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An Analysis of Sugars in Vein Tissue and Free Mesophyll Cells of Squash Leaves

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AN ANALYSIS OF SUGARS IN VEIN TISSUE AND FREE MESOPHYLL CELLS OF SQUASH LEAVES

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BY

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AN ANALYSIS OF SUGARS IN VEIN TISSUE AND FREE MESOPHYLL CELLS OF SQUASH LEAVES

The statement that translocated photosynthates move from the leaf to areas of high metabolic activity via the phloem in the higher plants is above question. From this point, however, researchers and scholars fall into several camps concerning the actual mechanism of translocation.

There are three major theories towards which most research is directed: (1) the electro-osmotic therory; (2) the protoplasmic streaming theory; and (3) the mass-flow theory. The first is based on a potassium pump mechanism and is supported by data from only a few researchers (23). The second theory, protoplasmic streaming, has been advanced by Thaine (26,27). His translocation theory is based on the alleged presence of transcellular strands within the sieve elements of phloem. It is believed that organic translocates move along these strands and that the flow of translocates can be bi-directional within the sieve element. Supporting evidence by other investigators (2,28) for bi-directional flow makes the protoplasmic streaming theory more acceptable, although Thaine's original framework has come under question. Esau et al. (5) have found Thaine's interpretation of transcellular strands to be inaccurate. She believes that they are artifacts of poor microtechnique and microscopy.

The last theory, mass-flow, is the one engendering most support and has been accepted by more students of translocation than any of the others. Proposed by Münch (18) in 1927, it remains, today, the best

explanation for most cases of translocation. Simply stated, mass-flow is the movement of translocates from an area of high photosynthate accumulation and high osmotic pressure (source) to an area of high metabolic activity and low osmotic pressure (sink). According to this theory, the osmotic potential difference occurs in only one direction and the corresponds to the direction of translocation. The explanation of bi-directional flow is not possible within the present limits of the massflow theory and is, therefore, the main point of objection to mass flow.

The theories, which are discussed above, are primarily involved with the long distance transport of assimilates; i.e., studies involving movement down (or up) the petiole and stem. In the past decade, much study has involved the actual movement of photosynthetic derivatives from areas of CO_2 fixation to areas of translocation within the leaf (6,9,10, 12,16,29). Many of the studies involve movement in and out of the blade, as well as phloem-loading mechanism (3,7,17). The sugars translocated by squash plants used in this study are stachyose, raffinose, and sucrose (21,32). The latter is the major translocated sugar of most higher plants. Beitler and Hendricks (1) have conducted experiments with squash involving analysis of C^{14} labelled products within the blade. They have indicated that stachyose, a major translocated sugar in Cucurbita, is a product of photosynthesis and is produced in the mesophyll cell. Webb has also made this assumption, but with no experimental evidence (30). Studies of this nature have incorporated the entire leaf blade. Although the sugars were produced as a result of photosynthesis, their exact location within the lamina could not be determined. Studies of isolated mesophyll cells, as well as other leaf cells, would be necessary to make this determination.

Much work has been accomplished with the isolation, both enzymatically and mechanically, of intact and metabolically active mesophyll cells from tobacco, soybeans, and other angiosperms (4,14,15,19,20,24). Racusen and Aronoff (20) found soybean mesophyll cells to be qualitatively similar to whole leaves in ethanol soluble compounds. There has been no report of isolation of squash mesophyll cells to the knowledge of the author. An analysis of sugars extracted from squash mesophyll cells may be of merit in studies of phloem-loading mechanisms when correlated with results of sugar analysis in laminar phloem.

It is the purpose of this study to quantitatively analyze the sugar content of mesophyll cells and leaf vein tissue, as well as to make comparisons and/or correlations between the two if possible. This project is a survey encompassing both non-reducing, translocated sugars and reducing, metabolic pathway sugars. The translocated sugars would be expected to appear in large quantities in the vein tissue and possibly in the free mesophyll cells, but the reducing sugars should predominate in the mesophyll cells.

METHODS AND MATERIALS

Seeds of straight-neck squash (Cucurbita melopepo torticollis Bailey) were planted at a depth of 2 cm. in clay pots filled with greenhouse soil. The soil was wetted and the pots were placed in one of two areas: (1) the constant temperature room with a temperature of 27C and a light intensity of approximately 800ft-c at the top of mature plants and 200 ft-c at the top of the pots. Light was produced from two florescent Grow-Lux 40 watt bulbs and four 40-watt incandescent bulbs and (2) A Sherer controlled environment chamber, also with a temperature of 27C, but with a light intensity of 1500 ft-c throughout most of the chamber. Light was emitted from four 40-watt incandescent bulbs and four 40-watt florescent bulbs. The photoperiod for both areas was 16 hours of light followed by 8 hours of darkness.

After germination, seedlings were reduced to four per six inch pot. This reduced competition but insured a high volume of leaves that was needed for mesophyll cell isolation. Leaves were harvested after the plants had flowered (28-36+ days). Flowering insured adequate "sink" areas, which facilitate production and movement of translocates. Main vein tissue did not include any tissue less than 0.5mm in width due to difficulty of dissecting any vein tissue less than this width. After dissection, the tissue was either stored at a -25C for a short time or was prepared immediately for sugar extraction. Sugars from pre-weighed vein

tissue were extracted with 80% (v/v) ethanol using a micro-Soxhlet extraction apparatus for three hours. At the end of this time, the volume of extracts were adjusted to 25 or 50 ml depending on the sample involved. The samples were stored at -25C.

Separation of individual sugars was accomplished by descending-flow method of paper chromatography. Approximately 50-200 µg of sugar was spotted on 19 x 46cm Whatman No. 1 paper chromatography strips. This sugar approximation was derived from a preliminary experiment which determined the approximate amount of sugars per ml of sample, based on 1 µg per .01 optical density unit. One ml of sample was usually spotted. 25 µg of known sugar solution (1%) was spotted on side strips for location of unknowns. The chromatograms were placed in a solution of 1-butanol, acetic acid and water (3:3:2) and allowed to develop for 24 hours at room temperature. This amount of time insured good separation of the sugars studied. After this time, chromatograms were dried for 10 minutes at 100C in a forced-draft chromatography oven. The side strips, 4.5cm in width, were cut from the chromatogram and sprayed with benzidine reagent (.5g genzidine, 10 ml (v/v) trichloroacetic acid, 10 ml glacial acetic acid and 80 ml 95% ethanol). Observable sugar spots developed within 5 minutes in a forced draft chromatography oven at 100C. Unknown sugars on the chromatogram were located with the aid of these side strips. The separated sugars, each on one inch wide paper strips, were eluted into test tubes with approximately one ml. of distilled water. Sugars were stored at -25C or were prepared immediately for analysis. This was accomplished by evaporating the eluate with a Rotary Evapo-Mix with reduced pressure and a water bath temperature of 85C. One ml of distilled water and 3 ml

of anthrone reagent (0.2g anthrone per 100 ml conc. sulfuric acid) were added to the dried sample and shaken by a Vortex Jr. mixer. Samples, thus prepared, were placed in a boiling water bath for 10 minutes, then placed in ice until they approached room temperature, and finally transferred to colorimeter tubes. The tubes were placed in a Bausch and Lomb Spectronic 20 spectrophotometer and their optical densities read at a wavelength of 620 nanometers. The spectrophotometer was zeroed with an anthrone-water blank. The sugars under examination in this study were stachyose, raffinose, sucrose, glucose, and fructose.

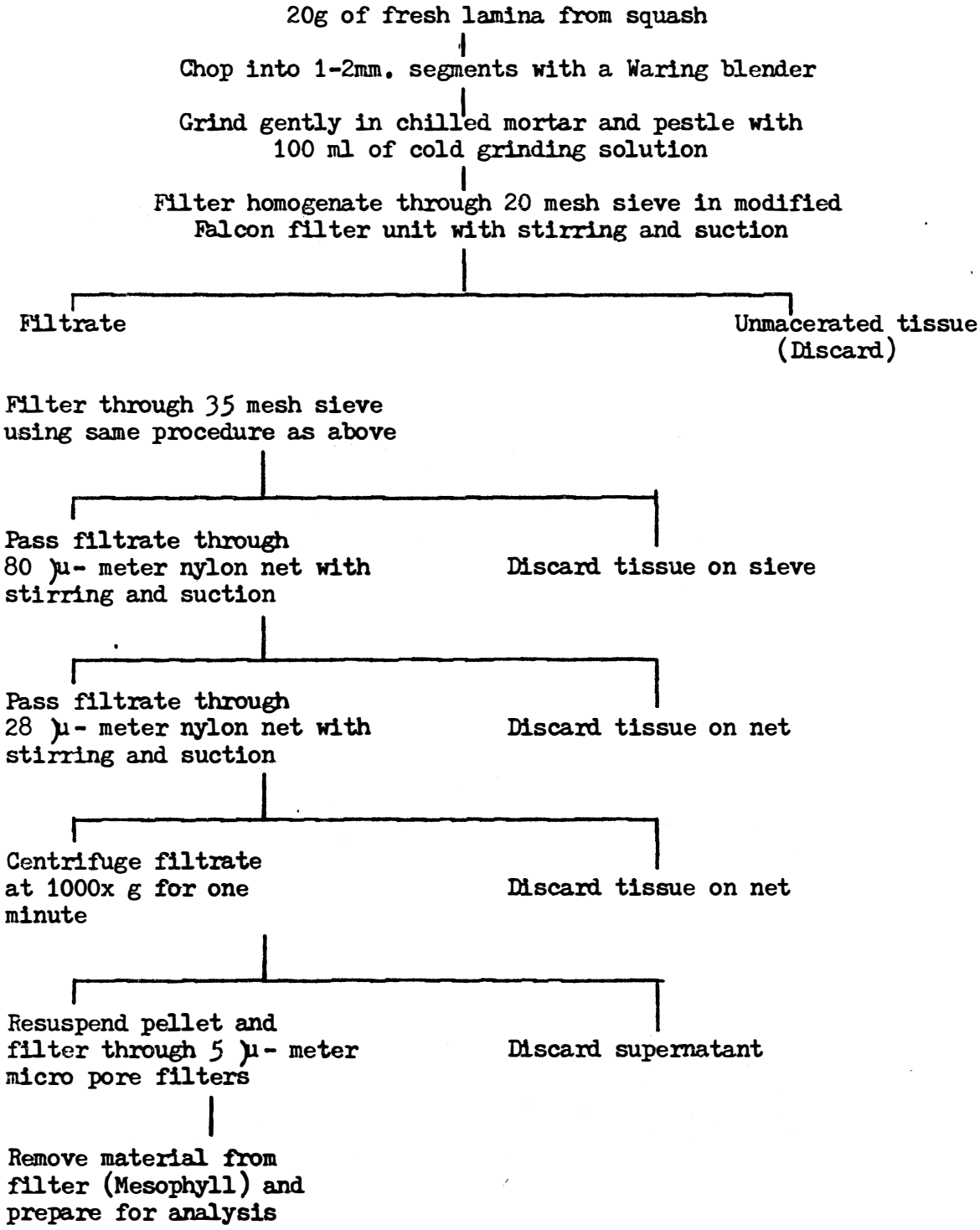
The remaining portion of the lamina, after dissection of the main veins, was stored at -25C until a large quantity (approximately 20 grams) was accumulated. This portion was used for the mesophyll-isolation phase of the experiment.

The technique for isolation used for this experiment was very similar to that of Edwards and Black (4) for isolation of spinach mesophyll cells. The mechanical method of isolation was selected instead of an enzymatic method due to the extensive amount of time (2 hours+) necessary for pectinase to complete its action with squash cells. During this time many metabolic changes could occur within the mesophyll. The mechanical method took no more than 45 minutes at the masimum. 20 grams of laminar strips were placed in a Waring blender and chopped into 1-2mm segments at low speed within several short time intervals. This phase produced much more area from which mesophyll cells could be freed. The segments were then transferred to a Coors procelain mortar and pestle, which was prechilled to 4C. 100 ml of cold granding solution (pH8) were added to the mortar. Edward's original solution was adjusted to 2mm $CaSO_4$ to insure

membrane stability. This leaf suspension was gently ground for 10 minutes and poured through a 20 mesh per inch stainless steel sieve. It was then poured through a 35 mesh per inch stainless steel sieve located in a modified Falcon filter unit. Suction and stirring were applied at this time. The filtrate within the filter unit was poured through an 80 μ-meter nylon mesh attached to a modified Buchner funnel. This mesh allowed the passage of mesophyll cells, but retained much of the minor veins, epidermis, attached mesophyll and ubiquitous epidermal hairs. Previous microscopic examination of squash mesophyll cells indicated that they were 14-20 μ-meters in diameter and the palisade cells were 40-50 μ-meters in length. Based on these dimensions, 28 μ-meter nylon mesh was selected for the next phase of filtration. This allowed passage of mesophyll and retained any fractions that may have passed through the previous filtration (usually epidermal hairs). This filtrate was poured into centrifuge tubes and centrifuged at 1000x g for one minute in a Sorvall SS-34 centrifuge rotor. This sedimented much of the free mesophyll cells, yet carried many free chloroplasts and cell fragments into the pellet as well. Microscopic examination showed that much of this material was less than 5 μ-meters in diameter. For this reason, Gelman micropore filters with pore diameter of 5 μ-meters were selected to filter the pellet remaining after centrifugation. The results yielded plasmopysed mesophyll cells and a few large chloroplasts and/or starch grains (Fig. 3). The yield from the original laminar weight was approximately 1%. Extraction and analysis methodology for mesophyll cells was the same as for vein tissue discussed earlier. Figure 1 consists of a flow chart of the isolation procedure for squash mesophyll cells.

Figure 1

Flow Chart for Isolation of Mesophyll Cells (Modified from Procedure of Edwards and Black)



RESULTS AND DISCUSSION

The samples were prepared of different days and from different sets of plants. This factor cannot be overlooked due to fluctuations in growth chambers and slight differences for each new set of plants. For these reasons, samples were placed in different groups based on these circumstances. All samples from Group I were prepared on the same day and from the same group of plants. This included both vein tissue and mesophyll cell samples. Groups I, II and IV were grown in the constant temperature room; Group III was grown in the controlled environment chamber. It was found in this study that an optical density of 0.06 is considered the amount of error within the method and technique used by the author. Although chromatograms were washed with distilled water to remove impurities before use, this did not guarantee freedom from error. To determine the final amount of error, a blank chromatogram was spotted with 80% (v/v) ethanol (the solvent for all samples in this study) and underwent the same procedures as the other chromatograms. The figure 0.06 was the average optical density reading obtained from material eluted from five locations on the chromatogram (Table 1). These five locations were areas for the sugars studied, had they been there. Corrected numbers of the results were derived after subtracting 0.06 from each original optical density reading.

Results of the sugar analysis for main vein tissue are found in Table 2. Some fluctuations can be observed in the amounts of stachyose, raffinose, and glucose. Three possible reasons can be cited to explain

Table 1: Optical Density of Blank Chromatogram

Position on Chromatogram	O. D.
Stachyose	0.045
Raffinose	0.08
Sucrose	0.055
Glucose	0.06
Fructose	0.06

Average O. D. - 0.06

Standard Deviation - 0.01

Table 2: Vein Tissue Sugars (Milligram per Gram Fresh Weight)

Group	Sample	Stachyose	Raffinose	Sucrose	Glucose	Fructose
I	1	6.95	3.53	1.24	1.82	1.90
	2	2.89	1.26	1.36	1.15	1.14
II	11	0.66	0.66	2.22	2.42	1.02
	12	0.94	0.32	2.62	3.50	1.54
III	18	0.89	0.94	2.34	3.68	1.06
	19	0.64	0.60	1.53	3.65	1.23
	20	0.86	0.71	1.34	3.33	1.08
	21	0.76	0.76	1.52	3.04	0.98
IV	22	0.29	0.30	1.16	0.37	0.00
	23	0.14	0.00	1.64	0.00	0.00
AVERAGE		1.50	0.91	1.70	2.30	1.00

this. (1) The oligosaccharides were hydrolysed and converted to glucose before the extractions took place. This assumption does not support observations by Webb (31) that stachyose accumulates in the blade and undergoes little further metabolism when translocation is reduced. (2) It is possible that the leaves which underwent dissection were still importing translocates at the basal end. The basal end of the leaf represented the greater portion of sample bulk due to its larger width. Although this section of vein tissue is larger, it is also younger than the apical end and, therefore, could possibly be importing sugars and hydrolysing them for growth requirements. Experiments with sugar beets by Fellows and Geiger (6) indicated import still occurred at 40% to 50% final laminar length although, export was rapidly increasing at this time. Kocher and Leonard (16) found that reduced leaf expansion did not affect transition from import to export in bean leaves. In studies of squash, leaves of approximately 10cm. in length and 10.5cm. in width imported only a small amount at the base of the lamina. Leaves 4cm. x 3.5cm. were importing in 50% of the laminar area. According to these investigators, transition from import to export involved two days' growth and occurred between 10% and 45% final leaf expansion (29). The use of a plastochron index to determine leaf age was a major factor which made their experimental situation much different than that of this study. Leaves used in this study were approximately 8-10cm. in width and length and three to six weeks of age. Any import would likely only have occurred in the most basal portions which, as indicated earlier, may have been a factor in the variations in the data. (3) The final explanation for the sugar fluctuations is that glucose is needed in large amounts for maintenance of the phloem and/or for energy in an activated flow of translocates. According to Biddulph

and Cory (2), movement of translocates up and down within one bundle sheath necessitates protoplasmic streaming and therefore the use of energy. Studies of sugar gradients in detached sugar cane leaves presented the possibilities of an activated mass flow which would also need a great deal of energy (12). In three of the four groups from Table 1, an increase of translocated sugars indicated a decrease in glucose and vice versa. This suggests that glucose may possibly be used for energy processes involved in translocation.

The results for analysis of sugars in mesophyll cells are found in Table 3. The procedure for grouping and determination of final data was the same as in the vein tissue. Group I in Table 2 was identical to Group I in Table 3 and so on. The much lower sugar concentrations in the mesophyll as compared to that in veins is supported by findings from Geiger et al (11). These findings also indicate that chloroplasts have a higher solute concentration in comparison with the rest of the cytoplasm. This correlates well with the results of Table 3. Groups I and II had a much higher concentration of free chloroplasts in the sample than Groups III and IV. The first two groups were collected without the last step of the isolation procedure which disposed of the numerous free chloroplasts alluded to in the methods. The higher concentration of chloroplasts, should have raised the sugar concentration of the sample which is what occurred.

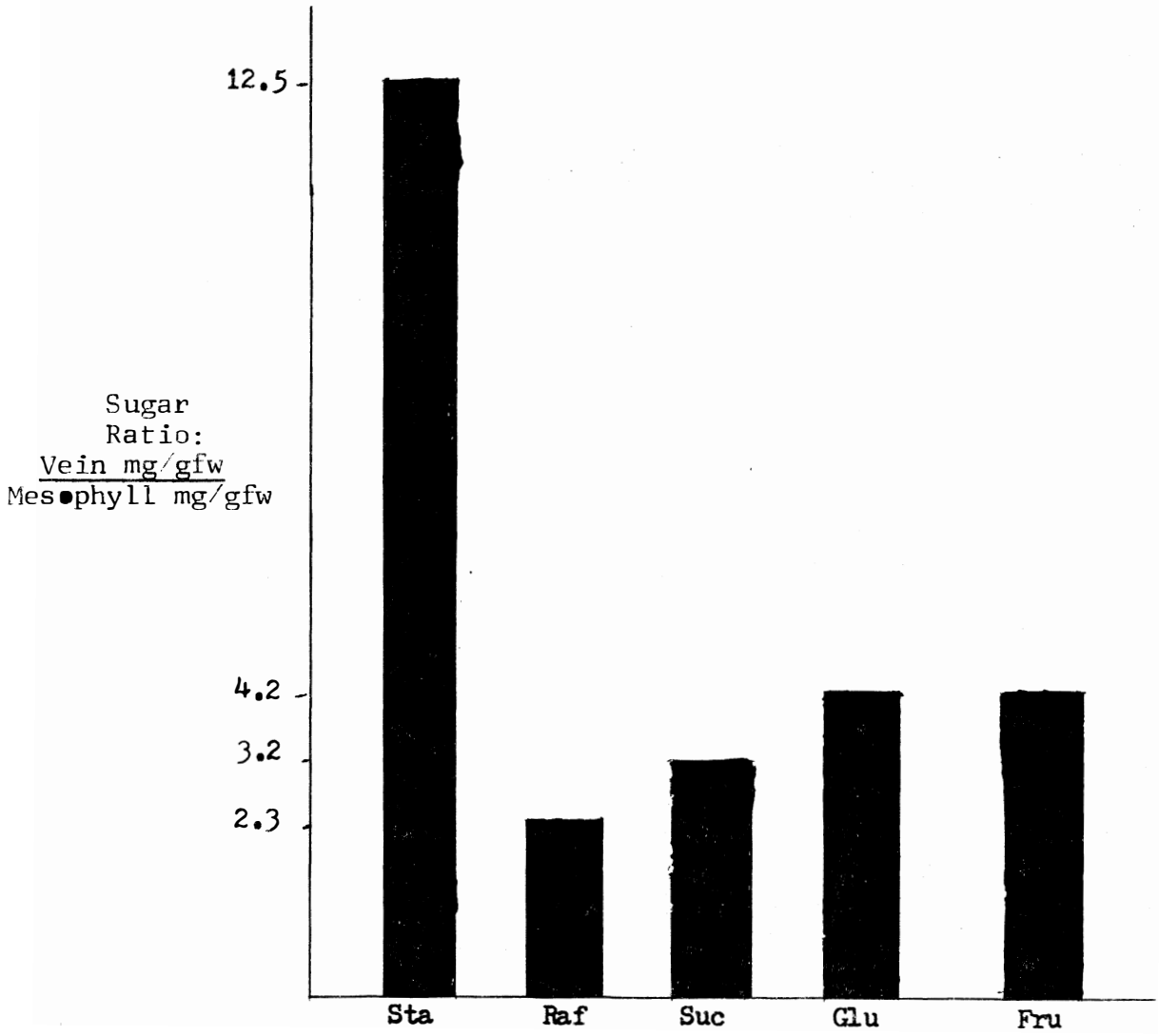
Stachyose, according to the findings of Beitler and Hendrix (1), the assumptions of Webb (30), and the findings of Jensen et al (14), was found in the mesophyll and is very likely produced in the chloroplast. This data indicates the same findings but the relatively small amount found within the mesophyll in this study merits further discussion.

Table 3: Mesophyll Cell Sugars (Milligram per Gram Fresh Weight)

Group	Sample	Stachyose	Raffinose	Sucrose	Glucose	Fructose
I	3	0.15	0.94	0.89	0.78	0.35
II	15	0.24	0.35	0.58	0.64	0.30
III	16	0.03	0.11	0.12	0.26	0.18
IV	25	0.07	0.12	0.52	0.46	0.07
AVERAGE		0.12	0.38	0.53	0.54	0.24

Figure 2 compares the ratios of sugar between the veins and mesophyll. Ratios were selected for comparison between vein and mesophyll because it was the best way to show the increase in concentration from the mesophyll to the main vein. It is clearly evident that stachyose, the major translocated sugar in squash, differs greatly between mesophyll and main vein concentrations. There are three possible reasons for this: (1) The loading mechanism, if between the mesophyll cells and the vascular tissue, as some authors indicate (3,7), is extremely selective for stachyose. Hendrix (13) has shown that externally added C^{14}-sucrose will load in the phloem quite easily. This indicates that the loading mechanism is not as specific for stachyose as the data in Figure 1 would suggest. (2) The stachyose may "leak out" of the mesophyll cells into the free space (apoplast) where it is accumulated and loaded into the minor veins via several different cell types (10,17). According to Sovonick et al (22), sugar loading into minor veins is an active process requiring the expenditure of ATP. This may add to further explanation for the high concentration of reducing sugars (Fig. 2). (3) The stachyose, although produced in the mesophyll cells, is produced in greater quantity upon entrance into the minor veins. Several investigators (8,12), conducting work with sugar cane, have found sucrose to be hydrolysed to hexoses in the free space and then reconverted upon entrance into the veins. Experiments by Yamamoto et al (33) with tobacco have indicated that smaller vascular bundles were used for sucrose synthesis rather than translocation. Studies with beanseed extracts (25) have found a specific enzyme which synthesizes stachyose by adding a galactose molecule to raffinose. The possibilities are apparent. It would seem logical that the constituent parts of the

Figure 2 Comparison of Sugars in Vein and Mesophyll Tissue.



stachyose molecule could be transferred through membranes much easier than the larger stachyose molecule (consisting of sucrose and two galactose molecules). Once the constituents are inside the conducting cells or their associated cells, they are combined to produce the larger more efficient carbon carrying stachyose molecule. If the molecule is produced in quantity once it is inside the conducting system, it could maintain the high concentration gradient necessary for flow much easier than if it was to be actively pumped into the system.

Finally, it must be admitted that although the mesophyll cells were isolated at a temperature of approximately 4C and in a solution isosmotic to hyposmotic (the exact osmotic potential of the cells being unknown) in relation to the cells, there is a possibility that mechanically isolated squash cells will leak. Preliminary experiments with a pectinase solution similar to that used by Jyung et al (14) produced cells of very good quality (Fig. 4). The introduction of C^{14} label into the atmosphere or medium of an isolated cell culture and the time sequenced removal of cell adiquots for analysis of photosynthates would give conclusive evidence in regards to the amount of stachyose in squash mesophyll cells. Isolation of other cells such as phloem parenchyma and bundle sheath cells would add much in investigating the locations of sugar synthesis. Experiments quantitatively analysing sugars in laminar free space of squash would also be beneficial in formulating a model for stachyose incorporation into the phloem.

This project, in its conclusion, suggests that stachyose, more than either of the lesser translocated sugars (raffinose and sucrose), is incorporated into the phloem in some fashion other than directly through the mesophyll. It is probably produced within the bundle sheath or

accumulated adjacent to it in the apoplast before loading.

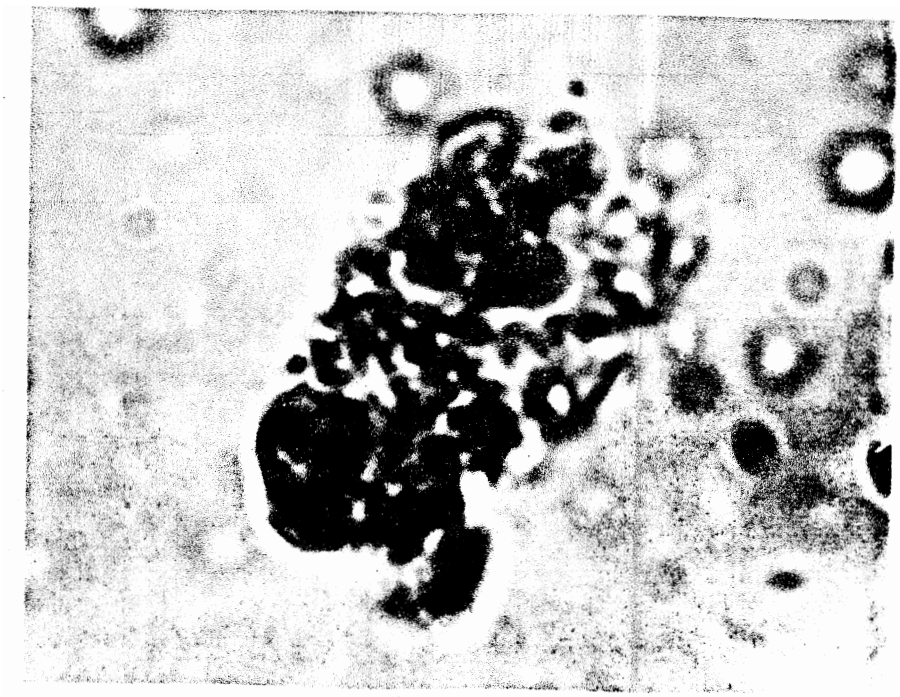


Figure 3. Squash mesophyll cell isolated by grinding with mortar and pestle. 4000X

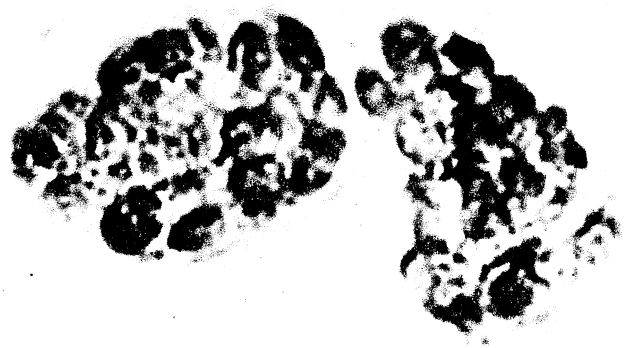


Figure 4. Squash mesophyll cells isolated by the use of a pectinase solution. 2800X

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