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THE USE OF PHLOEM EXUDATE IN THE STUDY
OF TRANSLOCATION IN *CUCURBITA PEPO*

by

Deborah Anne Baillie, B.Sc., B.L.S.

A thesis submitted to
The Faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of

Master of Science

Department of Biology
Carleton University
Ottawa, Ontario
November 1987

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acceptance of the thesis,

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of Translocation in *Cucurbita pepo*"

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ABSTRACT

Mature leaves of intact and pruned plants were exposed to $^{14}\text{CO}_2$ to study the movement of compounds from the leaf to the petiole and its exudate. Accumulation of ^{14}C in starch and other macromolecules was rapid in the leaf, slower in the petiole and minimal in the exudate. The short term distribution of radioactivity within the soluble fraction was similar in the leaf, petiole and exudate. The leaf and petiole were also similar to each other 24 hours later; the exudate differed dramatically. The total amounts of individual sugars, amino acids and malic acid were measured; other organic acids were semiquantified.

Reducing the source to sink ratio by pruning all the mature leaves except one decreased the sugar content of the remaining mature leaf, petiole, and exudate; increased the rate of CO_2 fixation; and enhanced the amount of ^{14}C in the neutral fraction of the petiole and exudate. Radioactivity in the leaf was unchanged. The activity of PEP-carboxylase was enhanced in the remaining mature leaf of pruned plants but unchanged in the petiole.

The exudate was alkaline with about 0.3% (w/v) sugar and 0.6% (w/v) amino acids. Invertase activity was absent; α -galactosidase activity negligible. The monosaccharides predominated as in the other tissues but contrary to the leaf and petiole these were not heavily labelled 24 hours

later. The organic and amino acid compositions also differed from the leaf and petiole. Slow turnover of P-protein occurred.

The exudate represents a specific part of the petiole but its exact origin within the tissue is unknown, as is the effect of sampling severed tissue on the composition of the exudate. A technique to easily sample the contents of *Cucurbita pepo* sieve tubes in the petiole of a source leaf while translocation is maintained needs to be developed. Exudate analyses from detached petioles should meanwhile be used only if augmented by other techniques.

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LIST OF ABBREVIATIONS

γ -ABA = γ -aminobutyric acid
2-D = two-dimensional
ALA = alanine
ARG = arginine
ASN = asparagine
ASP = aspartic acid
ATP = adenosine triphosphate
BSA = bovine serum albumin
CIT = citrulline
CYS = cysteine
CYSTATHIO = cystathionine
CYS-CYS = cystine
DCF = 2',7'-dichlorofluorescein
DPM = disintegrations per minute
EDTA = ethylenediaminetetraacetic acid
FRU = fructose
FRU-6-PO₄ = fructose-6-phosphate
GLN = glutamine
GLU = glutamic acid
GLY = glycine
GOL = galactinol
GOT = glutamate oxaloacetate transaminase
HIS = histidine
HPLC = high performance liquid chromatography

LIST OF ABBREVIATIONS

HSER = homoserine

HydroxyPRO = hydroxyproline

ILEU = isoleucine

LEU = leucine

LYS = lysine

MDH = malate dehydrogenase

MET = methionine

MYO = myoinositol

NAD = nicotinamide adenine dinucleotide

NADH = nicotinamide adenine dinucleotide, reduced

NADPH = nicotinamide adenine dinucleotide phosphate, reduced

OPA = o-phthalaldehyde

ORN = ornithine

PEP = phosphoenolpyruvate

PHE = phenylalanine

P_i = inorganic phosphate

PNPG = p-nitrophenyl- α -D-galactopyranoside

PPO = 2,5-diphenyloxazole

PRO = proline

RAF = raffinose

SER = serine

STA = stachyose

SUC = sucrose

SUC-6-PO₄ = sucrose-6-phosphate

LIST OF ABBREVIATIONS

TCA = tricarboxylic acid

THR = threonine

TLC = thin layer chromatography

Tris = tris(hydroxymethyl)aminomethane

TYR = tyrosine

UDP = uridine diphosphate

UDP-GAL = uridine diphosphogalactose

UDP-GLC = uridine diphosphoglucose

VAL = valine

INTRODUCTION

Evolution has physically separated production in the photosynthetic tissues of plants from consumption in the non-green tissues; hence, the processes by which organic compounds are moved or translocated throughout the plant have a direct bearing on growth and development. Changes in the distribution of translocates have, in fact, played an important role in the domestication and improvement of crops in the past. It is hopeful that elucidation of the processes by which photosynthetic products are mobilized from mature leaves (source) to storage and developing organs (sink) will reveal the limitations imposed on crop yields by the transport system and may even lead to the manipulation of transport and enhanced agricultural productivity. In addition, the development of effective agricultural chemicals, such as herbicides, pesticides, and fertilizers, often depends on the ability to assess and predict their phloem mobility (Lichtner, 1984).

Hartig first described the sieve tube in 1837 (for early literature see Crafts, 1932) but it is still not known precisely how it functions in the process of long distance transport. It is believed that a comprehensive picture of the contents of the sieve tubes in relation to their microstructure will lead to an understanding of their function. As a result, the chemical composition of the

phloem has often been studied in an attempt to determine the composition of the translocation stream and in turn, the mechanism of translocation.

The Cucurbits were widely used in the early work on phloem anatomy because they resulted in clear preparations for microscopic examinations (Crafts, 1932) and the ease with which they exuded sap also made them suitable candidates for physiological studies. Having been studied extensively in the past, the Cucurbits continue to be investigated, often fueled by the fact that they comprise an important economic crop in many parts of the world. In addition to these historical and economical considerations, however, some peculiarities in translocation in these plants have been reported and it remains to be seen whether the Cucurbits have a specialized transport system or represent translocation as it occurs in other angiosperms.

It is thought that a fairly reliable picture of translocates can be obtained through the analysis of phloem exudates, especially when combined with radioisotopic tracer experiments (Gunning, Swanson and Fisher, 1976). The results from recent studies on Cucurbit phloem exudate in which ^{14}C was used (Richardson, Baker and Ho, 1982; Richardson, Baker and Ho, 1984), however, have differed from other published research. Richardson, Baker and Ho (1984) found that 29% of the radioactivity recovered from the exudate was in the acidic fraction although it had been

previously reported to be 3% in legumes and *Nicotiana* (Pate, Sharkey and Lewis, 1974; Hocking, 1980). Unlike exudate analyses from many other species (e.g. Hall and Baker, 1972; Pate, Sharkey and Lewis, 1974; Hocking, 1980), Richardson, Baker and Ho (1982) reported that Cucurbit exudate contained large amounts of monosaccharides and higher concentrations of amino acids than sugars. In conclusion the authors suggested that the organic acids and amino acids may play as great a role as the sugars in carbon transport in the Cucurbits (Richardson, Baker and Ho, 1982). Previous research conducted by Dr. J. A. Webb on translocation in the Cucurbits (e.g. Webb and Gorham, 1964) using the analysis of petiole tissue rather than exudate had shown that the organic and amino acids played a negligible rôle in carbon transport. As a result of the differences observed through the analysis of exudate, Richardson, Baker and Ho (1984) proposed that the technique of sampling Cucurbit phloem exudate was superior to the analysis of extracts of petiole tissue. In addition, although not directly related to translocation, Richardson, Baker and Ho (1984) had found that only 2% of the radioactivity present in the Cucurbit source leaf was insoluble in ethanol whereas it had been previously reported as about 30% (Webb and Gorham, 1964). Moreover, the authors appeared to disregard this discrepancy, offering no explanation for the difference.

These differences may reflect natural flexibility in the transport system or the different experimental techniques used to study transport. In an attempt to reconcile data previously obtained in this laboratory with that of Richardson, Baker and Ho (1982, 1984) a research project on *Cucurbit* phloem exudate was undertaken. The objective was to investigate the chemical composition of phloem exudate in *Cucurbita pepo* with emphasis on carbon transport using ^{14}C as a tracer and in doing so, to determine whether or not the phloem exudate should be considered representative of the translocation stream and pursued as a viable research method in *Cucurbita pepo*.

Rapid incorporation of large amounts of radioactivity into malate is normally observed during exposure of this species to $^{14}\text{CO}_2$. During this study on translocation the production of labelled malate appeared to be enhanced by a reduction in the source to sink ratio. Considering the possibility that *Cucurbita pepo* might exhibit a C_4 pathway of photosynthesis, especially when under stress, additional research was conducted to investigate the level of PEP-carboxylase activity.

LITERATURE REVIEW

Methods of Studying Translocation

The analysis of phloem exudate is one of the oldest methods used to study the contents of phloem cells. When an incision is made in the bark of many angiosperm trees, the phloem sap will exude for several hours, even days (Milburn, 1972). Similarly, an incision into the bark of woody herbaceous plants like *Ricinus* can be made (Hall, Baker and Milburn, 1971). Some species of plants, such as the Cucurbits, will exude phloem sap wherever cut (Crafts, 1932), while other species will only exude from cuts made in specific organs; for example, the young inflorescent stalks of *Yucca* (Tammes and Van Die, 1964; Van Die and Tammes, 1975). Why this selective bleeding occurs is unknown. It has been attributed to variations in the phloem sealing mechanisms involving callose formation and protein accumulation and coagulation (Crafts and Crisp, 1971; Eschrich, 1975). Exudate was obtained from the fruit and young shoots of several legumes but not from their leaves, old stems or roots which suggested that the rates of phloem sealing with callose or protein varied (Pate, Sharkey and Lewis, 1974). Exudation may be species specific; however, it has been pointed out that studies in which exudation has been unsuccessful need to be examined closely as fruits which normally bleed fail to do so if grown out of season or

in an unsuitable artificial environment (Pate, Sharkey and Lewis, 1975). Consequently, exudation may be greatly dependent on external conditions as well as internal processes.

The presence of solutes within the semipermeable membrane of the sieve tubes results in hydrostatic pressure within the cells (turgor pressure) due to water osmotically entering the sieve tubes. Thus, translocation is thought to occur along a concentration and, in turn, pressure gradient. Water in the transpiration stream moves in response to more negative water potentials from the soil through the plant to the atmosphere, creating tension or negative pressure in the xylem vessels during the process. When the tissues are cut the pressures undergo rapid readjustment. No longer is there resistance to the uptake of water and the contents of the xylem vessels are pulled into the tissue (Crafts, 1936). Cutting the tissue steepens the pressure gradient in the phloem since the turgor pressure at the surface of the cut is reduced to zero (i.e. equal to atmospheric pressure). In contrast to the xylem, the phloem sap exudes at the cut surface due to the positive pressure within the phloem. This exudation lowers the turgor pressure thereby reducing the water potential; hence, water is osmotically absorbed from surrounding tissues. Provided that there is sufficient water in the surrounding environment and that sealing processes do not block the sieve tubes, exudation would

continue until the solute concentration in the sieve tubes is in equilibrium with that in the surrounding tissues. If solutes continue to be loaded into the phloem then exudation could continue indefinitely.

For whatever reason, exudation analyses cannot be applied to all species and when it is applicable, the amount of experimental material so obtained is often very small, necessitating either large amounts of plant material to harvest or extremely sensitive methods of microanalysis. The collection and analysis is further complicated in some species by the tendency of the exudate to gel upon contact with the air (Walker and Thaine, 1971; Walker, 1972). Successively cutting thin sections of the tissue can maintain bleeding (Crafts, 1932; Van Die and Tammes, 1975) and several researchers have used multiple cuts in the plant to increase the amount of exudate obtained for analysis. However, when the tissue is cut the turgor pressure is reduced and water moves into the area from the cells behind the cut in response to the decrease in water potential at the surface of the cut. Hence, it is likely that exudate obtained from a cut is more dilute than the sap *in vivo*. In fact, the concentration of solutes may be less during a second cut if the water content of the tissue had already been increased by a previous cut. Tingley (1944) found that this dilution effect resulted in decreasing solute concentrations with successive cuts and extended over a

distance of two feet in summer squash. Smith and Milburn (1980), on the other hand, found that incisions as little as 2 to 3 mm apart in the bark of *Ricinus* caused a sharp increase in the exudation rate accompanied by only a slight dilution of the total solutes suggesting that there was an influx of sap from other sieve tube elements which had not been previously contributing to the exudation. Hocking (1980) interpreted the increasing sugar concentration that he obtained from successive cuts up the stem of *Nicotiana glauca* to represent a concentration gradient. Due to the varying amount of dilution which may exist, Tingley (1944) cautioned that it was not likely possible to determine absolute measures of concentration within the plant.

It has also been cautioned that a cut into the translocation pathway may constitute a bottomless sink (Fellows, Egli and Leggett, 1978) and as such may change the processes involved in translocation. Abnormal flow can result from the creation of a new sink and the subsequent steepening of the gradient (Canny, 1973). An incision into *Ricinus* bark increased the translocation rate more than 16 cm hr^{-1} to a rate in excess of 100 cm hr^{-1} (Hall, Baker and Milburn, 1971). Smith and Milburn (1980), however, found that although *Ricinus* incisions resulted in increased exudation rates the solute flux also increased and the combined result was to keep the solute concentration relatively constant.

Pate, Sharkey and Lewis, (1974) found that successive cuts in the styler tips of legume fruit (Figure 1) when the fruit was attached to the plant did not decrease the rate of exudation and only caused a slight decrease in the sugar concentration from one collection to the next. Exudation ceased after two or three cuts in detached fruit although there were no significant differences in the concentrations of sugar and amino acids from the exuded sap of attached and detached fruit. This suggested that the continuance of sap exudation was dependent on the renewal of phloem contents by assimilates transported from other organs and/or a continuous flow of water through the tissue. Without this renewal, once the pressure is released the tissue ceases to bleed (Pate, Sharkey and Lewis, 1974). Smith and Milburn (1980) demonstrated the importance of water availability for exudation by obtaining more constant solute concentrations for prolonged periods in water cultured plants than in those grown in soil. They also found prolonged exudation in *Ricinus* to be dependent on fresh solutes being loaded into the phloem since the excision of source leaves reduced exudation whereas the removal of other leaves had no effect. Thus, exudate collection techniques fall into two classes: those in which the plant is left intact, such as incisions into the bark of trees or *Ricinus*, and those in which the sap is taken from an excised plant organ, for example, decapitated stem internodes or severed petioles.

Figure 1. Diagrammatic representation of vascular strands in fruit of *Lupinus albus* (from Pate, Sharkey and Lewis, 1974).

S = stylar tip from which phloem sap was collected

Some researchers have attempted to sustain exudation either by using mechanical stimulation before cutting, such as massage in *Ricinus* (Milburn, 1972), or by using chemical chelators like EDTA in the collecting medium (King and Zeevaart, 1974). *Lupinus* petioles continue to bleed for several hours into a 20 mM solution of EDTA, yielding more than 10 times the amount of sugar released with water (Pate, Sharkey and Lewis, 1975). It has been postulated that chelators sequester calcium ions and in doing so, possibly interfere with callose formation so that the phloem is maintained in a functional state, allowing continued exudation through unblocked sieve plates (King and Zeevaart, 1974). This is substantiated by the fact that excess calcium in the EDTA solution reduces exudation (Simpson and Dalling, 1981). The maximum exudation rate from strawberry pedicels when 10 mM EDTA was used, however, was only 25% of the growth rate of the fruit and most exudation rates were only 10% or less (Forney and Breen, 1985). The authors interpreted this decrease in translocation to imply that EDTA had not totally prevented phloem blockage. It is possible, however, that the EDTA had interfered with one or more of the exudation related processes, such as solute loading or water transport.

Due to the observation of membrane irregularities and cellular disorganization in calcium deficient plants (Marinos, 1962), Epstein (1973) postulated that a reduced

level of calcium in sieve tubes could be responsible for their relatively structureless condition and that the functional and structural integrity of the sieve tube plasmalemma might be maintained by the presence of external calcium. By sequestering calcium ions surrounding the plasmalemma, EDTA may cause the membrane to leak allowing solutes from surrounding cells to enter the sieve tubes. The fact that there are optimal concentrations of EDTA for stimulating exudation above which the exudation rate is reduced (King and Zeevaart, 1974; Tully and Hanson, 1979) is believed to indicate that EDTA selectively interferes with a specific process rather than causing general cell leakage. However, it is still possible that at higher concentrations EDTA not only affects the permeability of the plasmalemma but also processes related to exudation. Simpson and Dalling (1981) found that 2-20 mM solutions of EDTA not only enhanced exudation from leaves of wheat but also resulted in the preferential exudation of phenylalanine and tyrosine relative to the other amino acids. Not only is there the possibility that EDTA increases contamination of the exudate by apoplastic compounds, but it may also alter the concentration of compounds endogenous to the phloem. Consequently, the presence of EDTA in the collecting medium may influence the composition of the exudate through several processes.

The phloem tissue is a difficult tissue with which to work because it is under positive hydrostatic pressure. The osmotic potential of phloem sap from both pumpkin and cucumber was measured to be -7 bars (Crafts, 1932). More recently the sap of *Cucurbita maxima* was found to have an osmotic potential of -8 to -12 bars (Richardson, Baker and Ho, 1982). Similar but often slightly higher values have been obtained for other species, such as -13 to -15 bars in rice (Fukumorita and Chino, 1982) and -14 to -15 bars in *Ricinus* (Hall and Baker, 1972). The osmotic potential of the sieve tubes appears to be greater than the surrounding tissues. In sugar beet source leaves the osmotic potential of the sieve tube-companion cell complex was -30 bars and in the adjacent phloem parenchyma and mesophyll it was -7 and -13 bars, respectively (Geiger et al., 1973). Similarly, the osmotic potential of the sieve tubes and companion cells in corn was -18 bars in comparison to -11 bars in the vascular parenchyma and mesophyll cells (Evert, Eschrich and Heyser, 1978).

These high osmotic potentials indicate that the sieve tubes have the potential to contain high turgor pressure but in order to calculate the actual pressure the water potential must also be measured (Milburn, 1975). Using the osmotic potential, exudate flow rate, and the dimensions of the path of flow from aphid stylets, the hydrostatic pressure at the point of puncture has been calculated as

10 to 30 bars (Peel, 1975). Although the exact pressure may be in dispute, it is generally agreed that the phloem tissue is under positive hydrostatic pressure which results in the expulsion of exudate when the tissue is cut.

The pressure released when the tissue is cut can also disrupt the subcellular structures and hence may terminate exudation due to plugged sieve pores. Consequently, exudate analysis always has the possibility of being contaminated by dislodged non-mobile phloem constituents. In addition, the sap may be diluted by water and/or contaminated by solutes from surrounding cells. Lastly, there is the possibility that contamination occurs from tissue autolysis induced by the wounding (Tully and Hanson, 1979).

In an attempt to localize the pressure released by restricting the size of the cut, aphids feeding on phloem have been used to collect samples of sap, although only minute quantities of sap can be obtained by this method. Microscopic analysis of aphid penetrated tissue indicates that each stylet from which exudate is obtained, terminates in a sieve tube element (Zimmermann, 1961; Zimmermann, 1963; Evert et al., 1968). In cases of *bicollateral* phloem, the aphid, *Aphis nerii*, has been shown to preferentially feed on the internal/adaxial phloem of the stem, petioles, and leaves of *Gomphocarpus* (Botha, Evert and Walmsley, 1975). In *Cucurbita maxima* another aphid, *Rhopalosiphum maidis*, preferentially fed on the abaxial phloem of the leaves

(Botha and Evert, 1978). These findings support the concept that there may be differences between the composition of the internal/adaxial and external/abaxial phloem. These studies also suggest that there may be taxonomic differences in the composition of bicollateral phloem provided that the different preferences of the insects were not the result of variations in their nutritional requirements.

Sap can be collected from severed stylets (Kennedy and Mittler, 1953; Mittler, 1958; Barlow and McCully, 1972) or the aphid can be left intact and the honeydew collected (Mittler, 1958; Ho and Peel, 1969). The entire aphid is occasionally harvested and extracted, especially for radio-isotopic contents (e.g. Atkins et al., 1982). Although exuding stylets have appeared to be a promising method of continuously sampling the translocates, many problems have been documented (Fisher and Frame, 1984) not the least of which is an unpredictable exudation success rate. In addition to the difficulties of colonizing, manipulating and severing the insects there remains the possibility that the sap is no longer in an unaltered state due to the injection of saliva during penetration by the stylet and the continued presence of the stylet sheath (Dixon, 1975). Exudate obtained from aphid stylets may more closely represent the natural processes occurring within the intact plant since there is likely to be less dilution of the sap (Peel, 1975); nonetheless, aphids should not be thought of as a simple way

to delicately tap the translocation stream without disturbing it (Dixon, 1975).

Microscopic observations (Crafts and Lorenz, 1944; Pate, Sharkey and Lewis, 1974) have traditionally been used to argue that exudate collected from cuts or incisions originated from the phloem tissue. The sugar composition of phloem sap from fruit tips (10-21% [w/v] sugar; 98% of which is sucrose) when compared with that of the surrounding fruit tissue (0.5-1% [w/v] sucrose; 1-2.5% monosaccharides), however, also suggests that the exudate from the legume fruit tips is phloem derived (Pate, Sharkey and Lewis, 1974). There is an unusually high proportion of vascular tissue in the stylar tip of legume fruit (Figure 1) and within this vascular tissue, the ratio of phloem to xylem is also high. Consequently, although exudate from this location may be phloem derived that from another location may not. The sap from the fruit tips of *Spartium*, *Jacksonia* and *Lupinus* had an anthrone-positive sugar content of 10-21% (w/v) while that from the young shoot tips of *Spartium* and *Jacksonia* and from the top 3 or 4 internodes of *Lupinus* contained only 3-5% (w/v) sugar. There were no significant differences in the sap collected from the fruit stalk and from the proximal and distal ends of *Lupinus albus* fruit. There appeared to be more dilution of the sap with water from surrounding cells in the shoots than in the stylar tips of the fruit probably due to the high ratio of

phloem to other tissue in the latter (Pate, Sharkey and Lewis, 1974). Thus, the sap composition can vary with the plant part sampled.

In *Lupinus albus* consistently higher levels of sucrose and lower levels of amides were found in the phloem sap of petioles and stem bases than in stem tops, inflorescent stalks, and fruit, regardless of the time of sampling. This suggested that sucrose was being unloaded from, and amides loaded into, the phloem tissue along the stem (Pate et al., 1979). Thus the sap composition may also vary with the location due to differences in the interchange between the xylem and phloem along the translocation pathway.

There are some major differences between the sap collected by exudation and the xylem sap generated by root pressure in decapitated *Ricinus* plants (Hall, Baker and Milburn, 1971):

- 1) alkaline pH (8.2) as compared to an acidic pH (6.0) in the xylem sap
- 2) high concentration of dry matter (10-12%) as compared to the xylem sap (0.2%)
- 3) high concentration of sugar (8-10%), predominantly sucrose, which is not detected in the xylem sap
- 4) higher concentration of K^+ (60-80mM) than in the xylem sap (10-14mM)
- 5) lower concentration of Ca^{2+} (0.5-1mM) than in the xylem sap (3-4mM)

Such differences are often used to support the argument that the exudate is phloem derived and to indicate whether or not the exudate is contaminated with the contents of xylem cells. It is still not possible, however, to measure the amount of dilution which may have occurred nor to determine whether the exudate represents the contents of conducting cells and to distinguish between mobile and non-mobile constituents.

Following the Second World War researchers began to use radioisotopes (e.g. Hartt, 1963) to help identify the compounds being transported and their path of movement. This was virtually the only method used for phloem research conducted on the Cucurbits during the 1950's and 1960's. Usually plant leaves were exposed to $^{14}\text{CO}_2$ or their roots or other abraded surfaces allowed to take up exogenously applied substances. Although major advances have been made using radioisotopes these methods have not been without fault. The distribution pattern of a tracer may result from the method used to apply it (Crafts and Crisp, 1971). There is no proof that exogenous substances duplicate the transport of assimilates nor that endogenous compounds follow normal transport pathways when applied to external surfaces or at concentrations much higher than those found *in vivo*. Tracing substances synthesized from radioactive carbon dioxide and radioactive nutrients in the culture medium more closely resembles the normal situation in the

plant but tends to over-emphasize those compounds which quickly incorporate the label. An example of this is stachyose transport in the Cucurbits. Although stachyose and sucrose are labelled at equal rates in the leaf upon exposure to $^{14}\text{CO}_2$ (Webb and Gorham, 1964), there is more label in the stachyose transported during short term studies than in sucrose (Webb and Burley, 1964; Webb and Gorham, 1964). As a result, this can be interpreted as indicative of stachyose being the primary sugar translocated (Webb and Burley, 1964) when, in fact, there may be as much and possibly more unlabelled sucrose simultaneously moving in the phloem, the labelled sucrose to be transported at a later time. In fact, several processes which occur simultaneously influence the translocation of a ^{14}C -labelled compound (Nelson *et al.*, 1961) and differences in the rates of these processes make radioactive tracer analyses subject to similar misinterpretations:

- 1) CO_2 assimilation
- 2) synthesis of the labelled assimilate
- 3) mixing of the labelled assimilate with endogenous unlabelled pools of the assimilate
- 4) local metabolism of the labelled and unlabelled mixed pools
- 5) translocation of the assimilate from the site of synthesis to the veins
- 6) long distance translocation via the phloem

- 7) radial transfer from the phloem to the surrounding tissues
- 8) accumulation and metabolism within the phloem and surrounding tissues

The other major approach to phloem research utilizes cytological techniques, such as fluorescent dyes (for literature see Esau, 1969), histochemical reactions (e.g. Fisher, 1978; Pristupa, 1979a; Pristupa, 1979b; Pristupa, 1983), and/or tissue microautoradiography (e.g. Trip, 1969; Fritz, Evert and Heyser, 1983), to locate and quantify specific substances. These procedures easily allow structural observations, but often it is the distribution, quantification and movement of substances within these structures which need to be determined. In addition to the problems concerning the transport of exogenous substances, these methods often rely on reagents in which the substances being analyzed are soluble (Fisher, 1976; Zamski and Zimmermann, 1979).

Although the best indicator of phloem transport would be studies which combine several methods, these have rarely been performed (Ziegler, 1975). Improvements in analytical techniques, however, have resulted in a reappearance of exudate analysis in the Cucurbits (Richardson, Baker and Ho, 1982) occasionally combined with radioactive tracers (Richardson, Baker and Ho, 1984). Instead of increasing the

knowledge of translocation these new studies have opened the entire subject of Cucurbit exudate analysis to debate.

Carbohydrates in the Translocation Stream: ✓

The type of sugar found in the sieve tube sap has been used to classify the angiosperms into three groups (Ziegler, 1975):

- 1) Those with sucrose as the predominant sugar, although small quantities of additional oligosaccharides may be present. Sucrose is the only sugar present in rice sap (Fukumorita and Chino, 1982) and in *Ricinus* exudate (Hall, Baker and Milburn, 1971). In fact, most of the species examined fall into this category.
- 2) Those containing considerable amounts of oligosaccharides in addition to sucrose. These include the Cucurbitaceae (Pristupa, 1959) and *Salix viminalis* (Peel and Weatherley, 1959).
- 3) Those with considerable amounts of sugar alcohols in addition to sucrose and other oligosaccharides. For example, sorbitol has been found in the exudate of several varieties of apple (Webb and Burley, 1962) and mannitol in white ash (Webb and Burley, 1964; Zamski and Zimmermann, 1979).

Hexoses, in general, are not detected in phloem exudate (Hall, Baker and Milburn, 1971; Pate, Sharkey and Lewis, 1974; Hocking, 1980) although they have occasionally been reported in low concentrations (Zimmermann, 1961; Tammes and Van Die, 1964). Reports of higher concentrations presumably represent the hydrolysis of di- or oligosaccharides or contamination from other cells (Ziegler, 1975). Richardson, Baker and Ho (1982) found high levels of monosaccharides in the phloem exudate from Cucurbits and attributed this to the site sampled. They suggested that locations near active sinks had higher levels of monosaccharides but failed to explain why 21% of the total sugar in the stem internodes was monosaccharide. Since galactosidase appears to be absent from sieve tubes (Ziegler, 1975) it is not surprising that the authors did not find α -galactosidase activity in the Cucurbit exudate. The invertase activity in this exudate was not analyzed but considering that this enzyme also appears to be absent from sieve tubes (Ziegler, 1975), one can only assume that the monosaccharides found in the stem internodes resulted from contamination of the sap.

Allowing for the possibility that low concentrations of monosaccharides do exist within the sieve tubes, the transport sugars are thought to be nonreducing (Ziegler, 1975). The ratio of radioactivity in reducing sugars to that in nonreducing ones decreases with the distance from the radioactive source (Webb and Burley, 1964) and increases

with the amount of time elapsed before the samples are taken (Webb and Gorham, 1964). Hence, the reducing sugars are likely to be products of local metabolism of the transported nonreducing sugars.

The concentration of sucrose detected in phloem exudate (w/v) was 8-10% in *Ricinus* (Hall, Baker and Milburn, 1971), 10-21% in legumes (Pate, Sharkey and Lewis, 1974), 15-17% in *Yucca* (Tammes and Van Die, 1964), 14-24% in *Nicotiana glauca* (Hocking, 1980), and 17-25% in rice sap (Fukumorita and Chino, 1982). Fisher (1978) used negative staining to estimate the concentration of sucrose in *Phaseolus* sieve tubes and companion cells at 11% (w/v). In *Cucurbit* phloem sap, however, the carbohydrate concentration is much lower than in other plants, often less than 1% (w/v) (Ziegler, 1975; Richardson, Baker and Ho, 1982). This concentration, however, is based on the inclusion of P-protein in the exudate volume and if this protein is not mobile then the concentration of sugar in the translocation stream of *Cucurbits* is underestimated. If stachyose ($C_{24}H_{42}O_{11}$, molecular weight 666) is translocated instead of sucrose ($C_{12}H_{22}O_{11}$, molecular weight 342), there would be the same number of carbon atoms in a solution containing one-half the molarity. On a molar basis, the concentration in species which transport stachyose could therefore be one-half that of species which transport sucrose without affecting growth. In the *Cucurbits* the transport of more carbon per molecule

may somehow compensate for fewer sugar molecules in the phloem or vice versa.

Nitrogen in the Phloem Tissue: —

In the exudate from most species the concentration of nitrogenous compounds is much less than that of the carbohydrates but nitrogenous compounds predominate in the Cucurbits (Ziegler, 1975). This nitrogen is mainly in the form of protein although some free amino acids are also present. *Yucca* phloem exudate contains $0.5-0.8 \text{ mg ml}^{-1}$ protein (Tammes and Van Die, 1964) and that of legume fruit and *Ricinus* contains $1-3 \text{ mg ml}^{-1}$ protein (Hall and Baker, 1972; Pate, Sharkey and Lewis, 1974). The protein in *Cucurbita maxima*, on the other hand, has been reported to be $76-112 \text{ mg ml}^{-1}$ (Richardson, Baker and Ho, 1982). This phloem protein has been termed P-protein (Esau and Cronshaw, 1967) and although its function is unknown, P-protein is generally considered not to be mobile in the phloem but to be expelled from the sieve tubes following the release of the turgor pressure.

A variety of enzymes have been found to be associated with the sieve tubes. Most of the enzymes involved with glycolysis, the TCA cycle and gluconeogenesis have been identified although invertase and galactosidase have not been found (for a complete list, see Eschrich and Heyser, 1975). The inability of peroxidases to move across a

Cucurbit graft union (Vickery, 1978) supports the conclusion that enzymes present in phloem exudate are not mobile and like the P-protein are not constituents of the translocation stream. Their role in translocation is unclear because it is difficult to ascertain their exact location due to technical limitations. Two types of methods have primarily been used for the identification of these enzymes: histochemical reactions coupled with microscopic observations and biochemical assays on phloem exudate. In addition to the usual problems of accurately fixing phloem tissue, histochemical reactions tend to be unspecific. Phloem exudate, on the other hand, may be contaminated by the contents of parenchyma and more importantly, companion cells.

Since sieve tubes are deficient in ribosomes, it seems likely that the enzymes are synthesized in the companion cells and either naturally transferred to the sieve tubes or forced into the exudate by the surging. Although the enzymes are not translocates, being able to discriminate between biochemical reactions occurring in the sieve tubes and those in the companion cells might shed some light on the translocation processes and constituents. Comparative studies using both histochemical techniques and exudate analysis would probably prove the most useful but as yet these comparisons are only available for the few enzymes for which histochemical reactions exist.

Amino Acids in the Translocation Stream:

The concentration of amino acids (w/v) was found to be approximately 0.5% in *Ricinus* (Hall and Baker, 1972), 1% in *Nicotiana glauca* (Hocking, 1980), 3-8% in rice phloem sap (Fukumorita and Chino, 1982), and 1-4% in legume fruit sap, increasing as the fruit aged (Pate, Sharkey and Lewis, 1974). Similarly, the concentration of amino acids in Cucurbit exudate was 0.5-3% (Richardson, Baker and Ho, 1982) although this, like the sugar concentration, is likely to be underestimated due to the presence of P-protein.

The amino acid composition of phloem sap within a species appears to be very constant. Increasing the amount of nitrate in the rooting medium of nodulated *Lupinus* resulted in greater levels of total amino acids in the phloem sap but did not alter the balance among the constituent compounds (Pate, Sharkey and Lewis, 1975). These authors also found that an amino acid profile similar to that from nodulated plants was obtained from *Lupinus* phloem sap even when inorganic nitrate was taken up by the xylem in cut shoots. In another study the spectrum of amino acids in the phloem sap showed only slight diurnal changes in spite of the reduced transpiration and presumably lowered accessibility to nitrogen in the dark (Pate et al., 1979). These findings suggest that the loading mechanism can adapt to a wide range of nitrogen levels and is capable of

maintaining a standard mixture of amino compounds in the translocation pathway.

Although the amino acid composition differs both quantitatively and qualitatively among species (Table 1), asparagine, glutamine, aspartic acid, and glutamic acid are usually the predominant ones found in their exudate (Ziegler, 1975). Depending on the species, serine, threonine, proline, and valine may also be present at high concentrations. Although the hydrolysis of rice sap samples suggested that small amounts of glycylglycine and alanyl-glycine may have been present (Fukumorita and Chino, 1982); in general, the amino acids found in phloem exudate are thought to exist in free form and are considered to be constituents of the translocation stream.

Table 1. Amino acid composition in phloem exudate analyses reported in the literature.

Amino Acid	% on a molar basis						
ALA	0.7	4.4	3.7	0.7	1.5	5.4	2.6
ARG	NR	0.7	5.9	2.2	T	T	2.3
ASN	52.1 ^a	NR	NR	18.1	2.5	NR	3.9
ASP	4.9	27.5	2.2	1.4	1.9	23.5	19.6
CYS	NR	1.1	T	0.2	NR	NR	NR
CYS-CYS	NR	NR	NR	NR	T	NR	NR
γ -ABA	0.6	NR	NR	ND	1.4	NR	ND
GLN	14.3	NR	NR	15.5	35.0	NR	3.7
GLU	2.2	15.1	23.8	11.3	19.1	34.8	30.3
GLY	0.9	3.6	0.5	1.6	T	6.4	0.6
HIS	1.3	5.0	2.0	1.8	0.5	1.1	1.9
ILEU	2.5	1.0	2.6	3.2	3.0	2.7	4.1
LEU	0.5	1.0	1.8	3.0	ND	1.1	4.7
LYS	2.7	0.9	5.1	3.0	1.8	0.5	4.7
MET	0.4	0.9	0.5	0.2	ND	T	0.7
PHE	1.2	0.8	3.8	4.1	4.6	1.6	3.2
PRO	0.6	9.0	5.8	4.2	18.0	NR	NR
SER	6.9	12.4	10.5	13.6	7.0	4.3	5.7
THR	2.2	15.3	23.4	6.1	2.2	14.4	4.9
TYR	0.4	0.7	0.3	1.6	0.6	NR	2.3
VAL	5.6	1.1	6.4	6.4	ND	4.3	4.7
Others	NR	NR	1.6	1.7	NR	NR	NR
Total	100.0	100.0	100.0	99.9	100.0	100.1	99.9
Source	<i>Lupinus</i> ^a	<i>Cucurbita</i> ^b	<i>Pisum</i> ^c	<i>Oryza</i> ^d	<i>Nicotiana</i> ^e	<i>Ricinus</i> ^f	<i>Triticum</i> ^g

Notes: ND = Not detected; T = Trace amount; NR = Not reported

^aPate, Sharkey and Lewis, 1974 (Data recalculated)^bRichardson, Baker and Ho, 1982 (Data recalculated)^cBarlow and Randolph, 1978 (Data recalculated)^dFukumori and Chino, 1982^eHocking, 1980^fHall and Baker, 1972^gHayashi and Chino, 1986b (Data recalculated)

Some of the differences in the amino acid composition of the exudate may be attributable to the nitrogen nutrition of the plants. Asparagine and glutamine predominated in the phloem sap of rice grown with ammonium sulfate as a nitrogen source while aspartic acid, glutamic acid, and serine predominated in rice grown with sodium nitrate as a nitrogen source (Hayashi and Chino, 1986a). Similarly, water stress has been demonstrated to cause slight changes in the amino acid profile of barley plants. Among the five principal amino acids in the exudate; namely, glutamine, glutamic acid, serine, alanine, and aspartic acid, the only change in water stressed plants was an increase in serine and the appearance of small amounts of proline and γ -aminobutyric acid (Tully and Hanson, 1979).

Although a variety of amino acids are present in the exudate, only a few of these acids incorporate radioactivity during short term labelling studies using carbon dioxide. In the exudate from the pods of soybeans, 30% and 23% of the radioactivity recovered from the basic fraction was in serine and asparagine, respectively (Fellows, Egli and Leggett, 1978). This agrees with the earlier report that sucrose and serine accounted for 95% of the radioactivity extracted from soybean stems (Nelson et al., 1961). Webb and Gorham (1964) similarly found that radioactive serine/glycine and aspartate/glutamate were detectable in petiole tissue extracts of *Cucurbita pepo* within minutes of

exposing the leaf blade to $^{14}\text{CO}_2$, although the authors cautioned that there was no direct proof that these compounds were transported. Serine is easily synthesized from 3-phosphoglycerate, one of the photosynthetic products transported out of the chloroplasts (Ziegler, 1975) and consequently, the high levels of serine, especially radioactive serine, suggest that this amino acid may be one of the primary long distance translocates of nitrogen. After exposure of nodulated legumes to $^{14}\text{CO}_2$, the photosynthetic products, serine, alanine, and glutamic acid, were heavily labelled in the phloem exudate but the amides, despite being the most abundant amino compounds, were only weakly labelled (Pate, Sharkey and Lewis, 1974). Since asparagine and glutamine were typical end products of nitrogen fixation in root nodules, the authors suggested that some amino acids were easily exchanged between the xylem and phloem (Sharkey and Pate, 1975).

In short term labelling studies the amino acids in the exudate contain much less radioactivity than the neutral substances. Out of the radioactivity recovered from the exudate, the amino acid fraction in legume fruit contained 3.4% (Pate, Sharkey and Lewis, 1974). In *Nicotiana glauca* stem exudate it was 4.2% (Hocking, 1980) and 10-15% in *Cucurbita maxima* (Richardson, Baker and Ho, 1982; 1984). These small percentages indicate that there is a time lag between the synthesis of most of the amino acids and their

transport. Either the amino acids are not primary products of photosynthesis or after incorporating radioactivity are confined to specific pools and not immediately transported. In rice exudate the sugar fraction contained 4 times as much carbon as was present in the amino acid fraction (Fukumorita and Chino, 1982) and in wheat the sugar contained only 2 times that of the amino acids (Hayashi and Chino, 1986b). The authors suggested that the amino compounds played a major role in carbon transport in both these species. This idea was also proposed by Richardson, Baker and Ho (1982) for transport in the Cucurbits. When the low sugar concentration in Cucurbit exudate is considered, the amino acids may play a very significant role in carbon transport in these species.

Many plant species contain other nitrogenous substances in their phloem exudate although there is great variation both in the presence and concentration of these compounds. Ornithine in *Yucca* (Van Die and Tammes, 1975) and β -(isoxazolin-5-one-2-yl)-alanine in peas (Urquhart and Joy, 1981) comprised less than 1% and 2% of the total amino acids on a molar basis, respectively. On the other hand, two ureides, allantoin and allantoic acid, detected but not quantified in *Yucca* (Tammes and Van Die, 1964) were located in *Acer*, *Platanus*, and *Aesculus* at concentrations of 0.3-1 mg ml⁻¹, accounting for approximately 15% of their total nitrogen (Ziegler, 1975). Considerable amounts of

another ureide, citrulline, which was found but not quantified in the phloem exudate of *Cucurbita* (Kating and Eschrich, 1964) were detected in *Beta*, *Carpinus*, *Alnus*, and *Juglans* (for literature see Ziegler, 1975). Putrescine, a possible decarboxylation product of ornithine, was present at low concentrations of 15-70 $\mu\text{g ml}^{-1}$ in *Quercus*, *Robinia*, *Tilia*, and *Salix* while canavanine, an important nitrogen storage compound in legumes, was found at 300-400 $\mu\text{g ml}^{-1}$ in *Robinia* (for literature see Ziegler, 1975).

Most procedures which are capable of detecting amino compounds at low concentrations within very small quantities of exudate usually emphasize the detection of the protein amino acids and occasionally a few of the very common nonprotein compounds, such as γ -aminobutyric acid. Since most analyses do not routinely include detection of other amino compounds, these less common nitrogenous compounds may be more widely distributed than they are considered to be.

Organic Acids in the Translocation Stream:

Although detailed analyses of the carbohydrates and nitrogenous compounds in phloem exudate have been performed, there are few similar reports for the organic acids. Malate is often the predominant anion, occurring at concentrations much higher than those of the other acids (Table 2). This difference in concentration may be the reason why some studies, especially the early ones in which paper

chromatography was used, failed to detect a variety of acids. Malate was the only organic acid detected in *Ricinus* exudate by Hall and Baker (1972) but a more recent analysis of the same species found 10 acids present (Richardson, Baker and Ho, 1982). While malate in these studies of *Ricinus* occurred at concentrations greater than 2 mg ml^{-1} , a third study of *Ricinus* exudate, however, detected only $100\text{--}200 \text{ } \mu\text{g ml}^{-1}$ (Mengel and Haeder, 1977). Similarly, citrate, tartrate, and possibly oxalate, but not malate, were located in willow exudate (Peel and Weatherley, 1959). Thus the species or the environmental conditions may determine the organic acid composition.

There appears to be selective translocation of the organic acids. Labelled glycerate synthesized in the leaf of soybean was not found as a translocate (Nelson et al., 1961). Similarly, although labelled malate was detected in the tissue extracts of *Cucurbita* petioles as early as 5 minutes after exposure of the leaf blade to $^{14}\text{CO}_2$, labelled glycerate was still not detected after 15 minutes, although both organic acids were simultaneously labelled in the leaf (Webb and Gorham, 1964).

Table 2. Concentration of organic acids in phloem exudate analyses reported in the literature

Organic Acid	% on a molar basis		
Malate	82.8	65.8	54.1
Succinate	3.5	11.3	22.2
Citrate	3.1	7.6	23.7
Lactate	1.7	1.2	NR
Pyruvate	0.4	5.8	NR
Oxalate	0.2	4.3	NR
Malonate	5.6	0.3	NR
Fumarate	0.6	0.3	NR
2-Oxoglutarate	1.9	3.1	NR
Glutarate	0.2	0.3	NR
Total	100.0	100.0	100.0
Source	<i>Cucurbita</i> ^a	<i>Ricinus</i> ^a	<i>Oryza</i> ^b

Notes: NR = Not reported

^aRichardson, Baker and Ho, 1982
(Data recalculated)

^bChino, Hayashi and Fukumori, 1986
(Data recalculated)

Organic acids accounted for 2.5% of the ^{14}C activity recovered from exudate in legume fruit tips (Pate, Sharkey and Lewis, 1974) and 3.2% in *Nicotiana glauca* stem exudate (Hooking, 1980). In contrast, the label recovered from the organic acid fraction of Cucurbit exudate was approximately 29% (Richardson, Baker and Ho, 1984). This prompted the authors to suggest that in the Cucurbits the organic acids together with the amino acids could be responsible for the translocation of up to one-half of the newly assimilated carbon.

Organic Phosphate Compounds in the Translocation Stream:

Many forms of phosphate have been found in phloem exudate including nucleotides and their derivatives, such as ATP (Hall and Baker, 1972; Ziegler, 1975; Mengel and Haeder, 1977) which occurred at a concentration 5 times that in most other plant tissues (Bielecki, 1973). In addition, 80% of the adenosine nucleotides and 87% of the guanosine nucleotides in Cucurbit phloem exudate were in the "energy rich" triphosphate forms (Lehmann, 1981). This combined with the fact that the ATP concentration remains relatively constant during long periods of exudation (Ziegler, 1975) suggests that the sieve tubes are very metabolically active although the role of the companion cells in supplying the ATP is unknown.

Phosphorylated sugars were absent from the exudate of willow (Peel and Weatherley, 1959) but present in the exudate of many other species including *Cucurbita pepo* (Ziegler, 1975; Lehmann, 1981). Sugar phosphates found labelled with ^{14}C in the leaf of soybean were not detected as translocates (Nelson et al., 1961) and their role in transporting carbon from a labelled Cucurbit leaf was considered negligible by Webb and Gorham (1964). When $\text{K}_2^{32}\text{PO}_4$ was applied to the leaf of *Cucurbita maxima*, however, a variety of labelled phosphate compounds was quickly detected in the sieve tube exudate (Bieleski, 1969). Bieleski (1969, 1973) suggested that inorganic phosphate is the transport form of phosphorus in the phloem and that ATP and the other phosphate esters were located in non-mobile pools in the phloem and labelled by the incorporation of the mobile phosphorus. Considering that the enzymes required for the synthesis of phosphate esters are present in the sieve tubes (Bieleski, 1973), the organic compounds present in the exudate may not be mobile but instead may be synthesized locally from translocated photosynthetic products. Their primary function may not be to provide substrates for the metabolism of other translocates but rather to provide for the requirements of the sieve tubes themselves (Ziegler, 1975).

Inorganic Constituents in the Translocation Stream:

Potassium is usually the predominant cation in phloem exudate (Ziegler, 1975) as is the case in the exudate of *Lupinus albus* fruit (Pate, Sharkey and Lewis, 1974); inflorescent stalks of *Yucca* (Tammes and Van Die, 1961); and the stem exudate of *Nicotiana glauca* (Hocking, 1980), *Ricinus* (Hall and Baker, 1972), rice (Fukumorita and Chino, 1982), *Salix* (Peel and Weatherley, 1959), and *Cucurbita maxima* (Richardson, Baker and Ho, 1982). Sodium, magnesium, and calcium are found at much lower levels so that the ratio of potassium to the other cations is high (Hall and Baker, 1972; Pate, Sharkey and Lewis, 1974; Hocking, 1980).

Phosphate is usually the most abundant anion in phloem exudate and in combination with high levels of potassium is responsible for the alkaline pH (Ziegler, 1975). In several studies on *Ricinus*, *Nicotiana glauca*, *Cucurbita maxima*, wheat, and rice, however, chloride and not phosphate was found to predominate (Hall and Baker, 1972; Pate, Sharkey and Lewis, 1974, Hocking, 1980; Chino, Hayashi and Fukumorita, 1986; Hayashi and Chino, 1986b).

It is important to note that the intracellular levels of those ions which form phosphate salts of low solubility (e.g. calcium, barium and lead) must be relatively low and closely regulated. The concentration of calcium in *Cucurbit* exudate has recently been reported as approximately 1 mM (Sabnis and McEuen, 1985), similar to that found by

Richardson, Baker and Ho (1982). About one to two-thirds of this was bound to low molecular weight ligands, leaving the level of free calcium to be about 0.4-0.8 mM (Sabnis and McEuen, 1985). This is substantially higher than the 100 nM levels of free calcium found in the most cells (Thomas, 1985), but may include apoplastic calcium from cut cell walls in addition to calcium confined to cellular compartments.

Nitrate is usually not present in exudate (Tammes and Van Die, 1964; Hall and Baker, 1972; Pate, Sharkey and Lewis, 1974; Hocking, 1980) and this characteristic is often used to argue that the exudate is phloem derived (e.g. Fellows, Egli and Leggett, 1978). Having found nitrate in their Cucurbit exudate led Richardson, Baker and Ho (1982) to suggest that it represented contamination by the xylem. Nitrate has also been detected in the phloem exudate of rice (Chino, Hayashi and Fukumori, 1986) and wheat (Hayashi and Chino, 1986b).

Control of Translocation:

Pate, Sharkey and Lewis (1975) have identified four processes which contribute to the presence of a solute in the phloem sap:

- 1) direct loading of photosynthetic products into the phloem. The presence of labelled compounds in the exudate after exposure of the leaf to $^{14}\text{CO}_2$

represents the bulk transfer of photosynthetically fixed carbon.

- 2) direct transfer from xylem to phloem without any change in form. The phloem contains radioactive asparagine after ^{14}C -asparagine is fed via the xylem. Similarly, some inorganic elements, such as potassium, are found in higher concentrations in the phloem than in the xylem.
- 3) reduction or metabolic conversion during transfer from xylem to phloem. Nitrate, commonly present in high concentrations in the xylem, is not located in phloem while the products of nitrate reduction are.
- 4) mobilization of storage products in the stem or leaf. Leaf senescence results in an increase in the total amino acids as well as an increase in the spectrum of amino acids found in the phloem (Peel and Weatherley, 1959; Pate et al., 1979).

Any change in the plant or its environment which affects these processes will in turn affect the composition of the exudate. Various factors have already been demonstrated to affect the composition and/or concentration of the transport substances.

The developmental age of the plant affects translocation presumably through alterations in the source to sink ratio. Fruiting plants assimilate and export more ^{14}C

than those which are not fruiting while at the same time levels of radioactivity recoverable from the vegetative tissues are unchanged (Barrett and Amling, 1978). The comparison of studies involving the same species may consequently be hindered by differences in the developmental stage of the plants. Removing sugar cane leaves was found to increase the translocation of ^{14}C and the removal of those above the fed leaf produced a greater increase than the removal of those below (Hartt, 1963). This illustrates that differences can even occur among vegetative plants of varying ages. Perhaps more important is the fact that many studies on translocation utilize plants which are pruned in one fashion or another. Not only do experimental procedures in which plants are pruned have the potential to affect the exudate composition but the effects may be quite variable depending on the extent and site of the pruning.

It is not surprising that translocation is also dependent on the culture conditions of the plants. The importance of water availability and nitrogen source have already been discussed. The other obvious influence is nutritional status. Increased potassium was found to enhance the export of photosynthates from the leaves of *Ricinus communis* (Mengel and Haeder, 1977). Although the concentration of most compounds remained unchanged, that of potassium, raffinose, glucose-6-phosphate, fructose-6-phosphate, glutamine, glutamic acid, serine, leucine,

aspartic acid, and malic acid increased significantly. The authors suggested that potassium affected the production of ATP which in turn resulted in increased phloem loading.

Translocation is also affected by low temperatures (Kanahama and Hori, 1980), especially root temperature (Hartt, 1963). Other environmental growth conditions, such as the concentration of oxygen and carbon dioxide, also play a role in translocation. Increased oxygen concentration around the source leaf resulted in increased pool sizes of amino acids in the leaf and petiole of *Salvia splendens* as well as an increase in the label incorporated into the amino acids and a decrease in starch in the leaf (Madore and Grodzinski, 1984). Higher levels of carbon dioxide did not increase transport in soybean although there was an increase in assimilation (Nelson et al., 1961). In dwarf cucumber, however, an enriched environment of carbon dioxide appeared to increase the levels of endogenous starch and the amount of photoassimilates exported and to alter the distribution of ^{14}C within them (Madore and Grodzinski, 1985).

The diurnal periodicity also affected the composition of exudate obtained from *Nicotiana glauca* (Hocking, 1980). Potassium was found to fluctuate in phase with the sugar, both increasing from dawn to noon and then decreasing continuously. The amino acids behaved similarly, although their maximum and minimum concentrations were three hours behind the others. Glutamine, serine, and proline were the

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amino acids which displayed periodicity; the other amino acids did not.

Several general conclusions about the translocation stream can be made from exudate analysis. It appears to be alkaline and some solutes, such as potassium, are consistently high in concentration while others, including calcium and nitrate, are low. The sugar content is usually high with the exception of species containing large amounts of P-protein. Sugars, amino acids and organic acids may all contribute to the distribution of carbon throughout the plant with possibly the relative importance of these fractions differing among species. As to the variations obtained, it is possible that these result from the experimental sampling method, genetic variability, growth conditions or flexibility in the transport system itself. The interactions among the factors influencing the composition of the translocation stream are complex and thus difficult to identify and study individually. Progress in understanding the mechanism and control of translocation continues to be hampered by the lack of a method which conclusively samples only the mobile constituents.

MATERIALS AND METHODS

Chemicals:

^{14}C -sodium carbonate was obtained from Atomic Energy Canada; ^{14}C -fructose-6-phosphate and ^{14}C -sucrose were obtained from New England Nuclear. The scintillation solution used to assay radioactivity in aqueous samples was 10 ml of toluene:triton X-100 (2:1 by volume) containing 0.5% (w/v) PPO; 15 ml of 0.5% (w/v) PPO in toluene was used for nonaqueous samples. NCS Tissue Solubilizer was from Amersham/Searle Corporation. Prescored Avicel Uniplates (20x20 cm, 250 μm cellulose) were obtained from Analtech Inc. for thin layer chromatography. A kit for the determination of L-malate by the UV method and a suspension of malate dehydrogenase (from pig heart) in ammonium sulfate solution were purchased from Boehringer Mannheim. Sephadex G25 Medium (50-150 μl) was bought from Pharmacia. Bovine albumin (fraction V powder; 96-99% albumin), Trizma HCl, and Trizma Base were obtained from Sigma and the latter two compounds used to prepare Tris buffer (Sigma, 1967). Otherwise reagent grade chemicals were used wherever available.

- Plant Material:

Seeds of *Cucurbita pepo* var. *Melopepo torticollis* Bailey, Early Prolific Straight-Necked Squash, were germinated in vermiculite and 7 days after planting the seedlings were transferred to individual pots filled with perlite. The plants were watered twice a day, one watering consisting of a modified Hoagland's solution (5mM KNO_3 , 10mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 1mM KH_2PO_4 with 7.2 mg $\text{Fe}(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$, 15 μl Versenol 120 (Dow Chemical Co.) and 1.0 ml Arnon's A-5 minor element solution per litre) prepared with deionized water.

Both germination and growth occurred in a growth chamber at $25^\circ\text{C} \pm 1^\circ\text{C}$ with a 14 hour daylength composed of three periods: an initial hour of light from incandescent lamps supplying $200 \mu\text{E sec}^{-1} \text{m}^{-2}$, 12 hours of light from incandescent and fluorescent lamps supplying a total of $450 \mu\text{E sec}^{-1} \text{m}^{-2}$ between 400 and 700 nm, and a final hour of light from incandescent lamps supplying $200 \mu\text{E sec}^{-1} \text{m}^{-2}$, measured by a L1-190S quantum sensor, Lambda Instruments.

Plant samples were collected between 4 and 6.5 hours after the first light period commenced. The plants were 21 to 28 days old inclusive, unless otherwise specified.

The most recently matured leaf and/or its petiole or its petiolar exudate was used in all experiments. The leaves were numbered consecutively from the first one to appear and leaves 3, 4, and 5 were the ones generally used

in the experiments. The developmental stage of each leaf was assessed visually.

All experiments were done on unaltered plants and these have been designated "intact plants". Many experiments were also performed on plants which had been pruned by the removal of their cotyledons and all the mature leaves and associated petioles except for the most recently matured leaf. In addition, the semimature leaves and their petioles were also removed, leaving only the emerging and very immature leaves intact. These plants have been designated "pruned plants". The pruned plants were placed back in the growth cabinet for 22 hours before being used in experiments.

Measurement of Leaf Growth:

The length and width of leaves 3, 4, 5, and 6 from 10 plants were measured daily between 5 to 7 hours after the start of the photoperiod. The length of each leaf blade was measured from the apex to the junction of the petiole at the base of the lamina and the width was measured at the widest point of the lobes lateral to the junction of the petiole (Figure 2). The leaf area was calculated as the area of the triangle without a correction for that part of the leaf below the base of the triangle.

Figure 2. Location of leaf measurements used in monitoring leaf growth in *Cucurbita pepo*.

Arrows indicate length and width axes

Labelling with $^{14}\text{CO}_2$:

Plants were transferred to a laboratory fumehood and illuminated with water-filtered incandescent lamps supplying $100 \mu\text{E sec}^{-1} \text{ m}^{-2}$. After a 10 minute equilibration period the petiole of the most recently matured leaf was enclosed in a layer of aluminum foil and the blade enclosed in a polyethylene bag sealed at the top of the petiole with a paper covered wire. Fifty μl of ^{14}C -sodium carbonate (specific activity $1.6 \mu\text{Ci } \mu\text{l}^{-1}$) and excess 5N sulfuric acid were placed on the end of a 50 ml glass syringe plunger and kept separated by a strip of petroleum jelly. The barrel was carefully lowered over the plunger and the needle immediately inserted into the bag. The syringe was quickly shaken to generate $^{14}\text{CO}_2$ and pumped for 15 seconds before removing the needle from the bag. After exposing the leaf blade to $^{14}\text{CO}_2$ for one minute, the bag was removed and then the petiole uncovered. Samples from the plant were either collected 19 minutes after labelling or, having been left undisturbed for 9 minutes, the plant was placed back in the growth cabinet and samples collected 24 hours later.

Extraction of Plant Tissue:

Sliced leaf blades and 2 cm midsections of petioles were quickly plunged into boiling 80% ethanol for 1 minute. The tissue was removed from the ethanol and ground using a mortar and pestle with additional ethanol. The two ethanol

extractions were combined and the tissue further extracted for 1 hour in a 60°C water bath before being vacuum filtered through Whatman #1. During labelling experiments 50 μ l replicates of each filtrate were assayed for radioactivity in a LKB RackBeta Liquid Scintillation Counter (Model 1217) which had been quench corrected to measure DPM. The soluble compounds in each filtrate were then fractionated by ion exchange chromatography (described below) and the filtered residues dried for several weeks over phosphorus pentoxide in a desiccator.

Hydrolysis of Ethanol Insoluble Residues Containing ^{14}C From Leaves and Petioles:

Replicate samples weighing 5 mg or less, depending on the amount of residue, of the dried tissue were put into small test tubes. These were each extracted for 30 minutes in 2 ml of 80% ethanol in a 60°C water bath. The residues were centrifuged at 730xg for 5 minutes and the supernatants discarded. The pellets were extracted two more times and after the final centrifugation 1 ml of 1N hydrochloric acid was added to each pellet. The tubes were sealed and heated at 100°C for 3 hours, cooled to room temperature and centrifuged at 730xg for 15 minutes. Fifty μ l replicates of each supernatant were assayed for radioactivity. The pellets were washed with 2 ml of 20% ethanol and centrifuged at 730xg for 15 minutes. The supernatant was discarded and

the pellet washed two more times. After the final centrifugation, the pellet was transferred to a scintillation vial and the radioactivity assayed. Blanks were prepared using unlabelled dried petiole and leaf blade tissue.

Collection of Exudate:

A single transverse cut was made through the midpoint of the petiole of a recently matured leaf using a new breakable double-edged razor blade (e.g. Tatra blade available from J.B.EM Services, Montreal) which had been soaked in xylene and ethanol. Immediately after the tissue was cut the detached portion was blotted on Whatman #1 filter paper and the exudate droplets which formed on top of the severed vascular bundles of the detached tissue were collected into a microcapillary pipette of known volume and constant internal diameter (Dade Diagnostics, Inc.). Under the dissecting microscope the droplets were observed to emerge from both the internal and external phloem, eventually fusing to form one droplet on each vascular bundle. All the droplets which emerged after blotting the single cut were collected and the volume determined from the length in the pipette. The exudate was then expelled into ice-cold, 50mM 2-mercaptoethanol, less than 60 seconds after the tissue was cut. The exudate in 2-mercaptoethanol was held at 0°C while the exudate from several other plants was added to it. The bulked exudate was then placed in a

boiling water bath for 5 minutes, cooled to room temperature and left at -18°C for at least 30 minutes. The sample was centrifuged at $730\times g$ for 5 minutes and the supernatant removed. The pellet was resuspended in 2 ml of 80% ethanol and the centrifugation repeated. Both supernatants were combined and in labelling experiments 50 μl replicates of the combined supernatant were assayed for radioactivity. The combined supernatant was fractionated by ion exchange chromatography (described below). The pellets from labelling experiments were solubilized (described below) and assayed for radioactivity.

For some amino acid determinations exudate was collected into methanol and diluted to a final concentration of 15% methanol and for most enzyme assays exudate was collected into buffers. The details of these collection media are described below.

Solubilization of Exudate Pellets Containing ^{14}C :

Each pellet was extracted for 30 minutes in 2 ml of 80% ethanol in a 60°C water bath. It was centrifuged at $730\times g$ for 5 minutes and the supernatant discarded. The extraction was repeated two more times and after the final centrifugation the pellet was transferred with 80% ethanol to a 20 ml scintillation vial and taken to dryness under a stream of air in a 45°C water bath. Fifty μl of water and then 500 μl of 0.6N NCS Tissue Solubilizer in toluene were

added to the residue. The vial was tightly capped and placed in a 45°C water bath for 2 hours during which time it was occasionally swirled. The vials were cooled at 25°C for 1 hour before the aqueous scintillation solution was added. To reduce the chemiluminescence the vials were stored in the dark at 25°C for 24 hours before the radioactivity was assayed. - Blanks were prepared by omitting the plant material.

Ion Exchange Chromatography:

Batches of Rexyn 101 (H⁺ form; 16-50 mesh) and BioRad AG1-X8 (acetate form; 100-200 mesh) were prepared in advance by packing large columns with a slurry of each resin and washing each with 10 volumes of distilled water. Miniature columns for each sample were prepared by packing a 1 ml bed volume of each resin into Pasteur pipettes. Each column was then rinsed with approximately 30 ml of distilled water. The entire soluble extract from a petiole or exudate collection was reduced to less than 1 ml under a stream of air in a 45°C water bath while a 3 ml portion of each leaf extract was similarly reduced. Each reduced sample was loaded onto a column of Rexyn 101 resin which was connected directly to a column of AG1-X8 resin. Two rinses of the sample vial with 1 ml of distilled water were also applied to the columns, followed by 15 ml of distilled water. The material which passed through both columns was collected as

the "neutral" fraction. The columns were then disconnected from each other and the compounds adhering to each eluted. The Rexyn 101 resin was eluted with 10 ml of 4M ammonium hydroxide and the eluate was collected as the "basic" fraction. The AG1-X8 resin was eluted with 10 ml of 3N formic acid and the eluate collected as the "acidic I" fraction. All flow rates were 10.5 ml min^{-1} . In some experiments the AG1-X8 resin was further eluted with 10 ml of 6N formic acid and then 10 ml of 2N hydrochloric acid, the eluates being collected as "acidic II" and "acidic III" fractions. All fractions were evaporated to dryness under a stream of air in a 45°C water bath.

Analysis of ^{14}C in the Neutral Fraction using Paper Chromatography:

The dried neutral fraction was taken up in 50 μl of water and spotted on Whatman #4 chromatography paper. The sample vial was rinsed with an additional 50 μl of water and this superimposed on the spot. A mixture of known sugars was also spotted to provide a marker strip. The compounds were separated by descending chromatography for 14 hours in n-propanol:ethyl acetate:water (7:1:2 by volume) (Albon and Gross, 1952). The chromatograms were dried and redeveloped for another 14 hours using fresh solvent of the same composition. The chromatogram was cut into appropriate strips and those with radioactivity scanned on a Packard

Radiochromatogram Scanner (Model 7201) attached to a Packard Recording Ratemeter (Model 385). The paper strips were cut to correspond with the peaks of activity in the scans and the pieces were assayed for radioactivity using nonaqueous scintillation solution. The marker strips were visualized either by being dipped in alkaline silver nitrate reagent (Trevelyan, Procter and Harrison, 1950) and fixed in 5% sodium thiosulfate or by being dipped in naphthoresorcinol (Bryson and Mitchell, 1951). The unknown compounds in the plant extracts were identified by comparing their R_f values, with those of the marker sugars.

Quantitative Analysis of Sugars:

Areas of the paper chromatograms corresponding to known sugars on the marker strips were cut into pieces and put into 20 ml vials with either 15 or 20 ml of water. The vials were capped and swirled gently at 5 minute intervals for a total of 20 minutes. The solutions were filtered through glass wool and the filtrates immediately analyzed by the method of Dubois et al., 1956. Blank pieces of developed chromatograms were also eluted as controls. In the assay 2 ml of the filtrate was placed in a test tube (i.d. 16-20 mm) and 1 ml of 5% phenol was added, followed by 5 ml of 96% sulfuric acid and the reagents rapidly mixed. Each tube was left at room temperature for 10 minutes, then shaken and transferred to a 28°C water bath for 20 minutes.

The absorbances of the solutions were measured at 490 nm with a Bausch & Lomb Spectronic 1001 and corrected for absorbance due to the contaminants eluted from the paper. The amount of sugar was calculated from a standard curve of 0-80 μ g of each sugar. Due to the incomplete separation of galactose, glucose, and fructose on the chromatograms, the area containing the three monosaccharides was eluted as one sample and the total amount calculated from the average of the three absorption coefficients.

Analysis of ^{14}C in the Acidic Fraction using Thin Layer Chromatography:

The dried acidic fractions were taken up in 40 μ l of water and spotted on prescored TLC plates which had been stored in a humidity chamber for at least 16 hours. The sample vial was rinsed with additional water and this superimposed on the spot. A mixture of known organic acids was also spotted to provide a marker strip. The plates were developed in chloroform:tertiary amyl alcohol:formic acid (98%):water (2:2:1:2 by volume), a modification of Ranson (1955). Fresh solvent was prepared for each plate and the plate was developed twice, each time with a portion of the organic phase of the solvent. The aqueous phase was used to wet filter paper lining the chromatography tank while 15 ml of the organic phase was placed in a trough at the bottom of the tank. The tank was equilibrated for 20 minutes prior to

insertion of the plate. The plate was developed until the solvent ascended 15 cm. The plate was dried in the fumehood for 30 minutes, during which time another 15 ml of the solvent was put in the trough and the tank equilibrated for 20 minutes. The plate was then placed in the tank and redeveloped for 15 cm and dried in the fumehood overnight. After being dried the plate was split into strips and the strips scanned on a Packard Radiochromatogram Scanner (Model 7201) attached to a Packard Recording Ratemeter (Model 385). Sections of the TLC plates corresponding to the peaks of activity in the scans were scraped with a razor blade. The scrapings were placed in 20 ml scintillation vials and counted for radioactivity using the nonaqueous scintillation solution. The marker strips were sprayed with 0.012% (w/v) 2',7'-dichlorofluorescein (DCF) in 96% ethanol (Rasmussen, 1967) and viewed under ultraviolet light. Radioactive compounds were identified by comparing the peaks of activity in the scans with those of the marker acids since DCF caused colour quenching in assaying radioactivity (Whereat, Snyderman and Barnes, 1968).

Quantitative Analysis of Organic Acids:

Leaf blades, whole petioles and exudate from intact plants were extracted as previously described. Malic acid in the extracts was measured using a kit from Boehringer Mannheim. The procedure followed was exactly as directed by

the supplier and was based on the method of Möllering (1974). Malate is oxidized to oxaloacetate by NAD in the presence of malate dehydrogenase (MDH):

To displace the equilibrium completely to the right the oxaloacetate is converted to aspartate in the presence of glutamate and glutamate oxaloacetate transaminase (GOT):

The amount of NADH formed is stoichiometric with the concentration of malic acid. The increase in NADH absorbance was measured at 340 nm with a Bausch & Lomb Spectronic 1001. The other organic acids were semiquantified by TLC as described above except that the second portion of solvent contained 0.012% (w/v) DCF to ensure an even distribution of fluorescence. The size and density of their spots of absorption under UV light were visually compared to those of marker acids run at known concentrations.

Identification of Sugar Phosphates:

A prescored TLC plate was spotted with 1 μg and 5 μg amounts of glucose-6-phosphate, 1 μg and 5 μg amounts of fructose-1,6-phosphate, 0.5 μl and 1.0 μl amounts of ^{14}C -fructose-6-phosphate (specific activity 9.65 $\mu\text{Ci ml}^{-1}$) and a mixture of known organic acids. The plate was developed as described above, split into strips, the one

containing ^{14}C scanned as described above and the others sprayed with 0.012% (w/v) DCF in 96% ethanol.

A 100 μl solution containing 500 μg each of fructose-1,6-diphosphate and glucose-6-phosphate was fractionated using the system of ion exchange described above. The neutral, basic and acidic I fractions were evaporated to dryness under a stream of air in a 45°C water bath, taken up in water, spotted on a TLC plate, and developed as described above. The plate was sprayed with 0.012% (w/v) DCF in 96% ethanol.

A 2 μl solution of ^{14}C -fructose-6-phosphate (specific activity 9.65 $\mu\text{Ci ml}^{-1}$) was added to 500 μl of water and the solution applied to a 1 ml bed volume of AG1-X8 resin (acetate form) and washed with 15 ml water. The column was sequentially eluted with 10 ml of 3N formic acid, 10 ml of 6N formic acid and 10 ml of 2N hydrochloric acid. The three acidic fractions and the initial wash were each taken to dryness in a 40°C water bath under a stream of air, resuspended in water and the radioactivity in each assayed.

Identity of the Unknown Acid, Compound A:

Areas of TLC plates containing marker spots of malonic, maleic and 2-oxoglutaric acid as well as compound A from exudate samples and which had been developed in solvent containing DCF as described above were each scraped and put in 20 ml vial with 2 ml of water and gently swirled at

5 minute intervals for a total of 15 minutes. Each mixture was transferred to a centrifuge tube using an additional 3 ml of water and centrifuged at 730xg for 10 minutes. Each supernatant was removed with a pipette and reduced to dryness at 25°C under a stream of air. Each dried eluate was resuspended in 50 µl of water and spotted on a TLC plate. The plate was developed in one dimension in ethanol (96%):ammonium hydroxide (28%):water (8:2:1 by volume) (Goebell and Klingenberg, 1962). Each plate was dried overnight in the fumehood and then developed twice in the second dimension at right angles to the first solvent using the chloroform solvent with DCF as described above. After being viewed under ultraviolet light, if the contrast of the acids required improvement, the plates were sprayed with universal indicator (Harleco) and re-examined under ultraviolet light.

Analysis of ^{14}C in the Basic Fraction using Paper Chromatography:

The distribution of ^{14}C in the basic fractions was determined by autoradiography of paper chromatograms. The dried basic fractions of leaf blades, petioles, and exudate were taken up in either 50 or 100 µl of water and spotted on 25 cm² sheets of Whatman 3MM. The chromatograms were developed in two dimensions at right angles to each other using the solvents of Webb and Gorham (1964): n-butyl

acetate:glacial acetic acid:water (3:3:1 by volume) and pyridine:ammonium hydroxide (28% NH₃):2-methyl-1-propanol (4:2:1 by volume). Each solvent was allowed to travel 19 cm above the origin and the sheets were dried overnight in the fumehood after each solvent. The chromatograms were exposed to Kodak X-Omat AR film and left at room temperature for various periods ranging from several days to several months. The x-ray film was processed in Kodak GBX Developer and Replenisher and Kodak GBX Fixer and Replenisher according to the instructions from the manufacturer. A running water rinse was used before and after the fixer solution. The locations of radioactive spots were compared to known amino acids similarly run on 2-D chromatograms and dipped in 0.1% ninhydrin in acetone.

Quantitative Analysis of Amino Acids:

The dried basic fractions of leaf blades and petioles were taken up in 15% methanol and 10 μ l volumes were analyzed by HPLC (Waters Analyzer Model 721; RESOLVE™ 5 μ m C₁₈ 3.9 mm x 15 cm column). Automated pre-column derivatization of the samples with o-phthalaldehyde (OPA) (Roth, 1971) took place in a WISP sample injector (Waters Scientific, Mississauga) using the AUTO.TAG™ procedure. The dried basic fraction of one exudate collection was similarly analyzed while a second one was analyzed by both

HPLC and a Beckman 119BL Amino Analyzer, run in physiological mode with ninhydrin detection.

Exudate collected into 15% methanol was centrifuged at 730xg for 5 minutes. The pellet was resuspended in 15% methanol and the centrifugation repeated. Both supernatants were centrifuged again and the supernatants from these final centrifugations were combined. Without undergoing ion-exchange chromatography a 10 μ l volume of the supernatant was analyzed by HPLC, another 10 μ l volume was analyzed by HPLC without derivatization and the remainder of the supernatant was combined with 5-sulfosalicylic acid (final concentration 25 mg ml⁻¹) to precipitate any proteins present. The solution was clarified by centrifugation and the supernatant adjusted to pH 7 with 1N sodium hydroxide (final concentration 0.25 N) before being analyzed by HPLC.

Another exudate collection in 15% methanol was centrifuged at 15,600xg for 5 minutes and the supernatant taken to dryness over sodium hydroxide pellets under reduced pressure. The sample was resuspended in 300 μ l of water acidified to pH 2 with 6N hydrochloric acid. A 50 μ l aliquot was combined with 20 μ l of 2 N sodium hydroxide and 10 μ l of the solution analyzed by HPLC. The remaining 250 μ l was combined with an equal volume of concentrated hydrochloric acid, sealed in a small test tube, and heated at 105°C for 4 hours. The hydrolysate was taken to dryness over sodium hydroxide pellets in a desiccator, resuspended

in 250 μ l of 10% methanol, adjusted to pH 7 with 30 μ l of 0.1 N sodium hydroxide and then 10 μ l was analyzed by HPLC.

Analysis of α -Galactosidase and Invertase Activity in Exudate:

Exudate was collected from plants aged 3 to 5 weeks. Petioles from several mature leaves per plant were cut once at their midpoint.

Exudate for the galactosidase assay (modification of Thomas and Webb, 1977) was collected into either 0.2M McIlvaine buffer pH 7.5 (Dawson et al., 1969) or 0.2M succinic acid-NaOH buffer pH 5.5 (Dawson et al., 1969) each containing 10 mM 2-mercaptoethanol and kept cold on ice. One hundred μ l of 0.6% (w/v) p-nitrophenyl- α -D-galactopyranoside (PNPG) was added to an equal volume of the exudate-buffer solution and incubated for 30 minutes in a 35°C water bath. The reaction was stopped by adding 2.8 ml of 5% (w/v) sodium carbonate and the change in absorbance measured at 400 nm using either a Hitachi Perkin-Elmer 139 UV-Vis Spectrophotometer or a Bausch & Lomb Spectronic 1001. In the control tubes the sodium carbonate was added prior to the PNPG. The assay was performed four times at pH 7.5 and three times at pH 5.5.

Exudate for the invertase assays was similarly collected with the exception that 2-mercaptoethanol was not included and the succinic acid-NaOH buffer was pH 5.4.

Five μl of ^{14}C -sucrose (specific activity $24.0 \mu\text{Ci ml}^{-1}$) were added to 200 μl of the exudate-buffer solution and the tubes were incubated for 30 minutes in a 35°C water bath. The reaction was stopped with the addition of 200 μl of anhydrous ethanol. The assay tubes were put in the freezer for 10 minutes and then centrifuged at $180\times g$ for 4 minutes. As a control the exudate-buffer solution was heated for 5 minutes in a 90°C water bath before being used in the assay.

Exudate which was collected into 50mM 2-mercaptoethanol was also assayed for invertase activity using the same procedure with the following reagents: 200 μl of the exudate-mercaptoethanol solution, 200 μl of either 0.1M McIlvaine buffer pH 7.5 (Dawson et al., 1969) or 0.1M succinic acid-NaOH buffer pH 5.4 (Dawson et al., 1969) and 400 μl of anhydrous ethanol. Water replaced the exudate-mercaptoethanol solution in the control tubes.

To measure the invertase activity 100 μl of the supernatant from each assay was spotted on Whatman #4 and a standard solution containing 50 μg of sucrose superimposed on each sample. ^{14}C -sucrose and a mixture of known sugars were spotted on the chromatogram as markers. The chromatogram was developed for 18 hours as described above. The strips with radioactivity were scanned and all the strips were then dipped in fresh urea solution (Dawson et al., 1969) to visualize the sucrose markers.

Analysis of Invertase Activity in Petioles:

Mature and immature petioles from 3 week old plants were each ground at 4°C with a mortar and pestle in either 0.2M McIlvaine buffer pH 7.5 (Dawson et al., 1969) or 0.2M succinic acid-NaOH buffer pH 5.4 (Dawson et al., 1969), using a 1:4 ratio of tissue weight to buffer volume. The brei was filtered through cheesecloth and centrifuged at 12,000xg at 0°C for 20 minutes. Two hundred μ l of the supernatant (equivalent to 44-50 mg fresh weight) were assayed for invertase activity using the same procedure as for the exudate. As a control the supernatant was heated in a 100°C water bath for 10 minutes and centrifuged at 180xg for 4 minutes before being used in the assay.

PEP-Carboxylase Isolation from Leaves and Petioles:

Leaves and petioles from both intact and pruned plants were quickly weighed and ground with a mortar and pestle in 0.1M Tris buffer (pH 8.0) containing 10mM 2-mercaptoethanol, 10mM magnesium chloride, and 20% (v/v) glycerol. The brei was squeezed through 8 layers of cheesecloth and a portion of the filtered crude extract assayed for protein and enzyme activity. The remainder of the crude extract was centrifuged at 48,000xg at 0°C for 20 minutes and the supernatant brought to 30% saturation at 0°C with ammonium sulfate (Dawson et al., 1969) by the gradual addition of the salt with gentle stirring. After standing 15 minutes, the

suspension was centrifuged at 12,000xg at 0°C for 10 minutes and the supernatant brought to 60% saturation at 0°C with ammonium sulfate as described above. The precipitate from the 30-60% saturation was resuspended in a small volume of the grinding buffer. The resuspension was desalted on a column of Sephadex G25 Medium which had been previously equilibrated with grinding buffer. All procedures were done at 4°C, using prechilled glassware and solutions.

PEP-carboxylase activity in the crude extract and in the Sephadex eluate was measured by assaying 200 μ l volumes at pH 8.0 and 25°C in a total volume of 2.01 ml containing 50mM Tris buffer, 5mM 1,4-dithiothreitol, 10mM magnesium chloride, 5mM sodium bicarbonate, 0.14mM NADH, 60 units of malate dehydrogenase and 2 mM phosphoenolpyruvate (PEP). All the reagents except PEP were mixed in a cuvette. After 3 minutes the reaction was initiated by adding PEP and followed by the decrease in absorbance of NADH at 340 nm using a Bausch & Lomb Spectronic 1001. Samples were diluted to yield a linear decrease for at least 5 minutes. Control tubes contained water instead of PEP. All reagents were prepared as stock solutions except for the NADH which was prepared each day immediately prior to being used in the assays. One unit (U) of enzyme is defined as that amount which reacts with 1 μ mol of substrate per minute.

The amount of protein was determined using the Coomassie brilliant blue G assay (Bradford, 1976; Spector, 1978). Batches of the dye reagent were prepared by stirring a 0.08% (w/v) solution of SERVA Blue G in 96% ethanol for 30 minutes and then adding 2 volumes of concentrated phosphoric acid and 1 volume water and stirring for another 30 minutes. The solution was diluted 1:4 with water and filtered through Whatman #42. Two ml of the diluted, filtered dye reagent were added to a 100 μ l sample in a cuvette and after standing 5 minutes the absorbance change was measured at 590 nm using a Bausch & Lomb Spectronic 1001. The amount of protein was calculated from a standard curve of 0-20 μ g bovine serum albumin (BSA). To correct for changes in the dye reagent a new standard curve was prepared daily.

Statistics:

Lotus 1-2-3 Version 2.01 (Lotus Development Corporation) was used to produce the following analyses of data:

- 1) linear regressions of BSA and sugar standard curves
- 2) linear regressions of PEP-carboxylase assays
- 3) means and their standard errors
- 4) tests of significance using a significance level of 5%. The PEP-carboxylase data contained equal sample sizes and the Student-Newman-Keuls test was

used (Rohlf and Sokal, 1969; Sokal and Rohlf, 1969). The radioactivity data contained unequal sample sizes and after the GT2-, T'-, and Tukey-Kramer Methods were performed the latter consistently yielded the lowest Mean Significant Difference and hence was used to determine the statistical significance (Rohlf and Sokal, 1981; Sokal and Rohlf, 1981).

RESULTS

Collection of Exudate:

Preliminary experiments (data not shown) indicated that not blotting the cut petiole before collecting exudate increased the amount of glucose and fructose in the exudate. The petiole contains large amounts of these monosaccharides (Cooil, 1941; J.A. Webb, unpublished); thus, it is assumed that the increase of these sugars in the exudate results from their presence in the cut parenchyma cells or exposure of the exudate to hydrolytic enzymes. In order to reduce contamination from extraneous compounds and enzymes which may be present in the parenchyma cells it was decided to blot the tissue with filter paper as quickly as possible before collecting the exudate. The initial exudate which was removed during the blotting process was consequently lost.

Preliminary experiments also suggested that making multiple cuts along a petiole resulted in greater dilution of the exudate since a greater volume of exudate was obtained but the total amount of sugar in the exudate remained the same. Cutting with a double-edged breakable razor blade also produced more exudate than a heavier single-edged industrial type of blade, presumably due to less cell damage since the former is a much sharper blade. Consequently, for subsequent collections a single cut in one

petiole per plant was produced by a new double-edged razor blade, the tissue briefly blotted with filter paper, and the exudate collected. The cut petioles ceased to exude within 40 seconds of being blotted presumably due to sealing of the sieve tubes. The average amount of exudate obtained using this method was 8.2 μ l per petiole. Collections from unlabelled plants averaged 8 petioles; those from labelled plants averaged 4 petioles.

Composition of the Exudate:

The pH of the exudate (1-7% v/v) was determined using universal indicator and a pH meter. In addition, exudate droplets from cut petioles that had been blotted were placed directly on narrow range pH paper. The pH of the exudate was determined to be approximately 7.5, similar to the pH of 7.3 already reported for squash (Crafts, 1936) and to pH values found in the exudate of other plants: 7.8-8.0 in *Nicotiana* (Hocking, 1980), 7.5-8.3 in legume fruit tips (Pate, Sharkey and Lewis, 1974) and both 7.3-7.6 (Smith and Milburn, 1980) and 8.2 (Hall, Baker and Milburn, 1971) in *Ricinus*. The exudate contained an ethanol soluble fraction and an insoluble P-protein fraction.

A constant but minimal level of α -galactosidase activity was detected in the exudate. The change in absorbance in the assays after 30 minutes averaged 0.003 ± 0.001 O.D. units μ l⁻¹ of exudate at pH 7.5 and

0.001±0.000 O.D. units μl^{-1} of exudate at pH 5.5. The solutions being assayed were 32-42% (v/v) exudate at pH 7.5 and 33-35% (v/v) at pH 5.5. The greatest absorbance changes, however, did not correspond to samples with the greatest volume of exudate. Therefore, the amount of exudate did not appear to relate to the amount of enzyme present possibly because some collections were more dilute than others or because the activity observed resulted from some other factor in the assay. No α -galactosidase activity was found in Cucurbit exudate by Richardson, Baker and Ho (1982) but the phloem sap of *Fraxinus* species reportedly did contain α -galactosidase (unpublished work of Pavolina cited by Kursanov, 1963) although no details of the research were stated. Nonetheless, the enzyme is generally not considered to be present in phloem exudate (Eschrich and Heyser, 1975; Ziegler, 1975). In order to verify that the observed activity resulted from α -galactosidase and was not an artifact of the assay this analysis of *Cucurbita pepo* exudate should be repeated with larger quantities of exudate and perhaps a different assay; for example, one using labelled raffinose as the substrate.

Neither acid nor alkaline invertase activity was present in the exudate. The solutions assayed were 16% (v/v) exudate in 2-mercaptoethanol and 43% (v/v) in each buffer. Each invertase assay produced a single peak on the radiochromatogram with an R_f identical to the ^{14}C -sucrose

marker and superimposed on the sucrose which had been cochromatographed with the sample. The area of the ^{14}C -sucrose peak at pH 7.5 was 104% that of the control; at pH 5.4 it was 99%. Thus, there appeared to be no hydrolysis of the sucrose by the exudate.

Invertase activity was detected in petiole tissue at both an alkaline and acidic pH. In the mature petioles 36% of the sucrose was hydrolyzed at pH 7.5 and 49% at pH 5.4. In the immature petioles 32% of the sucrose was hydrolyzed at pH 7.5 and 58% at pH 5.4. It appears that more invertase activity occurs in the petiole under acidic conditions and that more hydrolysis may occur in the immature petioles. Alkaline invertase tends to have lower levels of activity as compared to acid invertase (Ap Rees, 1974). Extensive work from this laboratory has already been reported on the occurrence of both acid (pH optimum 4.0-5.3) and alkaline (pH optimum 7.0-8.0) α -galactosidase in this variety of squash (Thomas and Webb, 1977; Thomas and Webb, 1978; Gaudreault and Webb, 1982) and consequently, assays for this enzyme were not performed.

Distribution of Radioactivity between Ethanol Soluble and Insoluble Fractions:

The radioactivity recovered from *Cucurbita pepo* is shown in Table 3 and Figure 8. Within 20 minutes of labelling there is a large amount of ^{14}C incorporated into

the insoluble fraction of the leaf blade. The insoluble residues of the leaf blades and petioles were hydrolyzed in 1 N hydrochloric acid at 100°C for 3 hours. Under these conditions the starch would have been hydrolyzed but not the protein. Preliminary experiments with the solubilization of these residues with NCS Tissue Solubilizer indicated that only one-half as much radioactivity was released with the solubilizer as with the acid presumably due to the ability of the former to hydrolyze protein but not starch. The liberation of ^{14}C by mild acid hydrolysis indicated that there was a rapid synthesis of large amounts of starch in the leaf blade during the photoperiod. Twenty-four hours later the leaf blade displayed a significant decrease in the radioactivity in the soluble fraction (Table 3; Table 4) but the distribution of the percentage of radioactivity in each fraction (Figure 3) remained constant.

In the petiole there was minimal starch synthesis within 20 minutes of labelling but 24 hours later the radioactivity in the soluble and insoluble fractions of the petiole increased 3- and 8-fold respectively, both increases being statistically significant. Presumably this difference is due to the movement of translocates out into the cortical region (Webb and Gorham, 1965) and its subsequent metabolism. The resulting distribution of radioactivity, 62% in the soluble and 38% in the insoluble, closely resembles the profile in the leaf blade.

Table 3. Distribution of radioactivity in *Cucurbita pepo* after exposure to $^{14}\text{CO}_2$ for 1 minute. Mean $\pm$ standard error; n = number of replicates.

- a) 19 minutes after labelling intact plants
- b) 24 hours after labelling intact plants
- c) 19 minutes after labelling pruned plants

Sample	n	Total DPM Recovered per Plant	
		Soluble Fraction	Insoluble Fraction Hydrolyzed
a) Leaf Blade	9	$2.36 \times 10^7 \pm 0.29 \times 10^7$	$1.63 \times 10^7 \pm 0.33 \times 10^7$
Petiole	8	$8.04 \times 10^3 \pm 3.21 \times 10^3$	169 $\pm$ 40
Exudate	6	758 $\pm$ 126	2.8 $\pm$ 0.2
b) Leaf Blade	3	$8.46 \times 10^6 \pm 0.55 \times 10^6$	$6.04 \times 10^6 \pm 0.41 \times 10^6$
Petiole	3	$2.29 \times 10^5 \pm 0.20 \times 10^5$	$1.43 \times 10^5 \pm 0.14 \times 10^5$
Exudate	3	$6.35 \times 10^3 \pm 0.46 \times 10^3$	839 $\pm$ 105
c) Leaf Blade	6	$2.49 \times 10^7 \pm 0.11 \times 10^7$	$1.24 \times 10^7 \pm 0.09 \times 10^7$
Petiole	5	$2.88 \times 10^5 \pm 0.24 \times 10^5$	$1.61 \times 10^3 \pm 0.27 \times 10^3$
Exudate	3	$3.29 \times 10^3 \pm 0.27 \times 10^3$	11.5 $\pm$ 1.1

Notes: Blade and petiole hydrolyzed with acid
Exudate hydrolyzed with NCS Tissue Solubilizer

Figure 3. Distribution of radioactivity recovered from *Cucurbita pepo* after exposure to $^{14}\text{CO}_2$ for 1 minute.

L = leaves; P = petioles; E = exudate

- a) 19 minutes after labelling intact plants
- b) 24 hours after labelling intact plants
- c) 19 minutes after labelling pruned plants

Table 4. Statistically significant differences between pairs of means of the total radioactivity in the soluble, insoluble, neutral, acidic and basic fractions of *Cucurbita pepo*. (Data from Tables 3 and 6).

a = 19 minutes after labelling intact plants

b = 24 hours after labelling intact plants

c = 19 minutes after labelling pruned plants

Y = pairs are significantly different ($\alpha=0.05$)

N = pairs are not significantly different ($\alpha=0.05$)

Plant Site	Fraction	Pairs of Means		
		a and b	a and c	b and c
Leaf Blade	Soluble	Y	N	Y
	Insoluble	N	N	N
	Neutral	Y	N	Y
	Acidic	N	N	N
	Basic	N	N	Y
Petiole	Soluble	Y	Y	N
	Insoluble	Y	N	Y
	Neutral	N	Y	Y
	Acidic	Y	Y	Y
	Basic	Y	Y	N
Exudate	Soluble	Y	Y	Y
	Insoluble	Y	N	Y
	Neutral	N	Y	Y
	Acidic	N	N	N
	Basic	Y	N	Y

Virtually all the radioactivity in the exudate within 20 minutes of labelling was ethanol soluble. Within 24 hours this fraction showed a statistically significant 8-fold increase. There appeared to be continued transport of ^{14}C into the phloem from storage pools in the blade and/or petiole or from labelled compounds that were recirculated back into the petiole and leaf via the xylem. As in the petiole there was a significant increase in ^{14}C in the insoluble fraction within 24 hours. The ethanol insoluble residue of the exudate was completely hydrolyzed with the NCS Tissue Solubilizer, unlike that of the blade and petiole; hence, the insoluble fraction was likely composed of P-protein and possibly some starch. Starch grains visible in disrupted sieve tubes (Esau, 1969) are primarily amylopectin (Palevitz and Newcomb, 1970) and have not been detected in exudate of *Cucurbita maxima* (Eschrich, Evert and Heyser, 1971). Therefore, assuming that minimal amounts of starch are present, a continual but slow turnover of the P-protein appears to occur. Using ^{14}C -labelled amino acids applied to abraded cotyledons of *Cucurbita maxima*, Nuske and Eschrich (1976) also recovered labelled P-protein from the exudate of cut hypocotyls.

The changes seen in the leaf blades of the pruned plants (Table 3c) were not statistically significant (Table 4) and the distribution of radioactivity on a percentage basis in the leaf blade (Figure 3c) was quite

similar to that of intact plants. Accumulation of radioactivity in the petiole, however, increased 40-fold in the soluble fraction and 9-fold in the insoluble fraction. Similarly, exudate radioactivity increased 4-fold in the soluble fraction. The percentage distribution of radioactivity between the two fractions in both petioles and exudate, however, was similar to that in intact plants. There was no significant difference in the ^{14}C found in the soluble and insoluble fractions of the leaf blades of intact and pruned plants (Table 4) but the latter translocated more ^{14}C as evidenced by the significantly higher levels found in the petioles and exudate of pruned plants (Table 3c, Table 4). This increase is apparently not offset by a similar reduction in sugar and starch stores. Therefore, the leaf remaining on the pruned plant must have fixed more $^{14}\text{CO}_2$ than the equivalent leaf on the intact plant. Mayoral, Plaut and Reinhold (1985) also found that in *Cucumis sativus* the removal of all source leaves except one resulted in a 39% increase in the CO_2 fixation rate by the remaining leaf 3 days after the treatment.

Pruning enhanced the ability of the petiole to attract assimilates as significant increases in radioactivity were found there in the soluble fraction (Table 3c, Table 4). It is possible that either expansion of the petiole had been stimulated or the translocates were being metabolized and stored for future use.

Not all the dried residue of a leaf blade or petiole was hydrolyzed under the conditions used. The activity in the unhydrolyzed residues was counted but the DPM obtained (Table 5) is an estimate only, representing a minimum level of activity present since not all the ^{14}C was in contact with the scintillator. These estimates of radioactivity remaining in the tissue after acid hydrolysis suggest that a large portion of the carbon in the leaf blade is utilized in processes such as protein synthesis, lignification, cell wall expansion and thickening. This is supported by the pattern of leaf growth observed in *Cucurbita pepo* (Figures 4 and 5). The width and length were approximately equal at the youngest age when the width of the leaf blade could be accurately measured without damage due to the leaf being folded. For leaf 3 this was when the plant was 17 days old. After a period of rapid expansion which lasted about 5 days, the length and width continued to increase slowly for several days until the early chlorotic stages of senescence were observed. Hence the area of the leaf blade would be continuing to increase although the blade would be visually assessed as being fully expanded.

In the petiole ^{14}C was also incorporated into macromolecules within 24 hours (Table 5). This accumulation is not evident in short term labelling; hence, it occurs much more slowly than in the leaf blade. The accumulation was more rapid in petioles of pruned plants than intact plants.

Table 5. Minimum level of radioactivity remaining in the ethanol insoluble residues after hydrolysis.
 Mean $\pm$ standard error; n = number of replicates.

- a) 19 minutes after labelling intact plants
- b) 24 hours after labelling intact plants
- c) 19 minutes after labelling pruned plants

Sample	n	DPM
a) Leaf Blade	9	$8.16 \times 10^5 \pm 4.41 \times 10^5$
Petiole	8	33.3 ± 1.7
b) Leaf Blade	3	$1.05 \times 10^6 \pm 0.17 \times 10^6$
Petiole	3	$8.40 \times 10^4 \pm 0.44 \times 10^4$
c) Leaf Blade	6	$4.45 \times 10^5 \pm 0.30 \times 10^5$
Petiole	5	231 ± 54

2

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MicroD

Figure 4. Increase in length and width of leaf 3 in *Cucurbita pepo*.

Bars = $\pm$ standard error

Half bars = positive or negative standard error

Dotted lines = sampling period used in experiments
(21 to 28 days inclusive)

Figure 5. Increase in area of leaf 3 and leaf 4 in *Cucurbita pepo.*

Bars = $\pm$ standard error

Half bars = positive or negative standard error

Dotted lines = sampling period used in experiments
(21 to 28 days inclusive)

Distribution of Radioactivity Within the Ethanol Soluble Fraction:

The radioactivity recovered from the ethanol soluble fraction is shown in Table 6 and Figure 6. The amount of ^{14}C incorporated into the neutral fraction of the blade was significantly different between 20 minutes and 24 hours. In the petiole all three fractions differed significantly while in the exudate the neutral and basic fractions differed.

Twenty minutes after labelling over 60% of the radioactivity in the blades, petioles, and exudate of intact plants was located within the neutral fraction and the remainder was approximately equally divided between the acidic I and basic fractions. After 24 hours over 65% of the radioactivity in both the blade and petiole was found in the acidic fraction whereas in the exudate over 70% of the radioactivity was contained in the basic fraction. The exudate, it seems, must represent only a specific portion of the petiole.

There was little change in the distribution of ^{14}C within the leaf blade of pruned plants and no statistically significant differences from intact plants were found in the ^{14}C recovered from each fraction. Significantly higher levels of ^{14}C were found in the neutral, acidic, and basic fractions of the petioles; in the exudate only the neutral fraction increased. In all cases, a 1:1 ratio between the acidic and basic fractions appears to have been maintained.

Table 6. Distribution of radioactivity within the ethanol soluble fraction of *Cucurbita pepo* after exposure to $^{14}\text{CO}_2$ for 1 minute. Mean per plant $\pm$ standard error; n = number of replicates.

- a) 19 minutes after labelling intact plants
 b) 24 hours after labelling intact plants
 c) 19 minutes after labelling pruned plants

Sample	n	Total DPM In Each Fraction		
		Neutral	Acidic I	Basic
a) Leaf Blade	6	$1.51 \times 10^7 \pm 0.16 \times 10^7$	$4.98 \times 10^6 \pm 1.11 \times 10^6$	$3.13 \times 10^6 \pm 0.50 \times 10^6$
Petiole	5	$6.52 \times 10^3 \pm 2.25 \times 10^3$	$1.21 \times 10^3 \pm 0.38 \times 10^3$	$1.31 \times 10^3 \pm 0.39 \times 10^3$
Exudate	4	$4.00 \times 10^2 \pm 0.57 \times 10^2$	$1.45 \times 10^2 \pm 0.26 \times 10^2$	$1.12 \times 10^2 \pm 0.16 \times 10^2$
b) Leaf Blade	3	$1.08 \times 10^6 \pm 0.29 \times 10^6$	$4.34 \times 10^6 \pm 0.40 \times 10^6$	$1.03 \times 10^6 \pm 0.24 \times 10^6$
Petiole	3	$4.50 \times 10^4 \pm 0.73 \times 10^4$	$1.17 \times 10^5 \pm 0.09 \times 10^5$	$1.58 \times 10^4 \pm 0.19 \times 10^4$
Exudate	3	$8.47 \times 10^2 \pm 0.53 \times 10^2$	$4.42 \times 10^2 \pm 0.46 \times 10^2$	$3.13 \times 10^3 \pm 0.35 \times 10^3$
c) Leaf Blade	5	$1.46 \times 10^7 \pm 0.19 \times 10^7$	$5.54 \times 10^6 \pm 0.52 \times 10^6$	$4.81 \times 10^6 \pm 0.51 \times 10^6$
Petiole	5	$2.50 \times 10^5 \pm 0.32 \times 10^5$	$2.03 \times 10^4 \pm 0.47 \times 10^4$	$2.45 \times 10^4 \pm 0.38 \times 10^4$
Exudate	3	$2.15 \times 10^3 \pm 0.17 \times 10^3$	$3.25 \times 10^2 \pm 0.23 \times 10^2$	$3.66 \times 10^2 \pm 0.26 \times 10^2$

Figure 6. Distribution of radioactivity recovered from the soluble fraction of *Cucurbita pepo* after exposure to $^{14}\text{CO}_2$ for 1 minute.

L = leaves; P = petioles; E = exudate

- a) 19 minutes after labelling intact plants
- b) 24 hours after labelling intact plants
- c) 19 minutes after labelling pruned plants

Percent

The acidic II and acidic III fractions resulted in an increase of less than 5% of the radioactivity in the soluble fraction (Table 7). Based on the acidic I fraction, the total recovery of radioactivity from the soluble fraction was 102-107% for leaf blades and petioles in both intact and pruned plants. This dropped to 76% in the 24 hour samples. This lower recovery could be attributed to material being too strongly bound to the anion exchange resin but when the acidic II fraction was included, the recovery was only increased to 79%. Hence it suggests that within 24 hours ^{14}C progresses into cellular material which is either bound to the exchange resin by non-ionic forces or is filtered out by the resins. Considering that leaf expansion can continue after the leaf visually appears to be fully expanded these cellular components could include cell wall precursors, such as phenolics and lignin, and peptides.

Table 7. Increase in radioactivity recovered by changing the ionic strength of the anion exchange eluant.
 n = number of replicates; n.d. = not determined.

- a) 19 minutes after labelling intact plants
 b) 24 hours after labelling intact plants
 c) 19 minutes after labelling pruned plants

Sample	n	Increase in recovered DPM	
		Acidic II Fraction	Acidic III Fraction
a) Leaf Blade	4	0.4%	0.3%
Petiole	4	0.7%	1.6%
b) Leaf Blade	3	4.7%	n.d.
Petiole	3	1.4%	n.d.
Exudate	2	3.0%	n.d.
c) Leaf Blade	3	1.1%	0.6%
Petiole	3	0.5%	n.d.
Exudate	3	2.9%	n.d.

Notes: n.d. = no data

Recovery in the exudate from intact plants was 79% when based on the acidic I fraction. The recovery from pruned plants, however, was 94% and 97% with the acidic I and acidic II fractions, respectively. Since the samples from the pruned plants contained more total radioactivity than those from the intact plants the low recoveries from the latter probably represent losses due to transfers in processing such small amounts of radioactivity. The recovery from plants after 24 hours, however, was only 73% and in these samples the total activity was even greater than in the pruned plants. Hence the low recovery in the 24 hour exudate samples cannot be solely attributed to transfer loss. It appears that the recovery after 24 hours may be similar to the blade and petiole in that the exudate also contains macromolecules or other substances which bind to the resin by non-ionic forces. The macromolecules are unlikely to include peptides as the hydrolysis of deproteinated crude exudate produced only slight changes in the amino acid composition of the exudate. The hydrolysis of rice phloem sap has been reported to only slightly increase the amount of a few amino acids, indicating that most of the nitrogenous compounds in the exudate are in the form of free amino acids (Fukumorita and Chino, 1982).

Distribution of Sugars:

The distribution of radioactivity recovered from the neutral fraction is shown in Table 8 and the concentration of the predominant sugars is shown in Table 9. In the leaf blade of intact plants there was almost 2 times as much sucrose as stachyose but the amount of ^{14}C incorporated into stachyose in 20 minutes was similar to sucrose. The petiole had an even greater ratio of sucrose to stachyose and yet had more ^{14}C in the latter. This suggests that stachyose and sucrose were simultaneously synthesized in the blade but there appeared to be selective transport of the recently synthesized stachyose because the newly synthesized sucrose was either diluted by that already present in the blade or kept in a separate pool from the sucrose being transported. Regardless of large amounts of free monosaccharides in the blade, very little label was incorporated into them within 20 minutes.

After 24 hours the labelled stachyose in the blade was greatly reduced either by transport or further metabolism. A major portion of the labelled sucrose remained and significant amounts of labelled monosaccharides appeared. About 50% of the label in the "other" sugars of the leaf blade appeared to be myoinositol and another 25% had moved just off the origin of the chromatogram.

Table 8. Distribution of radioactivity within the neutral fraction of *Cucurbita pepo* after exposure to $^{14}\text{CO}_2$ for 1 minute. Mean $\pm$ standard error; n = number of replicates.

- a) 19 minutes after labelling intact plants
 b) 24 hours after labelling intact plants
 c) 19 minutes after labelling pruned plants

Sample	n	% Radioactivity in Each Sugar					
		STA	GOL	RAF	SUC	MONO	OTHER
a) Leaf Blade	4	37.9 $\pm$ 1.1	5.8 $\pm$ 0.5	5.3 $\pm$ 0.5	46.1 $\pm$ 0.3	2.0 $\pm$ 0.4	3.1 $\pm$ 0.2
Petiole	3	66.5 $\pm$ 5.3	3.9 $\pm$ 0.8	5.2 $\pm$ 1.1	17.0 $\pm$ 3.7	2.2 $\pm$ 0.4	5.1 $\pm$ 1.0
Exudate	3	71.9 $\pm$ 0.8	5.2 $\pm$ 0.4	41.8 $\pm$ 0.9	6.8 $\pm$ 0.6	0.4 $\pm$ 0.1	3.8 $\pm$ 0.1
b) Leaf Blade	3	6.1 $\pm$ 0.5	7.5 $\pm$ 1.0	4.4 $\pm$ 1.1	29.3 $\pm$ 2.9	34.3 $\pm$ 2.5	18.4 $\pm$ 0.9
Petiole	3	3.0 $\pm$ 0.7	0.9 $\pm$ 0.2	6.0 $\pm$ 0.6	4.2 $\pm$ 0.8	77.7 $\pm$ 3.2	8.3 $\pm$ 1.1
Exudate	3	12.1 $\pm$ 0.4	2.2 $\pm$ 0.1	13.6 $\pm$ 1.1	12.4 $\pm$ 2.3	12.0 $\pm$ 1.4	47.7 $\pm$ 2.0
c) Leaf Blade	3	48.9 $\pm$ 1.1	8.1 $\pm$ 0.0	2 $\pm$ 0.2	33.4 $\pm$ 0.7	1.5 $\pm$ 0.3	3.9 $\pm$ 0.1
Petiole	5	84.2 $\pm$ 0.3	2.5 $\pm$ 0.5	2.5 $\pm$ 0.2	6.0 $\pm$ 0.5	0.6 $\pm$ 0.0	4.1 $\pm$ 0.1
Exudate	3	76.0 $\pm$ 1.8	2.7 $\pm$ 0.2	5.6 $\pm$ 0.7	6.0 $\pm$ 0.9	1.5 $\pm$ 0.6	8.1 $\pm$ 0.6

Notes: STA = Stachyose GOL = Galactinol RAF = Raffinose
 SUC = Sucrose MONO = Galactose + Glucose + Fructose

Table 9. Concentration of the predominant sugars in *Cucurbita pepo*. Mean $\pm$ standard error; n = number of replicate analyses of the combined eluate from 3 or more chromatograms.

a) intact plants
b) pruned plants

Sample	Stachyose		Raffinose		Sucrose		Monosaccharides	
	n	$\mu\text{mol per g dry wt}$	n	$\mu\text{mol per g dry wt}$	n	$\mu\text{mol per g dry wt}$	n	$\mu\text{mol per g dry wt}$
a) Leaf Blade	7	23.9 $\pm$ 3.6	3	7.5 $\pm$ 0.5	5	54.1 $\pm$ 2.2	6	191.3 $\pm$ 19.2
Petiole	6	21.3 $\pm$ 0.9	3	6.1 $\pm$ 0.6	6	132.1 $\pm$ 9.1	6	4409.7 $\pm$ 114.1
b) Leaf Blade	3	3.2 $\pm$ 0.6	3	3.5 $\pm$ 3.4	6	25.7 $\pm$ 3.7	6	54.2 $\pm$ 11.4
Petiole	6	13.5 $\pm$ 1.4	3	3.6 $\pm$ 0.6	6	50.0 $\pm$ 3.9	6	846.7 $\pm$ 37.3
	n	$\text{nmol per } \mu\text{l}$	n	$\text{nmol per } \mu\text{l}$	n	$\text{nmol per } \mu\text{l}$	n	$\text{nmol per } \mu\text{l}$
a) Exudate	3	1.0 $\pm$ 0.1	3	0.7 $\pm$ 0.1	3	2.0 $\pm$ 0.1	3	5.5 $\pm$ 0.4
b) Exudate	3	0.4 $\pm$ 0.1	3	0.2 $\pm$ 0.0	6	1.2 $\pm$ 0.2	6	1.7 $\pm$ 0.5

Notes: Monosaccharides = Galactose + Glucose + Fructose

Intact plants contain extremely large amounts of free monosaccharides in the petioles and after 24 hours most of the radioactivity in the petiole is found in these compounds presumably due to lateral diffusion of sucrose and oligosaccharides from the phloem and their subsequent hydrolysis (Webb and Gorham, 1965). After 24 hours over 40% of the label in "other" sugars of the petiole had moved just off the origin of the chromatogram.

The ratio of monosaccharides:sucrose:stachyose in the exudate of intact plants is 5:2:1 (from Table 9) and yet 72% of the radioactivity is found in the stachyose (Table 8). After 24 hours the radioactivity in the neutral fraction of the exudate is evenly split among several sugars but approximately 40% is in an unidentified neutral compound, "Compound N", which stains with silver nitrate but not with naphthoresorcinol and has an R_f less than verbascose. The identity of Compound N has not been determined but it is water soluble, stable at -60°C , uncharged, does not contain a fructose moiety, and has a very small R_f in n-propanol:ethyl acetate:water (7:1:2 by volume). Paper chromatograms of petiolar extracts developed in the same solvent contain large amounts of an unknown substance which have a similar R_f and which does not incorporate ^{14}C during short term labelling studies. Further research is required to determine the identity of Compound N and whether or not it is the same substance as that found in the petiole.

Pruning a plant dramatically reduced the sugar content of the leaf blades, petioles, and exudate. The total sugar concentration in the exudate of intact plants was 0.3% (w/v). In the pruned plants this concentration was reduced to 0.1% (w/v). The reduced sugar concentration was accompanied by an increase in the percentage of ^{14}C incorporated into stachyose (Table 8). This appears to be a selective increase in the biosynthesis, loading and transport of stachyose. The percentage of radioactivity found in the petiole during the short term labelling of pruned plants is similar to that reported by Hendrix (1968).

An interesting finding for the record was the existence of an apparently aberrant leaf blade on one intact plant. Twenty minutes after labelling the blade had the usual distribution of radioactivity in its soluble and insoluble fractions as well as the neutral, acidic, and basic components of the soluble fraction. Sucrose contained, however, only 1.1% of the radioactivity in the neutral fraction while 48.8% was in the monosaccharides. This sample was processed as one of a group of samples and so it is unlikely that hydrolysis in this particular sample occurred during processing. The total radioactivity in the petiole of this plant was very low and there was an unusual distribution of ^{14}C among the individual sugars with greater amounts in sucrose and raffinose and less in stachyose than in other plants. The leaf blade also contained one-fourth

as much sucrose and 3 times as much monosaccharides as other blades. No other plant analyzed displayed a similar ^{14}C or sugar distribution; therefore, it was unlikely that this sample represented natural variability within a mature leaf and petiole. It is possible that the plant had a defective loading and/or transport mechanism in the blade resulting in reduced transport to the petiole and a buildup of sugars which could undergo further metabolism in the blade. This was not likely caused by damage to the blade when being moved to the laboratory or during the labelling procedure since the unlabelled sugars were also affected. It is more likely that either the leaf which was labelled was still rapidly expanding or that the development of the leaf was so delayed that the sugars were being metabolized within the blade rather than being exported. Consequently, the results of the analysis of this unusual leaf blade have been omitted from Tables 8 and 9 and the results of the analysis on the petiole omitted from all tables.

Distribution of Compounds within the Acidic Fraction:

The R_f values and anion exchange behaviour of known solutions of organic acids, glucose-6-phosphate, fructose-6-phosphate, and ^{14}C -fructose-1,6-diphosphate were used to determine that the acidic I fraction was composed of the organic acids and some sugar phosphates while the acidic II fraction contained the majority of the sugar phosphates. In

addition about 10% of the radioactivity recovered from the labelled diphosphate marker appeared in (the acidic III fraction. A variety of sugar phosphates and nucleotides have already been identified in the phloem exudate of *Cucurbita pepo* (Lehmann, 1981).

The distribution of radioactivity in the acidic I fraction is shown in Table 10. Within 20 minutes of labelling the radioactivity in the leaf blade was predominantly found in the sugar phosphates and malate. There appears to be an inverse relationship between the radioactivity in these compounds; for example, in four leaf blades processed simultaneously the percentage of ^{14}C incorporated into these compounds was:

<u>Sugar Phosphates</u>	<u>Malate</u>
90.3%	1.2%
85.7%	1.2%
27.8%	41.6%
15.1%	44.0%

As a result of these variations, the means in Table 10 have large standard errors.

Table 10. Distribution of radioactivity within the acidic I fraction of *Cucurbita pepo* after exposure to $^{14}\text{CO}_2$ for 1 minute. Mean $\pm$ standard error; n = number of replicates.

- a) 19 minutes after labelling intact plants
 b) 24 hours after labelling intact plants
 c) 19 minutes after labelling pruned plants

Sample	n	% Radioactivity in Each Acid						
		Sugar Phosphate	Citrate	Malate	Glyco-late	Malonate	Succi-nate	Other
a)								
Blade	4	54.7±19.4	8.0±2.3	22.0±10.4	2.0±0.9	1.2±0.3	0.7±0.2	11.4±3.4
Petiole	4	67.6±8.7	5.6±1.7	10.8±4.1	1.2±0.2	1.4±0.3	1.6±0.2	11.6±2.5
Exudate	3	63.8±2.5	8.7±1.7	18.4±3.2	0.0±0.0	0.8±0.4	0.3±0.2	7.9±3.1
b)								
Blade	3	5.4±0.9	44.2±1.3	39.7±1.4	0.5±0.0	1.6±0.2	2.8±0.5	5.8±0.8
Petiole	3	3.5±0.2	7.4±0.1	74.8±0.3	1.3±0.2	1.9±0.1	2.1±0.2	9.0±0.6
Exudate	3	34.2±1.3	5.3±0.8	11.9±0.6	3.9±0.8	27.1±2.1	2.2±0.3	15.3±1.1
c)								
Blade	3	29.1±2.3	8.4±0.7	51.2±1.4	1.3±0.2	1.0±0.0	1.0±0.1	8.2±1.5
Petiole	3	50.0±3.4	9.4±0.7	28.8±4.9	0.4±0.0	1.4±0.2	1.6±0.1	8.5±1.3
Exudate	3	50.7±3.0	6.0±0.5	20.0±1.8	2.2±0.3	3.0±0.3	3.1±0.5	14.8±2.3

Notes: Malonate may include radioactivity from unidentified acidic compound, Compound A (see pages 99 and 124)

At 3.27 mg per gram fresh weight, malate was the predominant organic acid anion found in the leaf blade (Table 11). Although there is 1-2 times more malate than citrate, the malate contained close to 3 times as much radioactivity (Table 10). Additional organic acid anions were present at a concentration of less than one-tenth that of malate and were labelled to a much lesser extent. Although 11.4% of the activity is designated as "other" in the blade (Table 10), 7.9% was found on the chromatogram between citrate and malate and 1.8% between malate and glycolate and together these most likely represented swamping of the plate with malate. Supporting this conclusion is the fact that when there was little ^{14}C in malate there was little activity in these two areas as well. Similar situations were found in the other samples and the data in Table 10 has been recalculated so that the portion of "other" which may represent swamping of the TLC plate with malate has been subtracted from "other" and added to malate. The recalculated values are presented in Table 12. How much citrate, if any, may have overlapped with this malate and been included in the adjusted portion is unknown. Nonetheless, the recalculated values illustrate more clearly those samples which contained a large percentage of radioactivity in distinctly other, unspecified, organic acids.

Table 11. Concentration of malic acid in *Cucurbita pepo*. Mean of 3 replicates $\pm$ standard error.

Sample	Concentration of Malic Acid	
	$\mu\text{g ml}^{-1}$	mg (g fr wt)^{-1}
Leaf Blade	191.9 $\pm$ 11.2	3.27 $\pm$ 0.25
Petiole		5.00 $\pm$ 0.20
Exudate		

Table 12. Distribution of radioactivity within the acidic I fraction of *Cucurbita pepo* after exposure to $^{14}\text{CO}_2$ for 1 minute. (Data from Table 10 recalculated for swamping by malic acid). Mean $\pm$ standard error; n = number of replicates.

- a) 19 minutes after labelling intact plants
 b) 24 hours after labelling intact plants
 c) 19 minutes after labelling pruned plants

Sample	n	% Radioactivity in Each Acid						
		Sugar Phosphate	Citrate	Malate	Glyco- late	Malonate	Succi- nate	Other
a)								
Blade	4	54.7±19.4	8.0±2.3	31.6±13.4	2.0±0.9	1.2±0.3	0.7±0.2	1.8±0.5
Petiole	4	67.6±8.7	5.6±1.7	18.0±6.8	1.2±0.2	1.4±0.3	1.6±0.2	4.4±1.1
Exudate	3	63.8±2.5	8.7±1.7	22.1±3.3	0.0±0.0	0.8±0.4	0.3±0.2	4.1±3.4
b)								
Blade	3	5.4±0.9	44.2±1.3	44.6±1.7	0.5±0.0	1.6±0.2	2.8±0.5	0.9±0.1
Petiole	3	3.5±0.2	7.4±0.1	82.2±0.2	1.3±0.2	1.9±0.1	2.1±0.2	1.5±0.1
Exudate	3	34.2±1.3	5.3±0.8	17.2±1.0	3.9±0.8	27.1±2.1	2.2±0.3	10.0±1.7
c)								
Blade	3	29.1±2.3	8.4±0.7	58.2±2.3	1.3±0.2	1.0±0.0	1.0±0.1	1.3±0.1
Petiole	3	50.0±3.4	9.4±0.7	36.4±3.6	0.4±0.0	1.4±0.2	1.6±0.1	1.3±0.4
Exudate	3	50.7±3.0	6.0±0.5	28.9±0.8	2.2±0.3	3.0±0.3	3.1±0.5	6.0±1.1

Notes: Malonate may include radioactivity from unidentified acidic compound, Compound A (see pages 99 and 124)

Within 20 minutes of labelling the petiole contained a ^{14}C profile similar to the leaf blade with the exception that there was less variation in the amount of ^{14}C incorporated into the sugar phosphates. In the petiole over 75% of the radioactivity in "other" appeared at the solvent front. Acids which run near the solvent front include lactate and fumarate but because these compounds were not regularly included in the marker mixture their presence can only be very tentatively suggested. The concentration of malate in the petiole was 5.00 mg per gram fresh weight (Table 11), more than 100 times the amount of citrate. It was not surprising that after 24 hours virtually all the radioactivity was in malate, whereas in the leaf blade it was divided between citrate and malate (Table 12).

The exudate of intact plants 20 minutes after labelling had a profile of ^{14}C in organic acids similar to that of the petiole (Table 12) including 4.1% in acidic compounds at the solvent front and consistently high levels of radioactivity in the sugar phosphates. The ratio of radioactivity found in malate and citrate was similar to the 2:1 ratio found in the conducting bundles of rhubarb leaves (Kursanov, Brovchenko and Pariiskaya, 1959).

It was estimated that the amount of citrate in the exudate was one-half that of malate. At $200\ \mu\text{g ml}^{-1}$, the concentration of malate (Table 11) was 30 times less than that found by Richardson, Baker and Ho (1982); however,

there was much less discrepancy between the values for citrate, approximately $100 \mu\text{g ml}^{-1}$ compared to $300 \mu\text{g ml}^{-1}$.

Twenty-four hours after labelling the majority of the ^{14}C recovered from the soluble fraction of the leaves and petioles was found in the acidic compounds but approximately only 5% of this radioactivity was in the phosphorylated sugars. In contrast the exudate had only 10% of the recovered radioactivity in the acidic fraction and over 30% of this ^{14}C was in the sugar phosphates. The appearance of a distinct peak of radioactivity close to malonate was noticed for the first time in exudate samples 24 hours after labelling. This peak overlapped the malonate marker but had a slightly lower R_f . In addition, 70% of the radioactivity in the "other" acids was found between glycolate and malonate. The appearance of this radioactivity between glycolate and malonate combined with the lower R_f for the "malonate" peak suggested that the radioactivity first interpreted as being malonate and reported as such in Tables 10 and 12 was not truly malonate but an unidentified acidic compound, "Compound A". The presence of Compound A was later confirmed by 2-D TLC of samples from unlabelled plants.

Compound A, with an R_f between glycolate and malonate (Table 13), was found in leaf blades, petioles, and exudate of unlabelled plants. Assuming that the sensitivity of the DCF detection of Compound A was similar to that of malonate,

its concentration in the blade appeared to be one-tenth that of malate. In the petiole Compound A was the second most abundant organic acid but its concentration was only about one-twentieth that of malate. The exudate, however, had about 5 times more Compound A than malate. Therefore, although Compound A is abundant in *Cucurbita pepo*, its greatest concentration relative to other organic acids is in the exudate and to a lesser extent the petiole. Considering that Compound A might overlap with glycolate as well as malonate, the amount of ^{14}C incorporated into Compound A would, at the most, be the sum of the radioactivity reported as glycolate, malonate and other in Table 12. In terms of radioactivity, then, the only sample in which Compound A appears significant is the exudate 24 hours after labelling where Compound A might contain up to 41% of the ^{14}C in the acidic I fraction. Compound A becomes labelled much more slowly than citrate and malate and because of this characteristic its role in translocation may have been previously underestimated. Not only did the R_f of Compound A differ from glycolate but its appearance using DCF also differed. The glycolate markers, like succinate, tended to appear as diffuse, hazy areas on the TLC plates; Compound A was a very distinct, dense spot, similar to citrate, malate, and malonate. The identity of Compound A was not determined but 2-D TLC showed that it was not malonate, 2-oxoglutarate, or maleate (Table 14).

Table 13. R_f values of Compound A and malonic and glycolic acid markers. Mean $\pm$ standard error; n = number of replicates.

Sample	Glycolic Acid		Compound A		Malonic Acid	
	n	R_f	n	R_f	n	R_f
Leaf Blade	4	64.0 $\pm$ 0.9	4	69.0 $\pm$ 0.4	4	74.0 $\pm$ 0.4
Petiole	3	65.0 $\pm$ 0.6	3	71.3 $\pm$ 0.9	3	74.7 $\pm$ 0.3
Exudate	4	61.0 $\pm$ 1.9	4	69.0 $\pm$ 1.3	4	74.3 $\pm$ 0.6

Notes: Solvent = chloroform:tertiary amyl alcohol:formic acid:water
(2:2:1:2 by vol)

Table 14. R_f values of Compound A and organic acid markers in 2-D TLC. n = number of replicates.

Sample	n	R_f Values	
		1-D	2-D
Compound A ¹	2	55	64
Malonic Acid	3	14	71
Malonic Acid ¹	1	19	72
2-Oxoglutaric Acid	1	34	70
2-Oxoglutaric Acid ¹	2	34	61
Maleic Acid ¹	1	27	67

Notes: 1-D = ethanol:ammonium hydroxide:water (8:2:1 by vol)

2-D = chloroform:tertiary amyl alcohol:formic acid:water (2:2:1:2 by vol)

¹Samples eluted from developed TLC plates before run on 2-D TLC (see Materials and Methods)

Pruning appears to have stimulated an increase in ^{14}C incorporation into malate in the leaf and petiole with a corresponding decrease in sugar phosphates. The distribution of radioactivity in the exudate appears to be unaffected by pruning. Seventy percent of the radioactivity reported as other organic acids in the exudate of pruned plants is at the solvent front.

Distribution of Amino Acids:

The results from the analyses of amino acids are shown in Table 15. Detection of secondary amines, such as proline and hydroxyproline, was not possible with the HPLC method used. Histidine was either not present or at such low concentrations that it was not detected.

The leaf blade and petiole contained two peaks which appeared to correspond to threonine and citrulline. In the exudate the threonine peak, identified by having a retention time similar to that of threonine in the standard mixture, was so broad that any citrulline which would have followed was hidden by the peak. Since any citrulline present in the exudate would have been included in threonine and because citrulline was not regularly included in the standard mixture and could not be quantified *per se*, the citrulline in the blades and petioles was also quantified as threonine. Citrulline has been identified in the phloem exudate of *Cucurbita ficifolia* (Kating and Eschrich, 1964) and the

results of the sample run on the Beckman Analyzer (Table 16) indicated that only a trace of citrulline existed in the exudate. Therefore, the amount reported in Table 15 as threonine likely included only very small amounts of citrulline and instead may have contained additional compounds. In the Beckman analysis threonine appeared as a minor peak attached to a much larger unidentified peak which preceded it. This seems to suggest that in the exudate, at least, that which has been quantified as threonine may actually be a composite substance and difficult to separate into its components.

After hydrolysis a slight increase in aspartic acid was observed presumably the result of hydrolysis of asparagine. Although the exudate did not contain any glutamine after hydrolysis the 5-fold increase observed in glutamic acid can not be explained by degradation of the amide. The peak that had been considered to represent threonine, citrulline and possibly additional compounds was present at only 10% of the original concentration. The homoserine peak was also reduced to 25%. Lysine appeared to have increased slightly.

Table 15. Concentration of amino acids in *Cucurbita pepo* as determined by HPLC. Mean of 2 replicates for blade and petiole, 4 for exudate.

Amino Acid	Leaf Blade		Petiole		Exudate	
	nmol per g fr wt	mol %	nmol per g fr wt	mol %	nmol per μ l	mol %
ASP	1535.6	20.8	324.6	12.4	1.65	3.8
GLU	2092.6	28.3	624.3	23.9	1.53	3.5
ASN	126.7	1.7	104.8	4.0	0.78	1.8
SER	459.0	6.2	393.0	15.1	1.03	2.4
GLN	562.7	7.6	507.0	19.4	0.97	2.2
HIS	ND	--	ND	--	ND	--
HSER	191.4	2.6	72.1	2.8	7.01	16.2
GLY	98.7	1.3	150.0	5.7	0.86	2.0
THR/CIT.	239.5 ¹	3.2	90.4 ¹	3.5	25.78 ¹	59.5
ARG	46.8	0.6	10.3	0.4	0.24	0.6
ALA ²	1619.1	21.9	139.1	5.3	0.65	1.5
TYR	14.7	0.2	10.4	0.4	0.72	1.7
MET/VAL	95.2/103.9	1.3/1.4	29.5/52.8	1.1/2.0	0.44 ³	1.0
PHE	43.8	0.6	18.8	0.7	0.35	0.8
ILEU	60.6	0.8	34.4	1.3	0.53	1.2
LEU	52.2	0.7	29.2	1.1	0.31	0.7
LYS	39.6	0.5	20.3	0.8	0.47	1.1
Total	7583.9	100.0	2664.1	100.0	45.06	100.0

Notes: ND = not detected, possibly hidden by adjacent peak
¹Not separated in some samples, combined amount quantified as THR
²L-ALA and β -ALA, quantified as L-ALA
³Not separated in some samples, quantified as an equal mixture of MET and Val

Table 16. Amino acid composition of one exudate sample from *Cucurbita pepo* determined by both HPLC and Beckman Amino Acid Analyzer.

Amino Acid	nmol per μ l	
	HPLC	Beckman
ASP	1.27	0.84
GLU	1.47	1.78
ASN	ND	ND
SER	0.52	0.76
GLN/HSER	0.44/8.54	5.18 ¹
HIS	ND	0.02
GLY	ND	0.27
THR/CIT	12.55 ²	NQ ³ /T
ARG	0.32	0.02
ALA ⁴	0.28	0.90
TYR	T	0.01
MET/VAL	0.54/0.20	0.06/0.13
PHE	T	0.55
ILEU	0.34	ND
LEU	T	0.19
LYS	0.69	0.63
CYS	ND ⁵	0.66
CYS-CYS	ND ⁵	0.30
CYSTATHIO	ND	0.00
PRO	ND ⁵	ND
HydroxyPRO	ND ⁵	1.16
γ -ABA	NQ	0.08
ORN	NQ	2.53
NH ₃ ⁺	NQ	2.50

Notes: ND = not detected, possibly hidden by adjacent peak

NQ = detected but not quantified

T = trace amount, too low to be quantified

¹Not separated, combined amount quantified as HSER

²Not separated, combined amount quantified as THR

³Present as a minor peak on preceding large unidentified peak, not quantified

⁴L-ALA and β -ALA, quantified as L-ALA

⁵Not detectable by OPA without prior derivatization

The concentration of the amino acids in the exudate listed in Table 15 was $5.4 \mu\text{g } \mu\text{l}^{-1}$. The weight of cysteine, hydroxyproline and ornithine as detected in the Beckman Analyzer (Table 16) was $0.6 \mu\text{g } \mu\text{l}^{-1}$. Therefore the total concentration of amino acids in the exudate of intact plants was approximately $6 \mu\text{g } \mu\text{l}^{-1}$ or 0.6% (w/v). This is similar to the amounts reported for the Cucurbits as well as other species (Hall and Baker, 1972; Hocking, 1980; Richardson, Baker and Ho, 1982).

Six unidentified peaks appeared in the blades and petioles and seven appeared in the exudate. Not every exudate sample contained all seven compounds probably because several of the amino acids were present at concentrations verging on the limit of detection. The unknowns were present in crude exudate and thus were not produced by the ion exchange technique or the other procedures used. The unknowns formed fluorescent derivatives with OPA which were confirmed by the lack of fluorescent peaks without OPA. There were also several ninhydrin-positive compounds detected in the Beckman Analyzer which did not match the retention times of the standard compounds used. Presumably some of these were identical to the unknowns found in HPLC. The fact that these substances exchanged on the cation exchanger, were not removed by deproteinization procedures, and changed only slightly in acid hydrolysis suggested that the unknowns were

free amino acids. Unidentified compounds have been reported in the phloem exudate of wheat (Hayashi and Chino, 1986b) and barley (Weibull, Brishammar and Pettersson (1986) although Richardson, Baker and Ho (1982) did not mention finding either nonprotein or unidentified amino acids.

The pattern of elution of these unidentified basic compounds, B₁ through B₇, from the HPLC column was:

ASP, GLU, B₁, B₂, ASN ... ALA, B₃, TYR, B₄, B₅, LEU, B₆, B₇, LYS
 ↑
 increasing retention time

The concentration (calculated as glutamic acid) of these unknown compounds is given in Table 17. These amounts have been calculated using the fluorescence of the OPA-glutamate derivative in the standard mixture. Therefore, these concentrations are only accurate if the unidentified compounds have a relative fluorescence similar to that of glutamic acid. In samples containing one of B₆ or B₇ it was difficult to determine which one was present; therefore, B₆ and B₇ have been combined and are referred to as B_{6.7}.

Table 17. Concentration of unidentified compounds within the basic fraction of *Cucurbita pepo*. Quantified as glutamic acid. Mean of 2 replicates for blade and petiole; 4 for exudate

Unknown Compounds	nmol per g fr wt.		nmol per μ l
	Leaf	Petiole	Exudate
B ₁	112.8	23.8	12.41
B ₂	80.7	20.1	10.87
B ₃	196.4	49.0	0.32
B ₄	10.2	8.0	0.21
B ₅	444.7	464.8	0.84
B ₆₊₇	3.2	12.4	0.14

The concentration of B_1 appeared to have been reduced by hydrolysis although exact quantification was hampered by the fact that B_1 was overlapped by the B_2 peak after hydrolysis. B_2 remained unchanged after hydrolysis. B_3 and B_4 could not be detected due to the presence of a very large peak which appeared between them. This may have represented the ammonia liberated by hydrolysis of the amides. B_5 decreased a little; B_{6+7} increased slightly. Otherwise the amino acid profile of the exudate appeared to be unchanged by the hydrolysis. It must be remembered, however, that these observations were based on the hydrolysis of only a single sample and certainly need to be repeated.

Regardless of the number of amino acids present, however, radioactivity during short term labelling was confined to only a few compounds. The radioactivity in the amino acids was based on the observation of only a few preliminary autoradiographs and was not quantified. In the leaf blade the majority of the ^{14}C was contained in aspartic/glutamic acid and although there was not complete separation of these acids, aspartic acid appeared much more heavily labelled than glutamic acid. The next level of radioactivity was in β -alanine, followed by serine, an undetermined compound which was possibly phenylalanine, leucine and/or isoleucine, and lastly, glycine. A lot more activity was in aspartic acid and β -alanine than the others. Even smaller amounts of ^{14}C were contained in a few other

compounds. Radioactivity was found in the same amino acids in the petiole but with a different distribution of the relative amount of ^{14}C . Aspartic acid and glutamic acid appeared more evenly labelled as were glycine and β -alanine. These were followed by serine and the same unidentified amino acid as in the blade. Overall, the ^{14}C was more evenly distributed among the labelled amino acids in the petiole than in the blade. Pruning the plant appeared to cause a decrease in ^{14}C incorporated into β -alanine as compared to the other amino acids in both the leaf and petiole. The profile of ^{14}C found in the amino acids in the exudate was similar to the petiole and, thus, distinctly different from the blade. No data was obtained for exudate from pruned plants. This area needs further research emphasizing the amino compounds present in the greatest concentrations, including the unidentified compounds, and their ^{14}C incorporation.

Determination of PEP-Carboxylase Activity:

The increase in the photosynthetic rate was accompanied by an increase in the percentage of radioactivity incorporated into malic acid and a decrease of that in the sugar phosphates. This was interpreted as a possible indication of an increase in PEP-carboxylase activity. The results of the enzyme purification are given in Table 18. There was a significant increase in the activity per gram

fresh weight in the leaves of pruned plants without an increase in the amount of protein. A significant increase in the activity per milligram of protein was not detected in the crude extract but such an increase was found in the desalted 30-60% ammonium sulfate fraction. There were no significant changes in the protein content or activity on either a fresh weight or protein basis in the petioles of pruned plants. Therefore, reducing the source to sink ratio did not appear to stimulate a greater synthesis of PEP-carboxylase but it did enhance its activity in the leaf. In general the activity per gram fresh weight is lower in the petiole than in the leaf but much greater on a protein basis. This activity is not affected by the reduced source to sink ratio.

Table 18. Purification of PEP-carboxylase from leaf blades and petioles of *Cucurbita pepo*. Mean of 3 replicates $\pm$ standard error.

Sample	Protein (mg per g fr wt)	Total Activity (U)	Specific Activity		% Yield	Purif ^a (fold)
			(U per mg protein)	(U per g fr wt)		
INTACT BLADES Crude Extract	11.8 $\pm$ 1.4	4.8 $\pm$ 0.4	0.04 $\pm$ 0.01	0.40 $\pm$ 0.02 ^b	100	-
30-60% Desalted	7.0 $\pm$ 0.0	4.0 $\pm$ 0.5	0.05 $\pm$ 0.00 ^a	0.34 $\pm$ 0.03 ^c	84 $\pm$ 3	1.4 $\pm$ 0.1
PRUNED BLADES Crude Extract	12.6 $\pm$ 1.1	5.9 $\pm$ 0.2	0.04 $\pm$ 0.00	0.55 $\pm$ 0.07 ^b	100	-
30-60% Desalted	7.7 $\pm$ 0.8	6.1 $\pm$ 0.5	0.07 $\pm$ 0.01 ^a	0.56 $\pm$ 0.04 ^c	104 $\pm$ 11	1.7 $\pm$ 0.2
INTACT PETIOLES Crude Extract	1.8 $\pm$ 0.3	2.2 $\pm$ 0.3	0.15 $\pm$ 0.01	0.27 $\pm$ 0.05	100	-
30-60% Desalted	0.3 $\pm$ 0.1	1.2 $\pm$ 0.2	0.44 $\pm$ 0.03	0.15 $\pm$ 0.02	54 $\pm$ 4	2.9 $\pm$ 0.3
PRUNED PETIOLES Crude Extract	1.5 $\pm$ 0.1	1.5 $\pm$ 0.3	0.13 $\pm$ 0.00	0.19 $\pm$ 0.01	100	-
30-60% Desalted	0.3 $\pm$ 0.0	0.8 $\pm$ 0.3	0.36 $\pm$ 0.06	0.10 $\pm$ 0.02	55 $\pm$ 14	2.8 $\pm$ 0.3

Notes: ^a^b^cSignificantly different pairs ($\alpha = 0.05$)

DISCUSSION

Origin of the Exudate:

Observations under the dissecting microscope and the differences found between the ^{14}C composition of the petiole and the exudate suggest that the exudate represents only a specific portion of the petiole and is phloem-derived. There is, however, no proof that the contents of the exudate represent only the constituents of the translocation stream. The brevity of exudation from the cut petiole implies that the exudate was expelled by the force of the pressure surge and did not result from the workings of an intact transport system. The amount and rate of exudation obtained using this method of detached Cucurbit petioles do not, therefore, indicate the amount of translocates nor their rate of translocation but rather represent only relative concentrations.

It is generally accepted that the P-protein is not mobile and that its presence in the exudate indicates that the exudate originates from the lumen of a sieve tube element. Microscopic examination of exudate has shown that no cytoplasmic structures except P-protein are present and an examination of cut sieve tube elements has shown that the parietal layer of cytoplasm with its mitochondria and endoplasmic reticulum remains in the original position (Eschrich, Evert and Heyser, 1971). If the pressure surge

does cause some non-mobile constituents, such as the P-protein, to be present in the exudate, it appears that there is selectivity in the components affected by the surge. Until it is known precisely what the exudate represents, misinterpretation of its composition in terms of translocation will undoubtedly occur.

Composition of the Exudate:

The exudate was similar to that obtained from other Cucurbits (Richardson, Baker and Ho, 1982) in that it was alkaline, contained P-protein, lacked invertase, and contained more amino acids than sugars. Although there may have been small amounts of other sugars present, the total of the predominant sugars - stachyose, raffinose, sucrose, and the monosaccharides was 0.3% (w/v), within the range of 0.05-1.2% (w/v) reported for the total concentration of sugar from Cucurbit stems (Richardson, Baker and Ho, 1982). Cucurbit exudate apparently differs from the exudate of other species by having a much lower concentration of total sugar. In contrast, the average sugar concentration in 250 species of trees was 10-30% (w/v) (Zimmermann, 1963). In willow where the exudate contains primarily sucrose with some stachyose and traces of raffinose, the sugar concentration varied from 25% in stem cuttings obtained from October to March to only 4% in those taken in the summer (Peel and Weatherley, 1959). Hence, although the Cucurbits

generally have much lower levels of sugar in their exudate, some other species which translocate oligosaccharides may have sugar concentrations somewhat similar to those of the Cucurbits during particular times of growth. The difference between the concentration of sugar in the Cucurbits and the other species is reflected in the individual sugars as well. Typical concentrations of stachyose, raffinose and sucrose in tree exudate are 250 mM, 100mM, and 100mM respectively (Zimmermann, 1961), a minimum of 50 times the amounts found in this study.

Richardson, Baker and Ho (1982) also found that the monosaccharides comprised 21% (w/w) of the sugar in the exudate from Cucurbit stem internodes. This was thought to be high considering that previous analyses of exudate had either not detected reducing sugars (Hall, Baker and Milburn, 1971; Pate, Sharkey and Lewis, 1974; Hocking, 1980) or found that they constituted only a small portion of the total sugar (Tammes and Van Die, 1964). In the present study 37% (w/w) of the sugar in the petiole exudate from intact plants was monosaccharide. This may be slightly overestimated because it is a percentage of the combined amounts of stachyose, raffinose, sucrose, and the monosaccharides, and there may have been small amounts of other sugars present but not analyzed. If the amount of monosaccharides obtained by Richardson, Baker and Ho (1982) is recalculated as a percentage of only stachyose, raffinose,

sucrose, and the monosaccharides, then the value changes from 21% to 36% (w/v) which is very similar to the amount obtained in the present study.

Sugar cane has been shown to contain several invertase isozymes. These may co-exist within the same tissue but exhibit different pH optima and properties. The cell wall of sugar cane leaf sheaths contains three different invertases with pH optima of 4, 5.5 and 7.5 (Vattuone, Prado and Sampietro, 1981). Invertases in the sugar cane have also been shown to differ in location. Parenchyma tissue of mature stalks contain both a soluble neutral invertase with a pH optimum of 7.0 and an insoluble acid invertase with a pH optimum of 3.8 attached to the cell wall (Hatch, Sacher and Glasziou, 1963; Hawker and Hatch, 1965). In contrast to the enzymes found in the mature stalks, immature stalks of sugar cane contain a soluble acid invertase with an optimal pH of 5.0-5.5 and a small amount of insoluble acid invertase with a pH optimum of 4.4 (Hatch, Sacher and Glasziou, 1963). Hence, the isozymes also differ according to the developmental age of the tissue. As a result all the organs of the sugar cane appear to contain at least cell wall invertases and each tissue appears to have one predominant distinctive isozyme which likely corresponds to the predominance of a different activity (Prado et al., 1982). Each invertase, however, has a broad pH range and often has some activity at the optimal pH of the other isozymes (Hatch, Sacher and

Glasziou, 1963; Vattuone, Prado and Sampietro, 1981; Prado et al., 1982). Therefore, although the conditions used in the invertase assay may not have been optimal for any invertase which may have been present in the phloem cells, it is unlikely that the inability to detect invertase activity in the exudate was due to an incorrect pH in the assays.

The monosaccharides contained less than 1% of the radioactivity in the neutral fraction of the exudate; therefore, it is unlikely that they resulted from hydrolysis during the collection and handling of the exudate. The monosaccharides may have resulted from sieve tube metabolism and if so, invertase and other hydrolytic enzymes may be immobilized; that is, in contact with the flow of translocates but not soluble or released by the pressure surge. Invertase has never been found in exudate (Eschrich and Heyser, 1975); however, sucrose synthetase has been located in the phloem exudate from *Cucurbita pepo* (Lehmann, 1973) and *Robinia* (Eschrich and Heyser, 1975). This enzyme catalyzes the reversible reaction of sucrose synthesis from UDP-glucose and fructose. Therefore, sucrose catabolism within the sieve tubes may be a result of sucrose synthetase rather than invertase (Eschrich and Heyser, 1975). Activity from this enzyme would likely not have been detected during the invertase assays because of the extraction and assay conditions required (Claussen, Loveys and Hawker, 1985).

If the exudate is not only sieve tube derived but contains the contents of companion and/or phloem parenchyma cells the monosaccharides may have originated from these other cells. Although microscopic observations indicate that the only structures in the exudate are P-protein from the sieve tubes (Eschrich, Evert and Heyser, 1971) there is no proof that the soluble fraction of the exudate is not contaminated by that of the surrounding cells. It should be noted that studies in which monosaccharides were either not detected or detected in only low amounts were not performed on the Cucurbits but usually on species in which sucrose was the predominant sugar. In addition, these studies did not sample exudate from a detached portion of the plant but instead used techniques which collected samples from feeding aphids or incisions into the bark. Consequently, the occurrence of high levels of monosaccharides may be species specific or an artifact of the sampling method. It is still unlikely that these monosaccharides are transport compounds since the radioactivity in the exudate monosaccharides of pruned plants did not increase although the monosaccharide pools of the leaf blade were greatly decreased. This conclusion is in agreement with other studies on the Cucurbits (Webb and Burley, 1964; Webb and Gorham, 1964) and supports the generally accepted view that reducing sugars are not translocated (Zimmermann, 1961; Ziegler, 1975).

The variation between ^{14}C incorporated into the phosphorylated sugars and other acidic compounds of the leaf and petiole as compared to that in the exudate 24 hours after labelling suggests that the acidic compounds in general, and the sugar phosphates in particular, are not important translocates. This agrees with the tissue extract studies performed by Nelson et al. (1961) and Webb and Gorham (1964). It contrasts with the conclusion of Richardson, Baker and Ho (1984), however, whose examination of radioactivity in Cucurbit exudate utilized only short term labelling. This discrepancy is seen when the profile of the exudate is examined 24 hours after labelling; hence, Richardson, Baker and Ho (1984) would have been unaware of these differences.

The differences among the phosphorylated sugars of the leaves, petioles, and exudate suggest that the sugar phosphates found labelled in the exudate were not transported but resulted from metabolism within the sieve tube elements. Enzymes responsible for the synthesis of organic phosphate compounds have been detected in the exudate from several species (Becker, Kluge and Ziegler, 1971). Hence, the presence (Ziegler, 1975) or absence (Peel and Weatherley, 1959) of phosphorylated sugars in exudate may not indicate differences in the translocation processes but rather differences in the need for these compounds along the translocation pathway.

Cucurbita pepo petioles contain a large amount of malic acid, presumably stored in the vacuoles of the parenchyma cells. The petiole also has an extremely high water content, 98% as compared to 91% in the blade. The storage of solutes such as malate is responsible for the retention of water in the succulent leaves of CAM plants (Matile, 1987). Similarly the large amount of malate found in the *Cucurbit* petiole may be partially responsible for the support of the leaves through turgor pressure, thereby reducing the expenditure of dry matter production required to maintain efficient light interception (Watson, 1971). Unlike the blade and petiole, malic acid is not the primary organic acid in the exudate nor does it contain more radioactivity than the sugar phosphates. Therefore, it seems likely that malate is not transported but synthesized throughout the plant either from transported metabolites or carbon dioxide. Assimilation of radioactive carbon dioxide by the petiole of *Cucurbita pepo* does occur, resulting in the rapid production of labelled sucrose and malate but not labelled oligosaccharides (J. A. Webb, unpublished).

It is possible that the inverse relationship between the percentage of radioactivity in malic acid and the sugar phosphates results from their relative positions in the pathway of sugar synthesis. Hence the increase in radioactivity in malic acid would not result from increased synthesis of the acid itself but from a more rapid cycling

of the ^{14}C so that the radioactivity progressed more quickly along the synthetic pathway. An increase in the rate of photosynthesis or an increase in the rate of pool turnover or a decrease in the size of the pools of compounds already present in the leaf blade could cause such a shift.

Identity of Unknown Acidic Compound, Compound A:

Malate is usually the predominant organic anion found in exudate but in this study the unidentified compound, Compound A, was found at higher concentrations than malate assuming that its sensitivity in DCF detection is similar to that of malonate. Not only did the R_f of Compound A differ from malonate but the latter is known to be a potent inhibitor of succinate dehydrogenase (Smith et al., 1983). Large quantities of such autotoxic compounds are often isolated in cell vacuoles (Matile, 1987). It is generally considered that mature sieve tube elements do not contain vacuoles (Esau, 1969) and reports of these organelles in sieve tubes are usually considered abnormalities (Evert, 1984). It is possible, however, that tonoplasts do exist in mature sieve tube elements and that their absence in micrographs is the result of trauma (Canny, 1973; Canny, 1977; Walsh, 1980). If this is so, then some malonate may be stored within these fragile organelles, but nonetheless, Compound A was found to differ from malonate by 2-D TLC. Examination of the various acidic compounds and solvent

systems cited in Ranson (1955) suggests that the general order of carboxylic acids during chromatography in acidic solvents is the same: 2-oxoglutaric, maleic, tricarballic, *cis*-aconitic and isocitric lactone run in the vicinity of glycolic and malonic while the vast majority of the organic acids do not. It was also confirmed by 2-D TLC that Compound A was not 2-oxoglutaric or maleic acid. Since R_f values tend to decrease in basic solvents as the number of carboxyl groups increase (Ranson, 1955), the tricarboxylic acids; namely, tricarballic and *cis*-aconitic acid would unlikely produce the high R_f value of Compound A. Such a high R_f value in the basic solvent suggests that Compound A has only one or two carboxyl groups and few hydroxyl groups (Ranson, 1955). The common plant acids which have these structural characteristics, however, do not have appropriate movement in acidic solvents (Ranson, 1955). It also seems unusual that an acid occurring in such high concentrations would not have been detected during other research on Cucurbit exudate. Therefore, Compound A may be an artifact from the sample preparation. Isocitric acid is known to convert to isocitric lactone when being dried with heat and the lactone runs close to glycolic and hence, malonic acid, in acidic solvents (Ranson, 1955). Isocitric acid itself does not have a high R_f in basic solvents (Ranson, 1955) due to the presence of three carboxyl groups, but the R_f of the lactone derivative is greater than that of 2-oxoglutaric

acid in the same basic solvent that was used in this study (Goebell and Klingenberg, 1962). This could also explain the observation that exudate samples tended to streak more than those of the leaf and petiole because of the interconversion of isocitric acid and its lactone occurring during chromatography in an acidic solvent (Ranson, 1955).

It appears that Compound A may possibly be isocitric lactone and if so; its concentration and ^{14}C incorporation should be considered part of citrate in Tables 10 and 12 since citrate and isocitrate do not separate in the solvent system used (Ranson, 1955). If high levels of isocitrate were present in the samples, in fact, then the area on the TLC plate between citrate and malate which was considered to have been swamped by malate may have contained isocitrate, in which case the recalculation of Table 12 would not be entirely correct. Additional analyses of the exudate from *Cucurbita pepo* should be performed with emphasis on the separation, quantification and incorporation of ^{14}C in citrate, isocitrate, isocitric lactone and malate.

Assuming that the radioactivity incorporated into Compound A represents radioactivity contained in isocitrate then there was little radioactivity in this compound in the exudate during short term labelling but the incorporation of radioactivity increased during the next 24 hours. This increase suggests that equilibration of ^{14}C among the pools of isocitrate was occurring. This isocitrate may be

responsible for the large amounts of amino acids found in the exudate for, regardless of the amount of dilution, there does appear to be more amino acids than sugar as reported by Richardson, Baker and Ho (1982). The incorporation of ^{14}C into the amino acids of the exudate needs to be further investigated to determine whether these compounds are translocates or the result of local metabolism within the phloem. Isocitrate dehydrogenase has been located in the exudate of *Robinia* and *Cucurbita pepo* and has been detected histochemically in all phloem cells of *Cucurbita pepo* (Eschrich and Heyser, 1975). Not only could this enzyme convert isocitrate to 2-oxoglutarate as part of the TCA cycle but also to provide carbon skeletons for amino acid synthesis. In view of this, it is worthwhile to note that aminotransferases have been detected in the exudate of *Robinia* (Eschrich and Heyser, 1975).

Even without considering isocitrate, the $100\text{ }\mu\text{g ml}^{-1}$ concentration of citrate observed in the exudate is about 2 times that of calcium, assuming calcium to be similar to that found in *Cucurbita maxima* (Sabnis and McEuen, 1985) and *Ricinus* (Hall, Baker, and Milburn, 1971). Although some of this citrate may be compartmentalized, citrate within the translocation stream may be partially responsible for lowering the level of free calcium since the most likely low molecular weight chelator of calcium is citrate (Raven, 1985).

Identity of Unknown Basic Compounds, B₁ to B₇

The identities of the unknown basic compounds can only be very tentatively postulated on the basis of their retention times on the HPLC column relative to the standard amino acids. These tentative identities can then be matched against the locations of unidentified peaks detected by the Beckman Analyzer. Matching of the unknown peaks on the basis of their size, however, is not possible because the compounds may differ in their relative sensitivities to OPA and ninhydrin.

B₁ and B₂ follow aspartic and glutamic acid and are likely to be the same compounds found in much smaller amounts but not identified by Weibull, Brishammar and Pettersson (1986) in the phloem sap of barley. They are most likely acidic amino acids and may be compounds such as homocysteic acid or α -aminoadipic acid, the latter occurring ubiquitously in higher plants (Larsen, 1980). An unknown compound also eluted very early in the Beckman analysis, well before aspartic acid. This location suggests a very strongly acidic compound, such as homocysteic acid, phosphothreonine, or phosphoserine (Adriaens et al., 1977). In an ion exchange analyzer α -aminoadipic acid appears in the vicinity of proline (Adriaens et al., 1977) and in the Beckman Analyzer, which uses a similar ion exchange procedure to separate the amino acids, an unidentified peak

which may have been α -aminoadipic acid was located between cysteine and proline.

B₃ is probably γ -aminobutyric acid which was detected by the Beckman Analyzer at 0.08 nmol μ l⁻¹ of exudate. This low concentration would explain why the peak was not present in all HPLC samples. Although γ -aminobutyric acid was not detected in rice phloem sap (Chino, Hayashi and Fukumori, 1986) it is commonly found in phloem exudate. Both the exudate and xylem sap of *Cucurbita ficifolia* stems contained γ -aminobutyric acid (Kating and Eschrich, 1964) as did the phloem exudate from peas (Urquhart and Joy, 1981), *Nicotiana* (Hocking, 1980), *Lupinus* (Pate, Sharkey and Lewis, 1974), and wheat (Hayashi and Chino, 1986b).

B₄ which follows tyrosine may be α -aminobutyric acid which is commonly found in plants (Bidwell, 1979) or it may be ethanolamine, an intermediate in the synthesis of plant lipids (Bidwell, 1979). Several unknown peaks appeared in the Beckman Analyzer between γ -aminobutyric acid and ammonia which is the relative position of ethanolamine (Adriaens et al., 1977). However, α -aminobutyric acid appears between citrulline and valine (Adriaens et al., 1977) and no peak was located there, although one did appear before citrulline.

B₅ is most likely ammonia. The concentration of this amino compound was more variable than the other unidentified compounds which suggests that it may have been an artifact

Arising during the processing of the samples. Its highest concentrations appeared to be in samples which had been in contact with the ion exchange resin; hence, it may have resulted from the ammonium hydroxide used to elute the resin.

B₆ or B₇ likely represents ornithine which was detected in the Beckman Analyzer and has been found in *Yucca* (Van Die and Tammes, 1975) and pea exudate (Urquhart and Joy, 1981). The other compound may be hydroxylysine or norleucine, another common plant acid (Bidwell, 1979). However, no unidentified peak was located between ammonia and ornithine or between leucine and tyrosine where hydroxylysine and norleucine, respectively, would have been expected in the Beckman Analyzer (Adriaens et al., 1977).

These amino acids are only tentatively suggested as possible identities of the unidentified compounds found in the HPLC analyses. There are many other possibilities including both common plant amino acids, such as glutathione, and other less common amino acids, such as cucurbitine, the amino carboxypyrrolidone isolated from *Cucurbita moschata* seeds (Fang et al., 1961; Sun et al., 1961) and β -pyrazol-1-ylalanine detected in the seeds and vegetative tissues of watermelon and other Cucurbits (Noe and Fowden, 1960). More research is required to positively identify the amino acid composition of *Cucurbita pepo*. The identities postulated above are merely intended to suggest

some amino compounds which should be considered when research into this topic is continued.

Other Amino Acids in the Exudate:

Although histidine and methionine derivatives, such as 1-methyl histidine or methionine sulfone, can occur in the vicinity of threonine in HPLC analyses, such a derivative is not thought to comprise a significant portion of the large threonine peak obtained during the analysis of the exudate. Very little histidine was found in the exudate and no unidentified peak was detected in the Beckman Analyzer in the vicinity of histidine where the derivative would have been expected to appear (Adriaens et al., 1977). Methionine sulfone which was detected in the exudate of peas (Barlow and Randolph, 1978), would likely run close to threonine in the Beckman Analyzer (Adriaens et al., 1977); hence, it may not separate in either procedure. Like histidine, however, one would expect more methionine to be present in the exudate if such large amounts of the derivative were present.

Other compounds which might occupy a position similar to threonine in the Beckman Analyzer include γ -glutamylcysteine (Adriaens et al., 1977) which has been isolated from wheat germ and is involved in glutathione synthesis (Giovanelli, Mudd and Datko, 1980). Degradation of this compound could explain the large increase in

glutamic acid observed during hydrolysis. Meanwhile, detection of an increase in cysteine would not have been possible in the HPLC procedure used.

The concentration of the sulfur containing amino acids; namely, methionine, cysteine, cystine, and cystathionine detected in the Beckman Analyzer was approximately 0.06 molar% (Table 16). This is one-tenth the amount generally found in exudate (for literature values see Table 1). Such a low concentration suggests that sulfur may be transported and/or stored in another form such as the γ -glutamylcysteine or homocysteic acid previously discussed. Sulfate itself is not a major translocate (Ziegler, 1975); therefore, there may be organic compounds which transport this element in *Cucurbita pepo* and which have not yet been identified.

Assimilation and Partitioning:

The distribution of radioactivity between the soluble and insoluble fractions of the leaf blade agrees closely with Nelson et al. (1961) who reported that 50% of the total ^{14}C assimilated by *Glycine* source leaves was located in the ethanol insoluble fraction. This accumulation of starch in the leaf blade contradicts the results reported by Richardson, Baker and Ho (1984) in which 98% of the ^{14}C recovered was ethanol soluble and only 2% was insoluble. It is possible that the genetics or growth conditions of the plants were sufficiently different to reduce the starch

accumulation. It is more likely, however, that the 2% radioactivity reported as being insoluble was underestimated due to the procedure used since at least twice as much ^{14}C was liberated from leaf blade residues by acid hydrolysis than by NCS Tissue Solubilizer apparently because the latter was unable to hydrolyze starch. Joy (1964) found a continual turnover of protein and carbohydrate in fully expanded sugar beet leaves and as the leaves aged there was less radioactivity incorporated into the insoluble material but the proportion in the protein fraction remained constant. Therefore, it seems likely that the insoluble radioactivity reported by Richardson, Baker and Ho (1984) represented, for the most part, only that radioactivity incorporated into protein which NCS Tissue Solubilizer easily digests.

Starch is mobilized during the dark period but may not be totally consumed if the ratio of source to sink activity is high. After 24 hours 80% of the carbon fixed remained in the source leaf of tomato (Khan and Sagar, 1966) while in the sugar beet 50% remained, more being in the ethanol insoluble than in the ethanol soluble fraction (Joy, 1964). Unlike the starch of the petiole, the radioactivity in the starch of the leaf blade did not differ after 24 hours. This suggests that petiolar starch may be more readily mobilized than that of the leaf blade. Alternatively, compartmentation of the starch in the blade may have

resulted in the starch containing unlabelled carbon being metabolized while that with radioactivity was not. This preferential mobilization most likely resulted from the relative times that the starch stores were synthesized. Starch accumulates by the deposition of successive layers and is utilized in the reverse order of being synthesized. The radioactive starch in the leaf blade was formed shortly after exposure to $^{14}\text{CO}_2$. Unlabelled starch would have been synthesized later in the leaf blade during the remainder of the photoperiod; hence, the unlabelled starch would have been deposited and mobilized before that containing ^{14}C . Similarly, radioactive petiolar starch was formed later than that in the leaf blade and used before the latter.

The accumulation of starch may be due to supply simply exceeding demand since the stimulation of sink activity has been shown to increase the amount of radioactive carbon exported (Khan and Sagar, 1966). Boussingault suggested in 1868 that such an accumulation may be responsible for a reduction in the net photosynthetic rate of the leaf (Neales and Incoll, 1968). The plants in the present study were pruned 22 hours before being exposed to $^{14}\text{CO}_2$ and during the intervening dark period their sugar content was greatly reduced as, most likely, was their starch. If the photosynthetic rate is under the control of such stores (Milford and Pearman, 1975; Nafziger and Koller, 1976), this depletion could account for the increase in fixation

obtained with the pruned plants. A similar experiment in which pruned plants are kept in continuous light for 22 hours before $^{14}\text{CO}_2$ exposure should be performed to test this hypothesis.

Another possible explanation is that the removal of source leaves may have reduced the competition for some factor that was under limited supply when all the leaves were present (Wareing and Patrick, 1975). The remaining source leaf would have had more access to such substances as the hormones from the roots and young leaves, the products of root metabolism, and other soil-derived nutrients. For example, phosphate has the potential to control the photosynthetic rate (Herold, 1980) and deficiencies in nitrogen, phosphorous, or potassium increase stomatal and/or mesophyll resistances thereby decreasing the rate of carbon dioxide absorption (Nair, 1975).

Under optimal growth conditions when the source to sink ratio is high, the rate of carbon fixation in a mature leaf blade is less than its potential. This was evident when the removal of all but one of the mature leaves was found to increase the amount of carbon fixed within 20 minutes by the one remaining leaf. The leaves and roots of apple trees normally operate at levels well below their maximal capacity (Maggs, 1964) and in partially pruned leaves of variegated coleus the remaining photosynthetic tissue can compensate for that removed (Fisher and Eschrich, 1985). The obvious

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purpose of such a characteristic is to allow the plant to survive periods during which sink activity is stimulated. Barrett and Amling (1978) found that all leaves on fruiting plants of cucumber, *Cucumis sativus*, increased their rate of assimilation rather than robbing vegetative tissues to supply carbon to the expanding fruit. This ability allows the plant not only to withstand changes in the source/sink ratio during development but also during temporary periods of environmental stress.

Exactly how the rate of fixation is increased during these stressful periods is not known; more research is needed to determine the identity and location of this control mechanism. Although it appears that it would be beneficial for crop breeding programs to attempt to bring the operating level of assimilation closer to its maximum they would do so at the risk of limiting the ability of the plant to cope with periods of increased demand for assimilates.

PEP-Carboxylase Activity:

PEP-carboxylase activity has been found in virtually all the C_3 plant tissues examined (Latzko and Kelly, 1983) and similarly it was located in the leaf blades and petioles of *Cucurbita pepo*. The specific activity was much lower than the 25 U mg^{-1} of protein generally found for the purified enzyme from C_3 , C_4 and CAM plants (O'Leary, 1982)

because only an ammonium sulfate fraction was used to isolate the enzyme. Additional purification steps, such as DEAE cellulose or Sephadex G-200 chromatography, would have undoubtedly increased the specific activity obtained. The activity on a milligram of protein basis in the ammonium sulfate fraction was similar to partially purified extracts of immature chickpea pods (Singal and Singh, 1986) and peanut cotyledons (Maruyama et al., 1966). The activity on a fresh weight basis in the crude extract was also similar to that reported for cotyledons of *Cucurbita pepo* (Leegood and Ap Rees, 1978). Comparisons with other reports in the literature, however, are limited by the tendency to express the enzyme activity on the basis of chlorophyll content which was not measured in this study.

The level of PEP-carboxylase in C_3 tissues is in the order of $0.5 \mu\text{mol min}^{-1} \text{g}^{-1}$ fresh weight while leaves from C_4 plants usually contain 20 times more activity (Ting and Osmond, 1973a; O'Leary, 1982; Latzko and Kelly, 1983). In contrast to the high levels of the enzyme in C_4 plants, the leaves and petioles of *Cucurbita pepo* seem to contain typical C_3 levels of PEP-carboxylase.

The specific activity of PEP-carboxylase found in *Cucurbita pepo* was 40 times less than that reported for leaves of *Cucurbita maxima* seedlings (Magyarosy, Buchanan and Schürmann, 1973) and this difference was most likely due to the assay conditions and procedures used to measure

protein in each study. However, since no detailed report on the characterization of this enzyme from Cucurbit leaves was found in the literature the optimal *in vitro* conditions for the isolation and assay of this enzyme were unknown, much less those conditions existing *in vivo*. Hence the methods used in this study may have either contained inhibitors or lacked activators specific to this enzyme (O'Leary, 1982).

Variations in the environmental growth conditions, leaf position and plant age can affect the enzymatic activity of PEP-carboxylase (Crespo *et al.*, 1979; O'Leary, 1982); therefore, these parameters as well as the assay conditions were kept constant between the intact and pruned plants. Although the exact level of PEP-carboxylase in *Cucurbita pepo* remains in doubt, it appears that the increase in the photosynthetic rate observed in the leaves of pruned plants was accompanied by a significant increase in PEP-carboxylase activity per gram fresh weight. Four general types of PEP-carboxylase have been identified (Ting and Osmond, 1973b): C₄ green leaf enzyme, high K_m PEP, high K_m M_g, high V_{max}; C₃ green leaf enzyme, low K_m PEP, low K_m M_g, low V_{max}; root enzyme, low K_m PEP, low K_m M_g, low V_{max}; and CAM leaf enzyme, low K_m PEP, low K_m M_g, high V_{max}. If the increase in PEP-carboxylase activity in the pruned plants was a result of a change in the type of enzyme; for example, a shift from C₃ to C₄ enzyme, then a significant difference should have been observed in the activity per milligram of

protein in the crude extracts. Therefore, it is unlikely that the form of the enzyme was altered. Instead there was likely an increase in enzyme synthesis. This increase would have had to have been offset by a reduction in the weight of some other protein, however, as there was no change in the total protein content of the leaves of intact and pruned plants. The desalted 30-60% ammonium sulfate fraction from the pruned plants showed a significant increase in activity presumably because in addition to the increase in the amount of enzyme, the purification process reduced unidentified inhibitor(s). It should be noted, however, that the level of activity occurring *in vivo* may not be represented by that measured *in vitro* due to differences in the concentrations of inhibitors or substrates. In general, the level of PEP-carboxylase observed *in vitro* is much higher than that measured *in vivo* (Guern, Mathieu and Kurkdjian, 1983).

Malate is an intermediate used in the conversion of CO₂ to carbohydrates in C₄ and CAM plants but the accumulation of malate and the increase in ¹⁴C found in malate during long term labelling suggests that like other C₃ species (Ting and Osmond, 1973a), the compound is an end product of photosynthesis in *Cucurbita pepo* rather than an intermediate. There are many possible roles for PEP-carboxylase in C₃ plants, the function perhaps being determined by the tissue type (Latzko and Kelly, 1983). Among the proposed functions are the replenishment of TCA intermediates, the

generation of NADPH, the recapture of respired CO_2 , the anaerobic conversion of glucose, and the maintenance of cytoplasmic pH and electroneutrality. Hence, in the leaf of *Cucurbita pepo* PEP-carboxylase may play a different role from that in the petiole. In the leaf the level of activity of the enzyme activity was somehow related to the increase in the assimilation rate observed when the source to sink ratio was reduced. The level of enzyme activity in the petiole, however, was independent of the source to sink ratio suggesting that the enzyme was fulfilling a function different from that in the leaf. In the petiole where large pools of monosaccharides and malate are found and where ^{14}C incorporation into these compounds increases with time, PEP-carboxylase could be responsible for the anaerobic conversion of glucose to malate (Figure 7).

Figure 7. Anaerobic conversion of glucose to malate by PEP-carboxylase (from Latzko and Kelly, 1983).

Sum of the reactions: $\text{glucose} + 2 \text{CO}_2 \rightarrow 2 \text{malate}$

Control of Translocation:

Sucrose and stachyose are synthesized simultaneously in the leaf blade but the recently synthesized stachyose is transported before the sucrose. This likely results from compartmentation of sucrose within the blade such that the newly synthesized sucrose is either separated from the transport pool or diluted by sucrose which had been previously synthesized. Based on the amount of total sucrose and stachyose found in the exudate, sucrose may be as important a carrier of carbon in the translocation of *Cucurbita pepo* as is stachyose. It has previously been suggested that the loading system does not control which nonreducing sugar is transported, but instead control is exercised by the process which supplies the nonreducing sugar (Hendrix, 1968; Hendrix, 1973; Beitler and Hendrix, 1974).

Webb and Gorham (1964) reported that in intact plants the galactose moieties of stachyose were labelled before the glucose and fructose. Hendrix (1968, 1973) and Beitler and Hendrix (1974) observed the same labelling pattern in pruned plants. Although pruning reduces the pools of sugar in the leaf blade prior to labelling it appears that stachyose synthesis still incorporates unlabelled sucrose. Pruning does not, therefore, seem to alter the pathway of stachyose synthesis. It does, however, change the relative rates at which ^{14}C is incorporated into the specific sugars and

hence, increases the rate at which stachyose is produced and decreases the rate of sucrose production. Sucrose synthesis depends on the supply of UDP-glucose:

either:

or:

Stachyose synthesis depends on the supply of UDP-galactose which results from the conversion of UDP-glucose:

Therefore, it may be that pruning somehow causes a shift in the amount of UDP-glucose being converted to UDP-galactose and in turn, stachyose. Hence, the preferential transport of stachyose may not result from selectivity in the loading process but the relative availability of the nonreducing sugar as previously suggested (Hendrix, 1968; Hendrix, 1973; Beitler and Hendrix, 1974).

The Translocation Stream:

Determination and quantification of the constituents of the translocation stream is complicated by the presence of both mobile and non-mobile substances within the sieve tubes. The sieve tube should not be regarded simply as a long distance conduit through which substances flow but

rather as a series of dynamic interchanges with specific substances entering and exiting the stream all along the pathway. The amount of carbon, nitrogen, and other elements which enter the stream is greater than that which is removed; hence, there is a net long distance transport of substances. This constant exchange of materials makes it difficult to identify which compounds are long distance translocates and which ones are the result of local metabolism within the phloem and surrounding tissues.

The solute concentration (%/v) within the exudate included 0.3% sugar, 0.6% amino acid, 0.02% malate, and 0.1% Compound A (assuming it to be 5 times that of malate). Thus, the total concentration of solutes in *Cucurbita pepo* exudate is approximately only 1%, similar to that of Richardson, Baker and Ho (1982), and very low in comparison to other species (Tammes and Van Die, 1964; Hall, Baker and Milburn, 1971; Pate, Sharkey and Lewis, 1974; Hocking, 1980; Fukumoriita and Chino, 1982).

If phloem transport in the Cucurbits is a result of osmotically generated pressure flow either some unmeasured constituent of the translocation stream is responsible for the turgor pressure necessary for transport or the exudate collected from the detached petioles is greatly diluted during sampling. This dilution may have arisen from the influx of water from surrounding tissues or it may have been from within the sieve tube elements themselves if only a

small portion of the water content of the sieve tubes is free and represents the solvent of the translocation stream. Possible sources of water from within the sieve tube which may contribute to the dilution of the exudate may include water from within the cytoplasm and water bound to the P-protein. Although the question of the location of P-protein in undisturbed, functioning sieve tube elements has not been equivocally answered, there is little doubt that the P-protein is very sensitive to pressure surge and other trauma (Fensom, 1981). If P-protein is so easily distorted in damaged cells, then it is possible that water which may be bound to the P-protein could be released during the disruption. The exudate may very well not be greatly diluted by cutting, otherwise the concentration of amino acids would be extremely high. On the other hand, a low concentration of solutes in the exudate would imply that the loading of sugar from the mesophyll into the phloem may not be against a concentration gradient, as is often cited in the argument against symplastic loading (Geiger, 1976). With such a low solute concentration, it is possible that not osmotically generated pressure flow but rather some other undetermined mechanism is responsible for translocation in the Cucurbits.

Regardless of whether exudate constituents are translocates or products of phloem metabolism there are two major differences between Cucurbit exudate and that of other

species which appear to be indisputable; namely, a low concentration of sugar and a high concentration of P-protein. These two characteristics may be connected in some, as yet, undetermined fashion. The P-protein in *Cucurbita pepo* gels on contact with the air. This is an oxidative process because 2-mercaptoethanol not only prevents but, reverses the gelling. Consequently the internal environment of the sieve tubes must be strongly reducing. In this respect, McEuen, Hart and Sabnis (1981) found that the ability of P-protein to bind calcium was dependent on 2-mercaptoethanol, providing further evidence that there is a reducing environment *in vivo*. In addition, callose synthetase which tends to precipitate when extracted, remains dissolved in Cucurbit exudate in the presence of sufficient amounts of dithioerythritol, another reducing agent (Eschrich and Heyser, 1975). Hence it may be significant that the reducing state of the sieve tubes is responsible for the calcium binding activity of the P-protein and the solubility of callose synthetase when divalent cations, such as calcium, enhance callose synthesis (Eschrich and Heyser, 1975). Thus, while P-protein may serve a general wound sealing function in all dicots by physically blocking the sieve pores, in some species it may serve additional roles in sealing through coagulation and possible callose regulation. In the Cucurbits the P-protein may serve an additional function, yet unidentified, which

enables translocation to occur in the presence of a low sugar concentration. Accurate observations of functioning sieve tubes and the ability to sample their contents may depend on the ability to preserve the reducing environment during manipulation.

CONCLUSION

Differences between the chemical composition of the exudate and that of the petiole suggest that exudate analyses can augment identification of the contents of the phloem. The chemical composition of either the sieve tubes or the translocation stream cannot be accurately determined from exudate analysis alone, however, since the exact source of the exudate has not yet been confirmed. While it may not be possible to sample the translocation stream itself it is imperative that the number of assumptions about translocation be reduced by determining, at the very least, which cells the exudate represent.

Nondestructive exudate sampling techniques, such as the cutting of fruit tips or bark incisions in *Ricinus*, appear to be more useful in that larger volumes of exudate can be obtained and the phloem system can be maintained in an operating state for some time. The advantage of cutting petioles, however, lies in their proximity to the source leaf. A research system for *Cucurbita pepo* in which the petiolar exudate can be sampled without being detached is still required. At the present, it is unknown whether the differences observed in Cucurbit exudate are species specific or a result of the sampling method. Although feeding aphids have been used to study the phloem within a Cucurbit leaf (Botha and Evert, 1978) it appears that, as

yet, they have not been used for exudate analyses in these plants. While they might provide one way of more accurately tapping the translocation stream they undoubtedly would not be an ideal method (Fisher and Frame, 1984). The use of a technique which maintains the reducing environment of the sieve tubes and enables translocation to continue functioning, while not expected to elucidate the entire process, should at least reduce the number of uncertainties about Cucurbit translocation. Until such a system is available, exudate analyses should be used in conjunction with, rather than a replacement for, petiolar tissue extracts.

LITERATURE CITED

- Adriaens, P., B. Meesschaert, W. Wuyts, H. Vanderhaeghe and H. Eyssen. 1977. Separation of ninhydrin-positive compounds on a single-column amino acid analyzer using lithium buffers. *J. Chromatog.* 140: 103-108
- Albon, N. and D. Gross. 1952. The chromatographic determination of raffinose in raw sugars. *Analyst* 77: 410-412
- Ap Rees, T. 1974. Pathways of carbohydrate breakdown in higher plants. In Northcote, D.H. (ed.). *Plant Biochemistry*. Butterworths, London. *Biochemistry, Series One*. 11: 89-127
- Atkins, C.A., J.S. Pate, A. Ritchie and M.B. Peoples. 1982. Metabolism and translocation of allantoin in ureide-producing grain legumes. *Plant Physiol.* 70: 476-482
- Barlow, C.A. and M.E. McCully. 1972. The ruby laser as an instrument for cutting the stylets of feeding aphids. *Can. J. Zool.* 50: 1497-1498
- Barlow, C.A. and P.A. Randolph. 1978. Quality and quantity of plant sap available to the pea aphid. *Ann. Entomol. Soc. Amer.* 71: 46-48
- Barrett, J.E. and H.J. Amling. 1978. Effects of developing fruits on production and translocation of ^{14}C -labeled assimilates in cucumber. *HortScience* 13: 545-547
- Becker, D., M. Kluge and H. Ziegler. 1971. Der Einbau von ^{32}P in organische Verbindungen durch Siebröhrensaft. *Planta* 99: 154-162
- Beitler, G.A. and J.E. Hendrix. 1974. Stachyose: an early product of photosynthesis in squash leaves. *Plant Physiol.* 53: 674-676
- Bidwell, R.G.S. 1979. *Plant Physiology*. MacMillan, New York. 726pp.
- Bieleski, R.L. 1969. Phosphorus compounds in translocating phloem. *Plant Physiol.* 44: 497-502
- Bieleski, R.L. 1973. Phosphate pools, phosphate transport and phosphate availability. *Ann. Rev. Plant Physiol.* 24: 225-252

- Botha, C.E.J. and R.J. Evert. 1978. Observations of preferential feeding by the aphid, *Rhopalosiphum maidis* on abaxial phloem of *Cucurbita maxima*. *Protoplasma* 96: 75-80
- Botha, C.E.J., R.J. Evert and R.D. Walmsley. 1975. Studies on *Gomphocarpus physocarpus*: further evidence of preferential feeding by the aphid *Aphis nerii* on the internal phloem. *Protoplasma* 84: 345-356
- Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Ann. Biochem.* 72: 248-254
- Bryson, J.L. and T.J. Mitchell. 1951. Improved spraying reagents for the detection of sugars on paper chromatograms. *Nature* 167: 854
- Canny, M.J. 1973. *Phloem Translocation*. University Press, Cambridge. 301 pp.
- Canny, M.J. 1977. Flow and transport in plants. *Ann. Rev. Fluid Mech.* 9: 275-296
- Chino, M., H. Hayashi and T. Fukumori. 1986. Cation and anion composition of rice phloem sap. In Cronshaw, J., W.J. Lucas and R.T. Giaquinta (ed.). *Phloem Transport*. Alan R. Liss, New York. p.463-464
- Claussen, W., B.R. Loveys and J.S. Hawker. 1985. Comparative investigations on the distribution of sucrose synthase activity and invertase activity within growing, mature and old leaves of some C₃ and C₄ plant species. *Physiol. Plant.* 65: 275-280
- Cooil, B.J. 1941. Significance of phloem exudate of *Cucurbita pepo* with reference to translocation of organic materials. *Plant Physiol.* 16: 61-84
- Crafts, A.S. 1932. Phloem anatomy, exudation, and transport of organic nutrients in cucurbits. *Plant Physiol.* 7: 183-225
- Crafts, A.S. 1936. Further studies on exudation in cucurbits. *Plant Physiol.* 11: 63-79
- Crafts, A.S. and C.E. Crisp. 1971. *Phloem Transport In Plants*. Freeman, San Francisco. 481pp.

- Crafts, A.S. and O.A. Lorenz. 1944. Composition of fruits and phloem exudate of cucurbits. *Plant Physiol.* 19: 326-337
- Crespo, H.M., M. Frean, C.F. Cresswell and J. Tew. 1979. The occurrence of both C_3 and C_4 photosynthetic characteristics in a single *Zea mays* plant. *Planta* 147: 257-263
- Dawson, R.M.C., D.C. Elliott, W.H. Elliott and K.M. Jones (ed.). 1969. *Data for biochemical research*. 2nd ed. Oxford University Press, New York. 654pp.
- Dixon, A.F.G. 1975. Aphids and translocation. In Zimmermann, M.H. and J.A. Milburn (ed.). *Transport in Plants*. I. Phloem Transport. Springer Verlag, New York. *Encycl. Plant Physiol.* NS I: 154-170
- Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28: 350-356
- Epstein, E. 1973. Flow in the phloem and the immobility of calcium and boron: a new hypothesis in support of an old one. *Experientia* 29: 133-134
- Esau, K. 1969. The Phloem. In Zimmermann, W., P. Ozenda and D.H. Wulff (ed.) *Handbuch der Pflanzenanatomie*. Vol. 5, Pt. 2. Gebrüder Borntraeger, Berlin. 505 pp.
- Esau, K. and J. Cronshaw. 1967. Tubular components in cells of healthy and tobacco mosaic virus-infected *Nicotiana*. *Virology* 33: 26-35
- Eschrich, W. 1975. Sealing systems in phloem. In Zimmermann M.H. and J.A. Milburn (ed.). *Transport in Plants*. Springer Verlag, Berlin. *Encycl. Plant Physiol.* NS 1: 39-56
- Eschrich, W., R.F. Evert and W. Heyser. 1971. Proteins of the sieve tube exudate of *Cucurbita maxima*. *Planta* 100: 208-221
- Eschrich, W. and W. Heyser. 1975. Biochemistry of phloem constituents. In Zimmermann M.H. and J.A. Milburn (ed.). *Transport in Plants*. Springer Verlag, Berlin. *Encycl. Plant Physiol.* NS 1: 101-136
- Evert, R.F. 1984. Comparative structure of phloem. In White, R.A. and W.C. Dickinson (ed.). *Contemporary Problems in Plant Anatomy*. Academic Press, Orlando. p.145-234
-

- Evert, R.F., W. Eschrich and W. Heyser. 1978. Leaf structure in relation to solute transport and phloem loading in *Zea mays* L. *Planta* 138: 279-294
- Evert, R.F., W. Eschrich, J.T. Medler and F.J. Alfieri. 1968. Observations on penetration of linden branches by stylets of the aphid *Longistigma caryae*. *Am. J. Bot.* 55: 860-874
- Fang, S.-D., L.-C. Li, C.-I. Niu and K.-F. Tseng. 1961. Chemical studies on *Cucurbita moschata* Duch. I. The isolation and structural studies of cucurbitine, a new amino acid. *Sci. Sinica* 10: 845-851
- Fellows, R.J., D.B. Egli and J.E. Leggett. 1978. A pod leakage technique for phloem translocation studies in soybean (*Glycine max* L. Merr.). *Plant Physiol.* 62: 812-814
- Fensom, D.S. 1981. Problems arising from a Münch-type pressure flow mechanism of sugar transport in phloem. *Can. J. Bot.* 59: 425-432
- Fisher, D.B. 1976. Histochemical approaches to water-soluble compounds and their application to problems in translocation. In Wardlaw, I.F. and J.B. Passioura (ed.). *Transport and Transfer Processes in Plants*. Academic Press, New York. p.237-247
- Fisher, D.B. 1978. The estimation of sugar concentration in individual sieve-tube elements by negative staining. *Planta* 139: 19-24
- Fisher, D.B. and J.M. Frame. 1984. A guide to the use of the exuding-stylet technique in phloem physiology. *Planta* 161: 385-393
- Fisher, D.G. and W. Eschrich. 1985. Import and unloading of ^{14}C assimilate into nonphotosynthetic portions of variegated *Coleus blumei* leaves. *Can. J. Bot.* 63: 1708-1712
- Forney, C.F. and P.J. Breen. 1985. Collection and characterization of phloem exudate from strawberry pedicels. *HortScience* 20: 413-414
- Fritz, E., R.F. Evert and W. Heyser. 1983. Microautographic studies of phloem loading and transport in the leaf of *Zea mays* L. *Planta* 159: 193-206

- Fukumorita, T. and M. Chino. 1982. Sugar, amino acid and inorganic contents in rice phloem sap. *Plant & Cell Physiol.* 23: 273-283
- Gaudreault, P.-R. and J.A. Webb. 1982. Alkaline α -galactosidase in leaves of *Cucurbita pepo*. *Plant Sci. Lett.* 24: 281-288
- Geiger, D.R. 1976. Phloem loading in source leaves. In Wardlaw, I.F. and J.B. Passioura (ed.). *Transport and Transfer Processes in Plants*. Academic Press, New York. p.167-183
- Geiger, D.R., R.T. Giaquinta, S.A. Sovonick and R.J. Fellows. 1973. Solute distribution in sugar beet leaves in relation to phloem loading and translocation. *Plant Physiol.* 52: 585-589
- Giovanelli, J., S.H. Mudd and A.H. Datko. 1980. Sulfur amino acids in plants. In Mifflin, B.J. (ed.). *Amino Acids and Derivatives*. Academic Press, New York. *Biochemistry of Plants* 5: 453-505
- Goebell, H. and M. Klingenberg. 1962. Dünnschichtchromatographie von tricarbonsäurezyklus-substraten mit autoradiographie. *Chromatographie Symposium II*. Societ Belge des Sciences Pharmaceutiques, Brussels. p.153-165
- Guern, J., Y. Mathieu and A. Kurkdjian. 1983. Phosphoenolpyruvate carboxylase activity and the regulation of intracellular pH in plant cells. *Physiol. Vg.* 21: 855-866
- Gunning, B.E.S., C.A. Swanson and D.B. Fisher. 1976. Long distance transport - summary and discussion. In Wardlaw, I.F. and J.B. Passioura (ed.). *Transport and Transfer Processes in Plants*. Academic Press, New York. p.369-371
- Hall, S.M. and D.A. Baker. 1972. The chemical composition of *Ricinus* phloem exudate. *Planta* 106: 131-140
- Hall, S.M., D.A. Baker and J.A. Milburn. 1971. Phloem transport of ^{14}C -labelled assimilates in *Ricinus*. *Planta* 100: 200-207
- Hartt, C.E. 1963. Translocation of sugar in the cane plant. *Hawaiian Sugar Tech. Rep.* 151-167

- Hatch, M.D., J.A. Sacher and K.T. Glasziou. 1963. Sugar accumulation cycle in sugar cane. 1. Studies in enzymes of the cycle. *Plant Physiol.* 38: 338-343
- Hawker, J.S. and M.D. Hatch. 1965. Mechanism of sugar storage by mature stem tissue of sugarcane. *Plant Physiol.* 18: 444-453
- Hayashi, H. and M. Chino. 1986a. Effect of nitrogen nutrition and light on the chemical composition of rice phloem sap. In Cronshaw, J., W.J. Lucas and R.T. Giaquinta (ed.). *Phloem Transport*. Alan R. Liss, New York. p.465-468
- Hayashi, H. and M. Chino. 1986b. Collection of pure phloem sap from wheat and its chemical composition. *Plant Cell Physiol.* 27: 1387-1393
- Hendrix, J.E. 1968. Labeling pattern of translocated stachyose in squash. *Plant Physiol.* 43: 1631-1636
- Hendrix, J.E. 1973. Translocation of sucrose by squash plants. *Plant Physiol.* 52: 688-689
- Herold, A. 1980. Regulation of photosynthesis by sink activity - the missing link. *New Phytol.* 86: 131-144
- Ho, L.C. and A.J. Peel. 1969. Investigation of bidirectional movement of tracers in sieve tubes of *Salix viminalis* L. *Ann. Bot.* 33: 833-844
- Hocking, P.J. 1980. The composition of phloem exudate and xylem sap from tree tobacco (*Nicotiana glauca* Grah.). *Ann. Bot.* 45: 633-643
- Joy, K.W. 1964. Translocation in sugar beet. 1. Assimilation of $^{14}\text{CO}_2$ and distribution of materials from leaves. *J. Exp. Bot.* 15: 485-494
- Kanahama, K. and Y. Hori. 1980. Time course of export of ^{14}C -assimilates and their distribution pattern as affected by feeding time and night temperature in cucumber plants. *Tohoku J. Agric. Res.* 30: 142-152
- Kating H. and W. Eschrich. 1964. Aufnahme Einbau und Transport von ^{14}C in *Cucurbita ficifolia*. II. Applikation von bicarbonat- ^{14}C über die wurzel. *Planta* 60: 598-611

- Kennedy, J.S. and T.E. Mittler. 1953. A method of obtaining phloem sap via the mouth-parts of aphids. *Nature* 171: 528
- Khan, A. and G.R. Sagar. 1966. Distribution of ^{14}C -labelled products of photosynthesis during the commercial life of the tomato crop. *Ann. Bot.* 30: 727-743
- King, R.W. and J.A. Zeevaart. 1974. Enhancement of phloem exudation from cut petioles by chelating agents. *Plant Physiol.* 53: 96-103
- Kursanov, A.L. 1963. Metabolism and transport of organic substances in the phloem. *Adv. Bot. Res.* 1: 209-278
- Kursanov, A.L., M.I. Brovchenko and A.N. Pariiskaya. 1959. Flow of assimilates to the conducting tissue in rhubarb (*Rheum rhaponticum* L.) leaves. *Soviet Plant Physiol.* 6: 544-552
- Larsen, P.O. 1980. Physical and chemical properties of amino acids. In Mifflin, B.J. (ed.). *Amino Acids and Derivatives*. Academic Press, New York. *Biochemistry of Plants* 5: 225-269
- Latzko, E. and G.J. Kelly. 1983. The many-faceted function of phosphoenolpyruvate carboxylase in C_3 plants. *Physiol. Vég.* 21: 805-815
- Leegood R.C. and T. Ap Rees. 1978. Identification of the regulatory steps in gluconeogenesis in cotyledons of *Cucurbita pepo*. *Biochim. Biophys. Acta* 542: 1-11
- Lehmann, J. 1973. Untersuchungen am Phloemexsudat von *Cucurbita pepo* L. II. Enzymaktivitäten der Gluconeogenese und des Auf- und Abbaus von Di- und Polysacchariden. *Planta* 114: 51-61
- Lehmann, J. 1981. Quantitative Bestimmung von Nucleotiden und Zucker phosphaten im Phloem-Exsudat von *Cucurbita pepo* L. *Z. Pflangen physiol.* 102: 415-424
- Lichtner, F.T. 1984. Phloem transport of xenobiotic chemicals. *What's New in Plant Physiol.* 15 : 29-32
- Madore, M. and B. Grodzinski. 1984. Effect of oxygen concentration on ^{14}C -photoassimilate transport from leaves of *Salix splendens* L. *Plant Physiol.* 76: 782-786

- Madore, M. and B. Grodzinski. 1985. Effects of CO₂ enrichment on growth and transport in a dwarf cucumber (*Cucumis sativus* L.) cultivar. *J. Plant Physiol.* 121: 59-71
- Maggs, D.H. 1964. Growth-rates in relation to assimilate supply and demand. *J. Exp. Bot.* 15: 574-583
- Magyarosy, A.C., B.B. Buchanan and P. Schürmann. 1973. Effect of a systemic virus infection on chloroplast function and structure. *Virology* 55: 426-438
- Marinos, N.G. 1962. Studies on submicroscopic aspects of mineral deficiencies. I. Calcium deficiency in the shoot apex of barley. *Amer. J. Bot.* 49: 835-841
- Maruyama, H., R.L. Easterday, H.-C. Chang and M.D. Lane. 1966. The enzymatic carboxylation of phosphoenolpyruvate. I. Purification and properties of phosphoenolpyruvate carboxylase. *J. Biol. Chem.* 241: 2405-2412
- Matile, P. 1987. The sap of plant cells. *New Phytol.* 105: 1-26
- Mayoral, M.L., Z. Plaut and L. Reinhold. 1985. Effect of translocation-hindering procedures on source leaf photosynthesis in cucumber. *Plant Physiol.* 77: 712-717
- McEuen, A.R., J.W. Hart and D.D. Sabnis. 1981. Calcium-binding protein in sieve tube exudate. *Planta* 151: 531-534
- Mengel, K. and H.-E. Haeder. 1977. Effect of potassium supply on the rate of phloem sap exudation and the composition of phloem sap of *Ricinus communis*. *Plant Physiol.* 59: 282-284
- Milburn, J.A. 1972. Phloem physiology and protective sealing mechanisms. *Nature* 236: 82
- Milburn, J.A. 1975. Pressure flow. In Zimmermann, M.H. and J.A. Milburn (ed.). Transport in Plants. I. Phloem Transport. Springer Verlag, New York. *Encycl. Plant Physiol.* NS I: 328-353.
- Milford, G.E.J. and I. Pearman. 1975. The relationship between photosynthesis and the concentration of carbohydrates in the leaves of sugar beet. *Photosyn.* 9: 78-83

- Mittler, T.E. 1958. Studies on the feeding and nutrition of *Tuberolachnus salignus* (Gmelin) (Homoptera, Aphididae). II. The nitrogen and sugar composition of ingested phloem sap and excreted honeydew. *J. Exp. Biol.* 35: 74-84
- Möllering, H. 1974. Determination with malate dehydrogenase and glutamate-oxaloacetate transaminase. In Bergmeyer, H.U. (ed.). *Methods of Enzymatic Analysis*. 2nd English ed. Academic Press, New York. 3: 1589-1593
- Nafziger, E.D. and H.R. Koller. 1976. Influence of leaf starch concentration on CO₂ assimilation in soybean. *Plant Physiol.* 57: 560-563
- Nátr, L. 1975. Influence of mineral nutrition on photosynthesis and the use of assimilates. In Cooper, J.P. (ed.). *Photosynthesis and Productivity in Different Environments*. Cambridge University Press, London. p.537-555
- Neales, T.F. and L.D. Incoll. 1968. The control of leaf photosynthesis by the level of assimilate concentration in the leaf: a review of the hypothesis. *Bot. Rev.* 34: 107-125
- Nelson, C.D., H. Clauss, D.C. Mortimer and P.R. Gorham. 1961. Selective translocation of products of photosynthesis in soybean. *Plant Physiol.* 36: 581-588
- Noe, F.F. and L. Fowden. 1960. β -Pyrazol-1-ylalanine, an amino acid from water-melon seeds (*Citrullus vulgaris*). *Biochem. J.* 77: 543-546
- Nuske, J. and W. Eschrich. 1976. Synthesis of P-protein in mature phloem of *Cucurbita maxima*. *Planta* 132: 109-118
- O'Leary, M.H. 1982. Phosphoenolpyruvate carboxylase: an enzymologist's view. *Ann. Rev. Plant Physiol.* 33: 297-315
- Palevitz, B.A. and E.H. Newcomb. 1970. A study of sieve element starch using sequential enzymatic digestion and electron microscopy. *J. Cell Biol.* 45: 383-398
- Pate, J.S., C.A. Atkins, K. Hamel, D.L. McNeil and D.B. Layzell. 1979. Transport of organic solutes in phloem and xylem of a nodulated legume. *Plant Physiol.* 63: 1082-1088

- Pate, J.S., P.J. Sharkey and O.A.M. Lewis. 1974. Phloem bleeding technique from legume fruits - a technique for study of fruit nutrition. *Planta* 20: 229-243
- Pate, J.S., P.J. Sharkey and O.A.M. Lewis. 1975. Xylem to phloem transfer of solutes in fruiting shoots of legumes, studied by a phloem bleeding technique. *Planta* 122: 11-26
- Peel, A.J. 1975. Investigations with aphid stylets into the physiology of the sieve tube. In Zimmermann, M.H. and J.A. Milburn (ed.). Transport in Plants. I. Phloem Transport. Springer Verlag, New York. *Encycl. Plant Physiol.* NS I: 171-195.
- Peel, A.J. and P.E. Weatherley. 1959. Composition of sieve-tube sap. *Nature* 184: 1955-1956
- Prado, F.E., O.L. Fleischmacher, M.A. Vattuone and A.R. Sampietro. 1982. Cell wall invertases of sugar cane. *Phytochem.* 21: 2825-2828
- Pristupa, N.A. 1959. The transport form of carbohydrates in pumpkin plants. *Soviet Plant Physiol.* 6: 26-32
- Pristupa, N.A. 1979a. Histochemical determination of keto sugars in lyophilically dried plant material. *Soviet Plant Physiol.* 26: 165-171
- Pristupa, N.A. 1979b. Localization of ketosugars in assimilating and conducting tissues of the sugar beet leaf. *Soviet Plant Physiol.* 26: 467-474
- Pristupa, N.A. 1983. Distribution of ketosugars among cells of conducting bundles of the *Cucurbita pepo* leaf. *Soviet Plant Physiol.* 30: 372-378
- Ranson, S.L. 1955. Non-volatile mono-, di-, and tricarboxylic acids - chromatographic and ion exchange methods. In Paesch, K. and M.V. Tracey (ed.). *Modern Methods of Plant Analysis*. Springer Verlag, Berlin. 2: 539-582
- Rasmussen, H. 1967. Separation and detection of carboxylic acids by thin-layer chromatography. *J. Chromatog.* 26: 512-514
- Raven, J.A. 1985. Long distance transport of calcium. In Trewavas, A.J. (ed.). *Molecular and Cellular Aspects of Calcium in Plant Development*. Plenum Press, New York. p.241-250

- Richardson, P.T., D.A. Baker and L.C. Ho. 1982. The chemical composition of cucurbit vascular exudates. *J. Exp. Bot.* 33: 1239-1247
- Richardson, P.T., D.A. Baker and L.C. Ho. 1984. Assimilate transport in cucurbits. *J. Exp. Bot.* 35: 1575-1581
- Rohlf, F.J. and R.R. Sokal. 1969. *Statistical Tables*. W.H. Freeman, San Francisco. 253pp.
- Rohlf, F.J. and R.R. Sokal. 1981. *Statistical Tables*. 2nd ed. W.H. Freeman, San Francisco. 219pp.
- Roth, M. 1971. Fluorescence reaction for amino acids. *Anal. Chem.* 43: 880-882
- Sabnis, D.D. and A.R. McEuen. 1985. Calcium and calcium-binding proteins in phloem. In Trewavas, A.J. (ed.). *Molecular and Cellular Aspects of Calcium in Plant Development*. Plenum Press, New York. p.33-40
- Sharkey, P.J. and J. Pate. 1975. Selectivity in xylem to phloem transfer of amino acids in fruiting shoots of white lupin (*Lupinus albus* L.). *Planta* 127: 251-262
- Sigma Chemical Company. 1967. Trizma. *Sigma Tentative Tech. Bull.* 106B 4 pp.
- Simpson, R.J. and M.J. Dalling. 1981. Nitrogen redistribution during grain growth in wheat (*Triticum aestivum* L.). III. Enzymology and transport of amino acids from senescing flag leaves. *Planta* 151: 447-456
- Singal, H.R. and R. Singh. 1986. Purification and properties of phosphoenolpyruvate carboxylase from immature pods of chickpea (*Cicer arietinum* L.). *Plant. Physiol.* 80: 369-373
- Smith, E.L., R.L. Hill, I.R. Lehman, R.J. Lefkowitz, P. Handler and A. White. 1983. *Principles of Biochemistry: General Aspects*. 7th ed. McGraw-Hill, New York. 886 pp.
- Smith, J.A.C. and J.A. Milburn. 1980. Phloem transport, solute flux and the kinetics of sap exudation in *Ricinus communis* L. *Planta* 148: 35-41
- Sokal, R.R. and F.J. Rohlf. 1969. *Biometry: the Principles and Practice of Statistics in Biological Research*. W.H. Freeman, San Francisco. 776 pp.

- Sokal, R.R. and F.J. Rohlf. 1981. *Biometry: the Principles and Practice of Statistics in Biological Research*. 2nd ed. W.H. Freeman, San Francisco. 859 pp.
- Spector, T. 1978. Refinement of the Coomassie blue method of protein quantitation. *Anal. Biochem.* 86: 142-146
- Sun, T.-T., S.-H. Loh, S.-W. Chow and Z.-Y. Kyi. 1961. Chemical studies on *Cucurbita moschata* Duch. *Sci. Sinica* 10: 852-859
- Tammes, P.M.L. and J. Van Die. 1964. Studies on phloem exudation from *Yucca flaccida* Haw. I. Some observations on the phenomenon of bleeding and the composition of the exudate. *Acta Bot. Neerl.* 13: 76-83
- Thomas, B. and J.A. Webb. 1977. Multiple forms of α -galactosidase in mature leaves of *Cucurbita pepo*. *Phytochem.* 16: 203-206
- Thomas, B. and J.A. Webb. 1978. Distribution of α -galactosidase in *Cucurbita pepo*. *Plant Physiol.* 62: 713-717
- Thomas, M.V. 1985. The definition and measurement of intracellular free Ca. In Trewavas, A.J. (ed.). *Molecular and Cellular Aspects of Calcium in Plant Development*. Plenum Press, New York. p.141-147
- Ting, I.P. and C.B. Osmond. 1973a. Photosynthetic phosphoenolpyruvate carboxylases: Characteristics of allo-enzymes from leaves of C₃ and C₄ plants. *Plant Physiol.* 51: 439-447
- Ting, I.P. and C.B. Osmond. 1973b. Multiple forms of plant phosphoenolpyruvate carboxylase associated with different metabolic pathways. *Plant Physiol.* 51: 448-453
- Tingley, M. 1944. Concentration gradient in plant exudates with reference to the mechanism of translocation. *Amer. J. Bot.* 31: 30-38
- Trevelyan, W.E., D.P. Procter and J.S. Harrison. 1950. Detection of sugars on paper chromatograms. *Nature* 166: 444-445
- Trip, P. 1969. Sugar transport in conducting elements of sugar beet leaves. *Plant Physiol.* 44: 717-725

- Tully, R.E. and A.D. Hanson. 1979. Amino acids transported from turgid and water-stressed barley leaves. 1. Phloem exudate studies. *Plant Physiol.* 64: 460-466
- Urquhart, A.A. and K.W. Joy. 1981. Use of phloem exudate technique in the study of amino acid transport in pea plants. *Plant Physiol.* 68: 750-754
- Van Die, J. and P.M.L. Tammes. 1975. Phloem exudation from monocotyledonous axes. In Zimmermann, M.H. and J.A. Milburn (ed.). *Transport in Plants. I. Phloem Transport*, Springer Verlag, New York. *Encycl. Plant Physiol.* NS I: 196-222.
- Vattuone, M.A., F.E. Prado and A.R. Sampietro. 1981. Cell wall invertases from sugar cane. *Phytochem.* 20: 189-191
- Vickery, R.S. 1978. Peroxidases do not move in phloem. *Planta* 138: 105-106
- Walker, T.S. 1972. The purification and some properties of a protein causing gelling in phloem sieve tube exudate from *Cucurbita pepo*. *Biochim. Biophys. Acta* 257: 433-444
- Walker, T.S. and R. Thaine. 1971. Proteins and fine structural components in exudate from sieve tubes in *Cucurbita pepo* stems. *Ann. Bot.* 35: 773-790
- Walsh, M.A. 1980. Preservation of the tonoplast in metaphloem elements of embryonic roots of *Zea mays* L. *Ann. Bot.* 46: 557-565
- Wareing, P.F. and J. Patrick. 1975. Source-sink relations and the partition of assimilates in the plant. In Cooper, J.P. (ed.): *Photosynthesis and Productivity in Different Environments*. Cambridge University Press, London. p.481-499
- Watson, D.J. 1971. Size structure and activity of the productive system of crops. In Wareing, P.F. and J.P. Cooper (ed.). *Potential Crop Production*. Heinemann, London. p.76-88
- Webb, J.A. and P.R. Gorham. 1964. Translocation of photosynthetically assimilated ^{14}C in straight-necked squash. *Plant Physiol.* 39: 663-672
- Webb, J.A. and P.R. Gorham. 1965. Radial movement of C^{14} -translocates from squash phloem. *Can. J. Bot.* 43: 97-103

- Webb, K.L. and J.W.A. Burley. 1962. Sorbitol translocation in apple. *Science* 137: 766
- Webb, K.L. and J.W.A. Burley. 1964. Stachyose translocation in plants. *Plant Physiol.* 39: 973-977.
- Weibull, J., S. Brishammar and J. Pettersson. 1986. Amino acid analysis of phloem sap from oats and barley: A combination of aphid stylet excision and high performance liquid chromatography. *Entomol. Exp. Appl.* 42: 27-30
- Whereat, A.F., D.R. Snyderman and L.A. Barness. 1968. Thin-layer chromatography of citric acid cycle intermediates, pyruvate and lactate. *J. Chromatog.* 36: 390-393
- Zamski, E., and M.H. Zimmermann. 1979. Sieve tube longevity in white ash (*Fraxinus americana*) studied with a new histo-chemical test for the identification of sugar. *Can. J. Bot.* 57: 650-656
- Ziegler, H. 1975. Nature of transported substances. In Zimmermann, M.H. and J.A. Milburn (ed.). Transport in Plants. I. Phloem Transport. Springer Verlag, New York. *Encycl. Plant Physiol.* NS I: 59-100
- Zimmermann, M.H. 1961. Movement of organic substances in trees. *Science* 133: 73-79
- Zimmermann, M.H. 1963. How sap moves in trees. *Sci. Amer.* 208: 132-142

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