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MECHANISM OF ORGANIC TRANSLOCATION IN STRAIGHT-NECK SQUASH

(TITLE)

BY

James F. Nicholson

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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CHARLESTON, ILLINOIS

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ABSTRACT

The mechanism of organic translocation in *Cucurbita pepo* L. variety melopepo torticollis was investigated. Two-centimeter petiole sections from nodes 2 and 3 of 22-day squash were analyzed colorimetrically for the concentration of stachyose and sucrose. Stachyose is an important transport molecule and, in contrast to sucrose, may be localized in the phloem. This investigation attempted to determine the possibility of stachyose being further localized in a particulate cell fraction.

The volume of the phloem was determined to be 20 mm^3 per petiole. The concentration of stachyose per petiole, assuming that all of the stachyose was in the phloem and that the stachyose was evenly distributed in the phloem, was calculated to be between 0.3 and 0.4 molar. The molar concentration of sucrose in the phloem was calculated to be between 0.25 and 0.35.

A fructose density-gradient with minimum and maximum amounts of fructose being 100 and 350 mg/ml, respectively, was used to isolate a fraction rich in stachyose. It was determined that 50 percent of the stachyose was localized in a 3 ml portion of the 14 ml gradient.

These data provide additional evidence to support the theory that phloem transport occurs by means of particulates which move in or on transcellular strands.

MECHANISM OF ORGANIC TRANSLOCATION IN STRAIGHT-NECK SQUASH

As photosynthesis proceeds, the photosynthates move out of the leaves toward other parts of the plant. Roots and stems require them for growth and other metabolic activities, and developing fruits and flowers will either metabolize these photosynthates or store them (20). Elucidation of the mechanism by which parts of the plant obtain the photosynthates is of special concern to plant scientists. There are three major hypotheses of how the photosynthates move long distances in the plant; these hypotheses may be grouped under the heading 'mechanisms of organic translocation.' They are: (1) mass flow, (2) electro-osmosis and mass flow, and (3) protoplasmic streaming. This report is concerned with protoplasmic streaming, a concept which has been extended by Thaine and others (7,13,21,22,23, 24,25) to include the transcellular streaming concept. If transcellular strands are present in the sieve elements, their presence could explain bidirectional translocation in stems (2,3,12,28,29) and would, therefore, indicate that bidirectional movement can occur in

the same phloem bundles (2,12,27). Trip and Gorham (26), using autoradiography, confirmed that longitudinal translocation occurs in sieve tubes rather than in other phloem cells.

Thaine (22), using phase-contrast microscopy, observed small plastids and mitochondria-like particles moving along the transcellular strands. The transcellular strands, 1 u to 7 u in diameter, were seen in sieve tube elements, phloem parenchyma, border parenchyma, and cortical cells (22). Transcellular strands have been observed in such plants as _Primula obcooica_ (21), _Populus_ (13), _Tilia_ (13), and straight-neck squash (22). Esau _et al_. (11) described the transcellular strands observed by Thaine as "merely lines caused by diffraction of light from walls out of focus." They suggested that the sieve plates described by Thaine (21,22) resembled end walls of parenchyma cells rather than those of sieve elements (11).

Trip and Gorham (27), using straight-neck squash, determined that most of the radioactivity supplied to the leaf as tritiated glucose was present in the form of the transported sugars stachyose, sucrose, and raffinose after analysis of petiole sections. It has been confirmed by others (19,29,30,31,32) that these three carbohydrates are the materials translocated in cucurbits.

Since stachyose has been established as a transport molecule in squash (19,29), straight-neck squash was selected as the experimental plant for the present study. Straight-neck squash also has other desirable characteristics which suggest its use as an experimental plant: (1) it has a long hollow petiole much of which is vascular tissue, (2) it can be grown in growth chambers rapidly and with relative ease, and (3) in recent years it has been studied extensively by plant physiologists.

Hendrix (16), by analysis of squash petiole sections, determined that stachyose incorporated 60-77% of photosynthetically fixed C^{14}. The amount of C^{14} incorporated into stachyose was dependent on the length of time of labelling. Stachyose, unlike sucrose, appears to be present only in the transport area of the sieve tubes.

No publications reporting attempts to isolate transcellular strands have been found. The present investigation utilized density-gradient centrifugation (4,5,6) to isolate pure transcellular strands or particulates on the strands. If a particulate fraction, rich in stachyose and derived from the phloem could be isolated, then the first concrete evidence pointing toward the involvement of transcellular strands and associated particulates in the translocation mechanism would have been obtained.

MATERIALS AND METHODS

Plant material. Seeds of Cucurbita pepo L. variety melopepo torticollis Bailey were germinated in a moist chamber for 4 days. They were maintained on cheese-cloth over an aerated, 10^{-4} M $CaSO_4$ solution for an additional 4 days. The seedlings were then transferred to an aerated mineral solution (Table 1), in a Sherer Controlled Environment Chamber, Model 25-7. On the day of each experiment (22 or 23 days from the time the seeds were put into the moist chamber), the petioles from nodes 2 and 3 (counting from the bottom of the squash to the top) were removed. These petioles were used because the blades were large and would be exporting more photosynthates than any of the other blades of this age of plant.

Experimental procedure. The petioles from nodes 2 and 3 were sectioned into several 2-centimeter lengths. The first centimeter of the petiole, that portion closest to the stem, was not used due to the reported accumulation of sugars in this region (15). The 2-centimeter sections were weighed on a Sartorius analytical balance to the nearest hundredth of a gram. Each section was then extracted separately in a soxhlet extraction bank

with 80% (v/v) ethanol in water for 3 hours. The extracts were diluted to 100 ml with 80% (v/v) ethanol in water and stored in a freezer at -25 C until sugar determinations were made.

Total sugar determination. One ml of each extract was evaporated in each of three test tubes by using a Rotary Evapo-mix and vacoum pump. One ml of distilled water was added to the dried extract. To this, 3 ml of anthrone reagent (100 mg of anthrone dissolved in 50 ml of concentrated sulfuric acid) was added and the contents stirred by a Vortex mixer. The mixture was heated to 100 C for 10 minutes in a water bath. The samples were cooled to room temperature and then transferred to Spectronic 20 tubes. The optical density of the extracts was read at 620 nm in a Bausch and Lomb Spectronic 20 and compared with an anthrone-water blank. Standard curves were prepared by testing known concentrations of stachyose and sucrose: 100, 50, 25, and 12.5 ug/ml. The optical density for each of the sugars was compared to the concentration. The results indicated that for each 0.01 optical density of total sugars there was approximately 1 ug of sugar. In addition there was 1.2 ug of stachyose and 0.7 ug of sucrose for each 0.01 optical density unit.

Table 1. EXPERIMENTAL PLANT ENVIRONMENT

Mineral Solution

$Ca(NO_3)_2$	3×10^{-3} M
KH_2PO_4	2×10^{-3} M
KNO_3	2×10^{-3} M
$MgSO_4$	2×10^{-3} M
H_3BO_3	800×10^{-7} M
$MnCl_2$	150×10^{-7} M
$ZnCl_2$	15×10^{-7} M
$CuCl_2$	8×10^{-7} M
MoO_3	7×10^{-7} M
Fe (as Fe-EDTA)	900×10^{-7} M (10 g Na_4EDTA/1)

Photoperiod	16 hours light, 8 hours dark
Light Period Temperature	24 C ± 1
Dark Period Temperature	19 C ± 1
Lights	Four-150 watt cool white fluorescent tubes. Eight-25 watt incandescent light bulbs
Light Intensity	1000 ft-c, six inches above the top of the culture tanks
Mineral Solution Change	Every 21 days

One ml of each petiole extract was spotted on each of three pre-cut Whatman #1 chromatography paper. Twenty-five ul of a 1% sucrose, 1% stachyose, and 1% raffinose solution was spotted on each side strip. The chromatograms were run in descending 1-butanol: acetic acid: water (3:3:2) solvent system (BAAW) for 24 hours. The chromatograms were removed from the chromatography jars and dried at 100 C for 15 minutes in a forced-draft National Chromatography Oven. The side strips with the known sugars were then cut from the chromatograms. The known sugars (stachyose, sucrose, and raffinose) were located on the side strips by using a benzidine spray reagent. The benzidine reagent was prepared according to the method of Bacon (1) and consisted of 0.5 mg of benzidine dissolved in 10 ml of 40 percent (w/v) trichloroacetic acid in water, 10 ml of glacial acetic acid, and 80 ml of 95 percent ethanol. The side strips were then developed in the forced-draft chromatography oven at 100 C for 15 minutes. The portions of each chromatogram containing stachyose and sucrose were cut out and eluted with approximately 1 ml of water by using an elution apparatus. The eluted solution was dried as before in the Evapo-mix and the amount of individual sugars was determined with the anthrone reagent and Spectronic 20 as outline above.

Preparation of fructose density-gradients.

Fructose density-gradients were used because it was determined that fructose separated from the stachyose on the chromatograms in the BAAW solvent system. Solutions of 100 mg fructose/ml and 500 mg fructose/ml were prepared for preliminary gradients. These solutions for the gradients were found to be too dense; therefore, solutions of 100 and 350 mg fructose/ml were used to prepare the gradients in order to isolate a fraction rich in stachyose. Fifteen ml of each solution was placed into the respective columns of an 60 ml capacity MSE Gradient Maker. The gradient maker was placed on a Thermolyne Stir Mate. A stirring bar was put into the less dense fructose solution to mix vigorously. Tygon tubing (inside diameter 2 mm) was connected to the gradient maker and positioned through a Buchler Polystaltic Pump. Glass tubing was pulled to a fine point and was used to extend to the bottom of 68 x 98-mm plastic test tubes from the Tygon tubing. The fine diameter glass tubing was used so there would be less mixing of the gradient when the tubing was pulled out of the prepared gradient. The speed control of the polystaltic pump was set on 4.5 and the motor was on low when the gradients were prepared. It took 10 minutes to prepare the gradients under these

conditions. The gradients were refrigerated for 1 hour before the petiole supernatant was layered on top the gradients. The gradients were refrigerated to insure a cold medium for the particulate fraction of the supernatant.

The uniformity of gradients was tested by using the same procedure as stated above, but $CoCl_2$ was substituted for fructose. The $CoCl_2$ solution was prepared by adding 23 g $CoCl_2$/l in 1% HCl. $CoCl_2$ was in one column and distilled water in the other column of the gradient maker. Three-ml fractions of the gradient were collected in Spectronic 20 tubes and read at 510 nm on a Spectronic 20. The results were graphed on linear graph paper and a straight line was obtained which indicated that a uniform gradient had been prepared by this procedure.

Preparation of petioles for grinding. A Ficoll-mannitol buffer (0.25 M mannitol, 2.5 g Ficoll/100 ml of solution, 5 mM $MgCl_2$, 0.025 M Tris-HCl (2-Amino-2-(hydroxymethyl)-1,3-propanediol), pH 7.0) was prepared by a modification of Honda's isolation medium (28). Petioles from nodes 2 and 3 were removed from 22-day squash. The two petiole sections, ten ml of cold buffer, and cold, washed sand were put into a cold mortar and pestle. The material was ground vigorously for 5 minutes. All grindate

was kept cold by placing it in an ice bath or by placing it in the freezer. The grindate was filtered through 4 layers of cheesecloth into a centrifuge tube which was placed in a HB-4 Sorvall swinging cup rotor in a RC2-B Superspeed refrigerated Sorvall Centrifuge. The grindate was centrifuged at 1000 x g for 5 minutes to remove all large cellular material (cell walls, cell membranes, etc.) and sand. The supernatant was layered onto the gradients by one-ml graduated pippettes. The gradients were then centrifuged at 25,400 x g for 3 hours.

Collecting gradient fractions. A rubber stopper with two holes was inserted into the centrifuge tube containing the gradient as is diagrammed below (Fig. 1).

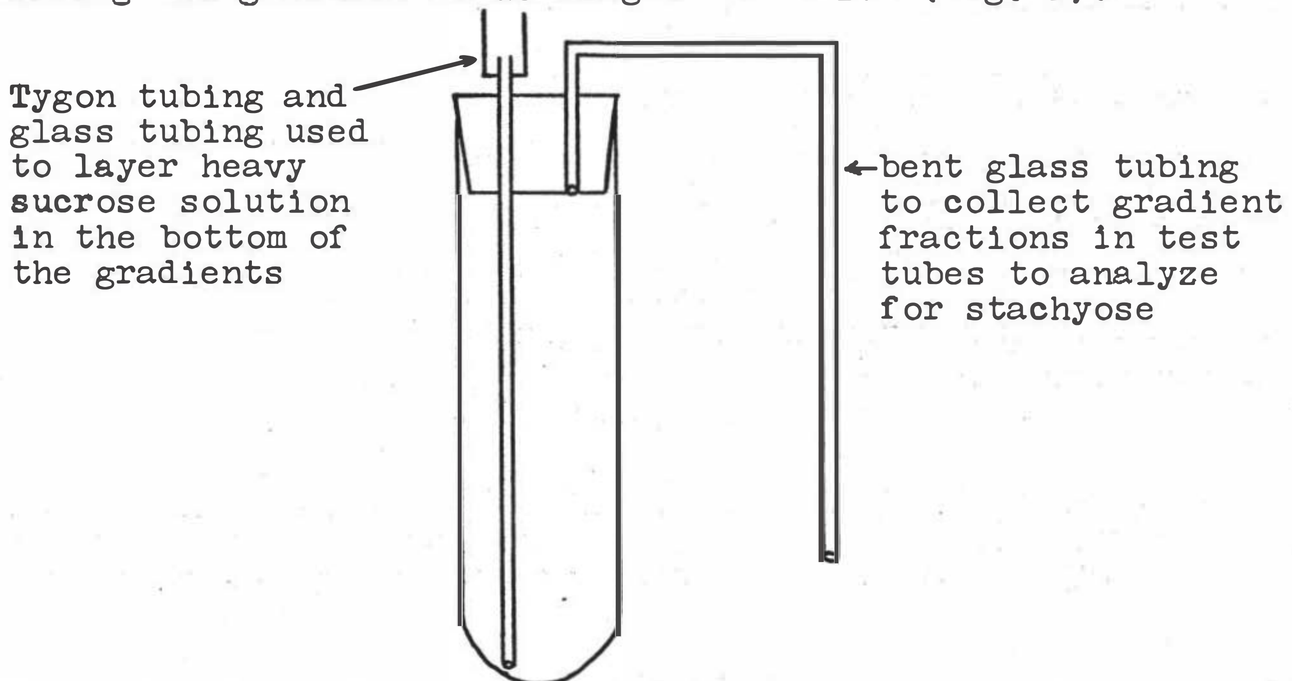


Figure 1. Apparatus constructed to remove fractions of gradients from the test tubes.

Glass tubing inserted through the stopper to the bottom of the tube was used to layer heavy sucrose (700 mg/ml with red food coloring added for visual observation) into the bottom of the gradient. The other hole contained a short piece of tubing which was used to collect the fructose gradient as the heavy sucrose forced the fructose out of the top of the tube. One-ml fractions of the gradient were collected in test tubes to be analyzed for the presence and amount of stachyose by descending paper chromatography.

Assay of gradient fractions. A 100 ul portion of each 1 ml fraction was spotted on Whatman # 1 chromatography paper as before. Only 100 ul was spotted per sheet because of the high concentration of fructose, which made large spots at the origins of the paper when the fraction dried. The chromatograms were developed for 24 hours in the same BAAW solvent system used earlier. Only stachyose-containing strips were eluted since the high concentrations of fructose did not separate well from the sucrose. The amount of stachyose per strip was calculated as stated earlier.

Volume of petiole and phloem. The petiole was cut into2-cm sections. Free hand cross sections were then made of the petiole and put on a slide with 1% analine blue.

Measurements of the height and width of the internal and external phloem were made using a Bausch and Lomb stereo-zoom phase microscope at a magnification of 430X. The phloem was observed to be triangular and became proportionally smaller from the base to the blade. Each 2-cm petiole section was then cut lengthwise and flattened so the width, length, and height could be measured. The outline of the petiole was conceived as a trapezoid and the volume obtained accordingly.

RESULTS AND DISCUSSION

Comparison of total sugars. The total sugars of each 2-cm petiole section were calculated to determine approximately the concentration of sugars along its length. The total sugars were approximations since it was not possible to determine the exact proportions of the individual sugars in the extract; this is because each sugar (on a per mg basis) yields a slightly different optical density with the anthrone reagent. For purposes of approximation, each microgram of sugar was assumed to result in a 0.01 optical density under the previously mentioned experimental conditions. The total sugars for every 2-cm section of petiole from nodes 2 and 3 (Table 2) were estimated by using this approximation.

A comparison of the total sugars from each petiole section of nodes 2 and 3 (Fig. 2*) indicates that the total sugars in each 2-cm section decreased in approximately the same proportions progressively from the base of the petiole to the blade. The data was as expected since the weight of each petiole section (Table 2, Fig. 3) also decreased from the base of the petiole to the blade.

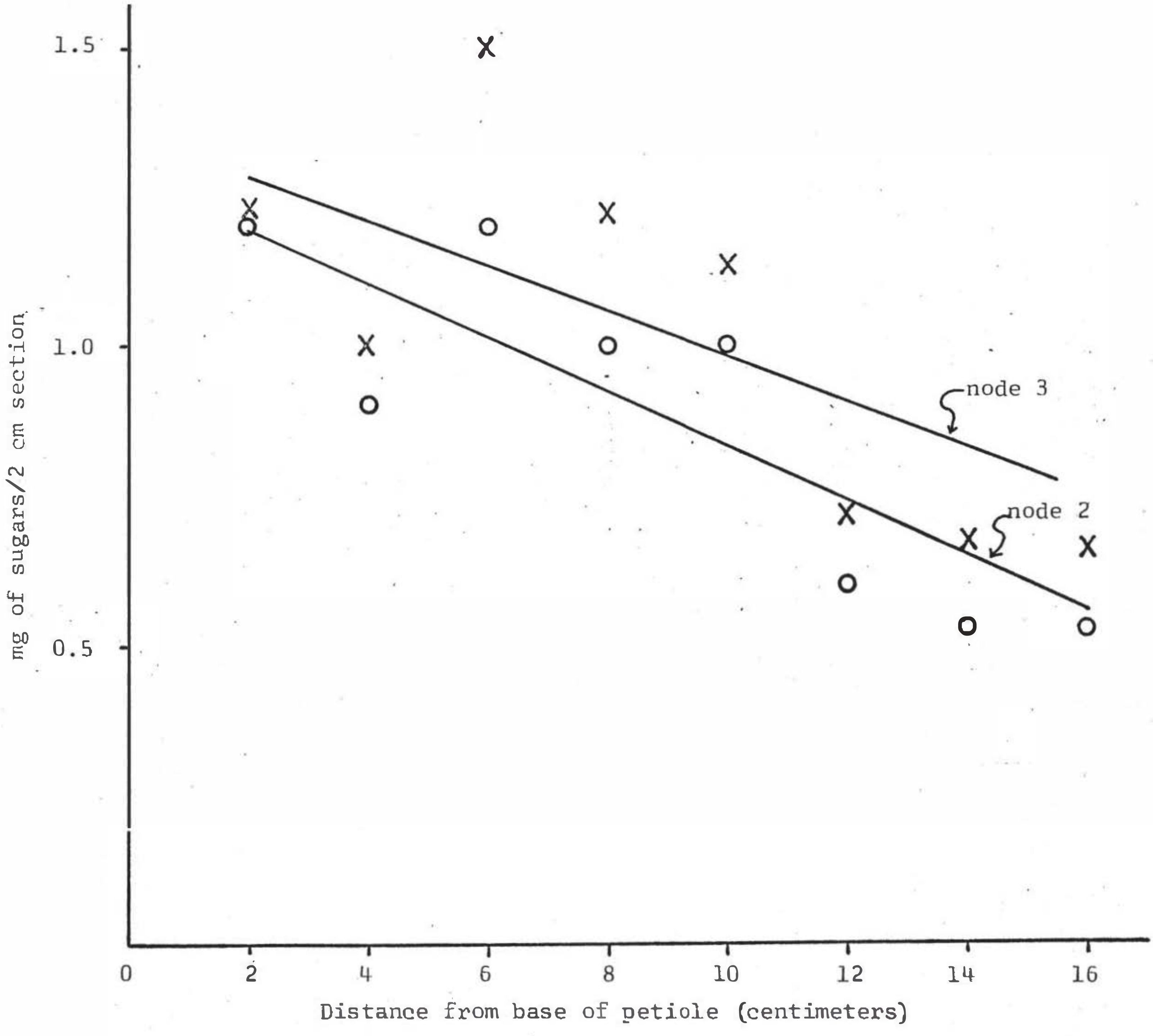
* Straight lines on graphs were calculated by the method of least squares.

Table 2. Comparison of total sugars and the transported sugars (stachyose and sucrose) in 2-cm sections of petioles from nodes 2 and 3 and in leaves from 22-day straight-neck squash.

node	cm section	weight (g)	mg total* sugars	mg stach-yose	mg sucrose
2	2	0.52	1.2	0.84	0.29
	4	0.43	0.9	0.63	0.21
	6	0.36	1.2	0.77	0.25
	8	0.31	1.0	0.77	0.25
	10	0.27	1.0	0.7	0.25
	12	0.23	0.6	0.45	0.17
	14	0.19	0.53	0.35	0.15
	16	0.17	0.53	0.35	0.17
leaf	--	1.0	7.0	0.88	1.58
3	2	0.58	1.23	0.84	0.21
	4	0.5	1.0	0.7	0.25
	6	0.42	1.5	0.98	0.33
	8	0.38	1.23	0.63	0.37
	10	0.33	1.13	0.77	0.37
	12	0.3	0.73	0.63	0.2
	14	0.26	0.67	0.56	0.33
	15.58	0.18	0.67	0.56	0.17
leaf	--	1.0	8.0	0.98	1.74

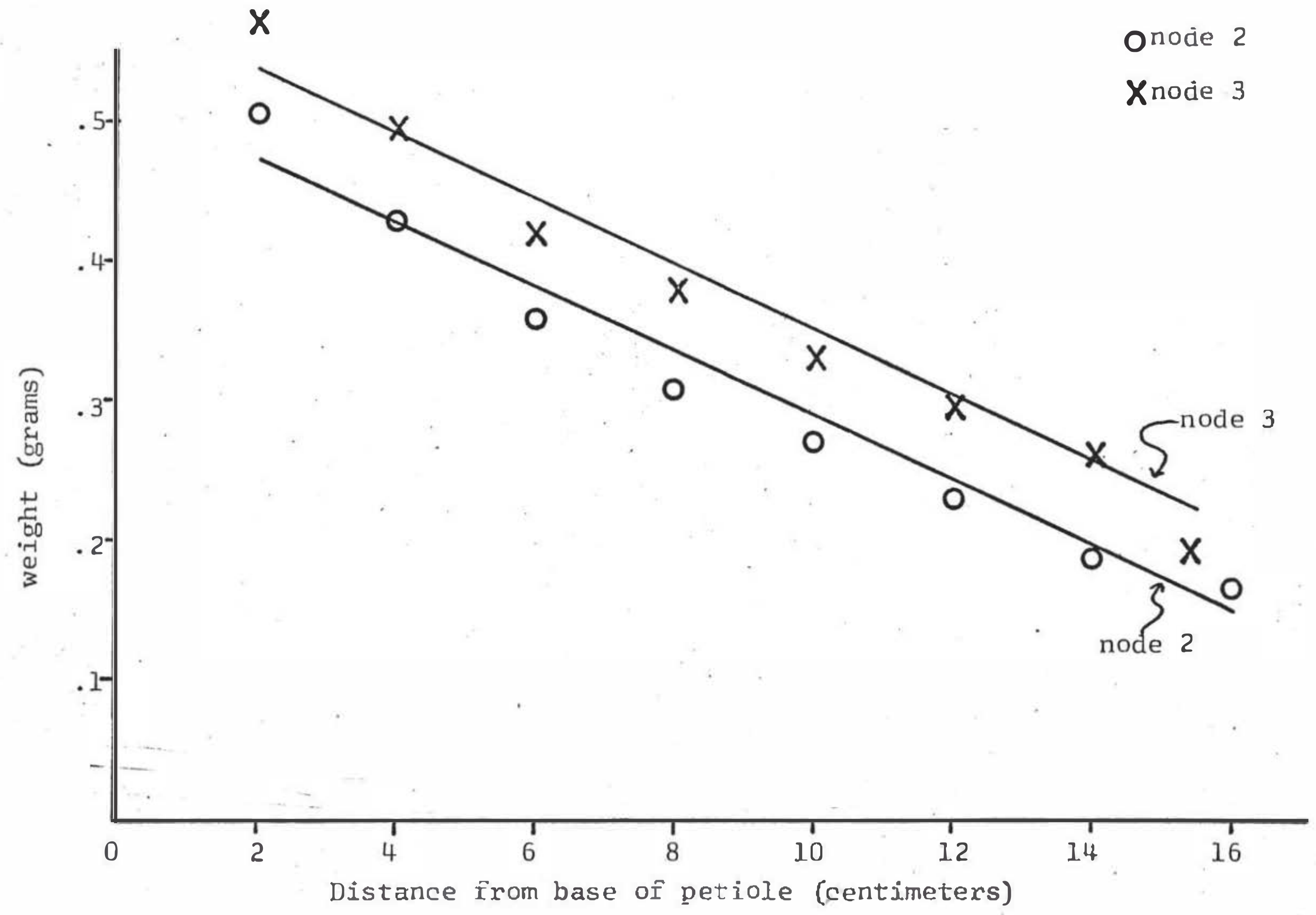
* approximate (See page 12)

Fig. 2--Total sugars in 2-cm sections of petioles from nodes 2 and 3 in 22-day straight-neck squash.



O node 2
X node 3
1.5
1.0
0.5
mg of sugars/2 cm section
node 3
node 2
0
2
4
6
8
10
12
14
16
Distance from base of petiole (centimeters)

Fig. 3--Comparison of the weights of 2-cm petiole sections from nodes 2 and 3 in 22-day straight-neck squash.



node 2
node 3
node 3
node 2
.5
.4
.3
.2
.1
weight (grams)
0
2
4
6
8
10
12
14
16
Distance from base of petiole (centimeters)

A number of calculations was made in an attempt to compare the total sugars obtained in each section with the structural features of the phloem. The results (Table 3, Fig. 4) indicate that the volume of the phloem and the total volume of each 2-cm petiole section decrease proportionally with the amount of total sugars and the weight (Fig. 3) of each petiole section.

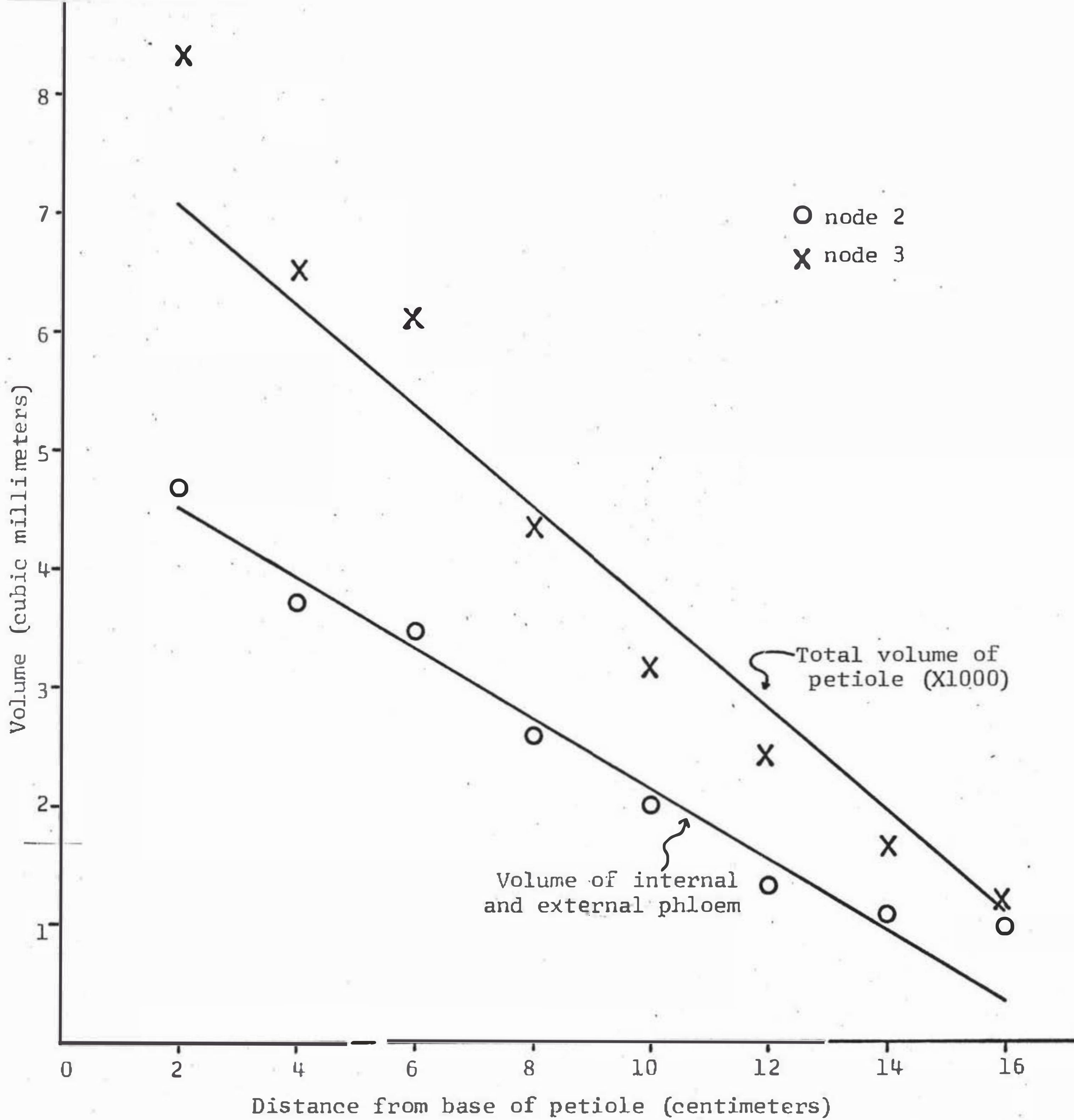
Comparison of stachyose and sucrose. Stachyose and sucrose accounted for approximately 90 percent of the sugars in the 2-cm petiole sections. In comparing the slopes of the lines in Figures 5 and 6, the sugars appear to decrease at approximately the same proportions progressively from the blade to the base of the petiole.

Calculations were made to compare the structural features of the phloem (8,9,10,14), outlined in Table 3, with the measured amounts of stachyose and sucrose obtained. The average volume of phloem per petiole was calculated to be 20 mm^3. A comparison of the amount of stachyose per petiole (experimentally determined to be 4.9 mg) with the volume of the phloem indicated that the molarity of stachyose fluctuated

Table 3. Total volume of 2-cm sections of petioles as compared to the volume of the internal and external phloem of 22-day straight-neck squash petioles.

cm section	volume of all tissue (mm^3)	volume of internal and external phloem (mm^3) int. + ext. = total (mm^3)
2	8,400	2.33 + 2.36 = 4.7
4	6,550	2.19 + 1.56 = 3.7
6	5,200	2.06 + 1.44 = 3.5
8	4,300	1.49 + 1.06 = 2.6
10	3,100	1.16 + 0.80 = 2.0
12	2,400	0.80 + 0.50 = 1.3
14	1,600	0.60 + 0.50 = 1.1
16	1,000	0.50 + 0.50 = 1.0

Fig. 4--Total volume of 2-cm sections of petioles compared to the volume of the internal and external phloem in 22-day straight-neck squash.



Volume (cubic millimeters)
8
7
6
5
4
3
2
1
0
2
4
6
8
10
12
14
16
Distance from base of petiole (centimeters)
O node 2
X node 3
Total volume of
petiole (X1000)
Volume of internal
and external phloem

Fig. 5--Stachyose in 2-cm sections of petioles from nodes 2 and 3 in 22-day straight-neck squash.

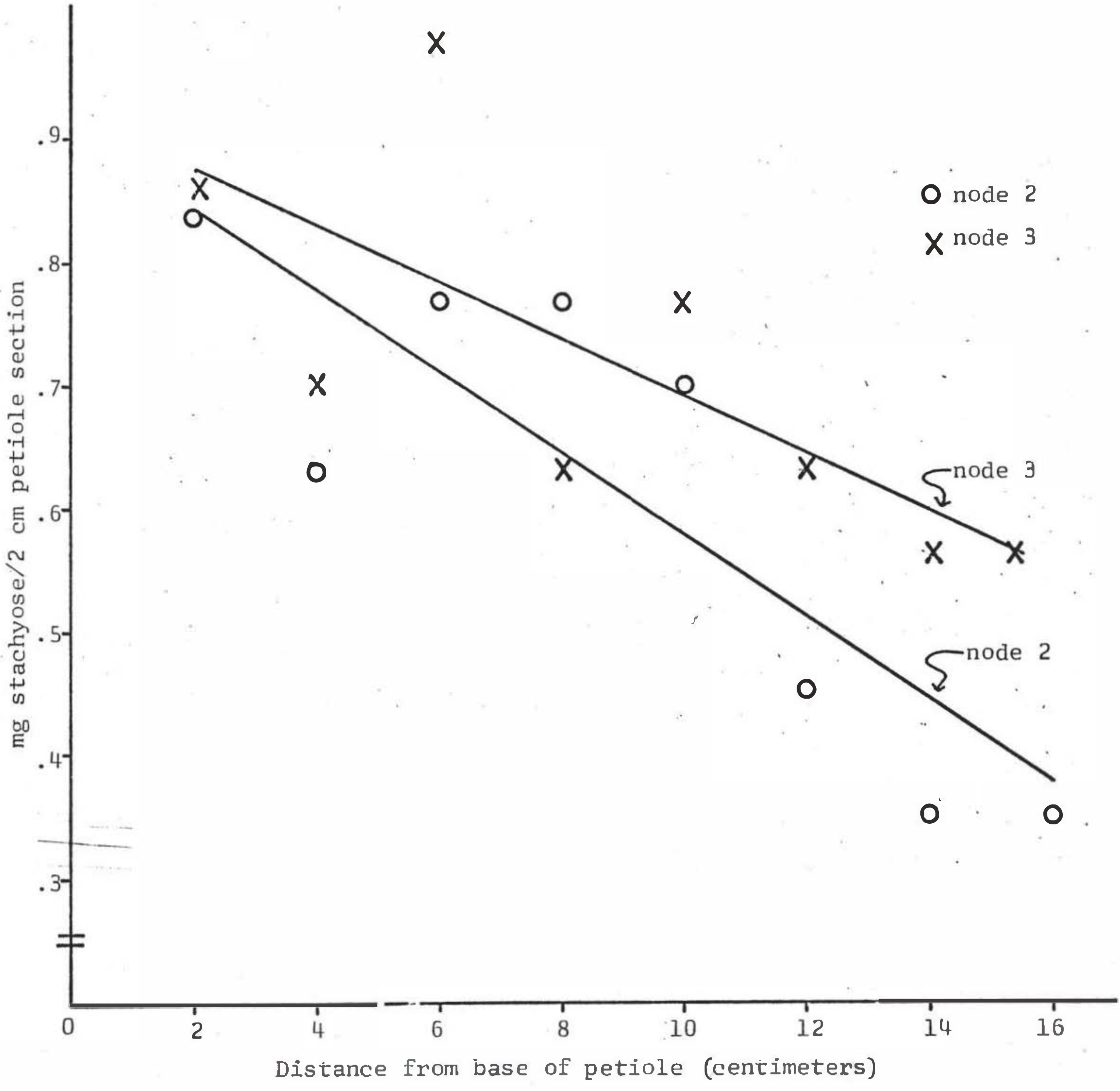
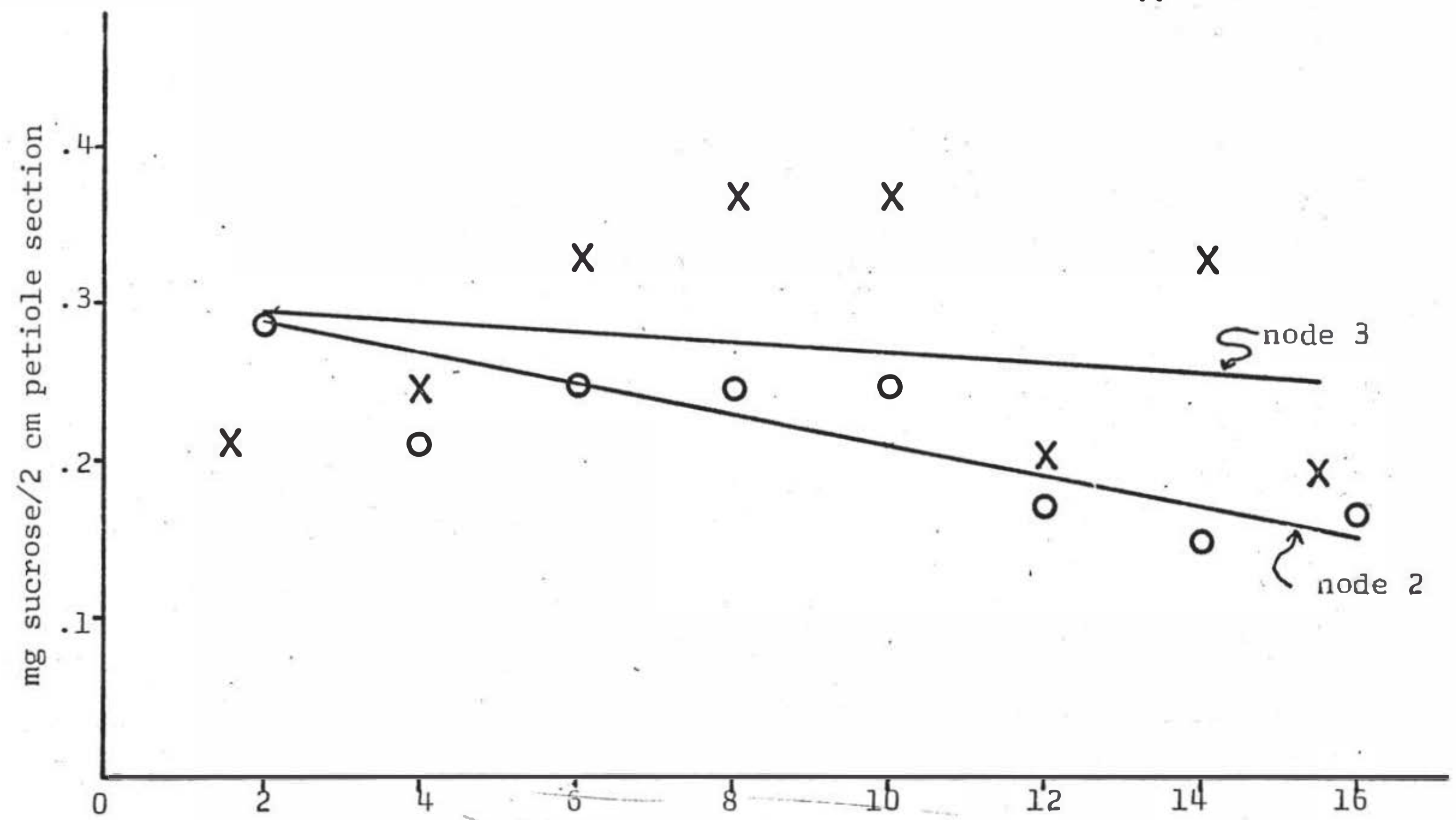

node 2
node 3
node 3
node 2
.9
.8
.7
.6
.5
.4
.3
mg stachyose/2 cm petiole section
0
2
4
6
8
10
12
14
16
Distance from base of petiole (centimeters)

Fig. 6--Sucrose in 2-cm sections of petioles from nodes 2 and 3 in 22-day straight-neck squash.


O node 2
X node 3
node 3
node 2
mg sucrose/2 cm petiole section
.4
.3
.2
.1
0
2
4
6
8
10
12
14
16
Distance from base of petiole (centimeters)

between 0.3 to 0.4, if all the stachyose were contained in the phloem. This appears to be consistent with Zimmerman's (32) analysis of sap exudate from white ash where he determined the molar concentration of stachyose to vary between 0.15 to 0.3 (depending on diurnal and seasonal fluctuations). The 0.3 to 0.4 molar stachyose calculated above was greater than that calculated by Zimmerman, but white ash has relatively high concentrations of mannitol (32), 0.05 to 0.2 molar, in the phloem along with stachyose. Thus, the total molar concentration of carbohydrates calculated by Zimmerman was similar to that obtained above.

Comparing the relative concentrations of stachyose and sucrose in the phloem of the leaves, it would appear the volume of the phloem per gram fresh weight of the leaf was proportionally less than the volume of the phloem per gram fresh weight in the petiole. This would explain the low concentrations of stachyose in the leaf as compared to the high concentrations in the petiole.

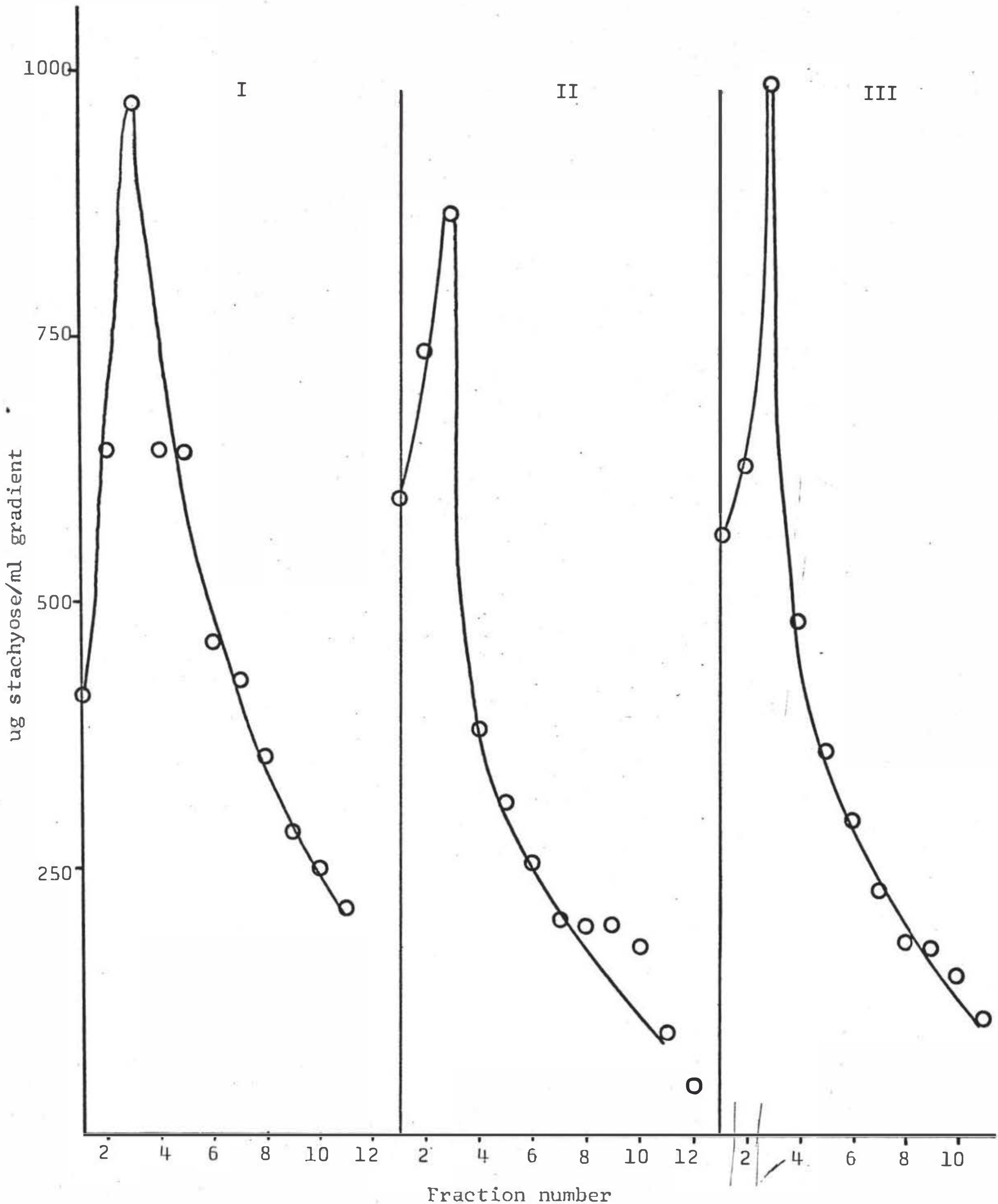
The calculated molar concentration of sucrose was determined to fluctuate between 0.25 to 0.35. This again was greater than Zimmerman's (32) results, but may be explained due to the high concentrations of mannitol present in white ash and not in squash (16).

Furthermore, not all the sucrose is likely to be in the phloem.

The translocation of higher molecular weight sugars has certain inherent advantages to the plant, for the osmotic value of the oligosaccharides does not become so high per hexose unit of carbohydrate, and a given amount of sap flow would result in a delivery rate proportional to the molarity; hence a given molarity of stachyose would contain twice as many hexose units as sucrose (18,32).

Gradient analysis. Stachyose has been established as a transport molecule in squash (19,29) and the above data appears to support the hypothesis that most of the stachyose is in the phloem. Therefore it seemed likely that any particulate matter rich in stachyose would be associated with the translocation mechanism. A fraction of the gradient was found which contained a high amount of stachyose (Fig. 7). The greatest amount of stachyose was found in the 3rd ml obtained from the upper portion of the gradient with a decline of stachyose in each subsequent fraction. Figure 7 shows there is a particulate fraction of fairly uniform density within the 2 to 5 ml fractions of the gradient since 50 percent of all the stachyose in the petiole was in these 4 ml. The remaining amount of

Fig. 7--Stachyose concentration in the different fractions of the density-gradients.



ug stachyose/ml gradient
1000
750
500
250
I
II
III
2
4
6
8
10
12
2
4
6
8
10
12
2
4
6
8
10
Fraction number

stachyose was distributed between the unlayered sediment and the rest of the gradient. The unlayered sediment contained 20 to 30 percent of the stachyose in the petiole and the remainder was in the fractions of the gradient other than the 2 to 5 ml fractions.

The fractions of the gradient were examined under a Bausch and Lomb stereo-zoom phase microscope at 430X and 970X. A 1 percent potassium permanganate stain was used to observe the streaming in transcellular strands (22) and was, therefore, used here. The results showed scattered bodies of approximately 1 u in diameter. The distribution of these bodies appeared to be rather uniform in the gradient.

Since a fraction with many particulates could not be found by microscopic examination, it is not possible from these methods to determine which particulates are associated with stachyose. This may be due to the dilution of the fraction samples for microscopic examination or to the lack of a proper staining technique. Certainly, further experimentation is necessary to substantiate this mechanism of organic translocation. Stachyose has been shown to be in the phloem by previous experimentation and a particulate fraction rich in stachyose thought to be from the phloem has been obtained by these

experiments. Further clarification of the mechanism of organic translocation in straight-neck squash by trans-cellular strands could be obtained by the following: (1) the vascular bundles could be dissected out of the squash petiole for sugar extraction and grinding; (2) the tissue, other than the vascular tissue, of squash petiole could be extracted and analyzed to confirm that the stach-yose is in the phloem; (3) the amount of phloem in the leaf of squash could be determined, then compared with the amount of phloem in the petiole to see if the amount of phloem is proportional to the amount of stachyose in the respective regions; and (4) radioactive tracers could be fed to the leaf so the sugars which incorporated the tracers could be more easily determined in the gradients.

SUMMARY

The mechanism of organic translocation in *Cucurbita pepo* L. variety melopepo torticollis was investigated. Two-centimeter petiole sections from nodes 2 and 3 of 22-day squash were extracted in 80% ethanol and the individual sugars separated by descending paper chromatography. The quantity of individual sugars was then determined colormetrically by using an anthrone reagent. Stachyose has been shown by previous studies to be the sugar which was translocated; therefore, any particulates rich in stachyose might be involved with the system of organic translocation.

The volume of the phloem was calculated to be 20 mm^3 per petiole. The concentration of stachyose per petiole, assuming that all of the stachyose was in the phloem and the stachyose was evenly distributed in the phloem, was calculated to be between 0.3 to 0.4 molar. The molar concentration of sucrose in the phloem was calculated, using the above assumption, to be between 0.25 to 0.35. These results appear to be consistent with the results of Zimmerman who analyzed sap exudate from white ash.

The supernatant from grinding the petiole in a Ficoll-mannitol buffer was layered on top of a linear fructose density-gradient with minimum and maximum amounts of fructose being 100 and 350 mg/ml, respectively. The density-gradients were then centrifuged for 3 hours at 24,500 x g. Analysis of 1 ml fractions of the density-gradients for stachyose by descending paper chromatography in a 1-butanol:acetic acid:water (3:3:2) solvent system and colormetrically showed the third ml of the gradient to be richer in stachyose than any other gradient fraction. This indicates a particulate fraction of uniform density, rich in stachyose, that may be associated with the system of organic translocation.

This report lends further credence to the theory of protoplasmic streaming in transcellular strands as originally proposed by Thaine.

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VITA

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