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# Solute Distribution in Sugar Beet Leaves in Relation to Phloem Loading and Translocation[1]

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## ABSTRACT

**The distribution of solutes in the various cells of sugar beet (*Beta vulgaris* L.) source leaves, petioles, and sink leaves was studied in tissue prepared by freeze-substitution. The differences in degree of cryoprotection indicated that sieve elements and companion cells of the source leaf, petiole, and sink leaf contain a high concentration of solute. The osmotic pressure of various types of cells was measured by observing incipient plasmolysis in freeze-substituted tissues equilibrated with a series of mannitol solutions prior to rapid freezing. Analysis of source leaf tissue revealed osmotic pressure values of 13 bars for the mesophyll and 30 bars for the sieve elements and companion cells. The osmotic pressure of the mesophyll of sink leaves was somewhat higher.**

**The sharp concentration increase at the membrane of the sieve element-companion cell complex of the source leaf indicates active phloem loading from the free space at this site. Active loading of the phloem is presumably needed to move the sugar from the chloroplasts of the mesophyll to the sieve tubes against the concentration gradient. The osmotic pressure of the mature sieve element-companion cell complex appears to be approximately the same in source leaf, path, and sink leaf tissue. There is a distinct difference in concentration between the mature sieve element-companion cell complex in the sink and the surrounding mesophyll. The solute distribution suggests that sugar is actively accumulated from the free space by the developing sink leaf tissue.**

**The osmotic values observed in the various cells are consistent with the operation of a mass flow mechanism of translocation driven by active phloem loading and by active accumulation of sugar by sink tissues.**

In 1930 Münch (20) described a mechanism of translocation whereby flow through the phloem is the result of a difference in osmotic pressure at the two ends of the translocation system. According to this model, synthesis of sugar in the source leaf gives rise to high osmotic pressure in cells of the blade while removal of sugar by utilization or storage lowers solute concentration in the sink regions of stems, roots, and fruits. Osmotic measurements by Roeckl (22) by plasmolysis, refractometry, and cryoscopic techniques revealed that the solute concentration of the sieve-tube sap is considerably higher than that of source leaf mesophyll cells of the same plant. Her findings suggested that high solute concentration in the mesophyll cells is not the basis of the proposed driving force; rather, the data indicated the existence of active transport of solute from mesophyll cells to sieve elements against a concentration gradient.

Advocates of Münch's mass flow theory proposed that phloem loading is maintained by metabolic activity of source region phloem, while in the sink region metabolism is some way responsible for removal of translocate (5). Numerous workers have postulated that an active process in the minor vein phloem serves to drive mass flow of solutes (1, 2, 4, 5, 7, 10, 14, 22, 25, 26). In support, various investigators have used autoradiography to demonstrate loading of minor vein phloem (9, 14, 16, 17, 23). Barrier and Loomis (1) used the term "loading" for the process and proposed that it is an essential step in the translocation process. Eschrich (6) advocated the term "phloem loading" for this step.

Although Roeckl (22) found that the sugar gradient is incompatible with passive loading of minor vein phloem, several key questions await more definitive answers, including the path of solute transport from mesophyll to the sieve elements, the site of phloem loading, and the feasibility of phloem loading as part of the driving force for translocation. In the present work plasmolysis was observed at the cellular level following equilibration of leaf tissue with solutions of graded osmotic pressure so as to measure osmotic pressure of the various cells proposed to be associated with phloem loading and translocation. The pattern of solute distribution observed suggests that sugar is loaded into the companion cell-sieve tube complex from the free space. The osmotic pressure values that were measured provide specific parameters for developing a model for mass flow translocation of solutes in the phloem.

## MATERIALS AND METHODS

Sugar beet plants (*Beta vulgaris* L., cv. Klein E type of monogerm hybrid) were grown in solution culture (11). Tissue was sampled from 10-cm long blades of the 7th or 8th leaves which were photosynthesizing in 2000 to 3000 ft-c of light. Similar leaves were used in previously reported translocation studies (9–12). Freeze-substitution was carried out by the method of Fisher (8). Tissue was quickly removed from the blades and cut into strips 0.5 mm wide and 5 to 8 mm in length. Thin strips were forcibly flicked from the end of a flexed microspatula into an isopentane-methyl cyclohexane mixture cooled with liquid nitrogen until it was slightly viscous (−175 C). Freezing of the isopentane mixture was carefully avoided to prevent tissue damage. Temperature was monitored with a thermocouple probe throughout the process to avoid warming the tissue above −70 C or cooling below −175 C.

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Tissue was picked up with a scoop partially filled with solid carbon dioxide snow and transferred to approximately 20 ml of −70 C methanol in screw-cap test tubes suspended in an acetone-Dry Ice slurry. Tissue which had not been transferred quickly enough to avoid excessive warming was discarded. After allowing tissue-ice to dissolve in methanol at −70 C for 3 days, the tissue was warmed to −30 C by removing the vial from the cooling mixture to a deep freeze for ½ hr. Caps were loosened to allow pressure release. All but approximately 1 ml of the methanol was decanted, and 1 ml of 2% osmium tetroxide in absolute methanol at −30 C was added. The tissue was slowly warmed to room temperature and allowed to stand 1 hr for staining. The strips were cut into 1- to 2-mm lengths with a razor blade without drying, transferred to absolute acetone, and embedded in Spurr's mixture. Tissue was sectioned with a diamond knife and viewed with an RCA EMU-4 electron microscope.

In plasmolysis studies the upper surface of the blade was gently rubbed with 600 mesh alundum-water paste to remove the epidermis, and the tissue was allowed to come to osmotic equilibrium in a given mannitol solution. After this equilibration period the tissue pieces were quickly frozen and freeze-substituted in methanol. Source leaf tissue pieces 0.5 mm × 5 to 8 mm were floated on a graded series of mannitol solutions which ranged from 0.3 to1.5 M in steps of 0.1 M. Slices 1 mm thick were removed from the top and bottom of source leaf petioles and equilibrated with solutions in a series from 0.9 to 1.2 M mannitol. Tissue strips 0.5 × 5 mm were removed from 4- to 5-cm long sink leaf blades and equilibrated in a series of 0.4 to 1.0 M mannitol.

Differences in the extent of ice crystal damage were used as an initial indication of solute concentration. Sucrose and other oligosaccharides, which are major constituents of the leaf, have been found to provide excellent cryoprotection while other organic solutes such as amino acids and peptides do so to a lesser extent (3). Plasmolysis data subsequently substantiated the distribution of solute inferred from cryoprotection patterns.

Incipient plasmolysis was determined as that point in an increasing concentration series at which a significant proportion of the cells of a given type first showed plasmolysis. Mannitol solutions of up to 1.5 M were prepared by gentle heating; crystallization did not occur in any of the solutions for at least 24 hr at 25 C. Below 1.2 M the solutions were stable indefinitely. The osmolality of the solutions, measured by freezing point depression, was found to be a linear function of the molar concentration from 0.8 M to at least 1.3 M, the highest concentration routinely used. Osmotic pressure was calculated from osmolality measurements according to Michel (19, equation 1) using an osmotic coefficient of 1.00 for mannitol. Freezing point depression measurements were made with an Advanced model 3W osmometer. The conventions for osmotic quantities used in this paper are after Noble (21).

Incipient plasmolysis of mesophyll cells was measured directly by light microscopy with living tissue and in sections prepared by the freeze-substitution/plasmolysis method. The direct method yielded an osmotic pressure equivalent to 0.6 M mannitol (0.64 molal) compared with 0.5 M mannitol (0.53 molal) for the freeze-substitution method and 0.45 to 0.50 osmolal by a freezing point depression method and homogenized tissue. The higher osmotic pressure value obtained with living cells probably results from the difficulty in detecting the earliest stages of plasmolysis by light microscopy. The agreement between the methods is reassuring.

The freeze-substitution/plasmolysis method is open to criticism because of potential problems resulting from tissue shrinkage, from the cutting of tissue, and from the relatively long equilibration time. Most of these are common to plasmolytic methods. Mesophyll cell shrinkage was found to be less than 3% of the cell diameter; the resulting osmotic pressure values could be up to 10% too high. Deplasmolysis, which would result in high osmotic pressure values, was not observed in fresh sections during a 60-min period in mannitol. Because of the difficulty in plasmolyzing sieve tubes reported in earlier literature (5), there was some question of the ability to use the plasmolytic method with tissue pieces. The relatively large pieces used in the freeze-substitution/plasmolysis method (over 15 sieve-element lengths wide) provide a sufficient number of sieve plates for sealing and lessen damage to the sieve tube membranes. Any turgor loss upon cutting would lessen the efflux of water required during equilibration, resulting in osmotic pressure values which are lower than would be expected if the turgor were not released. If sealing is delayed, dilution by lateral influx of water would further lower the osmotic pressure in the sieve tubes. The nearly universal plasmolysis of sieve tubes in tissues equilibrated at a mannitol molarity of 1.2 or above attests that sealing of the sieve tubes is sufficient to allow plasmolysis. However, the osmotic pressure values for sieve tube contents obtained by this method probably represent a low estimate.

The terminology for referring to the specialized cells associated with sieve elements is a source of difficulty. Esau (4) suggested that the functional aspect of the organelle-rich cells associated with the sieve tubes be emphasized rather than their ontogeny. If the term "transfer cell" is restricted to those cells which show wall protuberances (14), this term cannot be applied to the specialized cells associated with minor vein sieve elements in sugar beet. While the ontogeny of the specialized phloem parenchyma is not clear in every case, the term "companion cell" will be used in this paper until a suitable term such as intermediary cell (4) is agreed upon.

## RESULTS AND DISCUSSION

The cryoprotective action of organic solutes was used as a preliminary means of locating high solute concentration in the cells of translocating leaves of sugar beet. Tissue was sampled from blades of 7th and 8th leaves of sugar beet plants at a stage when the blades were 10 cm in length or approximately two-thirds of their final size. Following rapid freezing and preparation by a freeze-substitution method (8), ultrastructure of the tissue was studied. Cells exhibited varying degrees of ice damage, presumably because organic solutes were compartmentalized at different concentrations in various cells and

FIG. 1. Micrographs of sugar beet source leaf tissue prepared by freeze-substitution. Tissue in A and F are controls; the rest were equilibrated with mannitol solutions of known concentration for 30 min prior to quick freezing. c: Companion cell; m: mesophyll; p: phloem parenchyma; s: sieve element; u: unplasmolyzed cell; →: site of membrane pulling away from cell wall. Size markers represent 1 μm except parts A (5 μm) G, and I (10 μm). A: Cross section of minor vein showing variation in quality of preservation of structure. B and C: Cross section of minor vein in tissue equilibrated with 1.0 M mannitol solution. The sieve element-companion cell complexes in B are not plasmolyzed while those in C show incipient plasmolysis. D: Cross section of minor vein in tissue equilibrated with 1.3 M mannitol solution. Two sieve elements and the companion cells are extensively plasmolyzed while a third sieve element is unplasmolyzed. E: Sieve element of minor vein from tissue equilibrated with 1.0 M mannitol solution. F: Phloem parenchyma cell and an adjacent companion cell from a minor vein in untreated tissue showing an abrupt change in the degree of preservation at the cell boundaries. G and H: Nomarski and electron microscope views of mesophyll cells from leaf equilibrated with 0.5 M mannitol solution. I: Nomarksi microscope view of a cross section of sugar beet petiole equilibrated with 1.1 M mannitol solution. Electron micrographs revealed that approximately 90% of the sieve elements and companion cells with plasmolyzed.

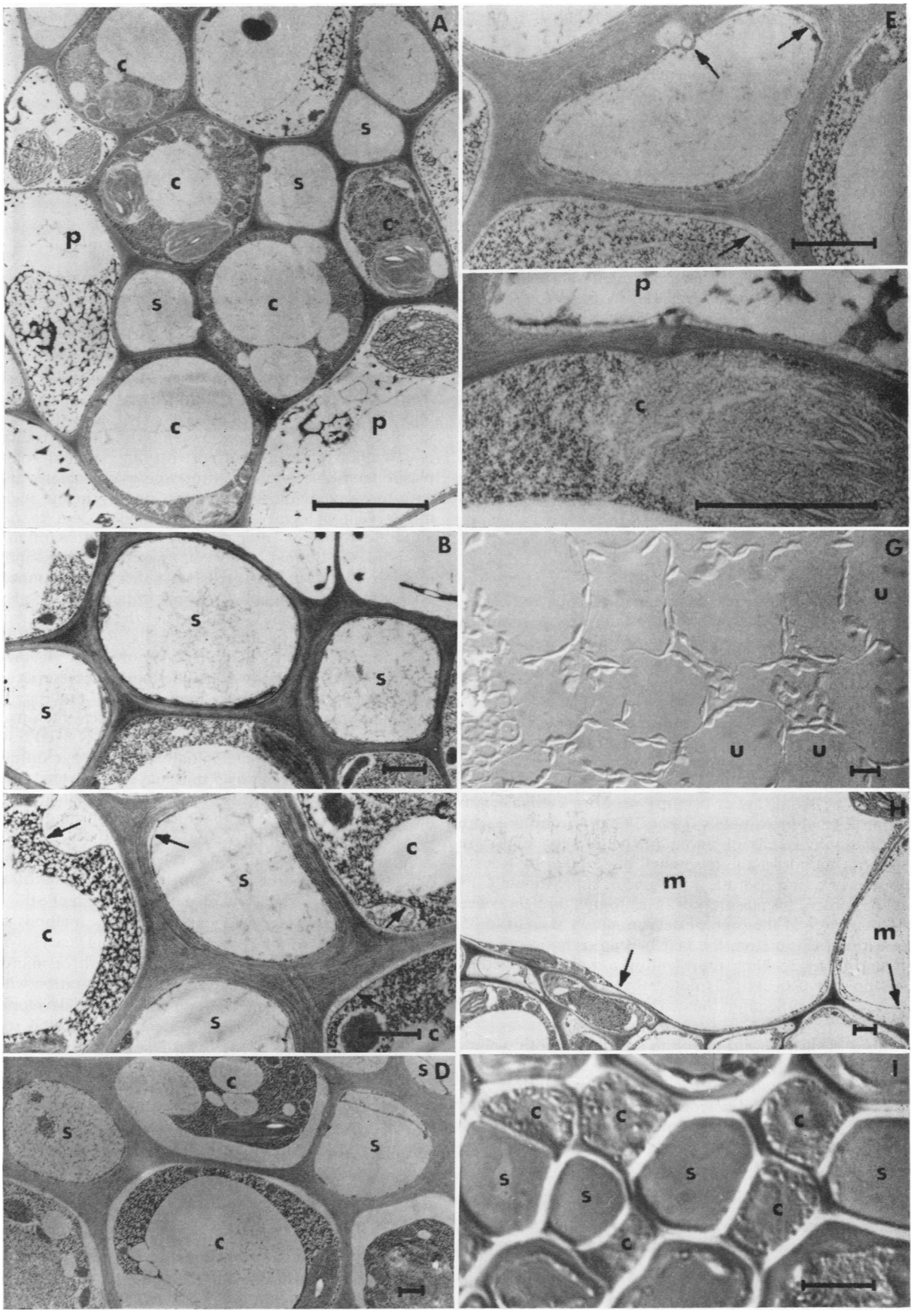


FIG. 1.

organelles and afforded differing amounts of protection. Figure 1A is a cross section of a typical minor vein.

Judged by the quality of preservation of structure, the highest organic solute content is in the companion cells and sieve elements. Chloroplasts in mesophyll cells are well preserved while most of the other cytoplasmic structures in the mesophyll are obliterated by ice damage. Preservation of chloroplast structure is indicative of high solute concentration which is expected in chloroplasts of a photosynthesizing leaf. Damage to the cytoplasmic region, as observed in the mesophyll cells, suggests a relatively low organic solute concentration in this portion of the protoplast. In companion cells and sieve tubes the cytoplasm and the plastids show excellent preservation, as would be expected if there is a high sucrose content in these cells. While cryoprotection offers a basis for suggesting possible sugar or organic solute distribution patterns, its reliability remains to be proven.

To investigate the pattern of solute concentration quantitatively, pieces of source leaf blades were floated on a series of solutions of mannitol ranging from 0.3 M to 1.5 M in 0.1 M concentration steps. After an equilibration period, the pieces floating on a given concentration were quickly frozen, prepared by freeze-substitution, and observed in order to determine the concentration of osmoticum which produced incipient plasmolysis in each cell type. The lowest mannitol concentration which produced incipient plasmolysis in the sieve elements and companion cells of the minor veins varied between 1.0 and 1.1 M or 28 to 30 bars (Fig. 1, C, E). Variations occurred between plants and between cells in the same plant. Figure 1, B to D, shows sieve elements and companion cells just below, at, and considerably beyond the point of incipient plasmolysis. Sieve elements and associated companion cells often show incipient plasmolysis at the same concentration, enabling us to infer a close physical and physiological connection between these cells.

When source leaf tissue was equilibrated with 0.5 M mannitol, a significant proportion of the mesophyll showed slight plasmolysis (Fig. 1, G, H), indicating an osmotic pressure of the mesophyll cells of approximately 13 bars. Phloem parenchyma cells show a still lower osmotic pressure equivalent to 0.3 M mannitol or approximately 7 bars. The osmotic pressure values for mesophyll cells observed in the present study are similar to those found for the mesophyll of *Robinia* by Roeckl (22), who measured an osmotic pressure of approximately 18 bars (0.6 M sucrose) by plasmolysis. She found that the sieve sap of the same tree at the time of determination was 30 to 35 bars. The expressed sap from the leaf had an osmotic pressure ranging from 13 to 23 bars, representing a weighted average of all cells present. Roeckl (22) noted that the values showed seasonal variations reflecting the carbohydrate status of the leaves.

The pattern of distribution of solute and the osmotic values for the various leaf cells provide a means of determining the site of phloem loading and give some data for developing a model for transport of solutes from chloroplasts to the minor vein phloem. The fact that solute concentration is much lower in the mesophyll than in the companion cells and sieve elements rules out the diffusion of sugar from the chloroplast into the sieve tube as envisioned by Münch (20). From the sharp change in concentration between the sieve tube-companion cell complex and surrounding parenchyma cells it appears that sugar does not enter the companion cells from surrounding cells via the plasmodesmata. Although it is possible that the plasmodesmata might produce this sharp a gradient by an active transport process, it seems more likely that the membrane of the companion cell-sieve element complex actively accumulates sugar from the free space. The ease with which sucrose enters the apoplast from the mesophyll has been demonstrated by Kursanov and Brovchenko (18). Physiological studies which will be reported in a subsequent paper give further evidence of the participation of free space in phloem loading.

Table I. *Relative Frequency of Plasmodesmal Connections between Various Types of Cells in a Leaf Blade of Sugar Beet*

| | Mesophyll | Phloem Parenchyma | Companion Cell | Sieve Tube |
|---|---|---|---|---|
| Mesophyll | Rare | Common | —[1] | —[1] |
| Phloem parenchyma | | Common | Abundant | Rare |
| Companion cell | | | Rare | Abundant |
| Sieve tube | | | | Rare |

[1] Cell combination rare or absent.

Given loading from free space, two pathways for transport from chloroplast to minor vein phloem seem plausible. Sugar may leak from the mesophyll cells (18), in which case the major flux would be by mass flow and diffusion through the apoplast with symplastic transport playing a minor role. Or symplastic transport may constitute the major means of transport with movement into the apoplast mainly in the region of the minor vein phloem. A survey of source leaf tissue fixed in glutaraldehyde or prepared by freeze-substitution revealed some plasmodesmata between mesophyll cells and between mesophyll and phloem cells. Whether these connections are quantitatively significant appears impossible to answer from present data. Table I summarizes the relative frequency of plasmodesmata between various cells. Presence of plasmodesmata argues for the possibility of movement via the symplast but does not allow us to conclude that it necessarily occurs. In a survey of literature and a mathematical analysis, Tyree (24) concluded that symplastic transport is a feasible means of cell to cell transport. Trip (23) observed that $^{14}C$ fixed during pulse labeling of photosynthate initially produced a diffuse pattern throughout the leaf but by 5 min had concentrated in the minor vein phloem. Symplastic movement at this velocity seems feasible because branching of the minor veins brings them within 2 to 3 cell diameters of all mesophyll cells (4, 9).

A comparison was made between the osmotic pressure values obtained from source and sink leaves. Analysis of the solute concentration in the cells of a developing leaf still in its importing or sink stage was carried out by methods similar to those followed for the source leaf. Young leaves with blades from 4.5 to 6.5 cm in length were prepared for microscopy by freeze-substitution, with and without equilibration with various mannitol solutions. The osmotic pressure of developing mesophyll in the sink leaf was equivalent to 0.5 to 0.6 M mannitol solution with values as high as 0.7 M being found on occasion. These osmotic pressure values are somewhat higher than those of the source leaf mesophyll. Diffusion-driven mass flow, based on an osmotic pressure gradient between source leaf mesophyll and the developing sink leaf tissue, appears unlikely from the observed pattern of solute concentration. Sieve elements and companion cells of the largest and presumably mature veins of the sink leaf have an osmotic pressure of 22 to 25 bars, lower than found in the source leaf. As observed in the source leaf phloem, there is a sharp transition in osmotic pressures from seive element-companion cell complex to the surrounding tissue. There is no indication of a gradient in adjacent cells which would suggest symplastic movement out of the phloem.

Petiole sections, of approximately 1 mm thickness, were floated on mannitol solutions and prepared by freeze-substitu-

tion. Some plasmolysis of sieve elements and companion cells was observed at 1.0 M while over 90% of the sieve elements showed plasmolysis when equilibrated with 1.1 M mannitol solution. Zimmerman (26) found osmotic pressure gradients of about 0.2 to 0.4 bar/m, similar in magnitude to the turgor pressure gradients measured by Hammel (15) in oak. The osmotic pressure gradient presumably results from the lateral influx of water as the turgor pressure is dissipated along the resistance of the path. Given the 20- to 30-cm path between source and sink leaves in sugar beet, the resulting osmotic pressure gradient would not be detectable by the present method. The 2-bar pressure decrease from source to sink predicted by the Poiseuille relationship from the dimensions observed in sugar beet sieve tubes is at the limit of detection of the method.

Measurement of the various osmotic parameters in cells associated with translocation is of assistance in assessing the feasibility of phloem loading as a source of driving force for translocation. The osmotic pressure values for the sieve tube-companion cell complex are of the order required to generate the turgor pressures measured by Hammel (15) for red oak phloem.

Aspects of the system which remain unknown include the water potential of the free space solution and the actual pressure drop along the transport system. Observations which appear to be significant elements in developing a model include: (*a*) The osmotic pressure of the sieve element-companion cell complex of the minor veins is higher than that of the source leaf mesophyll. (*b*) There is a sharp difference in solute concentration at the membrane of the sieve element-companion cell complex. (*c*) The osmotic pressure of the sieve tube contents is slightly lower in the mature sieve elements at the sink end of the path. (*d*) The osmotic pressure of the developing cells of the sink tissue appears to be higher than that of the source leaf mesophyll. These findings appear to be compatible with a pressure flow mechanism driven by active transport at both source and sink ends. Physiological studies related to elements of the proposed model are under way.

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