhouse were not controlled and were higher than the ambient, due to the glass walls of the greenhouse trapping radiation and thereby increasing temperatures.

Budbreak does not involve only morphological changes but also alterations in fructan and starch levels. For vegetative budbreak, vernalization increases the carbohydrate content at the shoot tips (SACHS,1987). In guayule, an increase in water-soluble fructans precedes vegetative budbreak and a decrease follows vegetative budbreak was observed in the greenhouse plants. The increase in water-soluble fructans was concentrated in the young stems which included the shoot region (Figure 53A-F). In the 1989 harvest (used as the control in this experiment), vegetative budbreak was associated with depolymerization of fructans and an increase in free fructose.

The initial speculations by KELLY AND VAN STADEN (1991) that starch was utilized as a carbon source for flowering were not unfounded as decreases in starch reserves in *Hevea* correspond with the period of development of young leaves and flowering (DE FÄY,1989). The plants which were moved into the greenhouse in January continued flowering and the level of starch in these plants was depressed thus substantiating the theory. All plants moved into the greenhouse after January did not flower and contained elevated levels of starch. However, these elevated levels may not have been due to non-utilization of starch, but rather due to the higher phytotron temperatures which would have promoted the activity of enzymes associated with starch synthesis.

FUCHIGAMI AND NEE (1987) proposed a degree growth model which can be applied to guayule (Figure 56). The model consists of 360° growth stages (GS), and the cyclic function passes through five distinct sequential growth phases: a) Spring budbreak (0°GS); b) maturity induction point (90°GS); c) vegetative maturity or the onset of rest (180°GS) ; d) maximum rest (270°GS); e) end of rest (315°GS); and f) the end of quiescence or onset of the spring budbreak (0°GS).

During the 0 to 90° growth stage in guayule, leaf or floral parts first emerge from the dormant buds, elevated temperatures influence growth. Photoperiod does not appear to influence vegetative budbreak as exposure of plants to

increased daylength did not promote budbreak although photoperiod may initiate flowering.

Plants then mature and reach the time at 135°GS when shorter photoperiods promote dormancy. Between 90 and 135°, in the case of guayule, from January to March, exposure of plants to long daylengths and elevated temperatures overcame the influence of the shorter photoperiod. However, there appears to be a critical period indicated by the hatching (**Figure 55**) when dormancy can be overcome, in this case represented by plants moved to the greenhouse in April/May. In June/July, plants had passed the 180°GS and acclimatized to the cold by producing fructans and rubber. From 270 to 360°GS the plants move from dormancy to budbreak whereafter manipulation of growth is impossible as plants approach budbreak.

5.5 CONCLUSION

Guayule appears to have developed a physiological ecotype suited to the latitude in which it exists. During Autumn, plants begin to lose leaves and enter the dormant state. Dormancy can, however, be averted as exposure of plants to long days prolonged the growth period in plants which were moved to the greenhouse in January, February and March. The induction of leaf fall and dormancy appear to be controlled by temperature rather than photoperiod. However, once the plant has entered dormancy (as demonstrated by the plants which were moved to the greenhouse in April, May, June and July), it remains in this condition until the maximum temperatures increase.

The fructans appear to provide carbohydrates for vegetative bud growth and thereafter for flowering. An increase in the number of leaves and stems in Spring would provide a larger area for photosynthesis and thus increase available carbohydrate. As starch synthesis depends on current photosynthetic carbon production, an increase in leaf number would elevate the level of starch

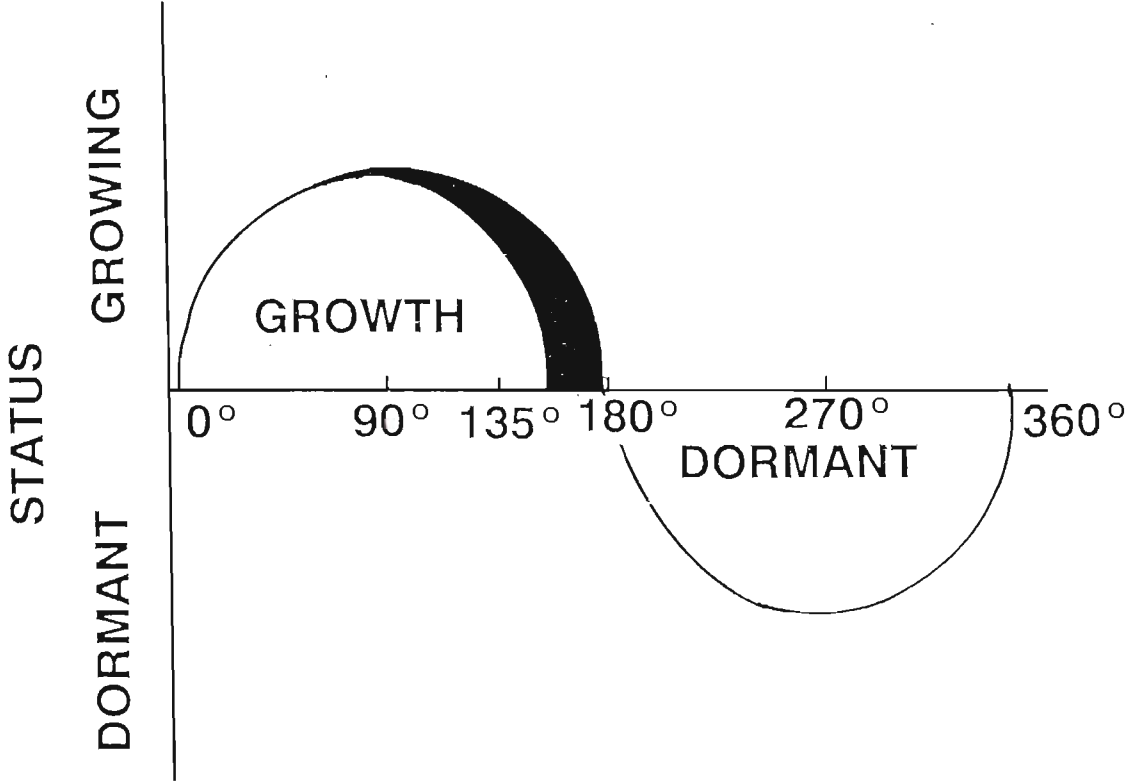


Figure 56. The model proposed by FUCHIGAMI AND NEE (1987) for plants which enter a dormant period during the Winter which is broken in Spring. Included between 135°C and 180 °C is a period during which time dormancy can be delayed.

after bud break. The newly-fixed carbon could also be utilized for rubber synthesis. However, associated with this increase in carbon is the appearance of peroxisomes and the subsequent carbon loss from leaves. Therefore in September the activity of the rubber transferase enzyme decreases and rubber production is substantially reduced. It can not be assessed whether this is the result of reduced carbon as a result of growth requirements or due to the change in environmental conditions.

However the practise of removing flowering stalks in many experiments would avail the plant of additional carbon in the form of fructans. This carbon would then be utilized for growth rather than rubber production. This in turn increases biomass, providing a greater area for rubber deposition which increases the final percentage rubber in the long term. Thus it has been interpreted that by preventing flowering, carbon is diverted from flowering into rubber production.

The breaking of dormancy in guayule appears to be controlled at the genome level, any attempt therefore, to increase rubber production by increasing the dormant period will have to take this into account. Budbreak and flowering do not effect rubber synthesis with respect to carbon supply.

CHAPTER 6

FINAL DISCUSSION AND CONCLUSION

The commercialization of guayule has been hampered by the low percentage rubber that is produced by the plants, by the great variability shown between plants with respect to rubber yield, and the fact that guayule plants have to be harvested destructively before the extraction of rubber can take place. Therefore any attempts to increase rubber yield would effect the economic prospects for guayule. These attempts have involved selecting and breeding plants which produce a high percentage rubber and /or spraying with bioregulators to increase rubber production. As our knowledge of guayule physiology increases, greater innovation with respect to increasing rubber production will occur.

In guayule, the isoprenoid pathway produces rubber during the winter months and volatile terpenes, sesquiterpenes, diterpenes and triterpenes throughout the year. This means that a considerable amount of carbon is channelled along this pathway. The fact that rubber is only synthesized during the colder months is due to the cold induction of the rubber transferase enzyme and not due to an excess of substrate. As this is the rate-limiting step for rubber production, there have been speculations that by altering the nature of the enzyme, rubber could be produced all year round.

Once rubber transferase is present in the cells of guayule, carbon partitioned to the stem and roots is utilized for the synthesis of rubber. It would appear therefore that the enzyme is either present in large amounts, utilizing substrate, or once present it is able to sequester carbon. It has emerged from this study that no plant component acts as a sink during Winter when the plant is supplied with excess [^{14}C] sucrose. Interestingly, although carbon is lost from the leaves (probably due to photorespiration or respiration) during the Summer, the

loss of leaves during the Winter months and the subsequent decrease in gross photosynthesis does not appear to effect the carbon economy of the plant adversely.

The translocated precursor in guayule is sucrose as application of [^{14}C] acetate to the leaves resulted in the labelled carbon being incorporated into the carbohydrates in Winter and Summer prior to translocation. Fructose, however, is the basic building block of the fructan polymer and is the principal free sugar in guayule throughout the year. Supplying plants with [^{14}C] fructose resulted in the translocation of the [^{14}C] fructose to the mature stem and roots which are the sites of fructan storage. The [^{14}C] fructose is incorporated into the acetone (resins) fraction in the Winter and Summer.

As the [^{14}C] sucrose and [^{14}C] fructose undergo different fates with respect to metabolism, it allows for some speculation as far as the compartmentation of the isoprenoid pathway is concerned. Perhaps the sucrose remaining in the cytosol is utilized for the synthesis of rubber molecules while the plastids of the resin canal epithelial cells are permeable to fructose which can be utilized for the synthesis of resins. If the plastids are processing the [^{14}C] fructose as fructose then this would provide evidence as to the autonomous nature of the plastids with respect to isoprenoid synthesis. If, on the other hand, the [^{14}C] fructose is converted to sucrose and then translocated, why is the plant metabolizing the [^{14}C] sucrose and [^{14}C] fructose in different ways? Perhaps by separating the processes of isoprenoid and *cis*-polyisoprene synthesis spatially an adequate supply of carbon is ensured.

The fructans which are stored in vacuoles in the mature stem and roots are polymerized and depolymerized when there is an excess or depletion in the carbon supply. The pith region emerges as the main storage area for fructans. This region contains no rubber although the isoprenoid enzymes are present. The localization of fructans in the pith allows for rapid lateral transport of

carbohydrate via the vascular rays from the pith to the cortex as these pith parenchyma cells are not filled with rubber and the cell vacuoles are available for fructan storage. The short distance translocation of these stored carbohydrates allows the processes of starch and rubber production to utilize a current photosynthetic source. Long distance transport from the leaves to the stem and roots of sucrose occurs via the phloem.

Fructans definitely act as the source of carbon for budbreak as at the time of its occurrence the water-soluble fructans are apparently depolymerized to balance the carbohydrate reserve. The presence of fructans rather than starch allows for the processes of reserve carbohydrate metabolism to continue during periods of low temperature and high irradiance. The loss of leaves in guayule results in a decrease in starch content which is corrected after budbreak, therefore the carbon required for new growth must originate from the fructans. The pattern of accumulation was not altered in the plants which were maintained at higher temperatures in long days in the greenhouse.

The occurrence of two types of fructan polymers ensures that there is always carbon available should adverse conditions prevail. The water-soluble polymers are depolymerized when the carbon supply is insufficient for the synthesis of ethanol-soluble fructans. The localization of a percentage of the water-soluble fructans in the roots provides another reserve, separated from "daily metabolism". The mature stem contains equal amounts of ethanol-soluble and water-soluble fructans probably indicative of the active metabolic role of this plant component.

Fructans have not been included in the by-product studies of guayule although they are commercially important sources of carbohydrate. Inulin forms part of a diabetic meal, it is used as a carrier substance for pharmacological products, a filler substance in the food industry, and in protectants against sunburn. Levulinic acid, a derivative of inulin, is used to make gasoline extenders,

polyesters and polyamides. Chicory extracts also contain fructans and Jerusalem Artichoke is being used in the production of ethanol (FRENCH,1989). Use could be therefore be made of the high fructan content of guayule.

This study has illustrated, once again, that the rubber-producing system in guayule is indeed complex. Tissue culture studies (NORTON, RADIN AND RODRIGUEZ, 1991ab) are not likely to succeed as most explants *in vitro* do not produce sufficient woody tissue to ensure that there is adequate space for rubber storage, that the ultrastructural requirements (compartmentation) for isoprenoid synthesis have been met, and that the characteristic nature of carbohydrate transport and metabolism is maintained.

TAIL PIECE

Most remarkable of all, despite the advent of a range of excellent synthetic alternatives, natural rubber has stood its ground, primarily because - surprising as it may seem - the overall balance of properties with which Nature has endowed natural rubber remains unsurpassed. This, plus the continued input of much thought and actions, will assure that, despite views that were held 20 to 30 years ago, natural rubber can face the future with confidence.

(ALLEN AND JONES, 1988)

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