ESTIMATION OF THE APOPLASTIC POTASSIUM CONTENT OF LEAFLET LAMINA TISSUE OF THE *ARGENTEUM* MUTANT OF *PISUM SATIVUM* L.

By

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ABSTRACT

ESTIMATION OF THE APOPLASTIC POTASSIUM CONTENT OF LEAFLET LAMINA TISSUE OF THE ARGENTEUM MUTANT OF PISUM SATIVUM L.

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The apoplastic K^+ content of leaflet lamina tissue of the Argenteum mutant of Pisum sativum L. was estimated by eluting K^+ at 1^o C into a 5 mM $CaCl_2$ solution bathing an area of tissue with abaxial epidermis removed. The elution time-course curve was interpreted as indicating an initial rapid diffusion from the apoplast followed by a slower, constant net rate of efflux across the plasmalemma. Extrapolation of the constant efflux rate to zero time was used to estimate apoplastic K^+ content. When plants were grown at 2 and 10 mM K^+, estimates were 88 and 142 ug K^+ gfw^{-1}, respectively. Assuming an apoplastic solution volume of 0.1 ml gfw^{-1}, these correspond to concentrations of 23 and 36 mM, respectively. Apoplastic K^+ represented approximately 2% of tissue K^+. Estimated K^+ bound by fixed negative charges in the cell wall during elution was 0.1 ueq gfw^{-1}, or 4 to 11% of apoplastic K^+.

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INTRODUCTION

Potassium (K^+) is considered the most important inorganic solute in higher plants (Lauchli and Pfluger, 1978; Marschner, 1983). Its roles in the production of cell osmotic potential (Mengel and Arneke, 1982), enzyme activation (Evans and Wildes, 1971) and electrical charge balancing (Smith and Raven, 1976) have major implications in plant growth, water use, metabolism, assimilate transport and pH regulation.

Numerous studies have been conducted to determine the total K^+ content of whole plants as well as of individual organs, tissues, cells and subcellular organelles. In contrast, the extracellular, or apoplastic, K^+ content of plant tissues has received relatively little attention. There are several reasons why the extracellular K^+ of leaves in particular is of interest.

Apoplastic K^+ may have specific functions in leaves. In some species, for example, it seems to be involved in one or more phases of phloem loading (Giaquinta, 1983). It is not known whether extracellular K^+ levels normally occurring in leaves play a regulatory role in this process (Doman and Geiger, 1979). If so, these levels may affect carbohydrate partitioning within the plant and, thus, quality and yield.

Apoplastic K^+ may also have enzyme-activation and pH-regulating roles.

Knowledge of the extracellular K^+ content of leaves would be helpful in delineating the relative importance of intra- and extracellular paths of K^+ transport between cells or tissues. To date, such studies have largely concerned roots, where ion movement from the soil solution to conducting tissue has been of interest. It is equally important to understand K^+ transport in the leaf with regard to overall ion distribution as well as that required for specific processes such as salt excretion, changes in guard-cell turgor pressure during stomatal movement, and turgor-mediated leaf movements.

The relationship between extracellular K^+ and regulation of total leaf content is of considerable interest. An optimum leaf K^+ status is important to plant growth and productivity. Yet the facility of K^+ retranslocation within the plant, particularly from older to developing tissues (Lauchli, 1972b), often leads to a decline in the K^+ content of mature leaves (Waughman and Bellamy, 1981). This may occur to the point of leaf K^+ deficiency, as during fruit development on some tomato cultivars (Lingle and Lorenz, 1969; Widders and Lorenz, 1982b). The existence of evidence against a significant loss in the ability of leaf cells to take up K^+ (Widders and Lorenz, 1983a) suggests that availability of the ion for uptake may be an important factor.

Robinson (1971) and Pitman (1975) proposed similar models for the regulation of leaf ion content in which the apoplastic and symplastic (intracellular) compartments are considered to be interdependent. According to these models, the symplastic ion concentration is the net result of fluxes through plasmodesmata and across the plasma membrane, the latter being dependent in part on the content of the apoplast. The apoplastic concentration, in turn, is the net result of import and export through vascular tissues, cell uptake and efflux, and ion exchange at the cell wall.

The influence of extracellular K^+ concentration on cell uptake has been demonstrated by Widders and Lorenz (1983a and b), who found K^+ fluxes in tomato leaf cells to depend on external K^+ concentration. The ability to determine how the extracellular ion concentration changes during plant ontogeny or under varying growing conditions may therefore be essential to understanding and potentially altering changes in leaf K^+ status.

Determination of extracellular K^+ content presents special problems for conventional methods of compartmental analysis. In leaves the problems are compounded by the presence of the cuticular barrier to diffusion. Kinetic analysis of radioactive tracer efflux from leaf tissue (discussed in Walker and Pitman, 1976) has required cutting the tissue as well as extensive presoaking to load the tracer, making the method unsuitable for quantitative determinations.

Ion loss during precipitation techniques (Harvey et al., 1979) and the low resolution of autoradiography for $^{42}K^+$ (Flowers and Lauchli, 1983) limit their use for extracellular K^+ quantitation. Several workers (Etherton, 1968; Lauchli, 1972b; Flowers and Lauchli, 1983) have cited the difficulty in manipulation of ion-sensitive microelectrodes as a limiting factor for analyzing compartments as small as the cell wall, where apoplastic K^+ is thought to be located.

In view of the above, the objective of the present study was to develop and evaluate alternate methods for estimating extracellular leaf lamina K^+ content. The research focused on two methods.

The first method considered was kinetic analysis of the elution of endogenous K^+ from leaflet lamina tissue of the Argenteum mutant of Pisum sativum L. A modification of previously used efflux techniques, it does not require cutting or presoaking of the tissue. It is an indirect method requiring little sample preparation and generally available instrumentation, and could be expected to yield information on overall apoplastic K^+ content.

The second method evaluated was X-ray microanalysis of freeze-dried Argenteum leaflet tissue. The potential for good spatial resolution and quantification of elemental content make X-ray microanalysis suitable for use in compartmental K^+ analysis (Lauchli, 1972a). It is a direct method requiring rigorous sample preparation and highly

sophisticated instrumentation. However, it has the capability of determining extracellular K^+ concentration at specific locations in the leaf.

The first method is described in the body of this paper. The second, which proved less successful, is discussed in the appendix.

LITERATURE REVIEW

Functions and Movement of Potassium in Plants

Potassium is an essential nutrient for higher plants, required for normal growth and development (Mengel and Kirkby, 1982). Though not a structural constituent of tissues or metabolites (Lauchli and Pfluger, 1978) it may account for up to 8% of the dry matter of higher plants, its concentration exceeding that of any other cation (Evans and Sorger, 1966). This high content attests to potassium's roles in numerous physiological processes.

As the principal osmotically active solute in plants, K^+ is required for attaining the turgor responsible for cell expansion during growth (Zimmerman, 1978; Mengel and Arneke, 1982). Potassium fluxes are also involved in the changes in motor-organ turgor causing nyctinastic leaf movements in several legume species (Satter and Galston, 1971; Kiyosawa and Tanaka, 1976; Campbell et al., 1981). Considerable work has established a central role for K^+ in the turgor-mediated opening and closing of stomates (Humble and Raschke, 1971; Raschke and Fellows, 1971; Edwards and Bowling, 1984), and therefore in the regulation of the plant's overall water status (Brag, 1972; Hsiao, 1976).

Numerous enzymes are activated by K^+, including a starch synthase (Hawker et al., 1974), pyruvate kinase, and some of those required for protein and nucleotide synthesis (Evans and Sorger, 1966; Suelter, 1970). A K^+-stimulated ATPase located at the plasmalemma appears to be involved in proton pumping and transport of other ions across the membrane (Hodges, 1976).

Potassium balances organic and inorganic anion charges in long-distance transport in the phloem (Marschner, 1983) and xylem (Kirkby and Armstrong, 1980). It serves as a counterion in active H^+ transport across membranes, which may have implications in overall regulation of intracellular pH (Smith and Raven, 1976). This role seems to be important in both oxidative (Kirk and Hanson, 1973) and photosynthetic (Steineck and Haeder, 1978) phosphorylation where proton gradients across mitochondrial and thylakoid membranes, respectively, may drive ATP formation (Mitchell, 1966).

Potassium has additional roles in photosynthesis. Its promotion of the synthesis of RuBP carboxylase (Steineck and Haeder, 1978) required for CO_2 reduction, as well as its role in stomatal movement, affect the rate of diffusion of CO_2 to chloroplasts in the leaf mesophyll (Peoples and Koch, 1979; O'Toole et al., 1980; Moorby and Besford, 1983). Potassium may also be required in chloroplasts for chlorophyll synthesis (Marschner and Possingham, 1975) and to maintain the structure of grana stacks of the thylakoid membrane (Penny et al., 1976).

There is considerable evidence that K^+ promotes the translocation of photosynthates (Hawker et al., 1974; Mengel and Viro, 1974). Its high phloem-sap concentration (Hall and Baker, 1972) contributes to the osmotic potential in sieve tubes, maintaining turgor at low sucrose concentrations (Giaquinta, 1983) and contributing to osmotically generated phloem movement (Moorby and Besford, 1983; Vreugdenhil, 1985).

Mengel and Haeder (1977) reported a positive relationship between K^+ supply and phloem loading in Ricinus. The extracellular K^+ content in particular seems to influence one or more aspects of loading from the apoplast thought to occur in some species. In Beta vulgaris, for example, sugars seem to be actively loaded into the phloem from the apoplast, having been released from the symplast in the vicinity of the phloem (Giaquinta, 1976; 1983). Several workers (Hawker et al., 1974; Doman and Geiger, 1979; Peel and Rogers, 1982) have reported stimulated sucrose efflux from mesophyll cells into the apoplast with increased external K^+ concentration. The work of Malek and Baker (1977) suggests that in Ricinus extracellular K^+ is directly involved in phloem loading from the apoplast. These workers and Hutchings (1978) have proposed that apoplastic K^+ is pumped into the companion cell-sieve element complex as protons are pumped out, the resulting proton gradient driving a proton-sucrose cotransport into the phloem.

The availability of K^+ for its many functions is enhanced by its high mobility in the plant. Potassium moves readily in both phloem and xylem, allowing it to freely circulate among organs (Lauchli and Pfluger, 1978). Its principal direction of transport is toward meristematic tissue which may be supplied to a large extent with potassium retranslocated from older plant parts (Greenway and Pitman, 1965). Fruit are particularly strong sinks for K^+. Hocking and Pate (1978) reported retranslocation of up to 73% of leaflet K^+ to fruit in Lupinus; similarly, Widders and Lorenz (1979, 1982b) found considerable K^+ redistribution from vegetative tissue to fruit in tomato.

Characteristics of the Apoplast

Potassium's high mobility is in part the result of its moving readily between the two major compartments of plant tissue, the symplast and apoplast (Lauchli and Pfluger, 1978). The symplast consists of the cytoplasmic continuum bounded by the plasmalemma, with intercellular connections in the form of plasmodesmata (Crafts and Crisp, 1971). Conversely, the apoplast is that portion of plant tissue lying outside the plasmalemma and consisting mainly of the cell wall continuum and intercellular space (Esau, 1977). Mature, non-living xylem vessel elements and tracheids are also considered part of the apoplast (Esau, 1977).

The cell wall consists of a cellulose framework embedded in a matrix of hemicelluloses, pectins and proteins

(Northcote, 1972; Haynes, 1980). The spaces between cellulose microfibrils, the units of wall structure (Esau, 1977), are large enough to accommodate water molecules as well as many solutes (Gaff et al., 1964; Franke, 1967; Lauchli, 1976). While the size of these spaces is reduced by the matrix substances, water and ions are able to migrate in cell walls unless diffusion is restricted by lignification or suberization (Haynes, 1980). The apoplast therefore represents a transport pathway parallel to the symplast in plants (Weatherly, 1970).

Pectic substances of the cell wall matrix are of particular interest in relation to ion transport in the apoplast. Pectic acid, a polymer of alpha-D-polygalacturonic acid, has free carboxyl groups which can provide fixed negative charges (Briggs et al., 1961; Luttge and Higinbotham, 1979). With the exception of protons, divalent cations are bound to these negative sites in preference to monovalent cations (Haynes, 1980). Proteins in the cell wall, which provide additional negative charges, may also have a small number of positively charged sites (Lauchli, 1976; Luttge and Higinbotham, 1979). The binding of ions to these fixed charges is significant in that it modifies ionic concentrations near the plasmalemma, which may ultimately influence ion transport and accumulation (Lauchli, 1976; Haynes, 1980; Greenleaf et al., 1980).

Besides serving as a transport pathway, the apoplast is the site of physiological processes (Preston, 1979). Due to

the presence of solutes and enzymes in the cell wall, biosynthetic and other reactions may occur there (Northcote, 1972). For example, sucrose hydrolysis in the apoplast may be a prerequisite for sugar transport in many plant tissues (Giaquinta, 1980). The apoplast is in communication with the symplast via the plasmalemma, and so is affected by cell processes (Preston, 1979). Its composition and content are determined in part by cell metabolism and seem to be maintained at a certain equilibrium with cell contents (Kursanov, 1984).

Apoplastic Transport

There has long been interest in the plant apoplast as a transport pathway for water and ions. As early as 1928, Scott and Priestley proposed that the soil solution diffuses into roots via the cell walls. In a review in 1951, Robertson suggested that ions move passively through the root along a concentration gradient in cell walls and water-filled intercellular spaces.

Jacobson et al. (1958) and Brouwer (1959) demonstrated the existence of a barrier between the root medium and xylem vessels, precluding an entirely extracellular pathway and requiring ion uptake across the plasmalemma. This barrier was further elucidated by Robards and Robb (1972), who studied ion movement in the apoplast of barley roots using an electron-dense stain. Except in the root tip, ion movement through cell walls was restricted by the

endodermis. Similar results were reported by Nagahashi et al. (1974) for corn roots.

Peterson et al. (1986) used lanthanum (La^{3+}) as an electron-dense marker of apoplastic solute movement in corn and barley roots. Within the root meristem solutes were found to move to the stele via the apoplast.

Several studies of apoplastic ion transport in roots have focused specifically on the movement of K^+. Vakhmistrov (1967) studied the ability of various root cells to absorb K^+ and concluded that the extracellular solution might serve as a source for its uptake. The root apoplast does not seem to be significant in K^+ transport, however. Van Iren and Van der Sluijs (1980) used autoradiography to localize potassium in barley roots. In mature roots, K^+ uptake seemed to be at the epidermis or hypodermis, the apoplastic pathway having little significance in radial transport. Similar results were reported by Chino (1981), who used X-ray microanalysis to determine K^+ distribution in the root cortex. Potassium was found mainly within cells, suggesting symplastic movement.

Plant tissues other than roots have also been studied with respect to extracellular transport. Priestley (1929) suggested that cell walls and intercellular spaces may be of importance in transport to the shoot apical meristem prior to differentiation of the vascular system. Hylmo (1953) studied changes in $CaCl_2$ uptake by intact pea plants at varying transpiration rates and concluded that the external

solution passes by mass flow through the plant apoplast coupled with movement of water alone through vacuoles.

In a recent study Erwee and Goodwin (1985) used fluorescent dyes to determine the extent of symplastic transport in various tissues of the aquatic plant Egeria densa. They reported barriers in the symplast between some cells connected by plasmodesmata. Their model for transport in the whole plant consists of a large number of symplast domains between which apoplastic transport may be necessary.

Studies of leaf tissue suggest the apoplast may be of considerable significance in water transport in some species. Levitt (1956) calculated that the rate of water movement from xylem endings through mesophyll cells to evaporative surfaces would be five orders of magnitude slower than the transpiration rate. On these grounds he concluded that water in the transpiration stream moves through leaf cell walls. Gaff and Carr (1961) studied water movement in Eucalyptus leaves. They estimated that as much as 40% of the water content of the turgid leaf could be in the cell wall. On this basis the wall was proposed as the main path for extrafascicular movement of water, possibly serving as a buffer to water loss from the protoplast.

In a review of plant water relations, Weatherly (1970) proposed that mesophyll cell walls represent the major pathway for water in leaves. The work of Burbano et al. (1976), who used a stain and light microscopy to study water

movement in cotton leaves, supports this hypothesis. In grasses, however, apoplastic water movement seems to be restricted to areas surrounding secondary veins due to the presence of a suberized bundle sheath surrounding major veins (Crowdy and Tanton 1970; O'Brien and Carr, 1970). These findings were more recently corroborated by Canny and McCully (1986) by means of a stain detected by electron microscopy in freeze-substituted, resin-embedded corn leaf tissue.

There may also be varying degrees of apoplastic transport of ions and other solutes in leaves. Kylin (1960) proposed that such transport involves both symplastic and apoplastic routes. This concept was supported by the work of Gunning and Pate (1969), who studied transfer cells in leaves. The primary role of these cells was proposed to be in solute exchange between xylem and phloem and their surrounding tissues. The extensive wall ingrowths characteristic of transfer cells were thought to provide an extracytoplasmic compartment from which solutes may be absorbed by cells. Differences among families in number and types of leaf transfer cells suggest the relative importance of apoplastic transport may also vary (Pate and Gunning, 1969).

Using precipitation of AgCl, Van Steveninck and Chenoweth (1972) examined Cl^- transport in the mesophyll of barley seedlings. They reported primarily symplastic

movement of the ion, but concluded that apoplastic transport may occur in certain areas of the cell wall.

Thompson et al. (1973) delineated an extracellular continuum for ion movement in Atriplex leaves using La^{3+} as an electron-dense tracer. Lanthanum was found to be distributed in walls throughout the mesophyll; however, since La^{3+} did not cross the plasmalemma, this demonstrated only that ions can move in the leaf apoplast and not the extent to which this occurs for those that can be taken up by cells. Campbell et al. (1974) used a similar technique to demonstrate an apoplastic pathway to salt glands in several halophytes.

Anatomical changes in the sunflower leaf during development were recorded by Fagerberg and Culpepper (1984). They reported a decrease with leaf age in the extent of the apoplastic path relative to the volume of tissue supplied, suggesting cell walls may be more important in water and ion transport in young leaves than in mature ones.

Evert et al. (1985) used ion precipitation to delineate a transport pathway in the transpiration stream of the corn leaf. Ions moved readily from vessels into the apoplast of phloem and bundle-sheath cells, but further ion movement was restricted by suberized bundle sheath walls.

A pressure-dehydration technique was used by Jachetta et al. (1986a) to determine the distribution of ^{14}C-labeled atrazine and glyphosate between the apoplast and symplast of sunflower leaves to help elucidate the transport pathways of

these herbicides. In another recent study the mode of transport of solutes in leaves of Ipomea tricolor was investigated by Madore et al. (1986) using liposome-encapsulated fluorescent dye injected into cells. These workers reported no apparent barrier to symplastic movement from mesophyll to minor veins that might necessitate apoplastic transport.

The extent of apoplastic ion movement to specific leaf tissues has received some attention. X-ray microanalysis was used by Campbell et al. (1981) to study the path of K^+ and Cl^- migration between opposite sides of the motor organ of the legume Samanea during turgor-mediated leaf movement. Results based on ion distribution between the protoplast and cell wall indicated an apoplastic path. In a related study, however, Satter et al. (1982) reported a barrier to apoplastic diffusion within the pulvinus, necessitating a partially symplastic pathway.

Hsiao (1976) reported guard cells of some plants are able to take up K^+ from an external medium, suggesting the apoplast may be a source of potassium for stomatal opening. Edwards and Bowling (1984) measured extracellular potassium activity across the stomatal complex of Tradescantia. The large electrical gradients observed between cells suggested little direct continuity between cells of the complex. These workers reported a large increase in K^+ activity in the guard-cell wall upon stomatal closure, indicating that potassium may move out into the apoplast.

Erwee et al. (1985) examined cell-to-cell communication in leaves of Commelina cyanea with fluorescent dyes. Dye injected into epidermal cells rarely moved into guard cells, where ion fluxes were proposed to occur via the apoplast.

Free-Space Studies

In addition to studies of ion movement within the apoplast, considerable work has investigated that compartment as intermediary for ion movement between cells and an external solution under experimental conditions.

Briggs and Robertson (1948) examined the uptake of various substances by potato tuber discs. Small, nonpolar molecules were found to freely penetrate the entire volume, while electrolytes and large molecules readily entered only part of the disc. Since the ions in this freely accessible phase diffused out of the discs into water, diffusion was assumed to be the principal mechanism for their entrance. These workers proposed the existence of a volume lying outside the vacuole whose contents readily exchange with an external solution.

The first reported measurements of the regions of the root accessible by free diffusion were by Hope and Stevens (1952), who studied the reversible diffusion of KCl between bean root tips and an aqueous solution. They estimated that 13% of the root volume was occupied by this easily accessible phase. This volume was termed "Apparent Free Space" (AFS) since the nature of ionic interactions between

the measuring solution and the compartment was not known, allowing only an estimate of its volume. Most of the AFS was believed to lie within the protoplasm, where these investigators proposed the existence of immobile anions constituting a Donnan phase.

Stiles and Skelding (1940) found the time course of Mn^{2+} uptake by carrot root tissue to be characterized by an initial period of rapid uptake followed by a much slower phase. Epstein and Leggett (1954) proposed two modes of ion uptake by roots corresponding to such flux rates. In the first, termed exchange adsorption, roots act as cation exchangers. This uptake, which is not linear with time, reaches equilibrium in about 30 minutes, involves readily exchangeable ions, does not require energy, and is not selective. In the second phase, active transport, ions are essentially not exchangeable. This uptake is linear with time, does not reach rapid equilibrium, requires energy and is selective. Epstein (1956) concluded that ion penetration into the "free" spaces of plant tissue, the first mode of uptake, is a prerequisite for active transport, the second mode. The model was later amended to include an initial diffusion phase to exchange sites (Epstein, 1962).

Briggs (1957) reviewed the concept of free space. The AFS was described as consisting of a "Water Free Space" (WFS) where the ionic concentration equals that of the surrounding solution, and a "Donnan Free Space" (DFS) where the ionic distribution is controlled by the fixed negative

charges of cell walls. The characteristic initial rapid phase of uptake was said to represent ion movement into the AFS and the slower phase, accumulation by the "Osmotic Volume."

Consistent with their earlier report, Briggs and Robertson (1957) proposed that free space includes the intracellular volume outside the tonoplast as well as cell walls and injected intercellular spaces. In contrast, Levitt (1957) argued that only the cell walls are available for diffusion into roots since the protoplasm is surrounded by a differentially permeable membrane which restricts free diffusion. Levitt's point of view was later corroborated by efflux studies by Pitman (1963) and Cram (1968) which indicated that the cytoplasm and vacuole are phases in series with the free space, the plasmalemma and tonoplast acting as boundaries to ion movement. There is now general agreement that the terms apoplast and free space are synonymous (Luttge and Higinbotham, 1979).

Early reports of free-space measurements for roots are fairly consistent. Hope and Stevens (1952) determined an AFS volume of 13% for bean roots, while a volume of 8 to 25% was estimated for roots of beans and corn by Bernstein and Nieman (1960). For wheat roots, Butler (1953) reported an AFS of 24 to 34% and later (Butler, 1959), 18 to 20%. Pitman (1965a) estimated an AFS volume in barley roots of 20 to 25%, while the cation exchange capacity of the DFS was 2 meq kg^{-1} fresh weight.

Briggs et al. (1958) studied radioactive tracer uptake by beet root discs. WFS was found to occupy 20% of the disc tissue, 15% attributable to cut cells and the remainder, to intercellular spaces. The amount of nondiffusible anions in the DFS was determined to be 10 to 14 meq kg^{-1} in a volume of about 2% of the root, for a concentration of 560 meq l^{-1}.

Briggs et al. (1961) extensively discussed estimation of free-space characteristics and the significant influence of experimental conditions on such determinations. This influence was demonstrated in a recent study by Shone and Flood (1985). Using mannitol along with substances of high molecular weight that did not penetrate the root, they corrected for the contribution of surface film to the measurement of AFS of barley roots. Their resulting AFS estimate was only 4.5 to 5.1% of root volume.

Leaf tissue has also been the subject of free-space investigations. Kylin (1957) demonstrated the presence of an "outer space" in the leaf of Vallisneria, an aquatic plant. Efflux of labeled sulphate from that tissue was characterized by rapid followed by slow, continuous rates, as found for roots. The AFS was estimated to be about 8% of leaf volume. Kylin and Hylmo (1957) reported similar results for wheat shoot tissue, leading Kylin (1960) to conclude that AFS is a property of green tissues as well as roots and storage tissue. Kylin (1960) reported WFS volumes of 4 to 5% for leaves of Crassula and 15 to 18% for wheat. Crowdy and Tanton (1970) studied the location of free space

in wheat leaves using precipitation of lead chelate and found it to be localized within cell walls. The free space was estimated to occupy only 3 to 5% of the tissue.

In another study of Vallisneria, Winter (1961) reported a Donnan phase for leaves which, like that in roots, is located in the cell wall and contains cations removable by exchange. Mecklenburg et al. (1966) also reported an exchangeable cation pool in the free space of leaves, while Van Steveninck and Chenoweth (1972) found Cl^- to be partially excluded from the cell wall of barley leaves, supporting the existence of a cation exchange system there.

Using ion uptake by Atriplex leaf slices, Osmond (1968) measured an AFS volume of 20% and estimated the amount of exchange sites in the DFS at 15 ueq g fresh weight^{-1} (gfw). Uptake was thought to be first into an exchangeable free-space fraction and then into a nonexchangeable compartment. On this basis it was suggested that ions transported to the leaf by the vascular system are delivered to the free space, from which they are absorbed by adjacent cells.

An examination of K^+ uptake by corn leaf slices (Smith and Epstein, 1964a) revealed kinetics and selectivity resembling those previously reported for roots, suggesting that ion carriers and their mode of operation are similar for leaves and roots. These workers (Smith and Epstein, 1964b) proposed that leaf free space may play the same role in cellular ion uptake as that of roots. They questioned

whether selectivity of the plant is due to mechanisms in the roots only, or in shoot cells as well.

Pitman et al. (1974a) used uptake of $^{86}Rb^{+}$ and $^{36}Cl^{-}$ to determine free-space characteristics of barley leaf slices. In support of previous findings, they reported that leaf free space could be considered to consist of WFS and DFS as in storage tissue and roots. The rapidly exchanging WFS was said to include cut cells, injected intercellular spaces and surface films of solution, while a more slowly exchanging inner component corresponded to the DFS. WFS was estimated to be 0.21 ml gfw^{-1}. The DFS contained exchange sites equivalent to 3.6 ueq gfw^{-1} in a volume of 0.013 ml gfw^{-1}, for a concentration of 280 ueq ml^{-1}. The pK_a of the fixed anions was confirmed to be 2.8.

The above authors discussed extrapolation to the intact plant of free-space determinations made on cut tissue. Since cutting does not destroy cell wall, DFS measurements using slices were thought to be valid estimates for the intact plant. The WFS volume, however, was considered to be different in the intact plant which would lack cut cells and free water in intercellular spaces. In the intact leaf WFS was felt to be limited to a portion of the cell wall.

In a review soon thereafter Pitman (1975) discussed the role of the free space in the regulation of leaf ion content and proposed the model presented earlier. He indicated that export from the leaf competes with cell uptake for ions in the free space and questioned whether the rate of influx to

cells is regulated by their vacuolar content or only by the availability of ions in the free space.

Smith and Fox (1975) determined the free-space characteristics of Citrus leaf slices using efflux of $^{36}Cl^-$ and $^{22}Na^+$ and reported a WFS of 0.2 ml gfw^{-1}. Exchangeable cations in the DFS were 20 to 25 ueq gfw^{-1}, considerably higher than the amount reported for barley leaves. These workers speculated that the leaf DFS might act as an extracellular cation reservoir, the significance of which would depend on the pH of the free space and changes in uronic acid levels in cell walls due to age or nutrition.

Widders and Lorenz (1983b) determined free-space characteristics of tomato leaf slices using uptake of $^{36}Cl^-$ and $^{86}Rb^+$. WFS volume, which was used to evaluate the integrity of the tissue, was estimated as 0.25 ml gfw^{-1}. There were 6.8 ueq gfw^{-1} fixed anions in the Donnan phase volume of 0.012 ml gfw^{-1} for an effective concentration of 550 ueq ml^{-1}.

A number of studies have more specifically concerned the fixed charges of the cell wall. Dainty and Hope (1959), working with the alga Chara, determined that DFS is located in the cell wall. In a related study, the indiffusible anions in the DFS were found to be proportional to wall thickness (Dainty et al., 1960). Pitman (1965b) determined that exchange sites in the DFS arise from bound uronic acids in the cell wall.

A metabolism-linked binding of some cations in the DFS was reported by Ighe and Pettersson (1974), while Luttge and Higinbotham (1979) suggested Donnan exchange in the free space may be indirectly metabolically controlled by maintenance of structure.

Pettersson (1966) and Persson (1969) proposed that exchange adsorption near the plasmalemma in the DFS may be the initial step in active ion uptake, forming the pool from which ions are accumulated. Kesseler (1980) examined the hypothesis that the preferential metabolic accumulation of K^+ by many algae may be enhanced by the preferential adsorption of the ion by the cell wall. In Valonia, K^+ was found to be enriched by such selective adsorption. It was proposed that by exchange of selectively adsorbed K^+ ions with protons liberated during cell metabolism, uptake of K^+ into cells might be facilitated.

Determinations of Apoplastic Solute Contents

A number of studies have been conducted to determine overall solute content of the apoplast, but quantitation of specific ions has been limited.

Klepper and Kaufmann (1966) measured the osmotic potentials of leaf guttation fluid and xylem exudate from the stem and petiole of tomato. The guttation fluid was found to be dilute compared to the exudate, suggesting that the xylem solution becomes depleted by cell uptake as it passes through the stem and leaf.

In a study of extracellular solute concentration as reflected by freezing-point depression, Scholander et al. (1966) collected fluid from leaves of several species using a pressure bomb. Solute concentrations were reported to be low in spite of possible contamination from damaged cells.

Oertli (1968) determined the osmotic potential of extracellular fluid from barley leaves grown under varying salt regimes. Salt accumulation in the apoplast was more pronounced under saline conditions and was attributed to the limited capacity of cells to take up salts. Robinson and Smith (1970) studied uptake of Cl^- by Citrus leaf slices in relation to external concentration. Their results suggested that an increase in cell uptake from the apoplast with increasing external concentration might contribute to the osmotic adjustment of leaf tissues under conditions of high salinity. They speculated that only if salt input to the extracellular compartment exceeded uptake would the osmotic pressure of the apoplast become high enough to cause salt damage.

Cosgrove and Cleland (1983) determined the overall solute concentration in the apoplast of herbaceous stem tissue using perfusion techniques. The apopolast of growing stem tissue contained a significant concentration of osmotically active solutes, about 25% of which were inorganic electrolytes. This was attributed to a high solute requirement for maintaining cell turgor as well as a high rate of transpirational water loss from cell walls. The

osmotic pressure of the free space ranged from 2.9 bars at the apex to 1.8 bars at the base. The significant osmotic pressure in the wall was said to offer an explanation for negative water potentials in nontranspiring plants.

Total concentrations of apoplastic solutes in sunflower leaves were determined by Jachetta et al. (1986b) using the osmolarity of sap fractions expressed over small increments of pressure. The sap concentration of the cell-wall/minor-vein fraction was approximately 8 MO kg^{-1}.

Jacobson (1971) analyzed extracellular fluid collected by centrifugation from Venus flytrap as a guide to formulating a physiological perfusion fluid compatible with isolated tissue. The estimated cation and anion concentrations in the free space were 27.9 and 16.5 meq l^{-1}, respectively, with an estimated K^+ concentration of 6.4 meq l^{-1}. A perfusion solution formulated on the basis of these results proved useful in maintaining isolated cells and studying responses of the trap to extracellular ion concentration.

Bernstein (1971) sought a direct method for determining solute potential in leaf cell walls. Vacuum perfusion was used to draw water through discs cut from amphistomatous leaves of several species, and successive fractions of the perfusate were collected. Based on the excess solute content in the first as compared to later fractions, the calculated total concentration of solutes originally present

in the cell walls ranged from 2 to 10 meq l^{-1}. Extracellular K^+ concentrations of 1 to 5 mM were reported.

An indirect approach to determining extracellular leaf K^+ concentration was taken by Pitman et al. (1974b), who studied KCl uptake by barley leaf slices from solutions of varying concentrations. These workers reported that cells appear to be in equilibrium with an apoplastic concentration of 5 mM K^+ (as KCl) and hypothesized an equivalent concentration in the intact leaf.

More recently, Widders and Lorenz (1982a) reported a decrease in the K^+ concentration of tomato xylem sap from 12 to 5 mM during plant development and speculated this might contribute to a reduction in extracellular leaf K^+ concentration. In a related study to estimate this concentration, these investigators (Widders and Lorenz, 1983a) reported zero net K^+ flux for tomato leaf slices at external concentrations between 1.0 and 3.5 mM K^+. They proposed that a decline in free-space concentration below this level might lead to net efflux from leaf cells.

X-ray microanalysis was used by Harvey et al. (1981) to determine ion compartmentation in Suaeda maritima leaves. Estimates were made of Na^+, K^+ and Cl^- concentrations in intracellular compartments as well as in cell walls. These workers reported K^+ concentrations of 13 to 17 mM for the cell-wall/intercellular-space. This paper was reported to be the first presentation of direct, absolute measurements

of ion concentrations in these compartments. As such, it may represent a landmark in compartmental ion analysis in plant tissue.

MATERIALS AND METHODS

Selection of Plant Material

The cuticle covering leaves is a relatively impermeable barrier to ion exchange with an external solution (Northcote, 1972). This restriction of free diffusion in the intact leaf precludes attainment of a rapid equilibrium between the tissue and solution (Luttge and Higinbotham, 1979) and has imposed difficulties on the study of ion relations in leaf tissue. As a result, special techniques have been required to expose interior leaf surfaces to exogenous solutions.

Some workers have used leaf slices to study ion fluxes (e.g. Smith and Epstein, 1964a and b; Pitman et al., 1974a and b; Smith and Fox, 1975; Widders and Lorenz, 1983a and b). Although freshly cut slices seem to retain valid uptake characteristics (Pitman et al., 1974b), there are serious problems due to tissue damage. Pitman et al. (1974a) reported that 30% of the cells in 1-mm leaf slices may be damaged by cutting. Similarly, leaf discs are subject to considerable cell damage during preparation (Smith and Epstein, 1964b; Osmond, 1968).

Cut tissue is not suitable for quantitative estimation of extracellular ion content due to ion leakage from cut cells (Robinson, 1971) and because resulting biochemical

changes in the tissue may affect the permeability of cell membranes still intact (Smith and Robinson, 1971; Ehwald et al., 1980).

A technique that increases access to the leaf apoplast is removal of the epidermis and, with it, the cuticle. Morrod (1974) used epidermis-free tobacco leaf discs to study cell membrane permeability, while Delrot et al. (1983) collected sugars from the apoplast of peeled Vicia faba leaves. The reported advantages to removal of the leaf epidermis are elimination of the cuticular barrier, an increase in the area for penetration of an external solution, and reduction of the diffusion pathway to the mesophyll (Morrod, 1974).

The leaflets of Argenteum, a mutant of the garden pea (Pisum sativum L.), have an epidermis that is easily removed by peeling. Hoch et al. (1980) reported large intercellular spaces between the epidermal layers and underlying mesophyll cells, apparently due to a weakened middle lamella, which give the leaflets a silvery appearance. These investigators found epidermal strips from Argenteum to be largely free of mesophyll cell-wall fragments. In other respects, there are no apparent differences between mutant and normal plants (Marx, 1982).

Peeled Argenteum and tomato lamina tissues were prepared for examination by scanning electron microscopy by fixation in 4% glutaraldehyde, dehydration in a graded ethanol series, critical-point drying and gold coating.

Scanning electron micrographs revealed negligible damage to underlying mesophyll cells when either abaxial (Figure 1a) or adaxial epidermis was removed from Argenteum leaflets. Damage observed was even less than to Vicia faba leaves, which are often used for epidermal peels. In contrast, many cells were ruptured when the lower epidermis was removed from tomato leaves (Figure 1b). Argenteum was therefore considered an ideal plant for the elution procedure used in this study.

The effects of eliminating the cuticular barrier to diffusion are demonstrated in Figure 2. $^{36}Cl^-$-labeled $CaCl_2$ solution moved into peeled leaflet discs at a significantly higher rate than for unpeeled discs (Figure 2a), in spite of the extensive cut edge available for diffusion. Figure 2b reveals the much more rapid elution of K^+ from peeled than from unpeeled areas of intact Argenteum leaflets. The methods used in both experiments are described later in this section.

Plant Culture

Seeds collected from Argenteum plants were sown in a sterile peat-based growing medium (Baccto Grower's Medium; Michigan Peat Co., Houston, Tex.) in 16-oz opaque plastic drinking cups with drainage holes. Soaking seeds in water overnight before sowing reduced germination time by one to two days. Batches of approximately thirty plants were

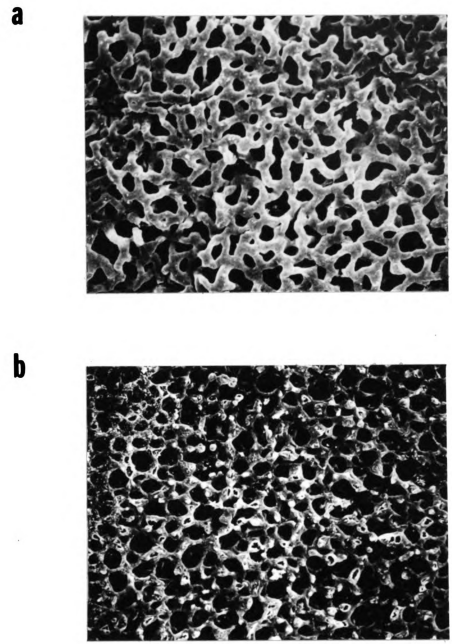


Figure 1. Scanning electron micrographs of leaf spongy mesophyll (200X) revealed when abaxial epidermis was removed from (a) Argenteum leaflet and (b) tomato leaf.

Figure 2. Effects of removal of abaxial epidermis on ion diffusion at 1° C into and out of Argenteum leaflet tissue. (a) Uptake of ^{36}Cl-labeled 5 mM $CaCl_2$ solution by discs with and without epidermis. Rates (peeled, 2.64×10^{-3}, and unpeeled, 1.38×10^{-3} ml gfw^{-1} min^{-1}) significantly different at 5% level. (b) Elution of K^+ from peeled and unpeeled leaflets. Constant rates (peeled, 1.72, and unpeeled, 0.15 ug K^+ gfw^{-1} min^{-1}) significantly different at 1% level. Points on both plots are means of six replications ± representative standard errors except where smaller than symbol.

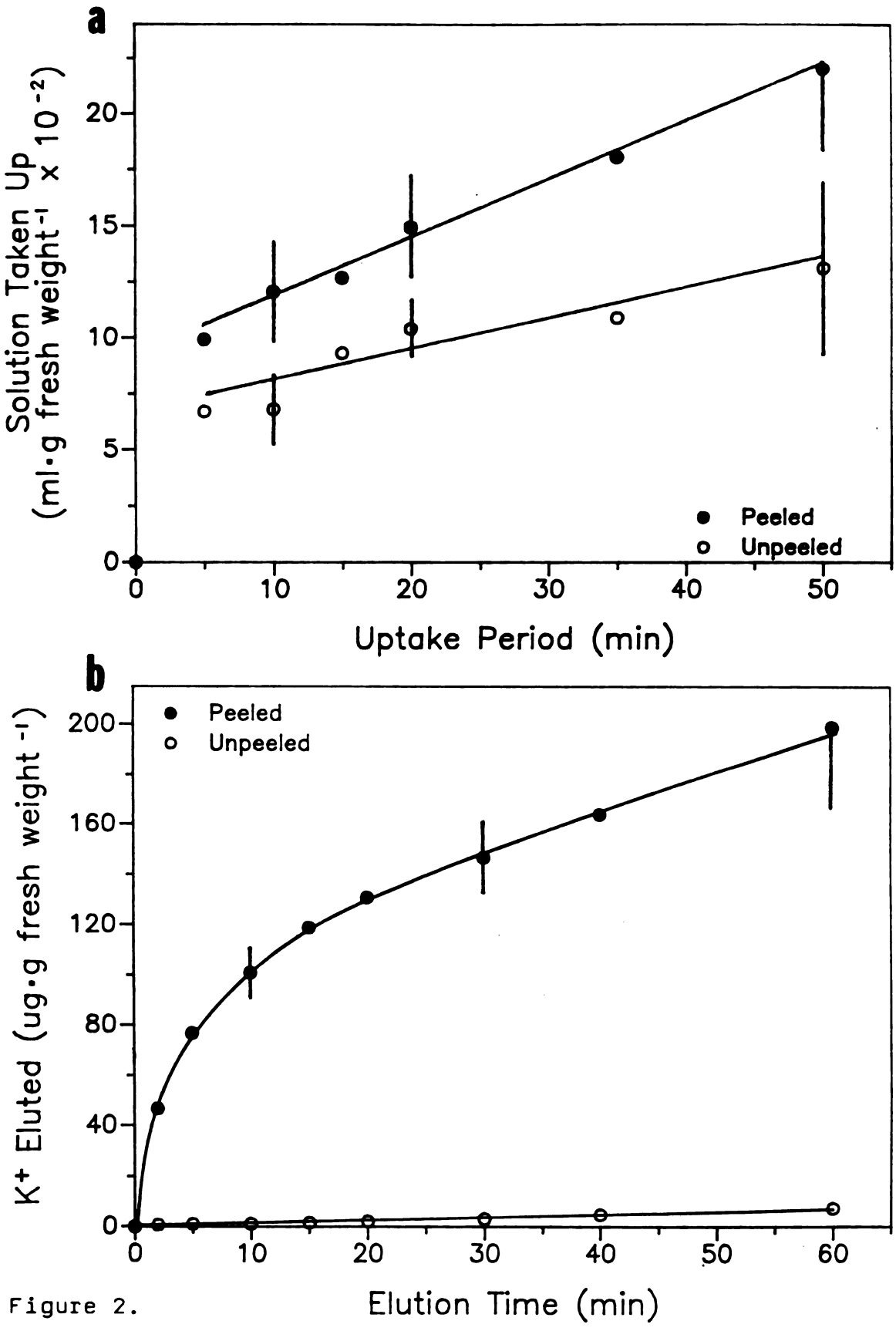

a
Solution Taken Up
(ml·g fresh weight⁻¹ x 10⁻²)
0
5
10
15
20
0
10
20
30
40
50
Uptake Period (min)
Peeled
Unpeeled
b
K⁺ Eluted (ug·g fresh weight⁻¹)
0
40
80
120
160
200
0
10
20
30
40
50
60
Elution Time (min)
Peeled
Unpeeled

Figure 2.

started at two-week intervals to provide a constant supply of plant material.

The growing medium was watered every third day until emergence. Thereafter, soluble fertilizer of 20-20-20 formulation (Peter's Soluble Plant Food; W. R. Grace and Co., Fogelsville, Pa.), mixed at a rate of 1 g l^{-1} and providing 4.25 mM K^+, was applied every other day to run-through.

Plants were grown in a controlled-temperature room at a constant temperature of 15 ± 2° C and average relative humidity of approximately 70%. A 15-hour photoperiod was provided by 400-watt mercury vapor lamps (model SON-T 400; Philips Electronics, Ltd., Bloomfield, N. J.) suspended 1 m above the bench. The average light intensity at plant level was 400 uE m^{-2} s^{-1}.

Powdery mildew was found to be a persistent problem, so plants were sprayed to runoff when two true leaves had formed with Bayleton 50% wettable powder (Mobay Chemical Corp., Kansas City, Mo.) mixed at the recommended rate of 0.15 g l^{-1}. One spraying per batch of plants was found to be sufficient. As an additional precaution, fertilizer solution was applied directly to the medium to avoid wetting the foliage.

Plants emerged five to seven days after sowing. Approximately two weeks after emergence, non-mutant (those lacking a silvery appearance) and stunted plants were removed. The remaining plants were supported with bamboo

stakes and branches were pinched off to maintain single stems. Plants were used four to six weeks after emergence, by which time they had developed nine to ten nodes.

For the sand-culture experiment, presoaked Argenteum seeds were sown in the same containers as above in a medium-grade white silica sand moistened with deionized water. Temperature, relative humidity and light conditions were the same as described above.

No additional water was supplied until several days after emergence, when seedlings were watered with a one-fourth strength modified Hoagland's solution supplying 1.5 mM K^+ as described below. The sand surface was then covered with a layer of cheesecloth and 1-mm plastic beads to prevent algal growth.

Ten days after emergence undesirable plants were removed as above. The remaining plants were randomly assigned to receive full-strength modified Hoagland's solution supplying either 2 mM (low) or 10 mM (high) K^+. As sources of macronutrients, the low-K^+ solution contained 2 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2 mM NaH_2PO_4, 1 mM $MgSO_4$ and 3 mM NH_4NO_3. For the high-K^+ solution, KNO_3 concentration was increased to 6 mM, NH_4NO_3 decreased to 1 mM, and 2 mM K_2SO_4 added. Both solutions included a full complement of micronutrients (Epstein, 1972). The one-fourth strength solution was a dilution of the high-K^+ formula minus K_2SO_4.

Plants were watered twice weekly until run-through with one of the above solutions. Approximately five weeks after

emergence, when plants were at the same stage as above, they were used in experiments.

Elution Procedure

Plants were randomly selected and transported to the laboratory just prior to each experiment. Recently fully expanded leaflets from leaves at node seven or eight were used. Since the first two nodes of pea stems produce only trifid bracts (Hayward, 1967), the above correspond to the fifth and sixth true leaves. Lower leaves were commonly too wrinkled to allow easy removal of the epidermis.

One leaflet was removed from each of six plants per treatment. The leaflets were rinsed with deionized water and immediately placed on a plastic weigh boat atop slushy ice. The abaxial epidermis was removed from an area near the base of each leaflet approximately 1.5 cm wide and extending from the midrib to one margin. Removal was accomplished by carefully slipping one point of sharpened forceps below the epidermis, grasping a portion of epidermis and gently peeling it away parallel to lateral veins. Peels were begun at the midrib or margin to avoid puncturing tissue in the center of the peeled area.

After all leaflets were peeled, a glass cylinder 0.8 cm high, cut from glass tubing of 1-cm internal diameter and polished on one end, was attached to the center of the peeled area of each leaflet in a method similar to that described by Greene and Bukovac (1971) for leaf discs.

The cylinders were attached using 100% silicone rubber (white General Purpose Sealant; Dow Corning Corp., Midland, Mich.). The sealant was spread in a very thin layer on a clean plastic weigh boat, the polished end of a cylinder placed on it and the cylinder rotated to achieve even coverage. The cylinder was then gently pressed onto a leaflet so that the entire circumference was sealed.

For experiments at 1° C, leaflets were transferred to individual weigh boats on the slush for elution. The elution solution was prechilled in a freezer until a thin ice layer had formed and kept on ice during the experiment. For experiments at higher temperature, the elution solution was equilibrated to that temperature and weigh boats containing leaflets were placed in a constant-temperature water bath during the experiment. In most cases the solution was 5 mM $CaCl_2$ prepared with the monohydrate form of the salt and having a pH of 5.5.

At 10-second intervals, 0.5 ml of solution was put into each cylinder using an autopipet. After predetermined periods (typically 2, 5, 10, 15, 20, 30, 40 and 60 minutes), a 200-ul sample was withdrawn from each cylinder with an autopipet at the same intervals and in the same order as the solution had been put in. Before removing a sample, solution was drawn into the pipet tip and released back into the cylinder to equilibrate the tip with the solution and mix the contents of the cylinder. Care was taken not to damage tissue within the cylinder while sampling. As soon

as all samples at a given time had been collected, 200 ul of fresh solution were pipetted into each cylinder.

Each sample was put in a 4-ml plastic vial containing a diluting solution. For K^+ analysis, this solution contained sufficient Cs^+ (as CsCl, to suppress K^+ ionization in the flame) and HCl to yield concentrations of 1000 ppm Cs^+ and 1% HCl after adding the sample; for Rb^+, the diluted sample was 2000 ppm K^+ (as KCl) and 1% HCl (Sotera et al., 1979 and 1981). The amount of dilution necessary depended on the nature of the experiment, but typical dilution factors were four for K^+ and three for Rb^+.

Whenever possible, experiments were conducted under a laminar flow hood. When samples were not being collected, leaflets and sample vials were kept covered to reduce evaporation and contamination. If it was not possible to analyze samples immediately, they were stored covered in a refrigerator no longer than overnight.

Samples were analyzed for K^+ or Rb^+ using an Instrumentation Laboratory (Andover, Mass.) Video 12 atomic absorption/emission spectrophotometer in emission mode. Standard operating conditions (Sotera et al., 1979) were modified to accommodate the small (generally <1 ml) sample size. Adjustments were made following observation of peak formation by the output signal during sample analysis. On the basis of the time over which maximum peak height was reached and sustained, an aspiration rate of 2.5 ml min^{-1}, a two-second delay between start of aspiration and readout

and a three-second analysis time were determined. Under these conditions it was possible to obtain two consecutive readings from a sample as small as 0.6 ml. The mean of two such readings was recorded for each sample.

Standards were prepared to contain the same concentrations of Cs^+ or K^+ and HCl as the diluted samples. When Rb^+ was present in samples analyzed for K^+, readings were greatly enhanced. It was therefore necessary that Rb^+ be included in those standards at the proper concentration.

Readings in ppm were corrected according to the dilution factor and converted to ug K^+ or Rb^+. The total amount of ion eluted from a leaflet by each sampling time was calculated by adding the amount removed in all earlier samples to the current content of the cylinder. The means of these values per gram fresh weight for all replications were plotted against time to produce an elution curve.

Fresh weight of the eluted leaflet volume was determined after each experiment was run. Since the eluted tissue was known to have taken up solution, fresh weight was recorded for a peeled disc of the same diameter as the cylinder, but cut from an analogous position on the opposite half of the leaflet. The weights of such discs from both halves of a leaflet were found to be nearly identical. Discs were not cut before elution since intracellular K^+ would have been released from cut cells into the apoplast.

The fresh weight of intact leaflet tissue changed very little over an hour-long period if kept ice cold. For

elutions over a longer term or at higher temperature, when weight did change, dry/fresh weight ratios of tissue from opposite, untreated leaflets were used to calculate fresh weights of dried eluted tissue.

In experiments involving elution from leaflet discs, a disc of the same diameter as a cylinder was cut from each leaflet using a cork borer. The disc was held with a dab of silicone rubber to the bottom of a plastic test-tube cap approximately 1.5 cm in diameter and 2 cm deep, and each cap was placed on a weigh boat. Other procedures were the same as above except that 2 ml of solution were pipetted over each disc at the beginning of the experiment.

Special Considerations for the Elution Procedure

Effects of sealant. The silicone rubber sealant used in this study had no apparent phytotoxic effect and was found not to be a source of K^+. It was, however, reported by the manufacturer to release acetic acid upon curing, which was found to reduce the pH of solution placed in a cylinder. By applying only a small amount of sealant to the cylinder, the pH change was kept at about one-half pH unit, resulting in a pH of approximately 5.0 when 5 mM $CaCl_2$ was used.

The pH of bathing solutions used in ion-flux studies is commonly in the range of 5.0 to 7.0 (e.g. Jackson and Edwards, 1966, 5.0; Smith and Epstein, 1964b, 5.2-5.8; Pitman, 1969, and Bernstein, 1971, 5.5; Doman and Geiger,

1979, 6.5). When 5 mM $CaCl_2$ was buffered with $CaCO_3$, there was not a significant difference between elution curves at pH's of 5.0 and 7.0 (Figure 3). Since all solutions used fell within this range even after exposure to the sealant, they were not buffered. The implications of elution solution pH are considered in the Results and Discussion section.

Vacuum infiltration. Subjection of leaf tissue to a vacuum helps to attain complete infiltration by an external solution (MacDonald and Macklon, 1972; MacNicol et al., 1973). In the present study, however, injection of solution into air spaces was not desirable since it could impede gaseous diffusion (MacDonald, 1975) and disrupt normal cell ion fluxes (Cosgrove and Cleland, 1983). In addition, vacuum treatment was found to promote leakage of the elution solution from cylinders. For these reasons vacuum infiltration was not used.

Sampling technique. The removal of aliquots of elution solution from a cylinder without replacement severely limited sample size and number. The entire volume of solution could not be collected due to the likelihood of damaging tissue with the pipet tip while removing the final samples. In addition, there was some drop in pH and the K^+ concentration of the solution was found to increase to an undesirable extent over the period of an experiment.

By replacing the solution removed in sampling, any number of 200-ul samples could be taken with little change in pH or K^+ concentration, or danger of rupturing cells.

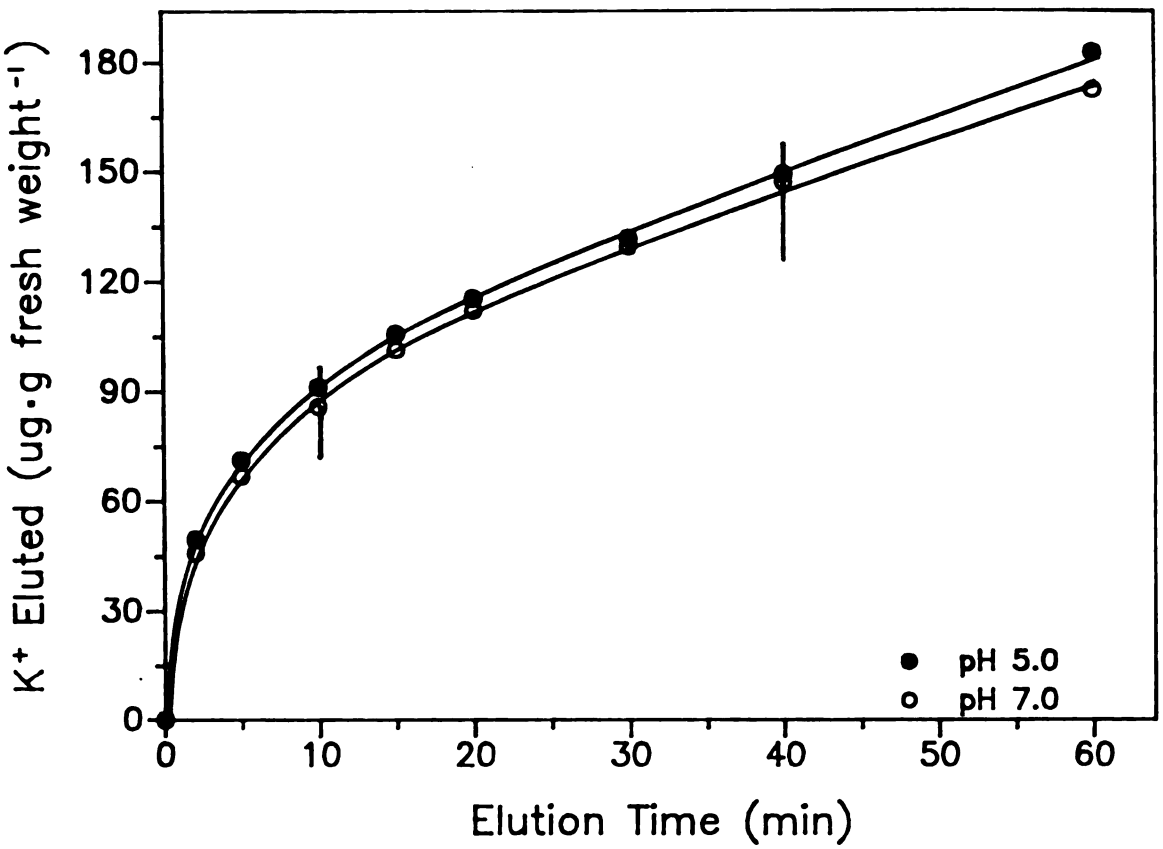


Figure 3. Elution of K^+ at 1° C into unbuffered (pH 5.0) and buffered (pH 7.0) 5 mM $CaCl_2$ solutions. Constant rates (pH 5.0, 1.68, and pH 7.0, 1.50 ug K^+ gfw^{-1} min^{-}) and Y intercepts (81.5 and 84.2 ug K^+ gfw^{-1}, respectively) not significantly different. Points are means of six replications ± representative standard errors.

While this method increased the possibility of volume changes due to pipetting error, it was found that with careful, uniform pipetting such changes were less than 2%.

The rate of evaporative loss from cylinders during elution was determined gravimetrically. Such loss was less than 1% of the original volume over a 60-minute period, but was considerably higher during long-term elutions. In these cases the volume of solution replacing samples was adjusted to maintain a more uniform volume.

Isolation of Cell Walls

Cell walls were isolated from Argenteum leaflets by homogenization and centrifugation as described by Bernstein (1971). After the final washing, however, the cell-wall preparation was not filtered but rather transferred to tared crucibles, dried at 90° C for 24 hours, and weighed.

Movement of Elution Solution into Tissue

Leaflets of the same age and position as those used for elution were randomly selected for determination of the volume of bathing solution moving into peeled leaflet tissue. Over the course of the experiment sixteen leaflets for each of six replications were required; since all could not be treated simultaneously, they were excised and prepared as needed. Each leaflet was cut in half lengthwise and the portion without midrib discarded to reduce the

volume of tissue analyzed. The remaining half was prepared for elution at 1° C as described above.

One-half ml of cold 5 mM $CaCl_2$ labeled with $^{36}Cl^-$ (as $H^{36}Cl$; New England Nuclear) was placed in each cylinder. The specific activity of the solution was 0.08 uCi ml^{-1}, and the change in Cl^- concentration due to labeling was calculated to be less than 1%. After 2.5, 5, 7.5, 10, 15, 20, 35 and 50 minutes the solution was poured out of two cylinders per replication. For one sample per replication, the entire half leaflet was prepared for counting; for the other, only the tissue covered by the cylinder. Since some leaflet tissue adhered to the cylinder upon removal, both the half leaflet or disc and cylinder were blotted and placed in a 20-ml plastic scintillation vial.

Vials were placed in a -20° C freezer overnight. To extract the $^{36}Cl^-$, 2 ml of deionized water were added to each vial. The loosely capped vials were then partially submerged in a water bath which was gently boiled for 10 minutes. After samples had cooled, 14 ml of scintillation cocktail (Safety-Solve; Research Products International Corp., Mt. Prospect, Ill.) were added to each vial and samples allowed to sit several hours. Standards, which contained 1 ml of labeled solution, a cylinder and leaflet tissue comparable to that in the samples, were treated in the same way.

Samples were counted for 10 minutes on an LKB Instruments (Rockville, Md.) model 1211 Rackbeta liquid

scintillation counter. The radioactivity of each sample was converted to millliliters of solution by dividing net counts per minute by the mean radioactivity of the standards, assuming the specific activity of solution within the tissue was the same as that in the bathing solution. The volume of solution that had moved into the tissue was then plotted over time.

To test the effect of removal of the epidermis on movement of solution into the tissue, a disc approximately 1 cm in diameter was cut from each of six peeled and six unpeeled leaflets. Each disc was placed on the bottom of a plastic test-tube cap for elution as previously described. Two ml of cold 5 mM $CaCl_2$ labeled as above with $^{36}Cl^-$ were pipetted into each cap. After 5, 10, 15, 20, 35 and 50 minutes the solution was poured off one disc per replication. The disc was removed, lightly blotted, and placed in a scintillation vial. Samples were prepared and counted as described above. The amount of solution in the tissue was plotted over time for both peeled and unpeeled discs.

To test for quenching that might result from release of pigments during sample processing, quantities of the labeled $CaCl_2$ solution ranging from 0.1 to 2.0 ml were placed in scintillation vials along with deionized water to make 2.0 ml and a peeled leaflet disc. Vials were prepared for analysis and counted in the same way as samples. When the radioactivity detected was plotted against quantity of

labeled solution (amount of isotope), they were found to be directly proportional within the lower range of the plot, where all samples fell. It was therefore not deemed necessary to adjust results for quenching.

Determination of Free-Space Characteristics

For determination of free-space characteristics of Argenteum leaflet laminar tissue, solutions of 0.5, 1, 3, 5, 10 and 20 mM KCl, all of which were also 0.1 mM $CaSO_4$, were labeled with either $^{86}Rb^+$ (as $^{86}RbCl$) or $^{36}Cl^-$ (as $H^{36}Cl$) (both from New England Nuclear) to specific activities of 0.1 and 0.08 uCi ml^{-1}, respectively. Leaflets of the same age as those used in elution experiments were randomly excised from five-week-old plants and prepared for elution in the usual manner at 1^o C.

Over the course of the experiment, 0.5 ml of each of the twelve labeled solutions was placed in the cylinders of four leaflets. After 5, 10, 20 and 40 minutes each solution was poured off one leaflet and the cylinder removed. The area covered by the cylinder was cut out with a razor blade, blotted and placed in a scintillation vial along with the blotted cylinder.

Samples were placed in a freezer overnight. Then 2.0 ml of deionized water were added to each vial, which was loosely capped and placed in a gently boiling water bath for 10 minutes. The $^{36}Cl^-$ samples were further prepared and counted as previously described. Each $^{86}Rb^+$ sample received

10.0 ml of 2.5 mM ANDA solution (7-amino-1,3-naphtralene disulfonic acid monosodium salt; Eastman Kodak Co., Rochester, N. Y.) as a wavelength shifter (Lauchli, 1969). Samples were allowed to sit for several hours before being counted for 10 minutes on the instrument described earlier.

Calculations to determine the volume of water free space (WFS), the amount of fixed anions in the Donnan phase (A^-), volume of the Donnan phase (V_D) and the effective concentration of fixed anions ($[A^-]$) were as per Briggs et al. (1958) and Pitman et al. (1974a) and as discussed in Briggs et al. (1961).

WFS was considered to be the mean volume of KCl solution at all concentrations taken up per gram fresh weight, calculated on the basis of $^{36}Cl^-$ activity of the tissue after 40 minutes.

The free-space contents of K^+ ($^{86}Rb^+$) and Cl^- ($^{36}Cl^-$) were estimated for each KCl concentration at each sampling time. This was done by separately plotting ueq gfw^{-1} of each ion in the tissue over time, regressing lines through the points for each KCl concentration, and extrapolating to zero time to find the Y-axis intercepts.

Plots of these free-space contents against KCl concentration yielded curves of different shape for K^+ and Cl^- (Figure 4). The K^+ curve represents total cation content, i.e. both that free in solution and that bound in the DFS. Since only a small portion of the fixed charges of the cell wall are positive (Lauchli, 1976; Luttge and

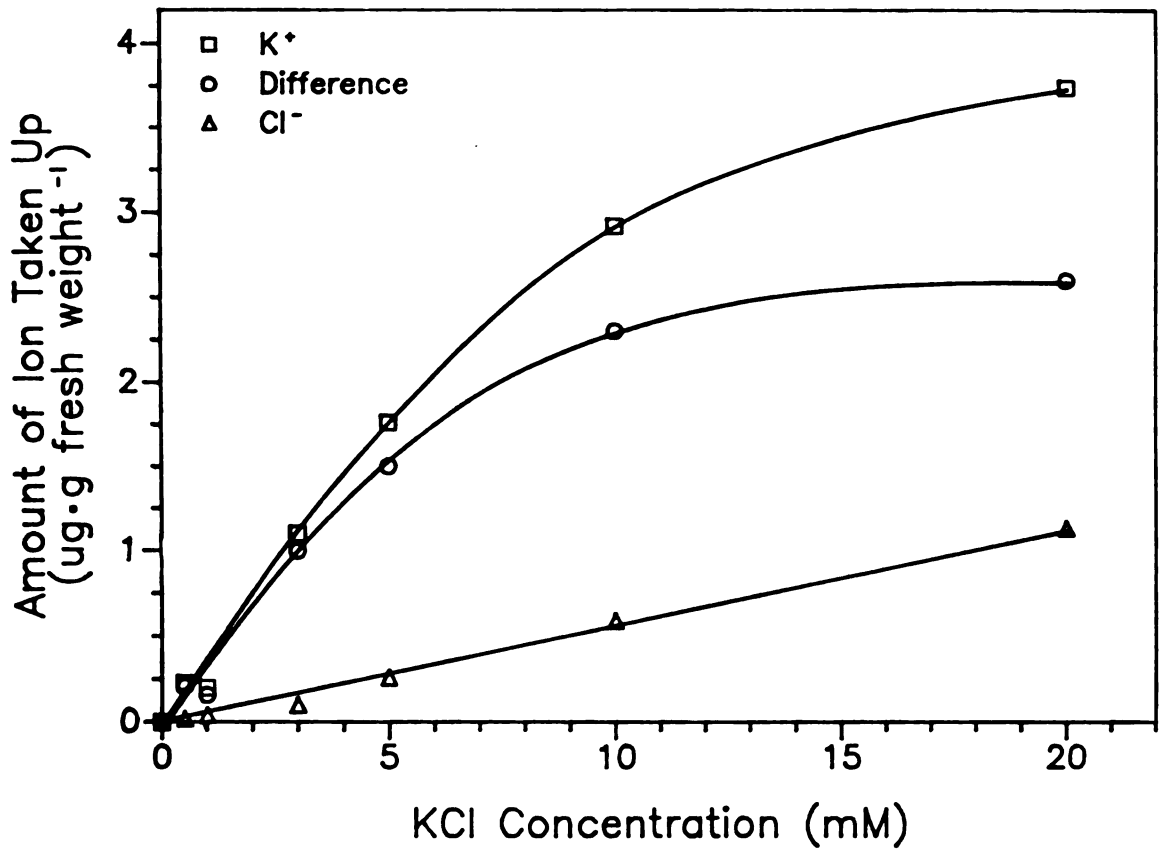


Figure 4. Potassium ($^{86}Rb^{+}$) and Cl^{-} ($^{36}Cl^{-}$) taken up at 1° C by peeled leaflet tissue from KCl bathing solutions of various concentrations. Difference curve, obtained by subtracting Cl^{-} curve from K^{+} curve, represents K^{+} bound in DFS.

Higinbotham, 1979), Cl^- would be bound in the Donnan phase to a considerably lesser extent than K^+. The Cl^- uptake curve should therefore represent principally free diffusion into the extracellular spaces of the tissue. By subtracting the Cl^- from the K^+ curve, a difference curve was obtained representing K^+ bound in the DFS at each KCl concentration (Figure 4).

To estimate the amount of fixed anions in the Donnan phase, for all KCl concentrations $K_i \times ([Ca]_o/[K]_o^2)$ was plotted against $1/K_i$, where $K_i = K^+$ in the DFS (from above), $[Ca]_o = Ca^{2+}$ concentration in the external solution (0.1 mM), and $[K]_o = K^+$ concentration in the external solution. A line was regressed through the points and the inverse of the X-axis intercept used as the estimate of A^-.

For estimation of V_D, $([Ca]_o/[K]_o^2) \times [K_i^2/(A^- - K_i)]$ was calculated for all external K^+ concentrations and the mean taken. The effective concentration of fixed anions, $[A^-]$, was calculated as A^-/V_D.

Collection and Analysis of Xylem Sap

Argenteum plants did not readily exude xylem sap, so 12 plants were randomly selected at each time of collection to assure that at least six replicate samples could be obtained. To induce exudation by positive root pressure, plants were heavily watered in late afternoon with the standard fertilizer solution, placed in a dark area of the

growth room, and covered with dark plastic bags to maintain high relative humidity around the plants.

At the beginning of the light period the next morning, the stem of each plant was cut approximately 3 cm above the planting medium with a razor blade rinsed with deionized water. The cut surface of each stem was also rinsed and blotted dry with a tissue. Decapitated plants were again covered with plastic bags supported by stakes left in the containers.

After several hours, exuding xylem sap had formed a droplet on some of the cut stems. A 10-ul sample was taken from each droplet using an autopipet. Each sample was placed in a 4-ml plastic vial containing 500 ul of dilution solution prepared as described above for K^+ analysis. In the laboratory samples were made to a final volume of 4.0 ml with the dilution solution and analyzed by atomic emission spectroscopy in the same manner as elution samples.

Determination of Tissue K^+ and Rb^+ Contents

For determination of total leaflet K^+ content, six leaves of the desired age and position were randomly collected from Argenteum plants. Leaflets were removed, rinsed in deionized water, and dried at 60° C for 48 hours. Leaflets from a given leaf were ground to a fine powder using a mortar and pestle and 0.2 g placed in a 100-ml volumetric flask. After the addition of 10 ml of concentrated HNO_3, flasks sat overnight at room temperature.

Digestion was completed by gently boiling samples down to a volume of approximately 0.5 ml followed by dropwise addition of H_2O_2 until the liquid remained colorless. The volume was brought up to 100 ml with deionized water; then 0.5 ml was further diluted to 50.0 ml with a solution containing 1000 ppm Cs^+ and 1% HCl. Samples were anayzed by atomic emission spectroscopy as described.

To determine K^+ remaining in the area of leaflet eluted, tissue covered by the cylinder was cut from each leaflet with a razor blade and the resulting discs were dried in individual coin envelopes for 48 h at 60° C. They were then weighed and placed whole into individual glass scintillation vials. Samples sat overnight in 1.0 ml of concentrated HNO_3, and digestion was completed as above. Samples were made to about 10 ml with dilution solution, quantitatively transferred to 50-ml volumetric flasks, and diluted to 50.0 ml.

Entire individual leaflets analyzed for Rb^+ were treated in the same manner except that 2.0 ml of concentrated HNO_3 were added to each vial. Dilution was to a final volume of 50 ml using a solution containing 2000 ppm K^+ and 1% HCl.

Solution Uptake through the Petiole

To follow the movement of labeled solution into excised leaves through the petiole, 24 leaves of identical position on randomly selected plants were excised near the stem.

Their petioles were immediately placed in deionized water and cut while submerged to a uniform length of 3.0 cm. If four leaflets were present, the two apical leaflets were removed to enhance solution movement into the remaining two.

Leaves were transferred from the deionized water to three 50-ml beakers, each containing 25 ml of 10 mM KCl solution labeled with $^{86}Rb^{+}$ (as $^{86}RbCl$; New England Nuclear) to a specific activity of 3 uCi ml^{-1}. The solution also contained 0.5 mM $CaSO_4$. The beakers were sitting in a 25° C water bath under incandescent lights providing 75 uE m^{-2} s^{-1} at leaf surface.

After periods ranging from 5 to 340 minutes, two leaves were randomly removed from the solution, their leaflets removed and weighed, and those from each leaf placed in a separate glass scintillation vial. Because of the large volume of material to be counted, samples were dry ashed at 480° C for six hours. When cool, 10 ml of 2.5 mM ANDA solution were added to each vial and samples were counted as above. Standards, each containing solution from one of the three beakers, were similarly prepared.

The volume of labeled solution taken up into leaflets by each sampling time was calculated by dividing the net counts per minute for each leaf by the activity of standards from the corresponding beaker. The mean volumes were then plotted over time.

In related experiments, excised leaves took up unlabeled RbCl solutions (all containing 0.5 mM $CaSO_4$) of

varying concentration for different periods of time. Six leaves per treatment were prepared as above and placed in 3.5 ml of the uptake solution contained in a 10 x 75 mm glass culture tube. The tubes were sitting in a 25° C constant-temperature water bath under lights, and each tube was covered with Parafilm with a hole pushed through it to receive the petiole. At the end of the desired uptake period, one leaflet was removed from each leaf and eluted as described earlier.

Pulse-Chase Experiment

Six recently fully expanded leaves per treatment (18 in all) were excised from node eight of randomly selected six-week-old Argenteum plants and prepared for uptake through the petiole as described above. Since all leaves could not be treated simultaneously, they were prepared and placed in the uptake solution as time allowed.

The petiole of each leaf was placed in 3.5 ml of a 50 mM RbCl solution (also 0.5 mM $CaSO_4$) contained in a glass culture tube under the conditions indicated above. After six minutes in the RbCl solution, leaves were transferred to culture tubes containing a chase solution of 50 mM KCl plus 0.5 mM $CaSO_4$, where they remained for 10 minutes.

Both leaflets were then removed from each leaf and placed between paper towels moistened with deionized water. Leaflets were subjected to elution immediately or after additional periods of 20 or 170 minutes, so the total

elapsed time between removal from the RbCl solution and elution was 10, 30 or 180 minutes. During the latter two treatments leaflets remained between the moistened towels and floated in plastic weigh boats atop a 25° C water bath.

At the time of elution, one leaflet from each leaf was transferred to a plastic boat on slushy ice, peeled and eluted at 1° C with 5 mM $CaCl_2$. Samples, 200 ul in volume, were taken at predetermined intervals up to 24 hours. They were placed in 4-ml plastic vials containing 400 ul of a solution 3000 ppm K^+ (as KCl) and 1.5% HCl and analyzed for Rb^+ as previously described.

Eluted leaflets were dried, weighed and analyzed for total remaining Rb^+. Fresh and dry weights were recorded for non-eluted leaflets, which were also analyzed for total Rb^+. The total Rb^+ eluted per gram fresh weight was calculated as for K^+ and elution curves plotted.

Glassware Washing Procedures

Because of the low K^+ and Rb^+ concentrations dealt with in this study, special procedures for washing glassware and sample vials were followed. All glassware used in preparing solutions and standards was washed in soap and water, thoroughly rinsed with deionized water, rinsed three times with concentrated HCl diluted with an equal volume of deionized water, and vigorously rinsed three more times with deionized water (Sotera et al., 1981).

Plastic sample vials were washed in the above manner initially and between series of elutions for K^+ and Rb^+. Between experiments involving the same ion, vials were rinsed vigorously several times in deionized water, once in the dilute HCl, and several more times in deionized water.

Silicone rubber was most easily removed from glass cylinders by peeling it off immediately after an experiment. If this was not successful, cylinders were soaked overnight in oleander mineral spirits. After removal of the sealant, cylinders were washed in the same manner as glassware.

Statistical Analysis

The experiments in this study were conducted using a completely randomized design. The optimum number of replications was determined as described by Little and Hills (1978). The use of six replications in elution experiments was found likely to detect a difference of 20% of the experimental mean. Variability among replications was reduced by using leaflets at the same stage of development from plants of uniform age.

The mean and standard error of the mean were calculated for the total amount of ion eluted from each replicate leaflet at each sampling time. For each leaflet a line was regressed through that amount for the 20- through 60-minute samples by the least-squares method and the slope and Y-axis intercept determined (Little and Hills, 1978).

For plotting, most elution curves were fitted to an equation of the form $Y = a \times (1-e^{-bX}) \times cX$ by nonlinear regression. The curve for the 24-hour elution at 20° C (Figure 10) and those for elution of frozen/thawed tissue (Figure 11) were better fitted to $Y = a + b \times \ln X$. The curve for rinsed discs (Figure 13) was fitted to the equation $Y = a/(1 + be^{-cX})$. First derivatives of the solved equations were used to determine non-steady-state elution rates at selected times (Protter and Morrey, 1967).

To detect differences between elution treatments, analysis of variance was conducted on ion quantities eluted by selected sampling times, on the Y intercepts of the linear functions, or on rates determined by nonlinear regression. Only two treatments at a time were compared by the F test, so mean separation was not necessary. Significant differences between slopes calculated by linear regression were determined using Student's t-test for homogeneity of slopes (Steel and Torrie, 1980). Experiments not involving elution were analyzed using one or more of the above procedures as appropriate.

RESULTS AND DISCUSSION

Characteristics of the Elution Curve

The typical 60-minute time course for the elution of K^+ from Argenteum laminar tissue is characterized by a rapid initial elution rate followed by a slower, steady rate after about 20 minutes (see Figure 2b curve for peeled tissue). Initial interpretation of the elution curve was based on previous kinetic analyses of curves of similar nature obtained in studies of radioactive tracer efflux from plant tissue.

Since no membrane or cuticular barrier to free diffusion exists between the apoplast and the bathing solution, the initial portion of the curve has been considered to represent processes involving direct interaction of the solution with the apoplast (Briggs et al., 1961). These processes include infiltration of the apoplast by the bathing solution, adsorption of introduced cations to negative charges in the Donnan phase in exchange for bound ions (Laties, 1959), and diffusion of ions into the external solution (Pitman, 1963). According to Pitman (1963), ions diffusing out during this first stage of elution come primarily from the apoplast, but also from the cytoplasm, with the elution rate controlled by diffusion in the apoplast. The above processes lead to the eventual

equilibration of ion concentrations in the solution with those in the apoplast (Pitman et al., 1974b; Lauchli, 1976).

After equilibrium has been reached, changes in the ionic concentration of the bathing solution are thought to reflect net efflux from cellular compartments (Mac Robbie and Dainty, 1958; Walker and Pitman, 1976; Lauchli and Pfluger, 1978). Pitman (1963) described a second stage of elution characterized by ion movement out of the cytoplasm and vacuole, the rate limited by net efflux across the plasmalemma, and a third stage with rate dependent solely on fluxes across the tonoplast.

In the present study, the period of rapid elution up to 20 minutes was hypothesized to correspond to Pitman's (1963) first stage, while the constant elution rate from 20 to 60 minutes was hypothesized to correspond to the second stage.

In excised root tissue, the reported time periods required for equilibration of the apoplast with a solution bathing the tissue range from eight (Briggs and Robertson, 1957) to 30 minutes (Butler, 1959). Hope and Stevens (1952) reported a constant rate of KCl efflux from bean roots within 20 minutes of placement in a bathing solution. Smith and Robinson (1971) observed an initial rapid elution of $^{42}K^{+}$ from orange leaf slices followed by a slower but constant rate of efflux after 15 to 30 minutes. Allowing for differences in experimental conditions, it therefore appears that the 20-minute period apparently required in the present study for equilibration of the external solution

with the tissue apoplast and achievement of a nearly constant rate of K^+ efflux is reasonable and consistent with previous studies.

Pitman (1963) estimated that by six hours the rate of $^{42}K^+$ efflux from beet root slices was controlled by vacuolar efflux. For $^{86}Rb^+$ efflux from tomato leaf slices, Widders and Lorenz (1983a) reported a vacuolar component that became evident after about four hours of elution. It therefore seems likely that steady-state vacuolar efflux could not be detected by elution analysis within only 60 minutes, and that the elution rate during the linear portion of the elution curve is determined by the net rate of efflux across the plasmalemma.

The experiments discussed below were conducted to test the above-hypothesized interpretations of the elution curve and to evaluate its use to estimate apoplastic K^+ content.

Principles of Ion Fluxes

As indicated, elution of K^+ from leaf tissue over time seems to involve both free diffusion from the apoplast and movement across cellular membranes. To identify and characterize the specific ion fluxes which comprise an elution curve, therefore, general principles of diffusion and transmembrane transport must be considered along with how they apply under experimental conditions.

When ions move by diffusion, they are transported from an area of higher to one of lower concentration by random

thermal motion (Mengel and Kirkby, 1982). Fick's first law describing the rate of ion diffusion through an unrestricted medium is as follows:

$$dQ/dt = -D \times A \times dc/dX$$

where dQ/dt = quantity diffused per unit of time

D = diffusion coefficient for a given ion and medium

A = cross-sectional area perpendicular to the direction of diffusion

dc/dX = change in concentration along a coordinate (X) in the direction of diffusion.

During an elution experiment, under conditions of constant temperature and pressure, the diffusion coefficient should not change (Briggs et al., 1961). However, the cross-sectional area for diffusion should increase as the external solution moves into the tissue, temporarily increasing the diffusion rate until a continuum between the tissue and solution is established. At the same time, the K^+ concentration gradient between the apoplast and external solution should decline as ions diffuse out of the tissue, reducing the diffusion rate. This would occur until achievement of a steady state where ions removed from the apoplast are replaced at the same rate by those from within cells. In the present study this is thought to occur by around 20 minutes.

When a membrane boundary occurs in the diffusion path, the diffusion coefficient in the above equation is replaced by a permeability coefficient which depends on the properties of the membrane and the solute (Briggs et al., 1961). Thus the quantity of an ion moving across a membrane per unit time depends on the membrane's permeability to the ion and the gradients across it (Briggs et al., 1961).

Membrane permeability might change over the course of an elution experiment if membrane properties are altered. In plants subject to chilling injury, for example, membrane lipids seem to undergo a phase change at temperatures below 10^{o} C which may lead to ion leakage (Lyons and Raison, 1973). Since temperate plants such as the pea are generally not subject to such injury (Fitter and Hay, 1981), the low temperature used in most experiments would not be expected to affect membrane permeability as significantly as in chilling-sensitive species.

Anaerobiosis due to infiltration of air spaces with solution may also affect membrane permeability. Mengel and Pfluger (1972) found anaerobic conditions to considerably increase the rate of K^{+} efflux from corn roots. In the present study, however, the intercellular spaces in the leaflet lamina tissue did not appear to be completely infiltrated with solution during the 60-minute elution period, so it was assumed that anaerobiosis was not a significant factor.

Due to the electrolytic nature of ions, their gradients across cellular membranes have both electrical and chemical potential components (Flowers and Lauchli, 1983). An ion can diffuse passively down an electrochemical potential gradient, but movement against such a gradient will only occur with the expenditure of energy (Luttge and Pitman, 1976b; Briskin and Poole, 1983). Passive ion movement against a chemical potential gradient can occur, however, if a sufficiently large electrical potential gradient in the opposite direction has been established (Luttge and Higinbotham, 1979).

During an extended period of elution of K^+ from tissue, the chemical potential gradient across the plasmalemma would be expected to be reduced as the cellular K^+ content was depleted, resulting in a lower net efflux rate. In addition, the electrical potential gradient might be affected by cell uptake of Cl^- from the elution solution or by the proton concentration of the solution. The implications of these influences, as well as the transport of specific ions, will be considered later in this discussion.

Interaction of Elution Solution with Tissue

Effects of ions in solution. The content and concentration of the elution solution used in the present study (5 mM $CaCl_2$) were evaluated with respect to their effects on K^+ elution.

Solutions bathing excised plant tissues under experimental conditions often include Ca^{2+} because of the physiological roles attributed to this ion which might affect ion transport. Calcium has been found necessary for maintenance of mechanical properties of the cell wall, perhaps by cross-linking carboxyl groups of pectic acids and hemicellulose chains (Luttge and Higinbotham, 1979; Nakajima et al., 1981). Similarly, by cross-linking membrane proteins and phospholipids, Ca^{2+} contributes to the maintenance of membrane integrity and function (Epstein, 1972). Calcium is also known to increase the ability of isolated plant tissue to retain soluble substances, particularly K^+ (Ehwald et al., 1980), perhaps due to its influence on membrane permeability.

Calcium has been found to influence the fluxes of other ions across the plasmalemma (Marschner, 1983). Viets (1944) reported that polyvalent cations, Ca^{2+} in particular, promoted ion absorption by barley roots. Mengel and Helal (1967) attributed the increase in net influx to reduced efflux, since low external Ca^{2+} was found to enhance the K^+ efflux rate.

An alternate hypothesis (Franklin, 1970) is that Ca^{2+} ions bound in the Donnan phase form a narrower layer than monovalent cations since fewer are required to cancel the negative charges. This would allow both cations and anions greater access to sites of absorption across the plasmalemma (Robson and Pitman, 1983).

Calcium binding in the Donnan phase is also significant because of its ability to exchange for K^+. According to Briggs et al. (1961), ion exchange readily occurs between bound and externally introduced ions, with divalent cations largely replacing monovalent if the concentration of the former is high enough. Under these circumstances there would be little reverse exchange of K^+ for Ca^{2+}, particularly at low temperature (Rains et al., 1964).

A Ca^{2+} concentration of 0.5 mM in the solution bathing excised plant tissue has been found sufficient to maintain maximum net influx rates of other ions (Epstein, 1961) and has been used in a number of studies (Smith and Epstein, 1964b; Brownlee and Kendrick, 1979; Widders and Lorenz, 1983a and b).

In the present study, however, another function of Ca^{2+} was to exchange for bound K^+. Since the amount of exchange of bound ions with those in an external solution increases with solution concentration (Briggs et al., 1961), it was felt a concentration greater than 0.5 mM might enhance this exchange. Calcium concentrations from 1 mM (Doman and Geiger, 1979; Osmond, 1968) to 10 mM (Hawker et al., 1974) have also been used in solutions bathing excised tissue. Drew and Biddulph (1971) suggested that 5 mM is more consistent with the actual apoplastic Ca^{2+} concentration. A compilation of xylem sap Ca^{2+} concentrations by Robson and Pitman (1983) lists concentrations up to 4.5 mM. While the relationship between xylem sap and apoplastic concentrations

is not known, a Ca^{2+} concentration of 5 mM was felt to be reasonable for the present study.

Calcium chloride was chosen as the elution solution rather than $CaSO_4$, which is often used in ion flux studies, due to the greater solubility of the former and the ease of working with $^{36}Cl^-$, which could be used to trace solution movement in the tissue. There are conflicting reports as to whether SO_4^{2-} interferes with normal K^+ transport across the plasmalemma. Epstein et al. (1963) reported that the mechanism for K^+ uptake at concentrations up to 0.2 mM was equally effective whether the anion was Cl^- or SO_4^{2-}, but uptake at higher concentrations was inhibited by the latter. Smith and Robinson (1971), however, found no difference in K^+ uptake by leaf slices in the presence of Cl^- or SO_4^{2-}.

In the present study when simultaneous elutions were conducted using 5 mM $CaCl_2$ or 5 mM $CaSO_4$ (Figure 5), there was not a significant difference in the amounts of K^+ eluted up to 60 minutes or in the slope of the linear function, thought to represent the rate of net K^+ efflux across the plasmalemma.

Figure 5 also presents an elution curve for deionized H_2O as the bathing medium. At 60 minutes significanty less K^+ had been eluted into H_2O as compared to $CaCl_2$, but the difference was not significant for $CaSO_4$. The constant elution rates were not different for any of the treatments.

A lower net K^+ efflux rate might be expected with Ca^{2+} in the bathing solution, but these results do not

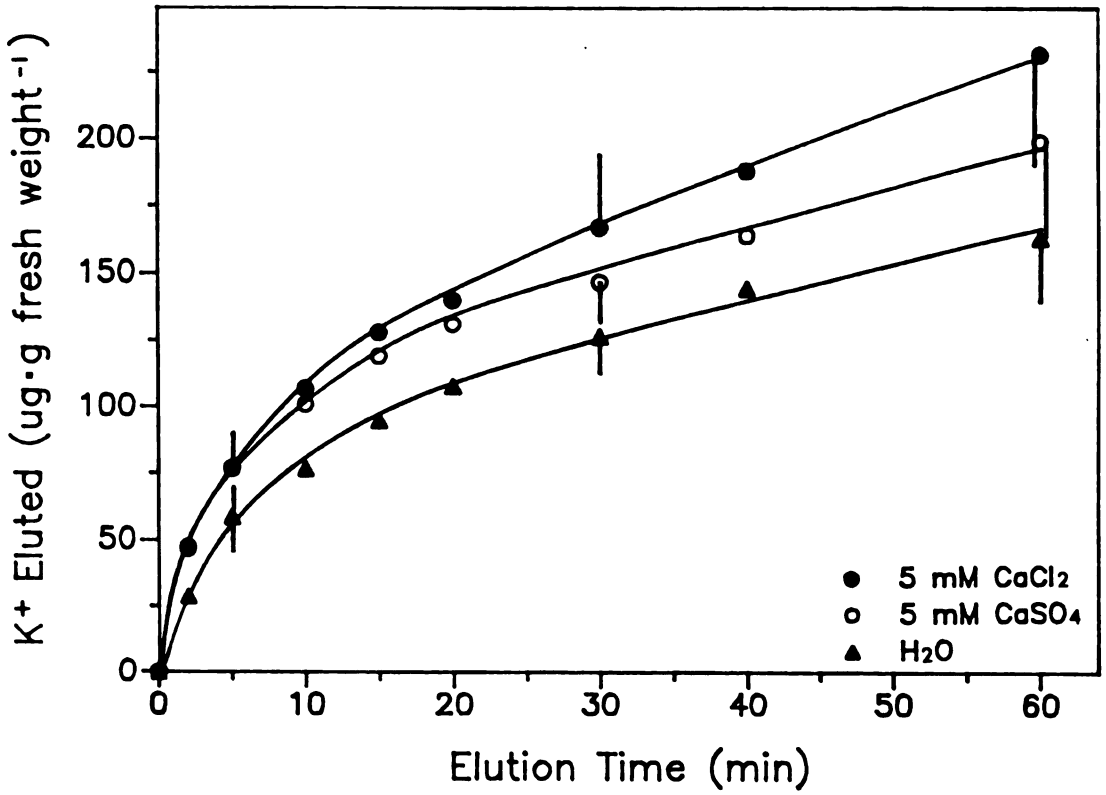


Figure 5. Potassium elution at 1° C into 5 mM $CaCl_2$ and 5 mM $CaSO_4$ solutions and deionized water. Constant rates ($CaCl_2$, 2.26, $CaSO_4$, 1.58, and H_2O, 1.38 ug K^+ $gfw^{-1}min^{-1}$) not significantly different. Y intercepts for $CaCl_2$ and $CaSO_4$ (96.6 and 99.4 ug K^+ gfw^{-1}, respectively) not significantly different. That for H_2O (83.7 ug K^+ gfw^{-1}) significantly different from both at 5% level. Points are means of six replications ± representative standard errors.

demonstrate such an effect. It may be that anion effects on K^+ fluxes influenced the results, or that under the experimental conditions Ca^{2+} already in the tissue was not depleted sufficiently to reduce membrane integrity. Bernstein (1971) reported little difference in solute concentration of perfusates collected from 97 mm leaf discs after infusion with water or 0.5 mM $CaSO_4$.

The effect of the concentration of the bathing solution on K^+ elution was tested by conducting 60-minute elutions at 1^0 C using 0.5, 5 or 50 mM $CaCl_2$ (Figure 6). With increasing $CaCl_2$ concentration, there was a decrease in the amount of K^+ diffusing into the external solution. Significant differences in the amount of K^+ eluted were observed from 10 through 60 minutes for all $CaCl_2$ concentrations. Elution rates from 20 to 60 minutes declined significantly with each increase in concentration.

Calcium uptake across the plasma membrane appears to be largely passive and at a relatively low rate (Hanson, 1984), so Ca^{2+} uptake probably did not significantly affect K^+ efflux. Since 0.5 mM Ca^{2+} is thought to be sufficient to maintain membrane integrity (Epstein 1961), higher concentrations would not be expected to affect this aspect of ion transport. The influence of Cl^- on K^+ fluxes may be more significant.

The chloride ion seems to be actively accumulated by cells (Pierce and Higinbotham, 1970; Campbell and Pitman, 1971), its uptake occurring against an electrochemical

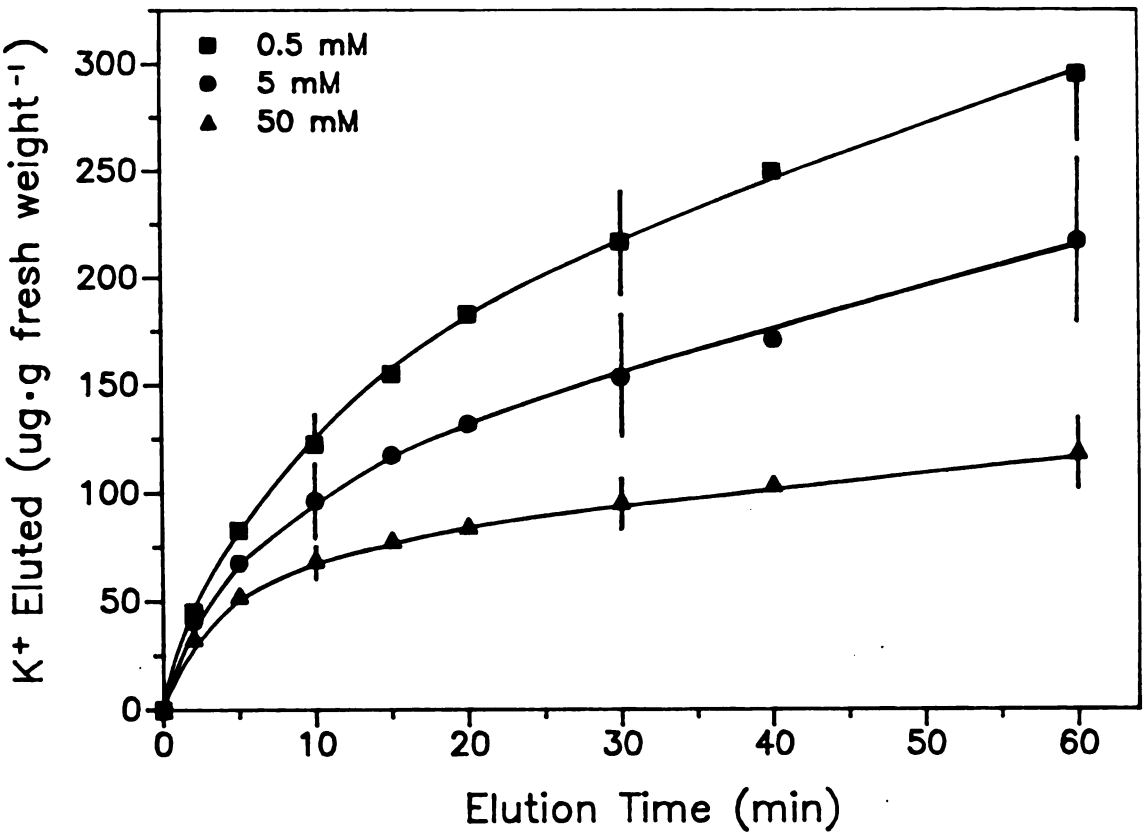


Figure 6. Potassium elution at 1° C into 0.5, 5 and 50 mM $CaCl_2$ solutions. Constant rates for 0.5 mM and 5 mM (2.79 and 2.12 ug K^+ gfw^{-1} min^{-1}, respectively) significantly different at 5% level. That for 50 mM (0.84 ug K^+ gfw^{-1} min^{-1}) significantly different from both at 1% level. Y intercepts for 0.5 mM and 50 mM (131.4 and 69.6 ug K^+ gfw^{-1}, respectively) significantly different at 5% level. That for 5 mM (88.9 ug K^+ gfw^{-1} min^{-1}) not significantly different from either. Points are means of six replications ± representative standard errors.

potential gradient into the negatively charged cell (Gerson and Poole, 1972). The plasmalemma is quite permeable to Cl^- and at high external concentrations it seems to be the tonoplast that limits influx of the ion (Cram, 1973).

At temperatures near 0^o C Cl^- uptake is limited (Pitman et al., 1974a). At the relatively high concentration of 50 mM, however, sufficient Cl^- may have accumulated in the cytoplasm to cause increased K^+ influx down the electrical potential gradient, leading to a reduced net K^+ efflux rate.

The effect of increased Ca^{2+} concentration on K^+ release from the Donnan phase is difficult to assess. It is possible that such exchange was promoted, but this cannot be discerned from the elution curves. Exchanged K^+ may represent only a small portion of the K^+ eluted and therefore not contribute signficantly to the results.

In another experiment to study the interaction between the ionic composition of the elution solution and K^+ efflux, simultaneous elutions were conducted at 1^o C into 5 mM $CaCl_2$ and 10 mM RbCl (plus 0.5 mM $CaCl_2$) solutions providing nearly equivalent cation charge and Cl^- concentration. Rubidium was selected as the cation because the mechanism of its cellular uptake is thought to be similar to that for K^+ (Smith and Epstein, 1964a; Lauchli and Epstein, 1970).

The mean constant K^+ efflux rate measured between 20 and 60 minutes was significantly higher for Rb^+ than for Ca^{2+} (Figure 7). In addition to the regular 60-minute elution, samples were taken after 24 hours to determine the

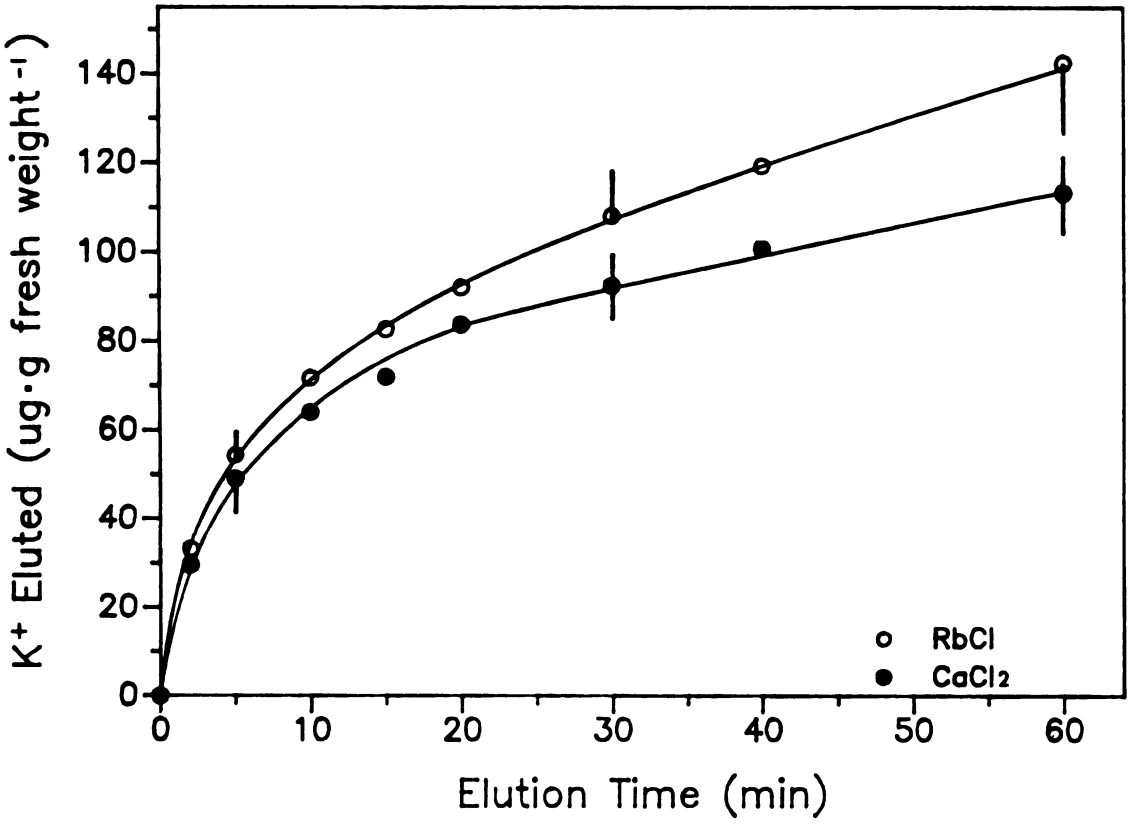


Figure 7. Potassium elution at 1° C into 5 mM $CaCl_2$ and 10 mM RbCl (plus 0.5 mM $CaCl_2$) solutions. Constant rates ($CaCl_2$, 0.73, and RbCl, 1.24 ug K^+ gfw^{-1} min^{-1}) significantly different at 1% level. Y intercepts (70.0 and 69.0, respectively) not significantly different. Points are means of six replications ± representative standard errors.

total amount of K^+ eluted into the two solutions. In that period almost twice as much K^+ was eluted into RbCl as into $CaCl_2$ (961 and 521 ug gfw^{-1}, respectively).

Rubidium has been found to competitively reduce K^+ absorption (Robson and Pitman, 1983), while Rb^+ uptake may result in the loss of K^+ from cells (Hosokawa and Kijosawa, 1985). Both of these factors would contribute to an increase in net K^+ efflux in the presence of Rb^+.

Effect of pH of the elution solution. The pH of the elution solution is of concern since a significant alteration from physiological pH might alter ion fluxes. As indicated, the pH of the 5 mM $CaCl_2$ solution was approximately 5.0 after exposure to the silicone rubber sealant. The pH of the leaf apoplast has been estimated to be between 5.0 and 6.0 (Glaquinta, 1976 and 1983). These estimates are consistent with reports of xylem sap pH's around 5.0 (Bollard, 1953; Ferguson and Bollard, 1976).

Ion binding in the DFS is affected by pH. Weak acids with a pK_a of approximately 3.0 provide the exchange sites in the Donnan phase (Pitman et al., 1974a; Morvan et al., 1980). At a pH of 4.5 or higher, the sites would be almost completely ionized (Briggs et al., 1961), while lower pH's would reduce the number of fixed negative charges and consequently the concentration of cations bound at the cell wall-plasmalemma interface (Pitman, 1969). Robson and Pitman (1983) suggested that the increase in root absorption

of cations with increasing external pH may reflect an increased accessibility to sites of ion adsorption.

There is evidence that ATPase-mediated ion uptake is affected by pH. The activity of the plasmalemma ATPase has been found to be greatly reduced at pH's below 5.0 and above 8.0 (Leonard, 1982; Sommarin et al., 1985). In addition, Epstein (1962) and Rains et al. (1964) reported that at low pH protons can interfere with the uptake of monovalent cations by competition for binding sites on carriers.

There may be a direct pH effect on membrane permeability. Campbell and Pitman (1971) and Luttge and Higinbotham (1979) reported that a low pH (less than 4) at the plasmalemma may increase passive membrane permeability. Injury was said to occur by denaturation of proteins, or by proton displacement of essential cofactors or functional groups.

The major deleterious effects of pH on ion flux seem to occur below 5.0 and above 8.0. At pH's within this range, which were characteristic of the solutions used in the present study, pH-induced changes in net K^+ efflux did not appear to be significant (Figure 3). These results indicate considerable buffering capacity of the cell-wall Donnan phase. Studies by Pitman (1969), Smith and Raven (1976) and Morvan et al. (1980) indicate that the cell wall may play an important role in regulating extracellular pH. This would be significant since the proton concentration of the relatively small volume of solution bathing cells might

otherwise change considerably due to proton fluxes across the plasma membrane. It appears that this buffering capacity was also significant under the present experimental conditions.

Infiltration of tissue by elution solution. In order to estimate the extent of tissue infiltration by the elution solution, 5 mM $CaCl_2$ labeled with $^{36}Cl^-$ was placed in elution cylinders as described earlier. The mean volumes of labeled solution taken up by only tissue within the cylinder and by the entire half leaflet to which the cylinder was attached are compared in Figure 8.

Elution solution might be expected to infiltrate lamina tissue outside the cylinder radius to some extent since there is no barrier to diffusion other than where the sealant contacts the tissue. Although the curves suggest that by 35 minutes solution had begun to move into tissue outside the cylinder, volumes taken up were not significantly different at any sampling time.

It is of interest to note that following a 24-hour elution only the tissue within the cylinder and a small area immediately surrounding it appeared water soaked. Therefore, even in the long term, lateral movement of solution in the lamina tissue may be restricted mainly to apoplastic regions in close proximity to the cylinder. This is supported by the finding that after a 24-hour elution period, the sum of K^+ removed by elution plus that remaining in eluted tissue within the cylinder was not significantly

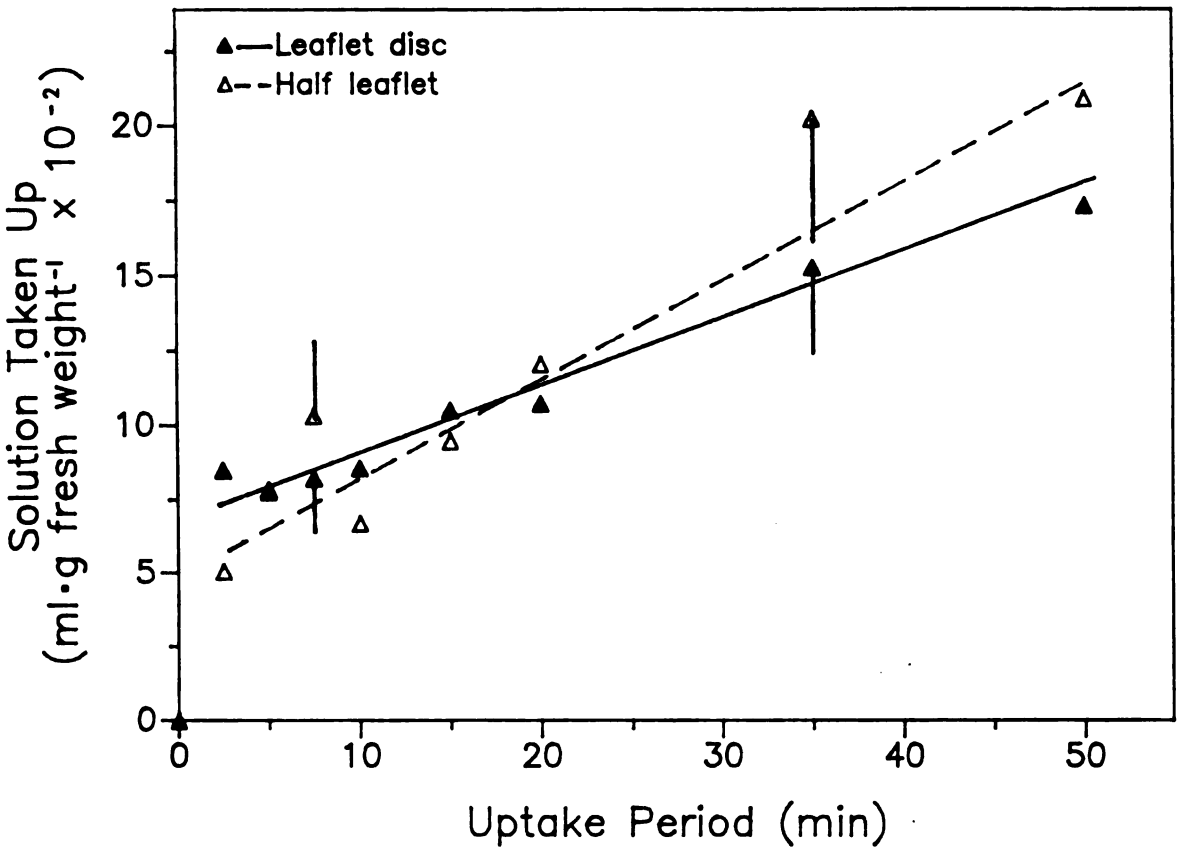


Figure 8. Infiltration of $^{36}Cl^-$-labeled 5 mM $CaCl_2$ solution at 1° C into only the peeled tissue within the cylinder (disc) and into the entire half leaflet to which cylinder was attached. Rates (disc, 3.44×10^{-3}, and half leaflet, 2.12×10^{-3} ml gfw^{-1} min^{-1}) not significantly different. Points are means of six replications ± representative standard errors.

different from the total K^+ contained in an analogous, noneluted tissue sample from the opposite half of the leaflet (Table 1). This provides evidence for limited K^+ diffusion from outside the cylinder area.

The infiltration rate appears to have been considerably higher during the first minutes of uptake than thereafter, when there was a fairly constant rate of label accumulation. This type of time course is characteristic of ion uptake by excised tissue (Briggs et al., 1958; Osmond, 1968) and indicates an initial rapid movement of solution into the most accessible portion followed by slower movement into additional areas.

Due to the hydrophilic nature of cellulose (Franke, 1967) and capillary attraction of interfibrillar spaces (Muhlethaler, 1967), it would be anticipated that solution would move readily into the cell wall. The apoplast is in free diffusional communication with the external solution, so ion movement is restricted only by the rate of diffusion in the cell wall (Haynes, 1980) which has been shown to be quite rapid (Pitman, 1965a).

Replacement of air trapped in intercellular spaces by the solution may account in part for the constant infiltration rate (Briggs et al., 1961). Cell uptake of the radioactive tracer, though reduced at low temperature, would also contribute to that rate (Pitman et al., 1974a).

By 5 minutes the volume of solution accumulated in both treatments in Figure 8 was approximately 0.075 ml gfw^{-1}. If

Table 1. Potassium removed by elution from Argenteum leaflets at 1° C in 24 hours plus that remaining in the eluted volume of tissue compared with total K^+ contents of analogous but noneluted tissue volumes from the opposite half of each leaflet. Results of two experiments are shown. Values are means of six replications per experiment ± standard errors.

Experiment	(A) Total K^+ eluted in 24 h (ug)	(B) K^+ remaining in eluted tissue[a] (ug)	(A) plus (B) (ug)	Total K^+ in noneluted tissue (ug)
1	7.7 ± 0.3	59.3 ± 6.5	67.0 ± 8.1	67.4 ± 5.0
2	14.1 ± 0.4	54.9 ± 4.4	69.0 ± 4.7	69.9 ± 5.8

[a]Eluted tissue is only that within the diameter of the cylinder containing eluting solution.

this represents infiltration of most of the available cell-wall volume, then 50% of this volume would have been filled in less than 2 minutes. Assuming the cell wall, thought to be the site of apoplastic K^+, is infiltrated this rapidly, then infiltration of the tissue would have a significant effect only on the early portion of the elution curve.

Analysis of Components of the Elution Curve

Temperature effects. Since temperature has been reported to have a lesser effect on the free diffusion of ions than on membrane-regulated fluxes (Luttge and Higinbotham, 1979), it was considered a useful treatment to differentiate the two hypothesized phases of the elution curve. Simultaneous sixty-minute elutions were therefore conducted at 1^{o} and 20^{o} C as previously described.

The total amounts of K^+ eluted at 20^{o} C were significantly higher starting at eight minutes (Figure 9). At this temperature, the constant elution rate between 20 and 60 minutes was approximately 7.5 times that at 1^{o} C. Earlier in the time course, however, smaller differences in elution rate were evident. At two minutes, the rate at the higher temperature was 1.4 times that at low temperature; by five minutes, the rate was 2.4 times, and by ten minutes, 5.2 times as great.

Temperature would be expected to have some effect on the physical processes thought to be most evident in the first part of the curve. The rate of diffusion of ions

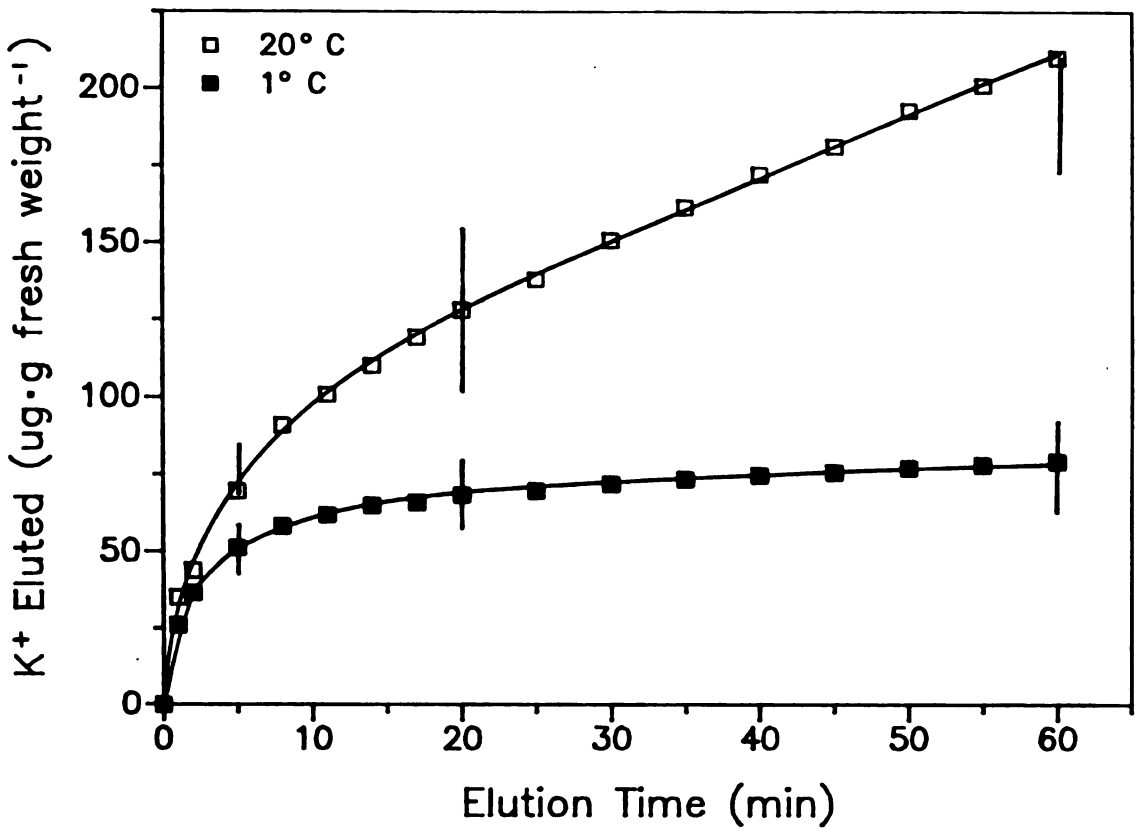


Figure 9. Potassium elution at 20° and 1° C. Constant rates (20°, 2.04, and 1°, 0.27 ug K^+ gfw^{-1} min^{-1}) significantly different at 1% level. Y intercepts (87.6 and 63.4 ug K^+ gfw^{-1}, respectively) not significantly different. Points are means of six replications ± standard error.

depends on their kinetic energy, the Q_{10} for diffusion in an aqueous medium being 1.2 to 1.5 (Briggs et al., 1961). The diffusion rate at 20° C would therefore be about 1.5 to 2.2 times that at 1°, and could account for the difference in rates up to about five minutes. Ion exchange in the Donnan phase may also be increased at higher temperature due to the increased kinetic energy of the ions (Briggs et al., 1961).

The greater difference in elution rates after five minutes indicates that membrane fluxes become increasingly important. To evaluate this factor, specific characteristics of membrane K^+ transport should be taken into account. Since the efflux rate evident in the elution curve is the net result K^+ influx and efflux, both processes must be considered.

Previous research has suggested that K^+ uptake by plant cells is active at K^+ concentrations less than 1 mM (Epstein, 1976; Poole, 1978; Cheeseman and Hanson, 1980) which were characteristic of the elution solution in the present study. The reaction providing energy for K^+ uptake is thought to be hydrolysis of ATP by a membrane-bound ATPase coupled to the extrusion of protons from the cytoplasm (Lauchli and Pfluger, 1978; Briskin, 1984). The ATPase may itself act as an ion carrier at low K^+ concentrations, or a different carrier may be involved (Luttge and Pitman, 1976b; Poole, 1978; Briskin, 1984).

Potassium transport may derive energy from either respiration or photosynthesis (Pitman, 1975), both of which

have reduced rates at low temperatures (Ting, 1982). In addition, ATPase activity decreases with temperature (Leonard, 1982; Sommarin et al., 1985), and reduced carrier turnover may be a factor (Brownlee and Kendrick, 1979). Drew and Biddulph (1971) observed that the rate of $^{42}K^+$ uptake by bean roots from solution containing 3 mM K^+ was 5.6 times faster at 25° than at 5° C. This is consistent with reported Q_{10} values of 2 to 3 for Rb^+ uptake by barley and corn roots (Carey and Berry, 1978).

In most cases K^+ efflux from cells seems to be passive (Satter and Galston, 1971; Van Steveninck, 1976). There is, however, evidence for active K^+ transport out of xylem parenchyma cells into vessels in roots (Lauchli and Pfluger, 1978) and for active efflux from guard cells during stomatal closing (Penny and Bowling, 1974). Even passive ion diffusion across a membrane may have a Q_{10} of 2 to 3 since hydrophilic particles must move between the aqueous media lying outside the membrane and the lipid phase within it (Luttge and Higinbotham, 1979). The rate of K^+ efflux across the plasmalemma at 20° C might therefore be four to nine times higher than at 1°, though reported temperature effects are somewhat lower. Pitman (1963) found that efflux of $^{42}K^+$ into the apoplast of beet root disc tissue was about three times more rapid at 25° C than at 2° C, while Brownlee and Kendrick (1979) presented similar results for $^{86}Rb^+$ efflux from mung bean hypocotyl segments.

Although K^+ uptake and efflux may be similarly affected by temperature, at the low K^+ concentrations in the bathing solution (typically <0.1 mM), a temperature-induced change in the amount of K^+ taken up would not have been great. In contrast, the high intracellular K^+ concentration (around 100 mM) could allow a considerable increase in K^+ efflux. Thus the difference in net efflux rates at the two temperatures is thought to reflect almost entirely the temperature effect on K^+ movement out of cells.

In order to evaluate the long-term effect of temperature on K^+ efflux, leaflets were eluted for 24 hours at 1^o and 20^o C (Figure 10). Although a significantly higher elution rate was observed at 20^o during the first two hours, by four hours the rates were nearly identical, and at eight hours that at 1^o C was approximately three times that at 20^o. Due to the higher initial rate of efflux at 20^o C, the intracellular compartment may have been considerably depeleted of K^+ within two hours. In a study of $^{42}K^+$ efflux from beetroot discs at 1^o and 25^o C, Pitman (1963) reported the loss of 60-90% of cytoplasmic K^+ within the first two hours at 25^o C, while only 10-40% had been lost at the lower temperature.

In the present experiment the intracellular K^+ concentration apparently became low enough that efflux was significantly slowed by the reduced chemical potential gradient across the plasmalemma. This effect was observed

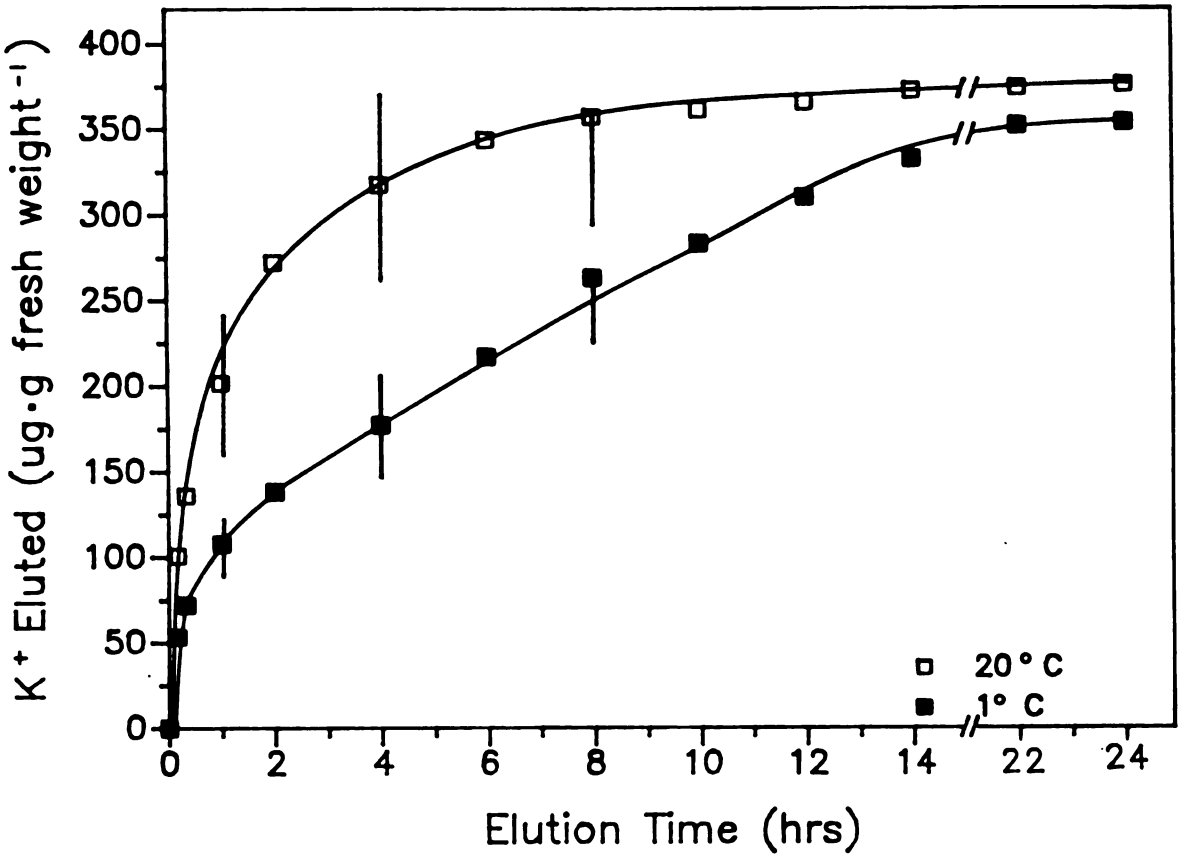


Figure 10. Twenty-four hour potassium elution at 20° and 1° C. Elution rates at one hour (20°, 1.55, and 1°, 0.33 ug K^+ gfw^{-1} min^{-1}) and at eight hours (0.088 and 0.28 ug K^+ gfw^{-1} min^{-1}, respectively) significantly different at 5% level. Rates at four hours (0.26 and 0.28 ug K^+ gfw^{-1} min^{-1}, respectively) not significantly different. Points are means of six replications ± representative standard errors.

after only four hours at the higher temperature, but was delayed until after 14 hours at 1° C.

Effects of altering apoplastic K^+ concentration. In an attempt to entirely disrupt the hypothesized membrane-regulated K^+ efflux, leaflets were frozen at -5° C for 30 minutes and allowed to thaw before eluting at 1° or 20° C. This pretreatment should have greatly increased the extracellular K^+ concentration as a result of leakage through ruptured cellular membranes.

The rates of elution at both temperatures were not significantly different (Figure 11), indicating that the temperature effects on elution described above were largely eliminated by the disruption of membranes. It is interesting that a period of 20 minutes was apparently still required for the extracellular compartment to equilibrate with the bathing solution. This indicates that equilibration is a physical process that occurs at approximately the same rate regardless of membrane integrity or the functionality of membrane-associated processes.

Over the entire course of the experiment a greater amount of K^+ was present in the bathing solution at 20° C, but the mean values for the two temperatures were not significantly different at any time. By 60 minutes at 20° C approximately 50 times more K^+ had been eluted from the frozen/thawed tissue than from the untreated tissue of the earlier experiment (Figure 9). This increased elution rate

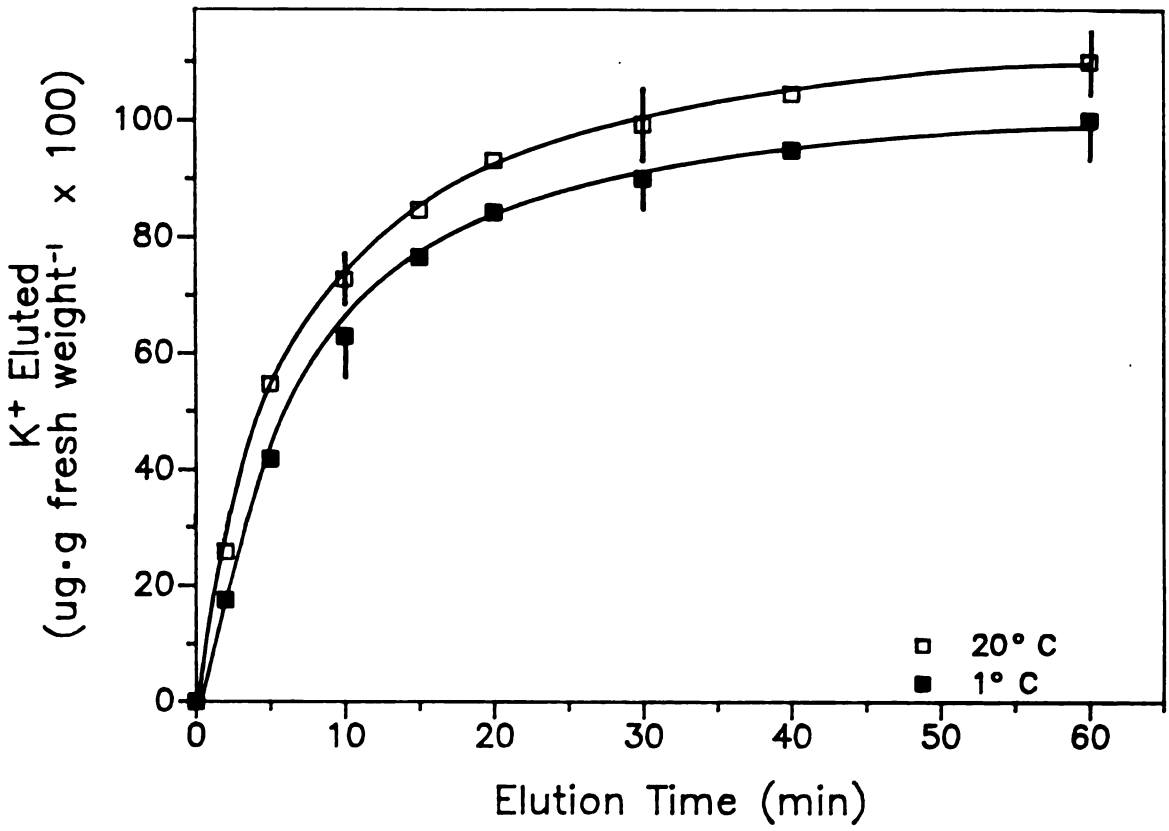


Figure 11. Potassium elution from frozen/thawed leaflets at 20° and 1° C. Rates at five minutes (20°, 673, and 1°, 597 ug K^+ gfw^{-1} min^{-1}), 20 minutes (113 and 89.1 ug K^+ gfw^{-1} min^{-1}, respectively) and 40 minutes (41.7 and 30.3 ug K^+ gfw^{-1} min^{-1}, respectively) not significantly different. Points are means of six replications ± representative standard errors.

is indicative of a much higher concentration gradient for diffusion between the tissue and the external solution.

Results of this nature have been reported in previous studies of ion loss from killed tissue. Osmond (1968) studied the elution of $^{22}Na^{+}$ from freshly cut versus previously boiled leaf discs. The rate of rapid loss occurring in the first four minutes was approximately tripled for the boiled discs. Cram (1968) reported a rapid rate of Cl^{-} elution from chloroform-killed carrot discs with a half-time of less than two minutes. This result was interpreted as supporting the hypothesis that the "fast component" of ion elution from living tissue is diffusion from the apoplast. Leopold (1980) investigated solute leakage from heat-killed versus live soybean cotyledons. The leakage rate from dead tissue was greatly increased during the first 10 minutes and somewhat less so for the remaining 30 minutes of the experiment. The overall rate of leakage was 10-fold greater from dead than from live cells.

To increase the K^{+} concentration of the apoplast without entirely disrupting cell membranes, a small portion of the exposed mesophyll tissue lying within the attached cylinder was abraded with a pipet tip to rupture cells. Abraded and nonabraded leaflets were then eluted at 1^{o} C for 60 minutes. As shown in Figure 12, there was a significantly higher initial elution rate from abraded than nonabraded tissue, but the constant efflux rates from 20 to 60 minutes were virtually identical. These results indicate

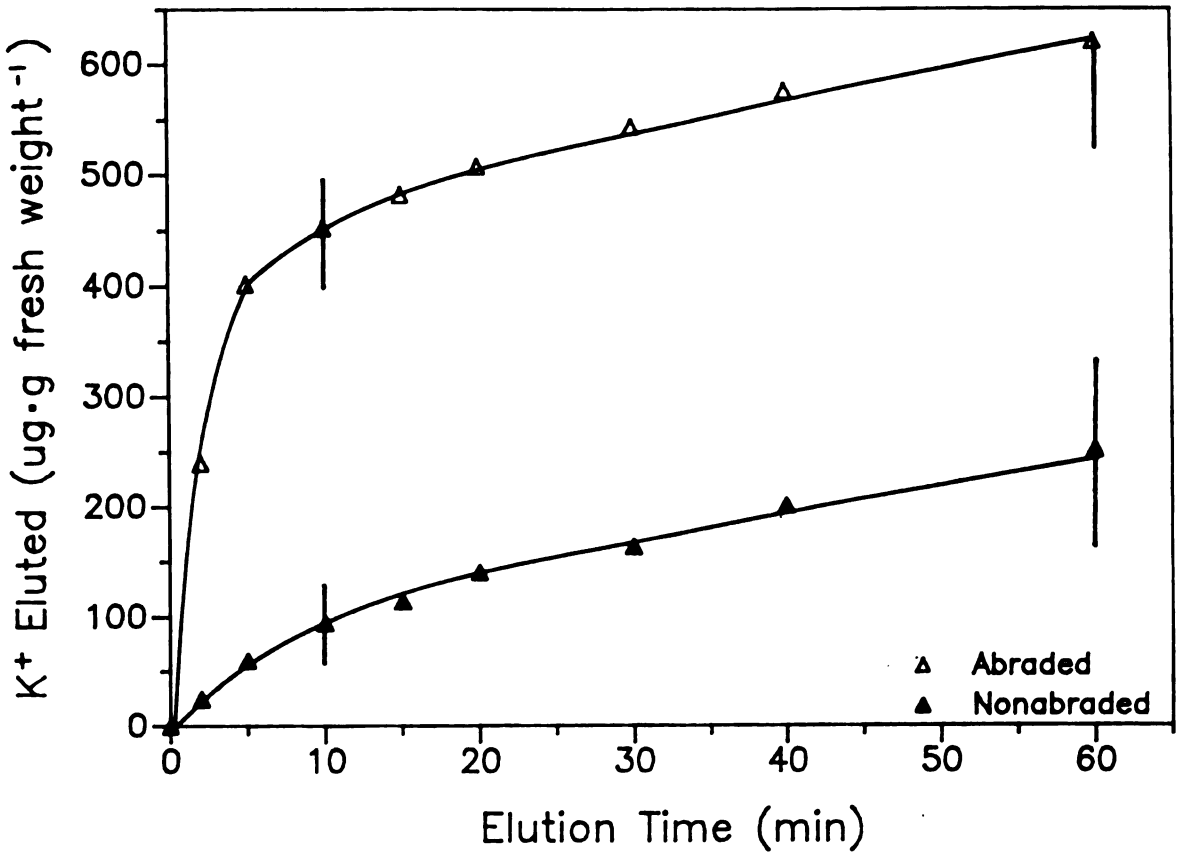


Figure 12. Potassium elution at 1° C from leaflet tissue with and without cells ruptured by abrasion. Rates at five minutes (abraded, 94.4, nonabraded, 10.2 ug K^+ gfw^{-1} min^{-1}) significantly different at 1% level, but constant rates (2.82 and 2.83 ug K^+ gfw^{-1} min, respectively) not significantly different. Points are means of six replications ± representative standard errors.

that K^+ lost from the tissue during the first stage of elution is largely from the apoplast and that this stage is completed within the first 20 minutes. They also demonstrate that inadvertent damage to leaflets can have a significant effect on results.

To reduce the extracellular K^+ content, peeled leaflet tissue within the cylinder was rinsed with 5 mM $CaCl_2$ for 20 minutes. The rinse solutions, replaced at five-minute intervals, were saved and analyzed for K^+ to assess the effectiveness of the rinse. Peeled leaflet discs received the same treatment for comparison. Sixty-minute elutions were then conducted using the rinsed discs as well as both rinsed and unrinsed leaflets.

The elution curve for unrinsed leaflets shows the typical trends (Figure 13). For rinsed leaflets, however, the rapid elution phase was essentially eliminated. The reduction in rate during the rapid phase is thought to be due to removal of a large portion of the extracellular K^+ in the rinses. The short duration of this phase (<5 minutes) is consistent with the establishment of a continuum of elution solution during the rinse period so that infiltration would not be a factor. The constant rate of elution from rinsed leaflets was not significantly different than that for the unrinsed tissue. This is interpreted as evidence that this rate is attributable to cell efflux.

The average rate of K^+ elution from rinsed discs during the first 20 minutes was only about 70% of that from 20 to

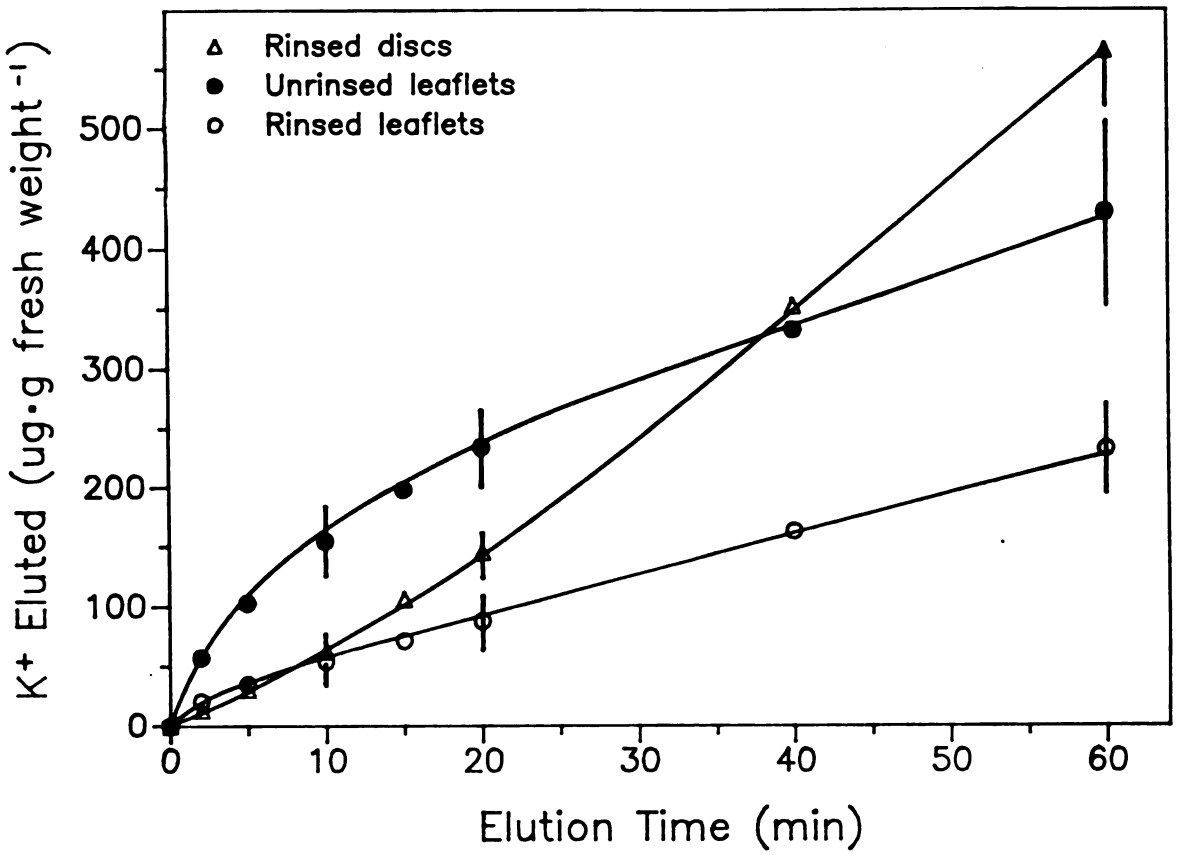


Figure 13. Potassium elution at 1° C from leaflet discs previously rinsed for 20 minutes in 5 mM $CaCl_2$ solution, and from rinsed and unrinsed peeled tissue of intact leaflets. Constant rates for leaflets (rinsed, 3.62, and unrinsed, 4.89 ug K^+ gfw^{-1} min^{-1}) not significantly different. That for rinsed discs (10.5 ug K^+ gfw^{-1} min^{-1}) significantly different from both at 1% level. Points are means of six replications ± representative standard errors.

60 minutes (7.4 vs. 10.5 ug K^+ gfw^{-1} min^{-1}) (Figure 13). This is thought to be due to more thorough rinsing of the disc tissue, where the cut edges provided additional area for diffusion. Analysis of the rinse solutions revealed that 75% of the total K^+ rinsed from discs was removed in the first rinse, while 50% was removed during the same period from lamina tissue with cylinders attached.

Since a solution continuum should have been established within the discs during the rinse period, the lag in appearance of K^+ in the bathing solution seems to represent the time required for intracellular ions to diffuse out of the innermost tissue. Again the 20-minute period appears to be critical.

The constant elution rate for discs was significantly higher than for intact leaflets. Membrane permeability in discs may have increased due to biochemical (Smith and Robinson, 1971) or structural changes resulting from damaged cells or anaerobiosis resulting from their more complete infiltration (Mengel and Pfluger, 1972). There may also have been a greater concentration gradient across the plasmalemma due to more rapid diffusion of ions away from cells, leading to a higher net efflux rate than in leaflets.

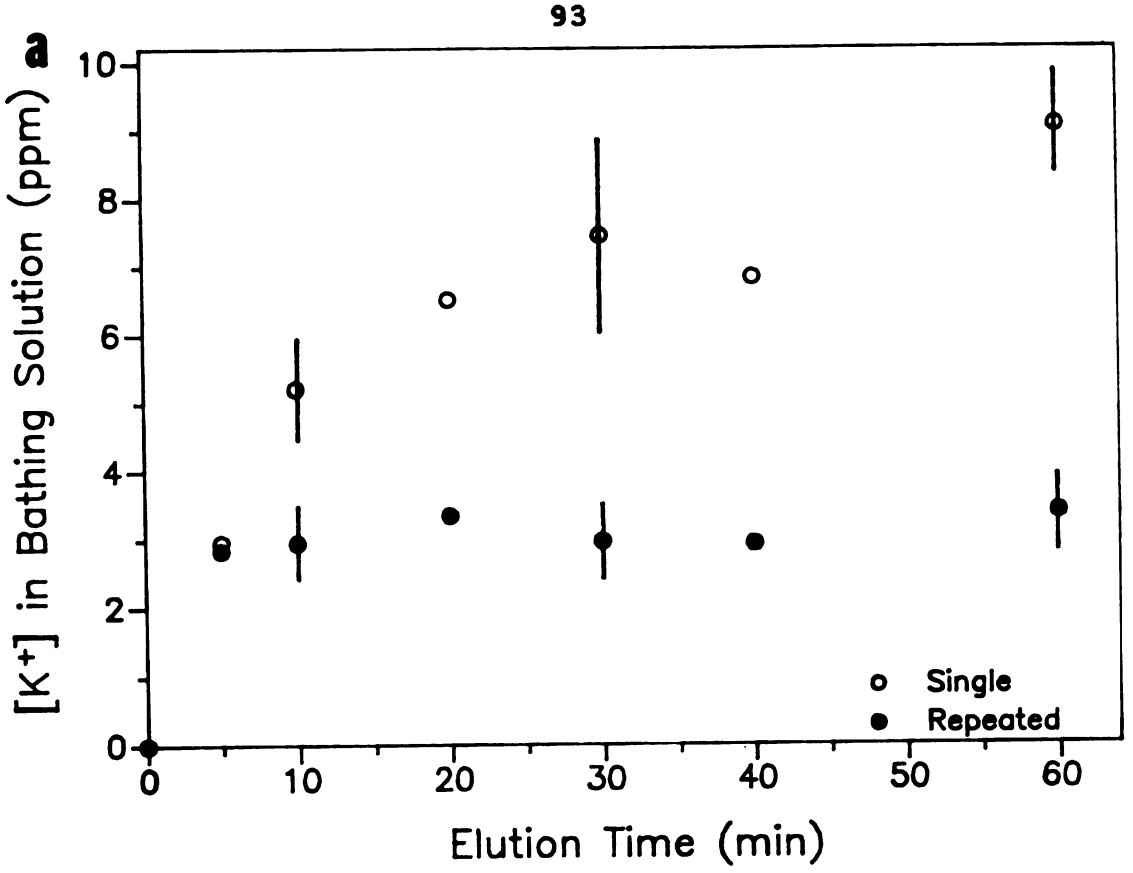
Effects of extra- and intracellular K^+ concentrations. The extracellular ion concentration is a major factor affecting fluxes across the plasmalemma (Robinson, 1971). Net $^{86}Rb^+$ influx to corn root cells was found to increase when external concentration was increased from 0.5 to 10 mM,

the most rapid change in rate occurring between 0.5 and 1 mM (Kochian and Lucas, 1983). Widders and Lorenz (1983a) reported an increase in vacuolar K^+ influx in tomato leaf slices as K^+ concentration in the bathing solution increased from 0 to 40 mM, while Atkinson et al. (1985) observed a 15% reduction in the rate of K^+ efflux from tobacco leaf cells when extracellular K^+ concentration was increased from 1 to 10 mM.

It would therefore be expected that a significant increase in the K^+ concentration of the elution solution over the course of an experiment might influence the time-course characteristics of the elution curve by reducing the net efflux rate. This effect was demonstrated by comparing elution with repeated sampling and replacement of solution (normal procedure) to that when only a single sample was taken from a given leaflet at one point in the time course (Figure 14 a and b). With repeated sampling, the K^+ concentration in the bathing solution remained at approximately 3 ppm (0.08 mM), while single sampling resulted in a continued increase to three times that concentration (Figure 14a). Elution with repeated sampling attained a steady rate after 20 minutes, while the rate with single sampling continued to decline, apparently due to increased extracellular K^+ concentration (Figure 14b).

Solution K^+ concentrations were examined for a number of typical elution experiments and were similar to those above for repeated sampling. The greatest increase in

Figure 14. Effects of single versus repeated sampling of solution eluting a given leaflet on (a) K^+ concentration in the solution over time and (b) time course of K^+ elution. Points on both plots are means of six replications ± representative standard errors.


a
[K+] in Bathing Solution (ppm)
10
8
6
4
2
0
0
10
20
30
40
50
60
Elution Time (min)
Single
Repeated

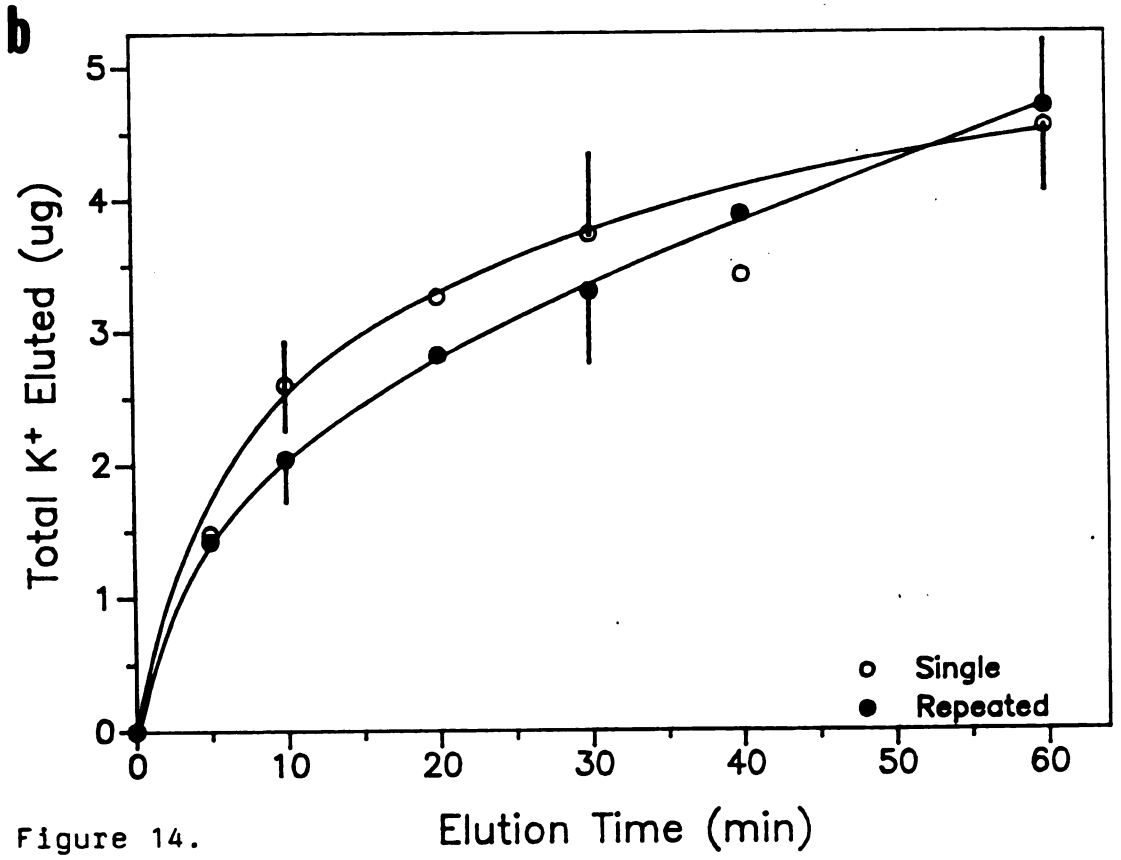

b
Total K+ Eluted (ug)
5
4
3
2
1
0
0
10
20
30
40
50
60
Elution Time (min)
Single
Repeated

Figure 14.

concentration, from 0 mM to approximately 0.1 mM, occurred during the first few minutes. Thereafter, the average change in concentration within a cylinder was only 0.01 to 0.02 mM. It therefore appears that any effect of change in extracellular concentration would be observed primarily in the early portion of the elution curve. Achievement of a steady cell efflux rate later in the curve would be promoted by this maintenance of a constant external K^+ concentration.

Changes in intracellular K^+ concentration might also affect net efflux. Glass (1976 and 1977) reported that the intracellular K^+ concentration of barley roots played a role in regulating uptake rate; a reduction in plasmalemma influx was associated with ion accumulation. Cram (1976), in a discussion of negative feedback regulation of ion transport in cells, cited a negative correlation between uptake and intracellular concentration. It was therefore of interest to estimate the change in intracellular K^+ concentration over the course of an elution experiment.

Intracellular K^+ concentrations of 90 and 190 mM were estimated for leaf lamina of Argenteum plants grown at 2 and 10 mM K^+, respectively. These values were based on total K^+ per gram fresh weight, assuming that most of the total was intracellular. They are in agreement with estimates of 100 to 200 mM K^+ in cells of other higher plants (Clarkson and Hanson, 1980; Flowers and Lauchli, 1983).

In the present study cellular K^+ concentrations were estimated after 20 and 60 minutes of elution by subtracting

the amount removed from the tissue from that initially present. On this basis the drop in cellular K^+ concentration during this period was only about 1.6% for plants grown at both K^+ levels. Thus the change in intracellular K^+ concentration during a 60-minute elution would not appear to significantly affect elution rate.

In order to better observe the effects of extra- and intracellular concentrations on K^+ elution, these were modified by allowing excised leaves to take up solutions of varying RbCl concentration via their petioles for various periods of time. Rubidium was used because its movement through the plant (Wallace, 1968; Hosokawa and Kiyosawa, 1985) and uptake by cells (Epstein, 1961; Smith and Epstein, 1964a; Lauchli and Epstein, 1970) are similar to K^+, but its endogenous concentration in Argenteum leaf tissue is below that detectable by the methods used in this study.

Excised leaves were placed in a 10 mM KCl solution labeled with $^{86}Rb^+$, as previously described, to follow the movement of Rb^+ into the leaflets. As seen in Figure 15, the rate of $^{86}Rb^+$ appearance in leaflets was essentially constant during the entire uptake period. Therefore the amount of Rb^+ in leaflets could be expected to increase with length of uptake period. The volume import rate of solution, the slope of the line regressed through the points, was 1.57×10^{-3} ml gfw^{-1} min^{-1}.

The effect of uptake time on the efflux kinetics of Rb^+ was examined by allowing excised leaves to take up unlabeled

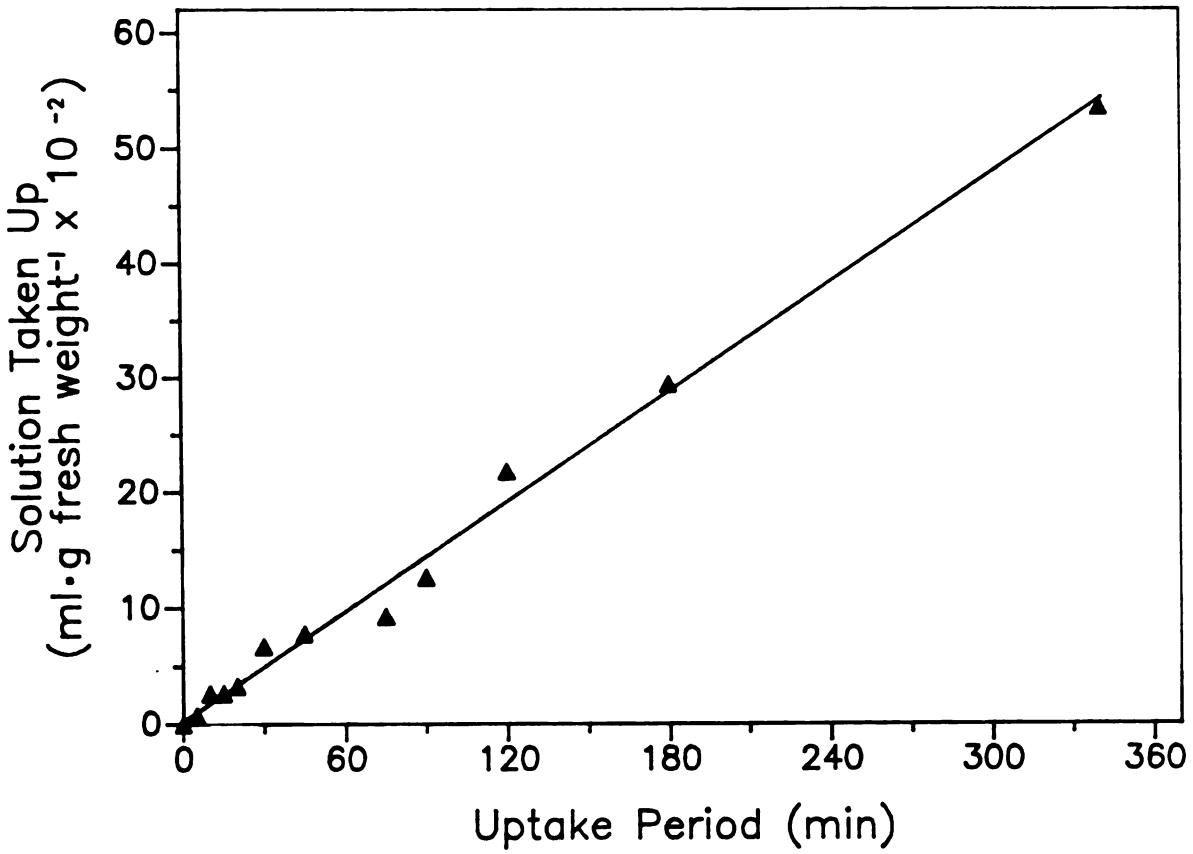


Figure 15. Appearance in <u>Argenteum</u> leaflets of $^{86}Rb^{+}$-labeled 10 mM KCl solution taken up at 25° C by excised leaves through petioles. Points are means of two replications.

20 mM RbCl through the petioles for 15 or 90 minutes, after which their leaflets were eluted for 60 minutes. The 15-minute uptake period was chosen because most of the Rb^+ taken up into leaflets could be removed from the tissue in a 20-minute rinse, suggesting that it was largely apoplastic. After 90 minutes, however, a considerable amount of Rb^+ remained in the tissue after rinsing, suggesting that cell uptake had occurred.

Following a 15-minute uptake, the constant elution rate observed after 20 minutes was only 0.22 ug Rb^+ gfw^{-1} min^{-1} (Figure 16). If most of the Rb^+ taken up was indeed apoplastic, such a low rate would support the hypothesis that the linear portion of the elution curve represents cell efflux. In addition, the fact that 10 to 15 minutes were required to elute Rb^+ from the apoplastic compartment is consistent with an apoplastic origin for K^+ ions eluted from lamina tissue during the initial 20 minutes of an experiment.

After a 90-minute uptake period the elution curve was more typical of those obtained for K^+, with a constant rate (1.94 ug Rb^+ gfw^{-1} min^{-1}) significantly higher than that for 15 minutes (Figure 16). Since a higher net cell efflux rate would be anticipated with increased intracellular concentration, this is further evidence that cell efflux is responsible for steady-state elution. The average elution rate for the first 10 minutes was also significantly higher for the 90-minute than for the 15-minute treatment, even

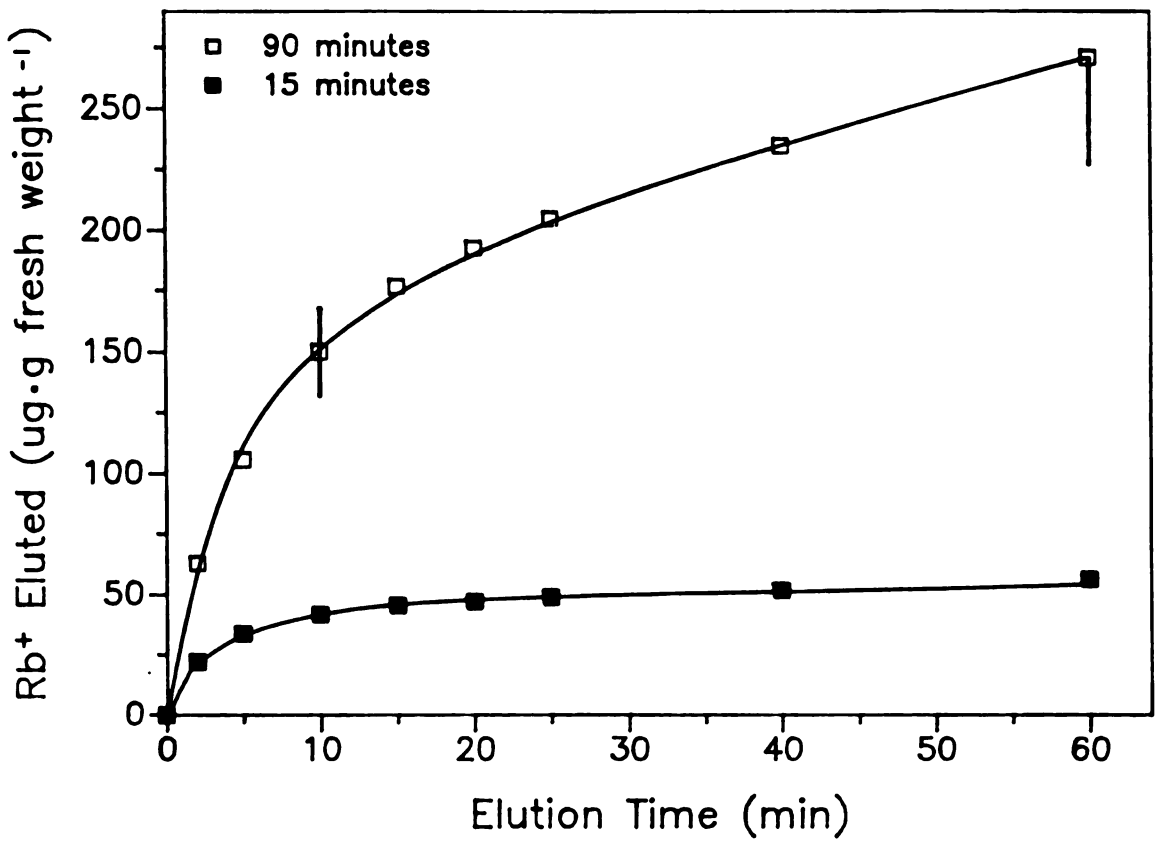


Figure 16. Elution of Rb^+ at 1° C from leaflets after excised leaves had taken up 20 mM RbCl solution through petioles for 15 or 90 minutes at 25° C. Rates for first 10 minutes and constant rates (given in text) significantly different at 1% level. Points are means of six replications ± representative standard errors except where smaller than symbol.

after subtraction of the constant rate (net rates of 13.9 and 4.83 ug Rb^+ gfw^{-1} min^{-1}, respectively). This indicates that extracellular Rb^+ content was also higher after the 90-minute uptake period.

Estimation of Apoplastic K^+ Content and Concentration

Estimation of apoplastic K^+ content using the elution curve.

The above-reported experiments suggest that apoplastic K^+ is removed from the tissue within the first 20 minutes of elution. Movement of the ion across the plasmalemma during this period, however, must still be accounted for (Briggs et al., 1961).

Since elutions were conducted at 1^o C, cellular K^+ uptake from the apoplast should have been minimal. As previously discussed, K^+ uptake at the concentrations found in the bathing solution seems to be active, and active ion accumulation is greatly reduced at low temperature. Ion efflux from cells is also reduced, but not completely eliminated, at low temperature.

In tracer efflux studies, which measure efflux rates of previously loaded radioactive ions from various tissue compartments, apoplastic efflux has been kinetically separated from movement across the plasmalemma by plotting the logarithm of tracer remaining in the tissue over time. The resulting slow, constant efflux rate from approximately 30 minutes to two hours has been attributed to the cytoplasm, and the extrapolate of that linear function to

zero time used to determine the initial content of the extracellular compartment (Pitman, 1963 and 1965a; Smith and Robinson, 1971; discussed in Clarkson, 1974; Walker and Pitman, 1976; Flowers and Lauchli, 1983).

In the present study, however, it seemed reasonable to estimate cytoplasmic efflux using the linear function regressed through nontransformed data representing total K^+ accumulated in the elution solution over time. This modification is justified by the following difference between tracer studies and the present one.

When excised tissue is loaded with a radioisotope from an external solution, the resulting concentrations within tissue compartments may not represent those of the endogenous ion. Such differences could affect elution kinetics. For example, they might result in a significant change in the specific activity of the cytosolic solution during an efflux experiment. Consequently the concentration gradient across the plasmalemma would change and tend to preclude determination of a long-term steady efflux rate without data transformation.

In contrast, in the present experiments the intracellular K^+ concentration and the gradient across the plasmalemma seem not to have changed significantly during an experiment. Consequently, a nearly constant rate of K^+ efflux could be observed.

It is believed that by the start of the steady-state elution phase, the rate of appearance of K^+ in the bathing

solution is controlled by movement across the plasmalemma. If this hypothesis is correct, the constant rate from 20 through 60 minutes may be considered to represent the net cellular efflux rate.

Since eluted K^+ includes ions of both extra- and intracellular origin, intracellular K^+ must be subtracted from the total to arrive at the net apoplastic content. Assuming that the net efflux rate is constant from the start of the elution period, this difference may be approximated by extrapolating the linear function to zero time, adjusting the Y intercept to 0,0, and subtracting values on the adjusted line from those of the original curve. The result is a difference curve representing K^+ elution from the apoplast (Figure 17). Its asymptotic maximum is equal to the point where the linear extrapolate intersects the Y axis, so the Y intercept is an estimate of apoplastic K^+ content.

To evaluate the effect data transformation would have on this estimate, the natural logarithm of K^+ eluted was plotted over time for two elution experiments. The 20- to 60-minute portions of the curves were still essentially linear, and when extrapolated to zero time, the Y intercepts were approximately 10% higher than those obtained with nontransformed data.

For the same two experiments, the natural logarithm of K^+ remaining in the eluted tissue was also plotted over time. In this case the 20- to 60-minute portions were not

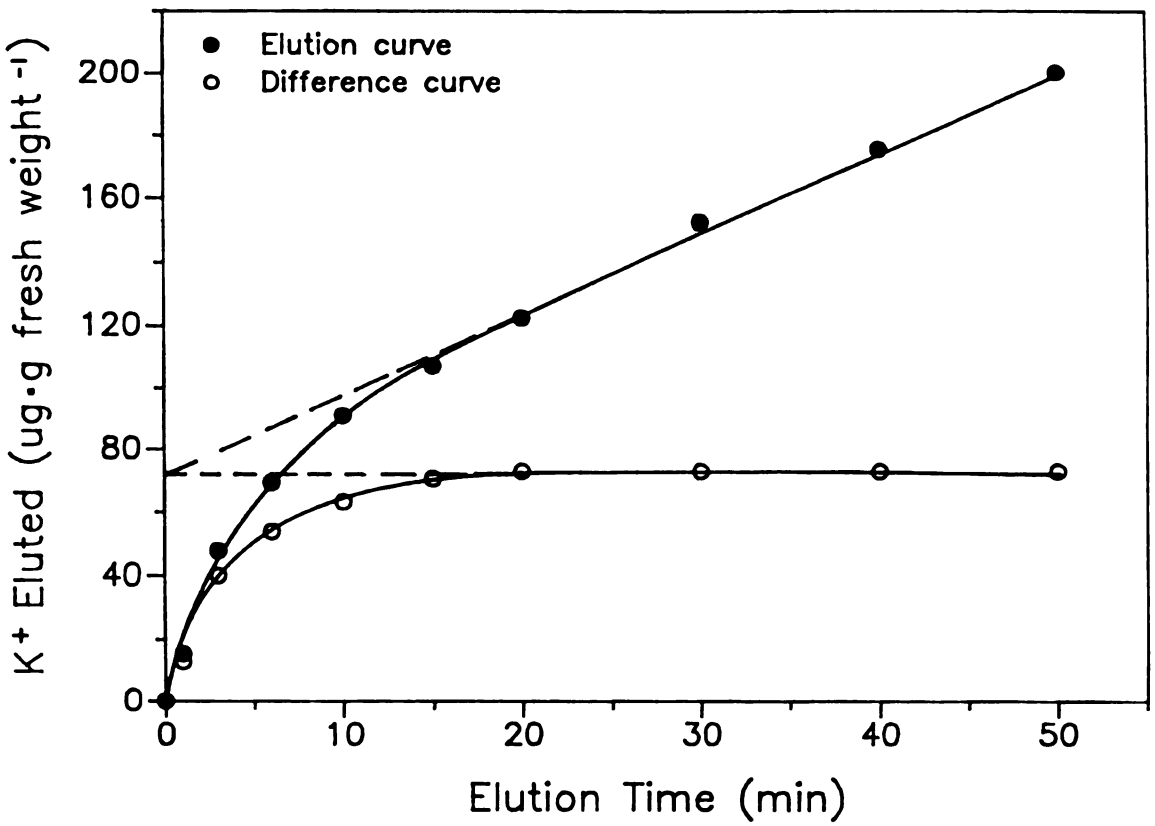


Figure 17. Typical curve for K^+ elution from <u>Argenteum</u> leaflet lamina tissue at 1° C and difference curve obtained by subtracting out constant cell efflux rate. Y intercept of linear extrapolations is estimate of apoplastic K^+ content.

quite linear, and the Y intercepts of lines regressed through these points were about 5% lower than for nontransformed data. It therefore appears that logarithmic data transformation is not necessary for estimation of extracellular K^+ content by the methods used in this study.

The assumption that the cellular efflux rate observed from 20 to 60 minutes is constant from zero time must be examined. The results of elutions conducted at 1^o and 20^o C (Figure 9) indicate that cell efflux may become an increasing portion of total elution after about five minutes. Similarly, the elution curve for rinsed lamina discs (Figure 13) suggests there is a lag period before the maximum cell efflux rate is evident. This could be expected to occur in unrinsed tissue if the extracellular ion concentration is progressively diluted during the period of infiltration by the solution, reducing the concentration gradient across the plasmalemma and leading to an increase in net efflux rate.

The estimated efflux rate for 0 to 20 minutes therefore represents a theoretical maximum, and the rate of appearance of intracellular K^+ in the bathing solution may actually be slower during the first 20 minutes than during the linear phase. If this is true, then extrapolation of the linear phase leads to an underestimate of the apoplastic K^+ content.

Without knowledge of exactly how the cell efflux rate changes early in the time course, it is difficult to make an

accurate adjustment. To determine how variation in cell efflux rate during the first 20 minutes might affect the extracellular K^+ estimate, Y intercepts were calculated assuming slower efflux rates during that period.

The rate of efflux from rinsed discs during the initial 20 minutes was approximately 70% of the constant rate. Applying this to a typical elution curve, extrapolation of the linear phase would result in a 15% underestimate of extracellular K^+ content. If the efflux rate for the first 20 minutes were 50% of the final rate, the underestimate would be by 25%. On this basis, it is doubtful that the method used in this study would underestimate apoplastic K^+ content by more than 15-25% if all other assumptions are correct.

Conversion of apoplastic K^+ content to concentration. Estimated extracellular K^+ content was converted to apoplastic solution concentration to make results more meaningful in terms of physiological processes and more suitable for comparison with the results of other studies. This required estimation of the volume of solution in the apoplast.

An attempt was made to arrive at this estimate gravimetrically by following the weight lost by evaporation from peeled leaflet tissue. However, no change in the rate of water loss was detected that might indicate apoplastic water was exhausted.

Cheung et al. (1975) determined the apoplastic water content of leaves based on the volumes of water expressed when leaves were subjected to increasing pressures using a pressure bomb. The accuracy of this method has been questioned (Tyree and Richter, 1982), though more recent modifications by Beeson et al. (1986) and Jachetta et al. (1986b) are reported to produce acceptable results. Due to the hollow and weak nature of Argenteum petioles, however, the leaves were not suitable for this procedure.

Pitman et al. (1974a) suggested that under normal circumstances free water in the leaf apoplast would be limited to a portion of the cell wall. Various means were therefore used to estimate the cell-wall water content of Argenteum leaflets.

Gaff and Carr (1961) and Bernstein (1971) determined that isolated cell-wall material can hold up to 150% of its dry weight in water. Due to the deposition of matrix substances in the intact wall, however, only one-half to two-thirds of the wall is thought to be available to water (Levitt, 1957; Muhlethaler, 1967). Dry cell walls isolated from Argenteum leaflets were found to constitute approximately 10% of leaflet fresh weight, somewhat lower than the mean value of 15% determined by Gaff and Carr (1961). If dry cell walls can hold 75-100% of their weight in free water (150% of one-half to two-thirds of wall volume), then water weighing 7.5-10% of the leaflet fresh

weight, or 0.075 to 0.1 ml gfw^{-1}, would be in Argenteum cell walls.

Another estimate is based on reports by Turrell (1936) and Weatherly (1970) that the internal surface area of dicot leaves may be approximated by multiplying the total external surface area by a factor of 10. Assuming that internal surface refers to the cell-wall surface and based on an average wall thickness of 1 um determined from electron micrographs, a total cell-wall volume of 0.016 cm^3 was calculated for the typical Argenteum leaflet used for elution. This represents about 16% of the total leaflet volume and is comparable to the value of 13% determined for the Eucalyptus leaf by Gaff and Carr (1961). By dividing the above volume by the average Argenteum leaflet fresh weight (0.173 g) a cell-wall volume of 0.092 cm^3 gfw^{-1} was obtained. Based on the water-holding capacities cited above, the cell wall would contain water amounting to 0.069 to 0.092 ml gfw^{-1} of leaflet.

The above estimates for extracellular water are in general agreement with that of Boyer (1967), who reported a value of 9% of total leaf water for sunflower, and with the 11% estimate of Jachetta et al. (1986b) for the same species.

Cheung et al. (1975) concluded that apoplastic water may constitute a minimum of 5% of total water in the fully turgid leaf. A similar estimate was proposed by Mengel and Kirkby (1982) for water in leaf mesophyll cell walls. Since

Argenteum leaflets are approximately 83% water, only 0.042 ml gfw^{-1} would be in the cell wall using this estimate.

The previously cited estimate of 40% by Gaff and Carr (1961) is subject to question since it was determined using ground cell-wall material which lacked the structural integrity of the intact wall. The above estimates suggest that this value may be a considerable overestimate of cell-wall water in Argenteum.

The above are clearly rough estimates of the volume of the apoplastic solution, but they suggest that a cell-wall water content in the range of 0.04 to 0.1 ml gfw^{-1} of Argenteum leaflet tissue may be realistic. The extracellular K^+ contents estimated from a number of elutions conducted under standard conditions are presented in Table 2 along with the corresponding calculated solution concentrations assuming the above two cell-wall water contents. Depending on the assumed volume, the concentrations range from approximately 10 to 62 mM. The variability of results among experiments is not surprising since these elutions were conducted using plants grown at different times and leaflets whose age varied somewhat. It is interesting to note that the results from the last four experiments, all conducted during a two-week period, are fairly consistent and may indicate changes in apoplastic K^+ as the plants aged.

Table 2. Apoplastic K^+ content and concentration of Argenteum leaflets from plants grown at 4.25 mM K^+ as estimated from various elution experiments. Values are means of six replications per experiment ± standard errors.

Date of experiment	Estimated K^+ content of apoplast ($ug \cdot gfw^{-1}$)	Calculated K^+ concentrations for assumed volumes[a] (mM)	
		0.04 $ml \cdot gfw^{-1}$	0.1 $ml \cdot gfw^{-1}$
7/20	54.5 ± 12.2	34.8 ± 7.8	13.9 ± 3.1
8/3	39.0 ± 9.4	24.9 ± 6.0	10.0 ± 2.4
11/7	88.8 ± 13.3	56.8 ± 8.8	22.7 ± 3.4
11/11	96.7 ± 16.5	61.8 ± 10.5	24.7 ± 4.2
11/13	81.5 ± 13.7	52.1 ± 8.8	20.8 ± 3.5
11/21	70.0 ± 12.3	44.8 ± 7.9	17.9 ± 3.1

[a]Volumes are estimates for apoplastic solution as explained in text.

Comparison with previous estimates. As previously indicated, estimates of extracellular ion concentration in the literature are limited and are based on a variety of experimental methods. Still it is interesting to consider them in light of the results of the present study.

Bernstein (1971) perfused cabbage, sunflower and castor bean leaves with water and found K^+ to represent about one-half of all ions removed from the apoplast. This corresponded to concentrations of 3.5 to 5 mM for cabbage, 1 to 5 mM mM for sunflower and 1 to 2 mM for castor bean. The plants in that study were grown with a half-strength Hoagland's solution and received 3 mM K^+.

Harvey et al. (1981) used X-ray microanalysis of Suaeda maritima leaf sections to estimate a cell-wall K^+ concentration of 13 to 17 mM. It is important to note that the plant under study, a halophyte, was grown under saline conditions (340 mM NaCl) known to cause Na^+ accumulation in leaves (Harvey et al., 1981). They may therefore have had a higher Na^+/K^+ ratio than would be expected in a glycophyte (Flowers and Lauchli, 1983).

Jacobson's (1971) report of 6.4 mM K^+ in extracellular fluid from trap tissue of the Venus flytrap is difficult to assess because K^+ may be involved in the trapping response and the cultural conditions are not known.

As indicated, Pitman et al. (1974b) found cells in barley leaf slices to be in equilibrium with an extracellular concentration of 5 mM KCl. The barley

seedlings used in the study had been supplied with 10 mM K^+. For tomato plants grown at 2 mM K^+, Widders and Lorenz (1983a) reported zero net K^+ flux from slices of fully expanded leaves at external KCl concentrations of 1 to 3.5 mM.

That previous results are generally lower than those proposed in this study may be a function of the method and/or the plant material. The extent to which ion fluxes in isolated, cut tissue represent those in the intact leaf is not clear. In addition, several factors might contribute to an overestimate of apoplastic K^+ concentration in the present study:

1. There may be considerable K^+ bound by the Donnan phase and not normally in solution that was released under the present experimental conditions.

2. Cells may be damaged during the elution procedure, releasing intracellular K^+. The effect of such damage was demonstrated in abraded tissue (Figure 12).

3. The estimate of cell-wall volume and/or water contained within the cell wall may be too low.

4. The apoplastic solution may occupy a volume greater than than the cell wall.

Comparison of xylem-sap and estimated apoplastic K^+ concentrations. Xylem exudate was collected a number of times over the course of the present study from plants four to five weeks old supplied with a nutrient solution containing 4.25 mM K^+. Potassium concentrations were

between 3.2 and 8 mM. These values are consistent with xylem-sap K^+ concentrations reported in the literature, compiled by Robson and Pitman (1983), ranging from about 0.5 to 12 mM. Argenteum plants did not exude sap readily and individual plants varied greatly in the degree to which exudation occurred, which may account for the variability in results. If the estimated apoplastic K^+ concentrations in Table 2 are correct, there would have to be a considerable increase in xylem-sap K^+ concentration within the leaf apoplast to reach those levels.

Xylem-sap ion concentration would appear to be related to that of the extracellular solution in the leaf since xylem empties into the apoplast (Esau, 1977). Determining the nature of this relationship, however, is hindered by lack of knowledge of the extent to which ions from the sap move in the apoplast before being taken up into the symplast (Lauchli, 1976). In addition, there may be changes in composition of the sap during its transport to the leaf lamina as well as during collection (Luttge and Higinbotham, 1979).

There is evidence that xylem-sap ion concentrations change over the path between roots and leaf cell walls (Oertli, 1968) so that sap collected at one point may not be representative of that at another. In addition, the ionic concentration in xylem exudate collected under conditions of a positive root pressure may exceed that in the xylem stream under normal conditions of transpiration due to reduced

water flux (Sutcliffe, 1976) and contamination from cut cells (Ferguson, 1980).

Movement of solutes through the xylem is affected by lateral diffusion, cell-wall binding, interchange between xylem and phloem, and uptake by neighboring cells (Peel, 1963; Neumann and Stein, 1983; Luttge, 1983). Hylmo (1953) reported that solute concentration of the xylem stream diminished over time due to absorption by tissue through which it flows. Klepper and Kaufmann (1966) found guttation fluid to be considerably diluted compared to stem or petiole sap. It should be noted, however, that in some guttating plants the fluid passes through a modified region of mesophyll which may consist of many compacted or transfer cells (Esau, 1977). These cells might remove a considerable quantity of solutes from the fluid before it reaches the leaf surface.

In the immediate vicinity of the leaf cell, uptake may alter the ionic concentration. Robinson (1971) proposed that the extracellular ion content of the leaf will depend on the rate of ion arrival relative to cell uptake. According to this worker, if ions are taken up by leaf cells at a rate higher than their arrival in the xylem sap, as in young, expanding leaves, the apoplastic concentration would be lower than that of the sap; if, however, ions enter the leaf as fast or faster than they are taken up, the xylem sap would represent a minimum extracellular concentration.

Evaporation is also a factor. If water near cells evaporates faster than solutes are taken up, as when the transpiration rate is high, solutes would accumulate in the cell wall (Cosgrove and Cleland, 1983).

Jacobson (1971) concluded that while xylem exudate may not closely resemble normal extrcellular fluid, it is useful as a standard for comparison with apparent apoplastic content. It is clear, however, that such comparison must be done with care.

Free-Space Characteristics of Argenteum Leaflets

The free-space characteristics of Argenteum leaflet tissue were determined as described for comparison with results of other tissue preparation methods. Of particular interest was the volume of water free space (WFS), which would be expected to differ in cut and intact tissues. Results are summarized in Table 3.

A WFS volume of 0.11 ml gfw $^{-1}$ was estimated for peeled Argenteum leaflet tissue. It is interesting that this value is consistent with estimates of cell-wall water volume previously discussed. The amount of fixed anions in the Donnan free space (A^-) was 2.8 ueq gfw^{-1}. Since the DFS volume (V_D) was estimated to be 7.8 ul gfw^{-1}, the effective concentration of fixed negative charges in the DFS (A^-/V_D) was approximately 360 meq l^{-1}.

This WFS volume is lower than reports of 0.20 to 0.25 ml gfw^{-1} for sliced leaf tissue (Osmond, 1968; Pitman

Table 3. Free-space characteristics of fully expanded Argenteum leaflet lamina tissue.

Water free space volume (WFS)	0.109 $ml \cdot gfw^{-1}$
Amount of fixed anions in Donnan phase (A^-)	2.82 $ueq \cdot gfw^{-1}$
Volume of Donnan phase (V_D)	7.78 $ul \cdot gfw^{-1}$
Effective concentration of fixed anions ($[A^-]$)	360 $meq \cdot l^{-1}$

et al., 1974a; Smith and Fox, 1975; Widders and Lorenz, 1983b). This is reasonable since there are fewer damaged cells to contribute to WFS in peeled intact Argenteum leaflets than in leaf slices.

The amount of fixed negative charges and volume of the DFS are also lower than in the above studies, where A^- ranged from 3.6 to 25 ueq gfw^{-1} and V_D, from 0.012 to 0.07 ml gfw^{-1}. Although slicing would not be expected to increase these values, the difference in methods cannot be discounted. Alternately, if Argenteum's loosely attached epidermis is attributable to weakened pectin middle lamella as suggested by Hoch et al. (1980), perhaps pectic substances within the cell wall are modified so that DFS volume and number of negative charges are reduced. Since both of these values were reduced to a similar extent in the present study, the calculated effective concentration of negative charges did fall within the range of those reported in the above studies (280 to 550 ueq ml^{-1}).

The measurement of free-space properties based on simultaneous movement into tissue of the anion and cation of a salt in solution presumes that the ions move at the same rate. According to Briggs et al. (1961) this is essentially true as long as the ions are free in solution; even though ionic mobilities may differ, electrostatic attraction causes them to move by diffusion at a fairly uniform rate. Charges within the tissue, however, alter their mobility. Cations are adsorbed to fixed negatively charged sites of the Donnan

phase (Briggs et al., 1961). They are therefore taken up to a greater extent than anions, whose uptake is proportional to their concentration in the external solution (Walker and Pitman, 1976).

Anion uptake alone has therefore been used to estimate the volume of the WFS (Briggs and Robertson, 1957; Walker and Pitman, 1976). By definition the WFS ion concentration equals that of the external solution, so WFS volume is simply the equilibrium anion content of the free space divided by the external concentration (Briggs et al., 1958). When mannitol, a nonelectrolyte, was used to measure WFS, results were similar to those obtained using Cl^-. Therefore anion repulsion by negative charges and binding to positive charges in the Donnan phase appear to have a negligible effect on WFS estimates.

Since the anion and cation move at essentially the same rate in solution, anion uptake also represents the amount of diffusible cation in solution in the free space (Briggs et al., 1958; Briggs et al., 1961). The excess of cation over anion taken up may therefore be used to estimate the amount of fixed anions in the DFS binding the cation (Briggs et al., 1961; Pitman et al., 1974a) as previously described (Figure 4).

It is likely that the free-space characteristics of a given tissue are not static, but rather change with growth and development. For example, they might be affected by increases in cell-wall volume (Robinson, 1971). Plant

growth regulators may also have an effect; gibberellic acid is reported to promote formation of cell walls low in pectic substances, while kinetin and ethylene have the opposite effect (Mondal, 1975). Such factors should be considered when free-space determinations are evaluated.

Estimation of K^+ Bound by the Donnan Phase

The difference curve in Figure 4 represents the amount of K^+ in the DFS in equilibrium with various KCl concentrations and 0.1 mM Ca^{2+}. A line was regressed through the portion of the difference curve from 0 to 5 mM KCl, where K^+ in the DFS was almost directly proportional to external K^+ concentration. This line was used to estimate K^+ bound in the Donnan phase during the six elution experiments presented in Table 2 based on the steady-state K^+ concentrations of the elution solution (mean concentrations between 20 and 60 minutes).

Results are presented in Table 4. The steady-state K^+ concentrations of the bathing solution ranged from 0.036 to 0.064 mM and corresponding estimates for K^+ in the DFS, from 0.098 to 0.106 ueq gfw^{-1}. The K^+ bound in the DFS during elution was 4 to 10.6% of the estimated apoplastic K^+. Since the values for bound K^+ did not vary greatly among experiments, the variation in the above percentages is attributable to the previously cited differences in estimated apoplastic K^+ content.

Table 4. Estimates of K^+ bound in Donnan phase in equilibrium with elution solution and as a percent of apoplastic K^+ content for experiments included in Table 2.

Date of experiment	Steady-state $[K^+]$ of elution solution[a] (mM)	Estimated K^+ content of apoplast (ueq·gfw^{-1})	Estimated K^+ bound in Donnan phase during elution[b] (ueq·gfw^{-1})	K^+ bound in Donnan phase as percent of apoplastic K^+ content
7/20	0.036	1.39	0.098	7.05
8/3	0.064	1.00	0.106	10.60
11/7	0.036	2.27	0.098	4.32
11/11	0.040	2.47	0.099	4.02
11/13	0.056	2.08	0.104	5.00
11/21	0.047	1.79	0.101	5.64

[a]Average solution concentration for the linear portion of the elution curve, from 20 to 60 minutes.

[b]Estimate is based on results of free-space study as described in text.

It should be noted that the above estimates for K^+ bound in the Donnan phase are not completely accurate since the solution used for elution was 5 mM Ca^{2+} while that used to establish the difference curve was only 0.1 mM Ca^{2+}. Pitman et al. (1974a) reported that K^+ bound in the DFS of barley leaf slices decreased with increasing Ca^{2+} concentration of the bathing solution.

The present estimates may not be indicative of bound K^+ in the intact leaflet since a considerable amount of the ion might have been removed during elution by exchange with Ca^{2+}. It is difficult to predict the degree to which the DFS binds K^+ under normal conditions without knowing the cations present in the extracellular solution and their concentrations. Epstein (1972) proposed that cation exchange at the cell well of roots is probably not significant for highly sqoluble monovalent cations such as K^+. Further study is required to determine whether or not this is true for leaves.

Elution Sensitivity to Changes in Xylem-Sap K^+ Concentration

If the elution method is to be of use in determining changes in apoplastic ion concentration, it must be sensitive enough to detect such changes under varying conditions of ion delivery to the leaf. To evaluate this sensitivity, excised leaves were allowed to take up through the petioles 1, 10 or 100 mM RbCl for 0.5, 3 or 22 hours.

The efflux rates for the 20- to 60-minute elution period and estimated apoplastic Rb^+ contents for the various treatments are presented in Table 5. Figure 18 presents elution curves after uptake of 1 mM RbCl for the three periods, while the curves in Figure 19 are for the three RbCl concentrations after a three-hour uptake period. There was considerable variability in results among replicate leaflets of the same treatment, suggesting that leaves differed in their resistance to xylem transport and therefore did not take up solution at the same rate. Such resistance could be attributed to xylem blockage by air bubbles or stomate closure due to stress.

It is significant that extracellular Rb^+ was detectable after only a 30-minute uptake of the 1 mM solution. This suggests that the elution method is quite sensitive, particularly since K^+ concentrations in the xylem solution are typically higher, often greater than 10 mM (Robson and Pitman, 1983).

The method was able to clearly differentiate extracellular Rb^+ concentrations resulting from the treatments. For a given RbCl concentration, the estimated apoplastic Rb^+ content increased with uptake time. The net efflux rate followed the same trend, consistent with an increasing intracellular concentration. These values also increased with increasing RbCl concentration at a single uptake time, suggesting that extracellular ion concentration

Table 5. Steady-state Rb^+ elution rates and estimated apoplastic contents of Argenteum leaflets from excised leaves that had taken up 1, 10 or 100 mM RbCl solution for 0.5, 3 or 22 hours. Values are means of six replications ± standard errors.

RbCl concentration (mM)	Uptake period (h)	Steady-state Rb^+ elution rate[a] ($ug \cdot gfw^{-1}\ min^{-1}$)	Estimated Rb^+ content of apoplast ($ug \cdot gfw^{-1}$)
1	0.5	0.059 ± 0.024	3.87 ± 1.19
	3.0	0.283 ± 0.076	17.9 ± 1.4
	22.0	0.915 ± 0.366	65.4 ± 21.8
10	0.5	0.751 ± 0.111	124 ± 9
	3.0	5.73 ± 0.85	486 ± 51
	22.0	90.5 ± 19.9	2,370 ± 510
100	0.5	6.29 ± 0.64	831 ± 80
	3.0	47.3 ± 4.6	4,340 ± 160
	22.0	836 ± 151	67,800 ± 10,500

[a]Slope of the linear portion of the elution curve, from 20 to 60 minutes.

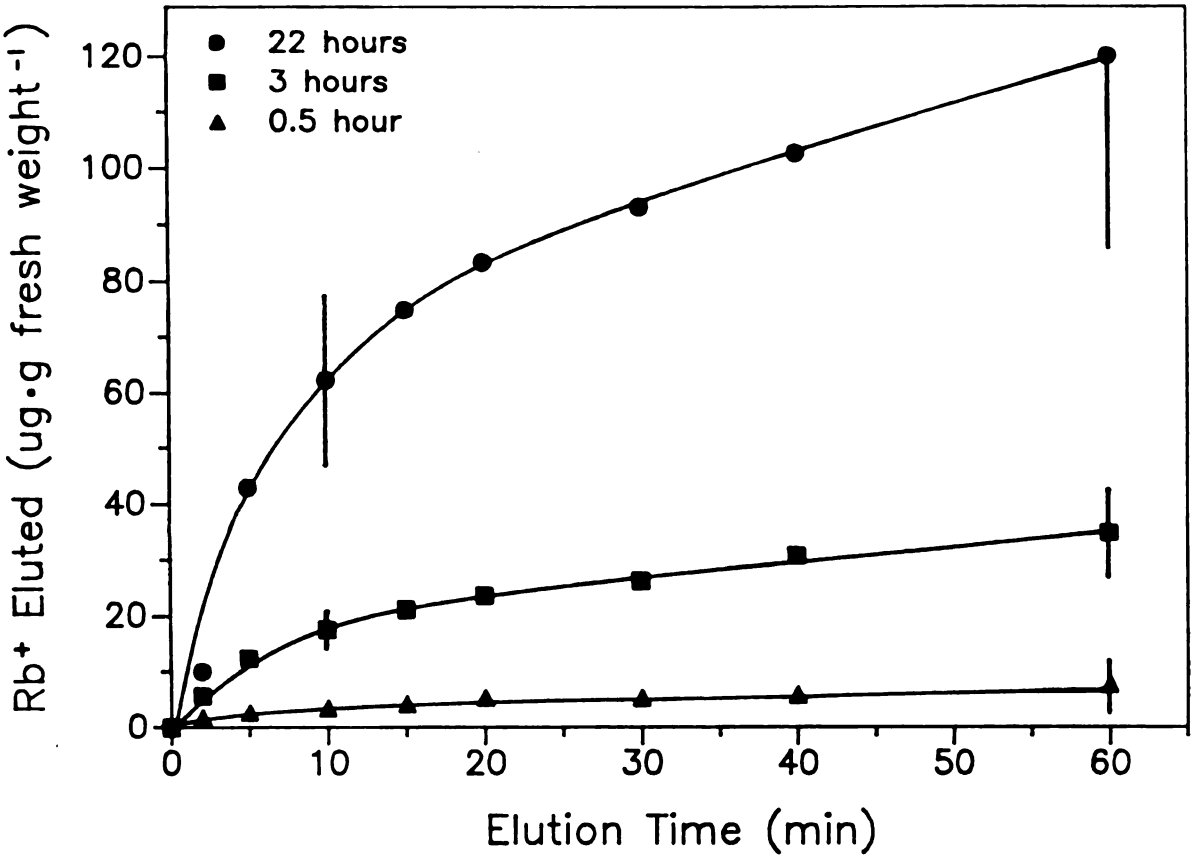


Figure 18. Rubidium elution at 1° C from leaflets after excised leaves had taken up 1 mM RbCl solution through petioles for 0.5, 3 or 22 hours at 25° C. Results in Table 5. Points are means of six replications ± representative standard errors except where smaller than symbol.

Figure 19. Rubidium elution at 1° C from leaflets after excised leaves had taken up 1, 10 or 100 mM RbCl solutions through petioles for three hours at 25° C. Results in Table 5. Points are means of six replications ± representative standard errors.

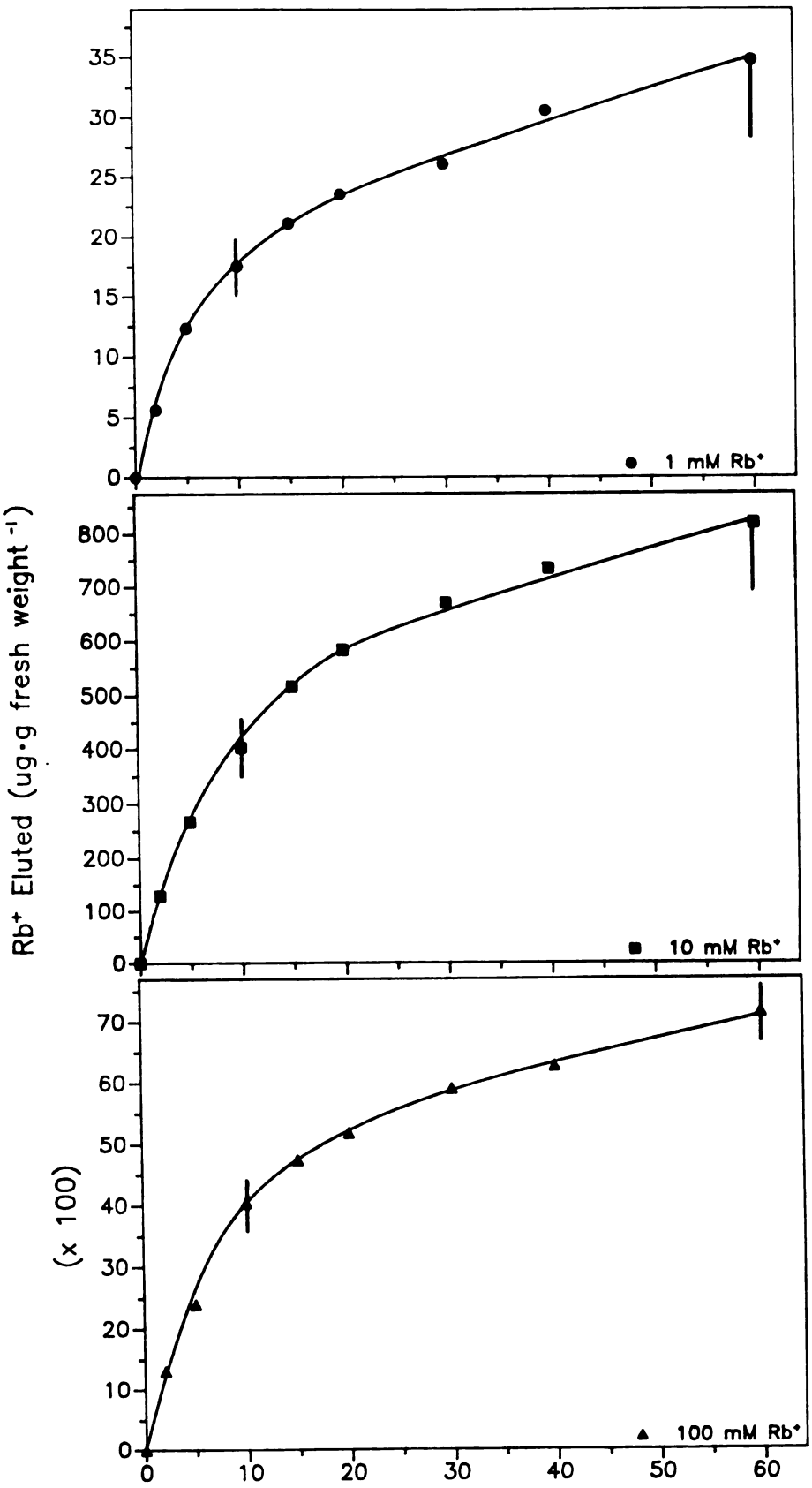

Rb+ Eluted (ug·g fresh weight -1)
(x 100)
1 mM Rb+
10 mM Rb+
100 mM Rb+
Elution Time (min)

Figure 19.

does significantly affect the rate of uptake and accumulation into the symplast.

It should be noted that the extent of leaflet ion accumulation seen in this experiment may not represent that in the intact plant. Since phloem continuity, and therefore function, had been disrupted, Rb^+ may have accumulated to a greater extent than it would have in intact tissue, where one would expect extracellular ion concentration to eventually stabilize.

Pulse-Chase Experiment

A pulse-chase type experiment was conducted as previously described to follow the movement of a uniform amount of Rb^+ into Argenteum leaflets over time. Preliminary rinse/elution experiments were conducted to determine the appropriate lengths of time for the uptake and chase periods. After a six-minute pulse of RbCl and a 10-minute chase, Rb^+ could easily be rinsed from peeled leaflet tissue, suggesting it was largely extracellular. With longer pulse and/or chase periods, cell uptake appeared to become increasingly significant. An uptake period of six minutes was therefore chosen to produce the Rb^+ pulse. A 50 mM RbCl solution was used to assure detection of Rb^+ by elution after the short pulse time.

When a water chase was used, there was considerable diffusion of Rb^+ from the petiole back into the water. This was reduced by using a 50 mM KCl chase solution, but some

loss of Rb^+ still occurred. This loss was minimized by using a uniform chase period of only 10 minutes, as described, after which all leaflets were excised to promote retention of ions that had been transported to the lamina. Variation in time for ion distribution within the lamina was achieved by waiting 0, 20 or 170 additional minutes before initiating the elution procedure. The total delay between the pulse and elution was therefore 10, 30 or 180 minutes.

Figure 20 presents six-hour elution curves for the three delay periods, with the results summarized in Table 6. Samples were also collected after 24 hours. Total Rb^+ in eluted leaflets was estimated by adding the amount removed by elution to that remaining in the entire leaflet. These calculated values were comparable to total Rb^+ in the opposite, noneluted leaflets.

Since the elution kinetics differed from those typically seen for endogenous K^+, the usual method for estimating extracellular ion content was not appropriate. The method used was that already described in connection with tracer efflux studies. The natural logarithm of Rb^+ remaining in the tissue was plotted over time, a line regressed through the points from 30 to 120 minutes and extrapolated to zero time. The Y intercept value was then subtracted from total Rb^+ present before elution to arrive at the estimate of extracellular Rb^+ content. The 30 to 120 minute portions of the log plots were essentially linear for all three treatments.

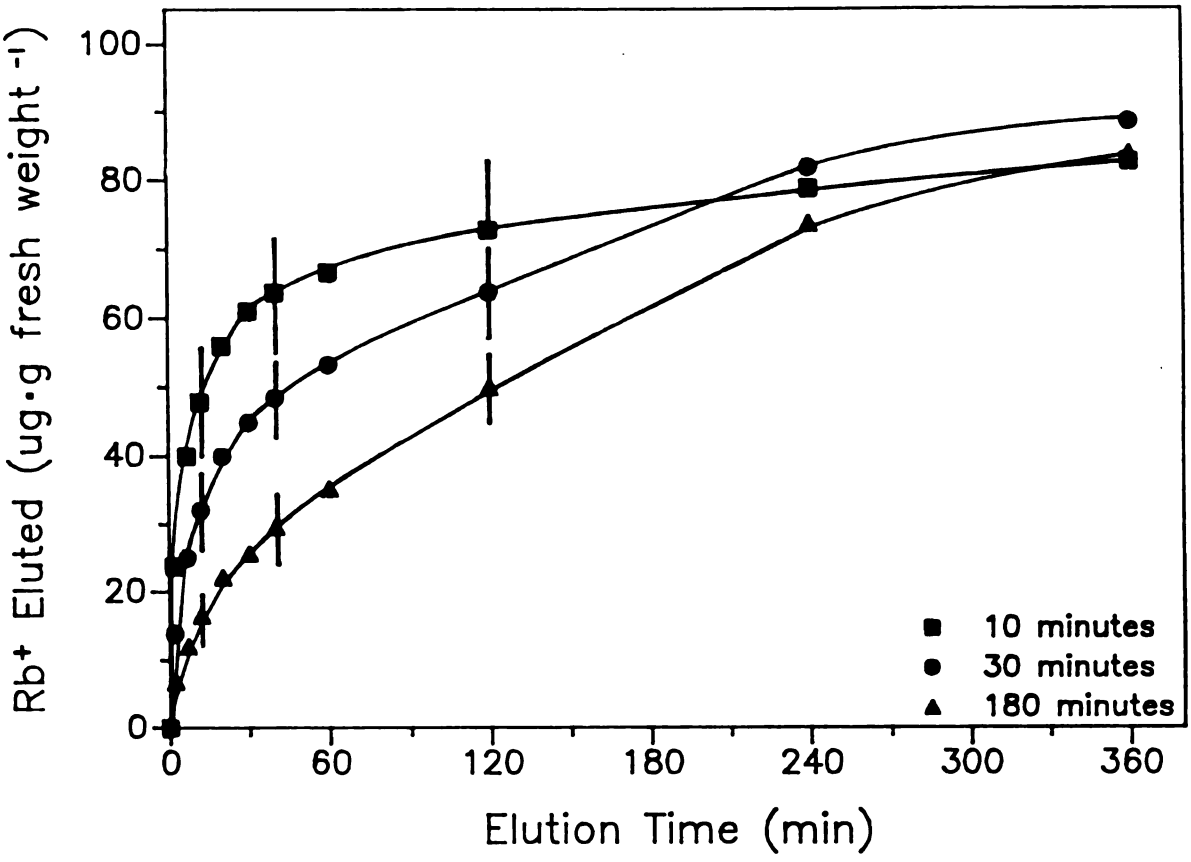


Figure 20. Rubidium elution at 1° C from leaflets after excised leaves had taken up a six-minute pulse of 50 mM RbCl solution through petioles at 25° C. Delays between pulse and start of elution were 10, 30 and 180 minutes. Results in Table 6. Points are means of six replications ± representative standard errors.

Table 6. Summary of experimental results when excised Argenteum leaves took up a pulse of 50 mM RbCl solution and Rb^+ was subsequently eluted from leaflets after a delay of 10, 30 or 180 minutes. Values are means of six replications ± standard errors.

Delay between uptake and elution	Total leaflet Rb^+ ($ug \cdot gfw^{-1}$)	0-20 min Rb^+ elution rate ($ug \cdot gfw^{-1}\ min^{-1}$)	1-4 h Rb^+ elution rate ($ug \cdot gfw^{-1}\ min^{-1}$)	Rb^+ content of apoplast ($ug \cdot gfw^{-1}$)	Apoplast Rb^+ as percent of total Rb^+	Total Rb^+ eluted in 24 h ($ug \cdot gfw^{-1}$)
10 min	344 ± 41	4.69 ± 1.37*[a]	0.064 ± 0.028*	60.4 ± 9.3**	17.6 ± 3.2**	129 ± 17
30 min	349 ± 65	2.87 ± 0.99	0.157 ± 0.061	40.1 ± 8.0*	11.5 ± 2.1*	110 ± 39
180 min	351 ± 44	1.40 ± 0.48*	0.211 ± 0.054*	16.6 ± 4.6**	4.7 ± 1.8**	128 ± 26

[a]Means within a column significantly different from each other at 1% level (**) or 5% level (*) by F test. Others not significantly different.

With an increase in delay from 10 to 180 minutes, there was a significant decrease in elution rate for the first 20 minutes, but a significant increase in rate for one to four hours. The period from 20 to 60 minutes was one of transition where average rates were approximately equal. As the delay period increased, there was a significant decrease in the estimated extracellular Rb^+ content. At four, six and 24 hours, however, the total amounts of Rb^+ eluted were not significantly different for the three treatments.

The initial rapid elution of Rb^+ followed by a slow rate of efflux observed after a 10-minute delay suggests that the Rb^+ ions were still localized principally in an extracellular compartment and consequently diffused readily into the elution solution. In contrast, the 180-minute curve is characterized by a lengthy steady elution rate which has been associated in the present study with long-term efflux from cells. This curve most closely resembles those obtained for K^+ elution. However, the steady-state efflux rate (0.21 ug gfw^{-1} min^{-1}) is only about 10% of that typically seen for K^+. While Rb^+ taken up by leaflets in this experiment represented about 0.2% of leaflet dry weight, K^+ content normally ranged from 4 to 6%; therefore, the low accumulation of Rb^+ in the lamina tissue most likely was responsible for the lower efflux rate.

Extracellular Rb^+ represented 17.6%, 11.5% and 4.7% of total Rb^+ for the 10-, 30- and 180-minute treatments, respectively (Table 6). These percentages may be somewhat

low for the lamina tissue alone since they were calculated using total Rb^+ of entire leaflets in which the midrib may have had a higher content than the remainder of the tissue. Nevertheless, these values support the hypothesis that the apoplastic ionic content was progressively reduced by cell uptake over time.

From K^+ elutions, extracellular K^+ was calculated to be approximately 2% of the total in the eluted tissue. Even after the 180-minute delay, therefore, the extracellular ion content seems to constitute a larger portion of the total than in the intact leaf. Apparently there was not sufficient time for cells to accumulate the ion to the same extent as in the intact leaf. This is consistent with the relatively lower constant efflux rate for Rb^+ as compared to K^+ discussed above.

Essentially the same amount of Rb^+ had been eluted by the end of the experiment for all three treatments but the elution kinetics varied, seemingly according to compartmentation of the ion in the tissue. These results suggest that with an increase in the time period between the Rb^+ pulse and elution, more of the ion had been taken up by cells, leaving progressively less in the apoplast. Thus, in the intact plant, a significant amount of ions being transported to the leaf lamina via the xylem stream may first diffuse into an apoplastic region of the tissue prior to being taken up and accumulated within individual cells. This hypothesis is supported by the finding of Jachetta et

al. (1986b) that under conditions of darkness and greatly reduced transpiration, leaf cells can deplete the cell wall of solutes almost entirely.

Plants Grown at Two K^+ Levels

To allow evaluation of the elution method under conditions of differing ionic levels in intact plants, Argenteum plants were grown as described by sand culture and supplied with nutrient solution containing either 2 or 10 mM K^+. Potassium concentrations in the soil solution generally range from 0.2 to 10 mM (Van Steveninck, 1962). Leaflets from both treatments appeared morphologically similar. All leaflets, however, were smaller than those of plants grown in the peat-based medium. The sand retained considerable moisture which may have restricted root growth and, consequently, that of the shoot.

Figure 21 presents four-hour elution curves for leaflets from high-K^+ (10 mM) and low-K^+ (2 mM) plants, with results summarized in Table 7. Total laminar K^+ in the tissue eluted, steady-state elution rates, estimated apoplastic K^+ contents and xylem-sap K^+ concentrations were all significantly higher for the high-K^+ treatment.

Assuming an extracellular solution volume of 0.1 ml gfw^{-1}, the calculated apoplastic solution concentrations were 36.2 and 22.65 mM K^+ for high- and low-K^+ treatments, respectively. Since the corresponding xylem-sap concentrations were 14.2 and 5.4 mM, an increase in sap

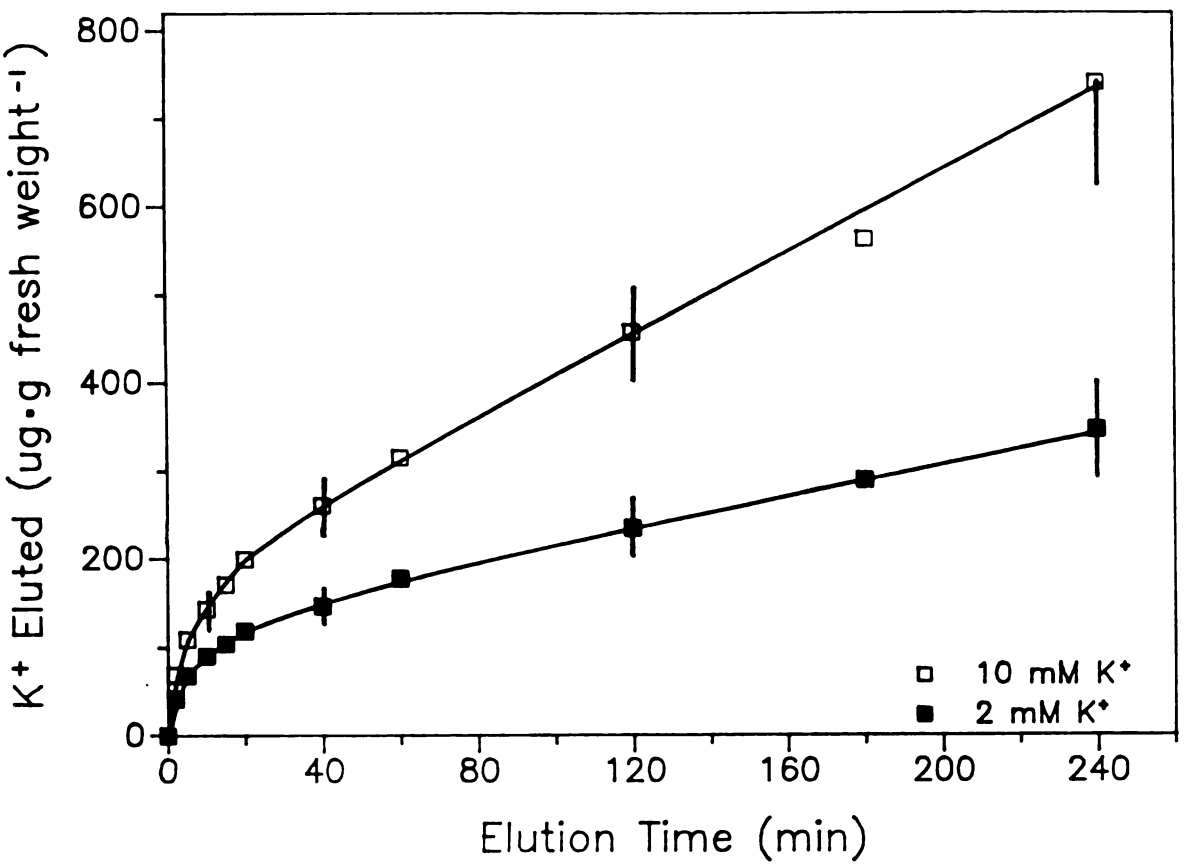


Figure 21. Potassium elution at 1° C from leaflets of Argenteum plants grown at 2 or 10 mM K^+. Results in Table 7. Points are means of six replications ± representative standard errors except where smaller than symbol.

Table 7. Summary of experimental results for leaflets of Argenteum plants grown in sand and supplied with nutrient solution containing 2 or 10 mM K^+. Values are means of six replications ± standard errors.

Nutrient Solution [K^+]	Total lamina K^+ (ug·gfw^{-1})	Steady-state K^+ elution rate[a] (ug·gfw^{-1} min^{-1})	K^+ content of apoplast (ug·gfw^{-1})	Estimated [K^+] of apoplast[b] (mM)	Apoplastic K^+ as percent of total K^+	Xylem sap [K^+] (mM)
2 mM	3832 ± 324	1.48 ± 0.31	88.4 ± 11.2	22.6 ± 2.9	2.31 ± 0.29	5.4 ± 0.5
10 mM	7780 ± 285	2.91 ± 0.51	141.6 ± 16.8	36.2 ± 4.3	1.82 ± 0.22	14.2 ± 1.6
F test[c]	**	**	*	*	NS	**

[a]Slope of linear portion of the elution curve, 20 to 240 minutes.

[b]Assuming an apoplastic solution volume of 0.1 ml gfw^{-1}.

[c]Means significantly different at 1% level (**), 5% level (*), not significantly different (NS).

concentration along the xylem path or in the leaf apoplast would have been required to result in the above values.

The K^+ content of lamina tissue from high-K^+ leaflets was approximately twice that of low-K^+ tissue. Since the estimated apoplastic K^+ content was 1.6 times as high, it represented a lower percentage of total K^+ in leaflets grown at 10 mM K^+ (1.82%) than at 2 mM K^+ (2.31%). These values are not, however, significantly different.

These results are in agreement with previous findings that the K^+ concentrations of xylem sap and plant tissue do not change in direct proportion to that supplied to roots. The concentration of solution bathing the root is not necessarily reflected in the xylem-sap concentration since uptake rate varies with external concentration (Pitman, 1975). Conti and Geiger (1982) reported that when K^+ supplied to roots of sugar beet was increased from 2 to 10 mM, the corresponding increase in leaf K^+ content was only two to three fold. According to these workers, at 2 mM K^+ leaf export and import rates were similar so K^+ did not accumulate. At the higher concentration, however, xylem import increased to a greater extent than phloem export and there was accumulation of the ion.

Further examination of the relationship between ion import and export rates, apoplastic ion concentration and cell uptake might aid in identifying those factors responsible for changes in leaf K^+ content. The ability of the elution method to detect changes in apoplastic K^+ in the

intact plant suggests that it might be useful in such studies.

SUMMARY AND CONCLUSIONS

Leaflets of the Argenteum mutant of Pisum sativum L. were found to be suitable material for estimating apoplastic K^+ content by elution since the abaxial epidermis may be removed without apparent injury to underlying mesophyll cells. This allows free diffusional access to the mesophyll apoplast with considerably less danger of contamination by intracellular contents than when leaf discs or slices are used.

The 60-minute time course for elution of K^+ from peeled Argenteum leaflet lamina tissue is characterized by a rapid initial elution rate followed by a slower, steady rate after about 20 minutes. Evaluation of the elution curve suggests that the rapid phase represents a period of ion diffusion from the apoplast as well as equilibration of that compartment with the bathing solution. The constant rate is thought to represent net efflux out of cells, which appears to begin at some point during the rapid elution phase.

Extrapolation of the linear portion of the curve to zero time seems to be a valid means of estimating the original apoplastic K^+ content. It is equivalent to subtracting K^+ thought to be of intracellular origin from the total eluted. A better understanding of cell efflux rates during the first 20 minutes of elution might make such

estimates more precise. Log transformation of data, as done in tracer efflux studies, is not thought to be necessary for compartmental analysis when eluting endogenous ions from leaf tissue.

Estimates of apoplastic K^+ content ranged from 39 to 97 ug gfw^{-1} for plants grown at 4.25 mM K^+. When plants were grown at 2 and 10 mM K^+, estimates were 88 and 142 ug K^+ gfw^{-1}, respectively; in both cases, apoplastic K^+ was found to represent approximately 2% of total laminar K^+.

Concentrations calculated based on an estimated apoplastic solution volume of 0.1 ml gfw^{-1} were 10 to 25 mM for plants grown at 4.25 mM K^+, and 23 and 36 mM for those grown at 2 and 10 mM K^+, respectively. These values are higher than those from previous studies, which may be due to experimental conditions or underestimation of extracellular solution volume in the present case. There is a need to better define the volume of extracellular solution so that more exact concentrations may be determined.

The elution method allows the determination of changes in apoplastic K^+ contents corresponding to xylem-sap concentrations typically found in plants. It may also be used to detect differences in ion compartmentation within leaf tissue. On this basis, ion movement into the leaf from the xylem seems to be first into an extracellular compartment from which it is progressively taken up by cells.

Free-space characteristics of Argenteum leaflet lamina tissue differed in some respects from past reports. In particular, the lower WFS estimate may be attributable to reduced tissue damage as compared to previously used methods. Estimated K^+ bound by negative charges in the cell-wall Donnan phase during elution was approximately 0.1 ueq gfw^{-1}, or 4 to 11% of apoplastic K^+. Knowledge of the extent of K^+ binding in the intact leaf would help define the role of the DFS in transport of the ion.

The method presented in this study is limited in that it is not applicable to most other species, where removal of the epidermis results in severe tearing of mesophyll cells. In addition, it is not suitable for estimating apoplastic K^+ near particular cells since only an overall estimate is obtained. Nevertheless, the elution method may be useful in future studies.

While the determination of absolute apoplastic ion concentration by elution needs refinement, the method seems appropriate for comparing relative concentrations under varying conditions. Argenteum could therefore be used as a model for the study of leaf apoplastic concentrations of K^+ and other ions. The elution method might serve as an overall estimator, complementary to a detection method with greater resolution, such as X-ray microanalysis.

Using these methods, it would be of interest to look at changes in apoplastic ion concentration during leaf and plant ontogeny, with changes in xylem import and phloem

export rates, and under varying growing conditions. Matters that might be addressed include leaf extracellular ion concentration in relation to cell fluxes and regulation of intracellular concentration; effects of apoplastic ion concentration on specific physiological functions in the leaf; the importance of extracellular transport in general ion distribution within the leaf or in movement to specific sites; and the role of the Donnan phase in ion transport and cell uptake in the leaf. Knowledge gained in these areas would contribute greatly to our understanding of leaf ion relations and their role in determining plant productivity.

APPENDIX

APPENDIX

X-RAY MICROANALYSIS OF FREEZE-DRIED LEAFLET LAMINA TISSUE

Introduction

The development of X-ray microanalysis as an analytical tool in conjunction with electron microscopy, allowing identification and analysis of very small volumes of tissue, has increased the potential for refining our knowledge of the distribution and concentration of ions in plant tissue. X-rays, produced when primary electrons of the beam generated by an electron microscope displace electrons of atoms in a sample (Marshall, 1980c), may be attributed to a particular element by their energy or wavelength (Flowers and Lauchli, 1983). Since the intensity of radiation is directly proportional to the concentration of the assayed element, this method is useful for quantitative as well as qualitative analysis (Marshall, 1980c).

There seems to be general agreement that for X-ray analysis of biological materials, sample preparation is the limiting factor (Chandler, 1979; Morgan, 1979; Flowers and Lauchli, 1983). This is particularly true for plant tissue, with its high water content (Echlin and Saubermann, 1977; Morgan, 1980). The purpose of this study was to evaluate

frozen sectioning followed by freeze-drying as a method of preparing leaf tissue for X-ray microanalysis of K^+ in the apoplast.

Sample Preparation Methods

The two principal criteria for evaluating sample preparation for ion localization by X-ray microanalysis are that elements be retained essentially *in situ* and that morphological detail be sufficient for clear identification of the structures to be analyzed (Chandler, 1979). The difficulty lies in satisfying both criteria in the same sample. The progress made in this regard for biological tissue has been reviewed by Lauchli (1972a), Van Steveninck and Van Steveninck (1978), Morgan (1980) and Spurr (1980).

Conventional sample preparation methods for electron microscopy involving fixation and dehydration in liquids may result in retention of only a small percentage of soluble ions (Hall *et al.*, 1974; Lott *et al.*, 1978; Morgan, 1980). Alternate methods have therefore been developed specifically for X-ray microanalysis, the primary method of fixation being rapid freezing at liquid-nitrogen temperature (around -180° C). This has the advantages of stopping physiological processes, diminishing movement of elements, stabilizing cell structure and providing mechanical strength for sectioning (Seveus, 1980).

The simplest technique involves analysis of the tissue in the frozen-hydrated state, either bulk-fractured or

sectioned (Markhart and Lauchli, 1982). This method requires the use of a cold stage to maintain the sample at low temperature, an apparatus not available for the present study.

Drawbacks to analyzing bulk frozen tissue are that the large mass fraction of water reduces the signal-to-background ratio of X-rays generated (VanSteveninck and Van Steveninck, 1978) and spatial resolution is poor (Chandler, 1979; Marshall, 1980a). These problems are reduced by analyzing thin sections of frozen tissue (Barbi, 1979; Satter et al., 1982). In both sample types, however, a localized rise in temperature due to electron irradiation may result in ion redistribution (Marshall, 1980b).

In freeze substitution, the ice in rapidly frozen tissue is slowly replaced at low temperature by a dehydration agent such as acetone or acrolein in diethyl ether, and the sample is then embedded in a plastic medium (Marshall, 1980d). Embedded tissue is generally more easily sectioned than frozen (Marshall, 1980d) and this method allows good preservation of morphological detail (Morgan, 1980). It is said to retain in situ a large percentage of mobile ions as long as the process is totally anhydrous (Pallaghy, 1973; Harvey et al., 1976; Marshall, 1980d). There is, however, danger of ion redistribution if the substituting fluid takes up water from the atmosphere or the embedding medium is not properly polymerized (Morgan, 1980; Markhart and Lauchli, 1982). Exogenous ions may be

introduced in both of these steps (Morgan, 1980), and even anhydrous solvents have been reported to alter X-ray spectra (Marshall, 1980d). Appleton (1977) reported considerable loss or redistribution of elements by freeze substitution.

In freeze-drying, ice is removed from the frozen sectioned or bulk tissue by physical dehydration at atmospheric pressure or under vacuum (Morgan, 1980). This method has the advantage of not requiring the use of solvents, so that maintenance of chemical integrity is theoretically possible (Morgan, 1980). Appleton (1978) reported better resolution in freeze-dried than frozen tissue due to reduction of electron scatter caused by water. While ions cannot be analyzed in solution, they seem to remain compartmentalized within and outside of cells by precipitating onto the nearest structure (Gupta et al., 1976). A major disadvantage has been poorer structural preservation than with other methods (Morgan, 1980).

All of the above methods have been used to prepare plant tissue for ion localization by X-ray analysis, as reviewed by Spurr (1980). The freeze-drying method used in the present study was based on that of Satter et al. (1982). That study was selected as a model because its objective was specifically to analyze for apoplastic K^+ and Cl^- in the Samanea pulvinus. The integrity of both protoplasts and cell walls, spatial resolution and elemental compartmentation were reported to be adequately maintained in freeze-dried tissue.

Materials and Methods

Plants of the Argenteum mutant of Pisum sativum L. were grown as described for the general elution experiments and leaflets used at the same stage of development.

Prior to freezing the tissue, freshly broken glass knives were prepared. Several layers of masking tape were placed on the back of each knife 2 mm below the cutting edge to support a grid (Appleton, 1978). In addition, a "shelf" of tape was added further down to keep grids from sliding down the back of the knife.

A knife was inserted at a 4° angle into the holder within the cryobowl of a Sorvall-Christensen FTS/LTC-2 frozen thin sectioner with low-temperature controller mounted on a Sorvall Porter-Blum MT-2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.). The coolant was vaporized liquid nitrogen. The bowl, knife and specimen chuck holder were then allowed to cool down and equilibrate to -80° C (measured at the chuck-holder assembly) for approximately 20 minutes before sectioning was begun. During the last five minutes of cooling the motor advancing the specimen was turned on to insure temperature equilibration. All tools used during freezing and sectioning were prechilled for several minutes in liquid nitrogen or in the cryobowl.

Liquid propane (-190° C), the freezing medium, was prepared by allowing propane gas from a cylinder to condense in a metal container sitting in liquid nitrogen in a Dewar flask.

A leaflet was gently clamped in a copper specimen holder at an area of the margin near the base of the leaflet (Figure 22). This positioning was so that the area analyzed would roughly correspond to that used in the elution studies. A drop of 50% sucrose solution was placed in the holder before inserting the leaflet to help support the tissue after freezing.

The mounted leaflet was rapidly cut using a razor blade to leave a triangular piece of tissue approximately 3 mm high (Figure 22) and immediately plunged into the liquid propane so the holder was completely submerged. After 15 seconds the sample was transferred to liquid nitrogen and carefully fractured using chilled forceps until a triangular piece of tissue approximately 1 mm high was left.

The specimen and holder were then transferred in liquid nitrogen to the cryobowl and mounted in the chuck holder with the specimen perpendicular to the knife edge. The specimen was trimmed by cutting 1-um sections until a smooth cutting surface appeared.

Before sectioning was begun, a 2 x 1 mm oval copper slotted grid (Ernest F. Fullam, Inc., Schenectady, N. Y.) was placed on the tape support below the knife edge to chill. The grid had been coated on one side with 0.5% Formvar (Fullam) to provide a film support over the slot for the sections. In this way the grid composition would not interfere with analysis (Lauchli, 1972a).

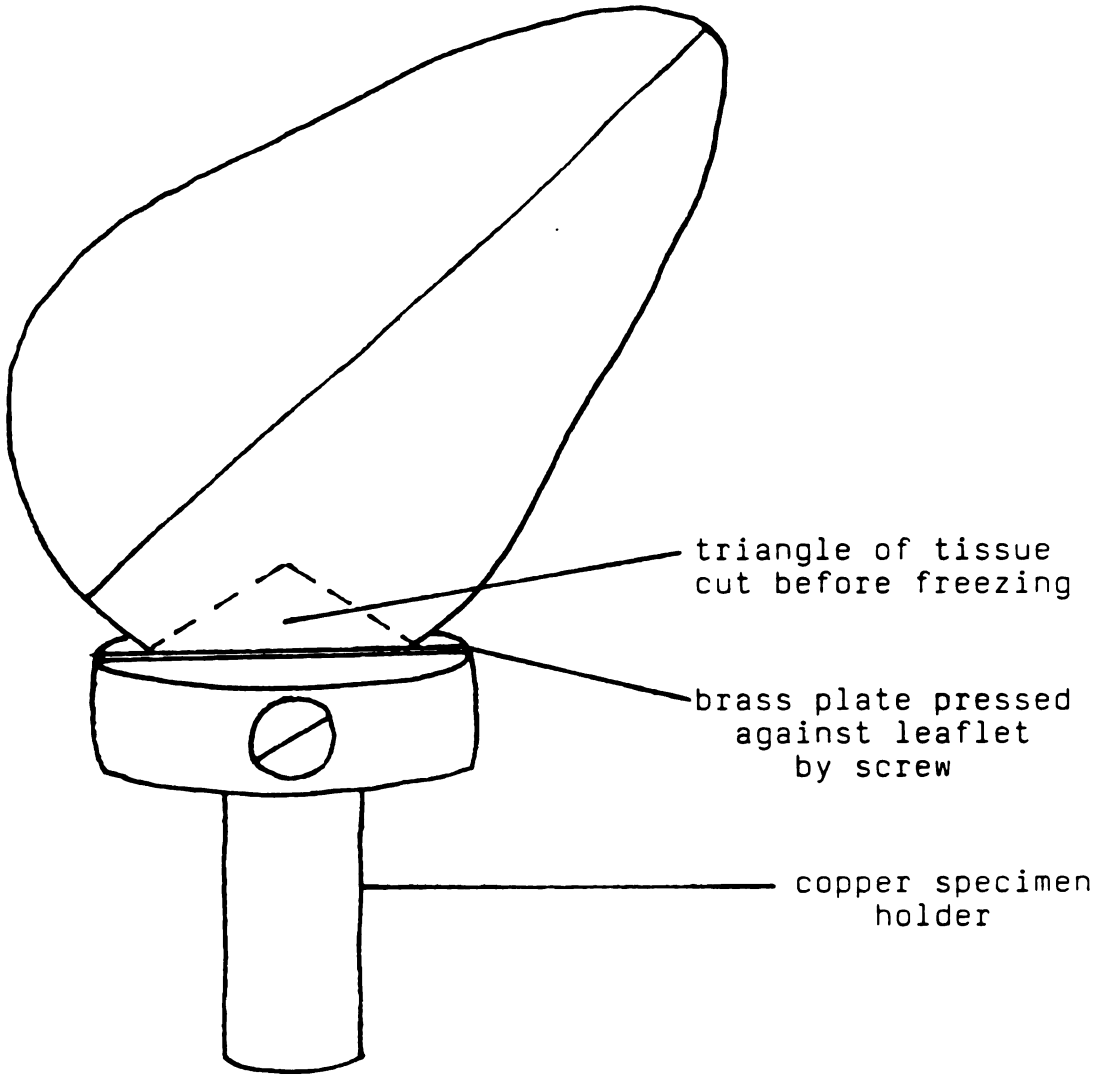


Figure 22. Positioning of leaflet in specimen holder and area cut out before freezing in liquid propane.

Sections 0.3 um thick were cut at a temperature of -80° C and cutting speed of 0.75 mm sec^{-1}. Frost was removed from the cut surface using a chilled eyelash brush. Sectioning seemed to be most successful when the bowl was covered and the microtome allowed to run undisturbed for one minute or more. If the knife edge became dull, the knife was replaced with one that had chilled in the cryobowl for at least 20 minutes.

When a number of sections had collected at the knife edge, they were moved to a grid using a chilled eyelash brush. This procedure was most easily accomplished under conditions of low humidity and low static electricity.

With the grid still on the knife, the sections were flattened and pressed gently onto the Formvar using the flat end of a chilled, polished copper rod 2 mm in diameter. The grid was picked up on the bent end of small metal spatula and placed on a 9-mm unpolished carbon planchet (Fullam) which had been cooled in the cyrobowl. A second cold planchet was placed on top to keep the specimen flat and help insulate it from temperature change during transfer to the freezer.

When all grids for a given session were complete, they were transferred to a -80° C freezer for drying. To maintain low temperature during the transfer, the planchet-grid sandwiches were placed over liquid nitrogen on a perforated metal plate mounted in a metal cylinder. A cardboard grid held the planchets in place. The cylinder

and samples were transferred to a styrofoam-lined metal can containing P_2O_5 as desiccant. The can had been allowed to cool in the freezer at least 30 minutes.

After 24 hours at -80° C, the can containing samples was transferred in a styrofoam cooler to a freezer at -25° C, where it remained for an additional 24 hours. It was then allowed to come to room temperature before opening.

For mounting, a carbon planchet was fixed atop a 9 x 4.5 mm aluminum stub (Fullam) using an adhesive tab (M. E. Taylor Engineering, Wheaton, Md.). Grids were attached to the planchet with a small amount of Television Tube Koat (G. C. Electronics, Rockford, Ill.). They were then carbon coated using a Ladd Research Industries (Burlington, Vt.) vacuum evaporator. One 3/16 point carbon rod was used to coat eight samples at a time, providing a fairly heavy coating. Carbon, which does not contribute significantly to background, makes the sample conductive to minimize heating and local charge buildup (Echlin and Saubermann, 1977) and protects against humidity (Appleton, 1977). Samples were stored in a desiccator at atmospheric pressure over P_2O_5.

Samples were subjected to energy-dispersive X-ray microanalysis using a Tracor Northern (Middleton, Wis.) TN-2000 X-ray analyzer mounted on a JEOL JSM-35C scanning electron microscope (Japan Electron Optical Laboratories, Tokyo) operating in spot mode. An X-ray spectrum was collected from cell wall for 100 seconds (dead time 20-30%)

using an accelerating voltage of 15 KeV (Lauchli et al., 1970) and beam current of 240 picoamps. Specimen tilt was 0^{o} from horizontal, and takeoff angle was 40^{o}.

Spectral peaks for K^{+}, Cl^{-}, P and S were identified by their K_{α} lines and counts above background recorded. Although K^{+} was of principal interest, data for the other elements were collected to compare relative contents.

Results and Discussion

Although the above steps were carefully carried out, most of the grids prepared were not suitable for analysis. In some cases, nothing remained on the grid by the time it was ready to be analyzed; in others, what was there was not identifiable. In no case was a complete section obtained, but scattered groups of cells were occasionally visible. Table 8 presents results for four samples in which cell wall could be distinguished, while Figure 23 illustrates a typical X-ray spectrum. Since it is not reasonable to draw conclusions based on so few samples, these results are presented only for general interest.

As indicated by the X-ray spectrum (Figure 23), background was quite high relative to peak height for Cl^{-} and S. Some coolants have been reported to cause Cl^{-} contamination, but this generally is not a problem with propane (Markhart and Lauchli, 1982). The Formvar film (Table 8) may have been contaminated during application. The higher K^{+} and P counts for analysis 2a as compared to 2b

Table 8. Results of X-ray microanalysis of cell wall in freeze-dried Argenteum leaflet lamina tissue.

Analysis[a]	Net Counts			
	K^+	Cl^-	S	P
1a	6719	471	894	3058
1b	6698	351	962	1713
Formvar film supporting above sample	189	60	207	126
2a	11133	134	1963	3302
2b	8976	158	1864	1802
3	5368	378	411	1225
4	6850	402	840	1689

[a]Analyses designated a and b are of different points on the cell wall of the same cell. Samples from four separate leaflets were analyzed.

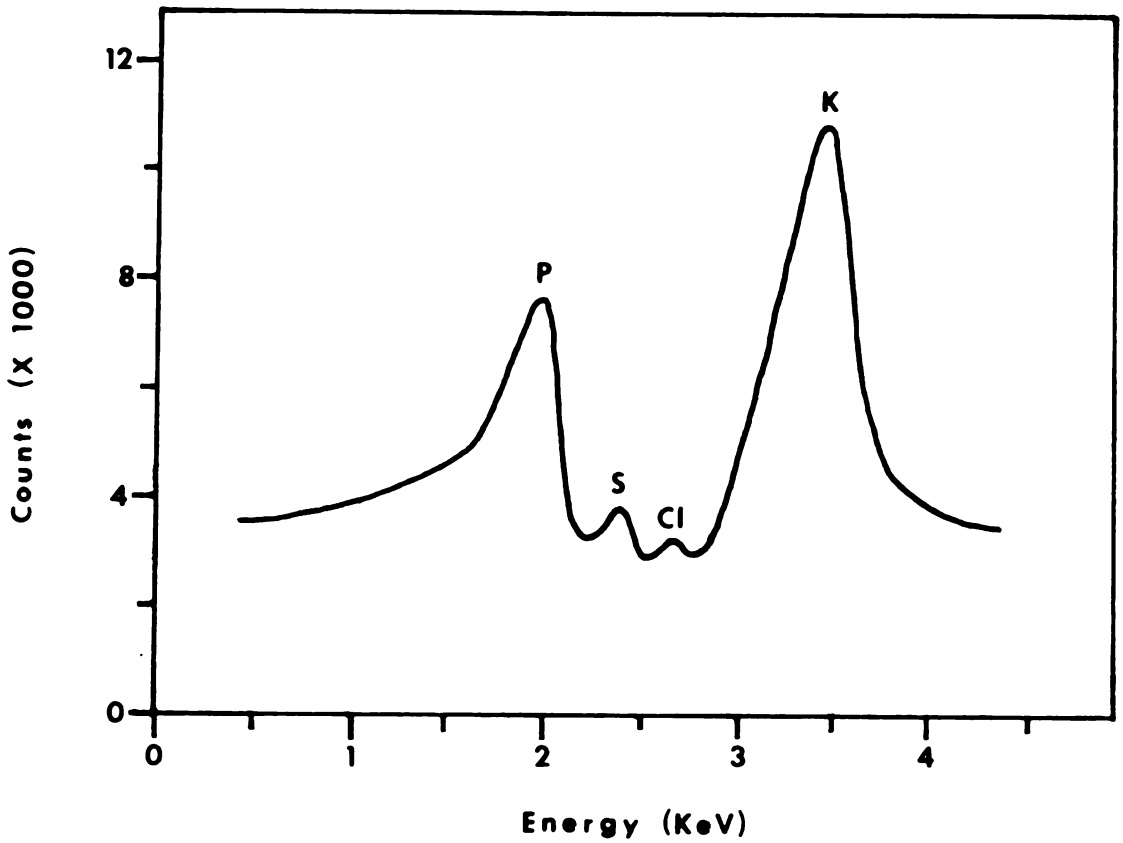


Figure 23. Portion of X-ray spectrum for cell wall of freeze-dried Argenteum leaflet lamina tissue corresponding to analysis 1a in Table 8.

from the same cell may be due to inadvertent analysis of a portion of plasmalemma.

The consistency in the relative amounts of the four elements detected and the fact that K^+ concentration in the cell wall seems to be well within the range of detection suggest that with some modification this method may be useful. Factors that might have affected the results of this study are briefly considered below.

Freeze fixation acceptable for X-ray analysis requires a rapid cooling rate (Morgan, 1980). Since biological tissues exhibit poor thermal conductivity (Seveus, 1978), it is recommended that only a small volume of tissue (1 mm^3) be frozen (Appleton, 1977). It is often necessary, however, to compromise between wound effects and rapid freezing (Van Steveninck and Van Steveninck, 1978). This is particularly true for highly mobile ions like K^+, which can quickly diffuse to neighboring tissue (Marshall, 1972).

In the present study, to minimize the possibility of analyzing tissue in which the apoplast had been contaminated with intracellular K^+, a somewhat larger piece than desired was frozen and subsequently reduced in size for sectioning. To minimize K^+ movement, only uncut tissue was exposed to the sucrose solution, and the time from cutting to freezing was no more than two seconds.

The use of a larger piece of tissue may, however, have reduced the freezing rate. The advantage of rapid freezing is that it minimizes the formation of ice crystals which can

rupture membranes and displace unfrozen solution, enriched in solutes (Nobel, 1975; Morgan, 1980). The presence of ice crystals may also make sectioning of frozen tissue more difficult (Marshall, 1980b).

Vitrification, the freezing of water without ice-crystal formation, can occur if cooling is rapid enough; but even under ideal conditions it may only occur in the outer 10 um of a sample since the loss of heat inside that volume is too slow (Marshall, 1980a). Argenteum leaflets are approximately 125 um thick, so that even under ideal freezing conditions ice crystals probably would have formed in the inner tissue. In addition, crystallization of vitrified ice may occur with a temperature rise (Marshall, 1980a). Temperatures below -60° C are thought necessary to prevent ice crystal formation (Morgan, 1980).

Liquid propane is reported to provide a faster cooling rate than other coolants commonly used (e.g. liquid or slushy nitrogen and freon-22) (Costello, 1980). Although liquid propane and liquid nitrogen are at essentially the same temperature, formation of an insulating layer of gas around the specimen in the latter results in poor cooling (Marshall, 1980d). Marshall (1980d) reported that regions relatively free of ice-crystal damage have been obtained using liquid propane. The use of cryoprotectants (e.g. gylcerol) has been reported to provide good structural preservation, but may result in ion movement across membranes (Appleton, 1978).

Even well frozen plant tissue seems to pose problems for sectioning. Echlin et al. (1982) observed that cutting thin sections of such tissue is a "difficult and troublesome procedure." They attributed this difficulty to the high water content of vacuoles relative to cell walls, resulting in a discontinuous material when frozen. The presence of large intercellular air spaces in leaves compounds the problem. A light micrograph of the Samanea pulvinus studied by Satter et al. (1982) reveals it to be more uniform and compact than leaf tissue, perhaps making it easier to section.

In spite of the difficulties, sectioning offers certain advantages. One is that the surface analyzed is smoother than that resulting when tissue is fractured (Echlin et al., 1982; Marshall, 1980a). Irregular specimen topography can result in loss of resolution, lower detection efficiency and generation of spurious signals (Hess, 1980). Differential drying of structures may itself result in an irregular surface, but sectioning can minimize this effect (Appleton, 1978).

As indicated, improved spatial resolution is obtained with sections. In bulk specimens which are too thick to allow the transmission of electrons, the electron beam penetrates in a teardrop form so that X-rays are generated from a volume larger than the surface feature focused on (Barbi, 1979; Morgan,1980). Echlin et al. (1982) reported

spatial resolution of 2 to 8 um in bulk specimens versus 0.05 to 0.2 um in sections 0.1 to 1.5 um thick.

Even if primary electrons can pass through a bulk specimen, they lose energy, lowering the efficiency of X-ray production (Marshall, 1980c). Lower elemental concentrations (down to around 100 ppm) may therefore be detected in thin sections (Van Steveninck and Van Steveninck, 1978).

Echlin and Saubermann (1977) recommended a section thickness of 0.2 to 2 um to attain good morphological information, sufficient material for analysis and adequate spacial resolution. Sections were cut at 0.3 um in the present study because tissue appeared to fracture at greater thickness and to be merely scraped off at lesser thickness.

Similarly, a sectioning temperature of -80° C was used in the present study because it was the lowest temperature at which the tissue appeared not to fracture. This is slightly lower than the -70° C at which Satter et al. (1982) sectioned.

Cutting at very low temperatures (<-100° C) may cause fracturing rather than true sectioning (Seveus, 1980), resulting in uneven section surface and thickness. Marshall (1980b) indicted that while sectioning below -100° C avoids recrystallization and thawing, sections cut at -80° C are more uniform in thickness. He questioned whether there is sufficient thawing at the higher temperature to cause redistribution of diffusible elements.

Dempsey and Bullivant (1976) and Appleton (1977) reported no ice recrystallization or significant ion redistribution in biological tissue at -70° to -80° C, the lowest temperatures at which Appleton (1977) felt true sections were obtained. Others, however, recommend lower temperatures. Hodson and Marshall (1970) suggested cutting at or below -100° C. Seveus (1980) has recommended a specimen temperature of -140° C with the knife and bowl at -100° C. It is likely that the optimum cutting temperature varies with tissue type and therefore must be individually determined.

There was some loss of samples during transfer to the grid due to electrostatic forces and air movement in the cryobowl. Even after being pressed against the Formvar film, samples did not adhere well. Some may have stuck to the carbon planchet placed atop the grid, a problem that might have been aggravated by a temperature increase while samples were transferred to the freezer.

Like the sectioning temperature, that used for freeze-drying (-80° C) was somewhat lower than in the method of Satter et al. (1982). It is possible that ice recrystallization occurred at this temperature, though Ingram et al. (1972) and Morgan (1980) have reported successful freeze drying below -60° C.

Appleton (1977, 1978) recommended slow drying at atmospheric pressure since surface-tension forces of rapid sublimation under vacuum might disrupt cells. Campbell et

al. (1981) reported that drying under vacuum disrupted plant tissue structure and so dried specimens at atmospheric pressure over phosphorus pentoxide, a desiccant with a strong affinity for water. Gradual warming has been recommended so tissue does not suffer thermal shock (Appleton, 1978).

Conclusions

The freeze-drying method of sample preparation used in this study appears to have merit, but requires modification if reliable results are to be obtained. A freezing method that allows vitrification of a greater volume of tissue is desirable. In addition, a lower sectioning and freeze-drying temperature than used in this study may be required to avoid ice recrystallization. It is important that utmost care be used to avoid any rise in temperature when samples are transferred between preparation steps.

Freeze-substitution merits further investigation since it may allow improved sectioning and retention of morphological detail. If substitution and embedding can be accomplished without ion distribution, this method may prove preferable to freeze-drying.

Perhaps the greatest possibility for success lies in analysis of frozen sections, provided the necessary equipment is available and problems of sectioning frozen leaf tissue can be overcome.

Continued work in this area is essential for the identification of reliable sample preparation methods that will allow realization of the potential offered by X-ray microanalysis for elemental analysis of plant tissue.

LITERATURE CITED

LITERATURE CITED

Appleton, T. C. 1977. The use of ultrathin frozen sections for X-ray microanalysis of diffusible elements. pp. 247-268. In: G. A. Meek and H. Y. Elder (eds.), Analytical and Quantitative Methods in Microscopy. Cambridge University Press, Cambridge.

Appleton, T. C. 1978. The contribution of cryo-ultramicrotomy to X-ray microanalysis in biology. pp. 148-182. In: D. A. Erasmus (ed.), Electron Probe Microanalysis in Biology. Chapman and Hall, London.

Atkinson, M. M., J. S. Huang and J. A. Knopp. 1985. The hypersensitive reaction of tobacco to Pseudomonas syringae pv. pisi. Plant Physiol. 79:843-847.

Barbi, N. C. 1979. Quantitative methods in biological X-ray microanalysis. Scanning Electron Microsc. 1979:659-672.

Beeson, R. C., Jr., J. M. Montano and W. M. Proebsting. 1986. A method for determining the apoplastic water volume of conifer needles. Physiol. Plant. 66:129-133.

Bernstein, L. 1971. Method for determining solutes in cell walls of leaves. Plant Physiol. 47:361-365.

Bernstein, L. and R. H. Nieman. 1960. Apparent free space of plant roots. Plant Physiol. 35:589-598.

Bollard, E. G. 1953. The use of tracheal sap in the study of apple tree nutrition. J. Exp. Bot. 4:363-368.

Boyer, J. S. 1967. Matric potential of leaves. Plant Physiol. 42:213-217.

Brag, H. 1972. The influence of potassium on the transpiration rate and stomatal opening in Triticum aestivum and Pisum sativum. Plant physiol. 26:250-257.

Briggs, G. E. 1957. Some aspects of free space in plant tissues. New Phytol. 56:305-324.

Briggs, G. E., A. B. Hope and M. G. Pitman. 1958. Exchangeable ions in beet discs at low temperature. J. Exp. Bot. 9:128-141.

Briggs, G. E., A. B. Hope and R. N. Robertson, 1961. Electrolytes and Plant Cells. Blackwell Scientific, Oxford.

Briggs, G. E. and R. N. Robertson. 1948. Diffusion and absorption in discs of plant tissue. New Phytol. 47:265-283.

Briggs, G. E. and R. N. Robertson. 1957. Apparent free space. Annu. Rev. Plant Physiol. 8:11-30.

Briskin, D. P. 1984. A current perspective of the plasma membrane ATPase of higher plant cells. pp. 531-537. In: W. J. Cram, K. Janacek, R. Rybova and K. Sigler (eds.), Membrane Transport in Plants. John Wiley and Sons, New York.

Briskin, D. P. and R. J. Poole. 1983. The plasma membrane ATPase of higher plant cells. What's New Plant Physiol. 14:1-4.

Brouwer, R. 1959. Diffusible and exchangeable rubidium ions in pea roots. Acta Bot. Neerl. 8:68-76.

Brownlee, C. and R. E. Kendrick. 1979. Ion fluxes and phytochrome protons in mung bean hypocotyl segments. I. Fluxes of potasssium. Plant Physiol. 64:206-210.

Burbano, J. L., T. D. Pizzolato, P. R. Morey and J. D. Berlin. 1976. An application of the Prussian blue technique to a light microscope study of water movement in transpiring leaves of cotton (Gossypum hirsutum L.). J. Exp. Bot. 27:134-144.

Butler, G. W. 1953. Ion uptake by young wheat plants. II. The "apparent free space" of wheat roots. Physiol. Plant. 6:617-635.

Butler, G. W. 1959. Uptake of phosphate and sulphate by wheat roots at low temperature. Physiol. Plant. 12:917-925.

Campbell, L. C. and M. G. Pitman. 1971. Salinity and plant cells. pp. 207-224. In: T. Talsma and J. R. Philip (eds.), Salinity and Water Use. John Wiley and Sons, New York.

Campbell, N., W. W. Thomson and K. Platt. 1974. The apoplastic pathway of transport to salt glands. J. Exp. Bot. 25:61-69.

Campbell, R. A., R. L. Satter and R. C. Garber. 1981. Apoplastic transport of ions in the motor organ of Samanea. Proc. Natl. Acad. Sci. 78:2981-2984.

Canny, M. J. and M. E. McCully. 1986. Locating water-soluble vital stains in plant tissues by freeze-substitution and resin embedding. J. Microsc. 142:63-70.

Carey, R. W. and J. A. Berry. 1978. Effects of low temperature on respiration and uptake of rubidium ions by excised barley and corn roots. Plant Physiol. 61:858-860.

Chandler, J. A. 1979. Principles of X-ray microanalysis in biology. Scanning Electron Microsc. 1979:595-606.

Cheeseman, J. M. and J. B. Hanson. 1980. Does active K^+ influx to roots occur? Plant Sci. Letters 18:81-84.

Cheung, Y. N. S., M. T. Tyree and J. Dainty. 1975. Water relations parameters on single leaves obtained in a pressure bomb and some ecological interpretations. Can. J. Bot. 53:1342-1346.

Chino, M. 1981. Species differences in calcium and potassium distributions within plant roots. Soil Sci. Plant Nutr. 27:487-503.

Clarkson, D. T. 1974. Ion Transport and Cell Structure in Plants. John Wiley and Sons, New York.

Clarkson, D. T. and J. B. Hanson. 1980. The mineral nutrition of higher plants. Annu. Rev. Plant Physiol. 31:239-298.

Conti, T. R. and D. R. Geiger. 1982. Potassium nutrition and translocation in sugar beet. Plant Physiol. 70:168-172.

Cosgrove, D. J. and R. E. Cleland. 1983. Solutes in the free space of growing stem tissues. Plant Physiol. 72:326-331.

Costello, M. J. 1980. Ultrarapid freezing of thin biological samples. Scanning Electron Microsc. 1980:361-370.

Crafts, A. S. and C. E. Crisp. 1971. Phloem Transport in Plants. W. H. Freeman, San Francisco.

Cram, W. J. 1968. Compartmentation and exchange of chloride in carrot root tissue. Biochim. Biophys. Acta 163:339-353.

Cram, W. J. 1973. Chloride fluxes in cells of the isolated root cortex of Zea mays. Aust. J. Biol. Sci. 26:757-779.

Cram, W. J. 1976. Negative feedback regulation of transport in cells. The maintenance of turgor, volume and nutrient supply. pp. 284-316. In: U. Luttge and M. G. Pitman (eds.), Transport in Plants II. Encyc. of Plant Physiol., New Series, Vol. 2A. Springer-Verlag, Berlin.

Crowdy, S. H. and T. W. Tanton. 1970. Water pathways in higher plants. I. Free space in wheat leaves. J. Exp. Bot. 21:102-111.

Dainty, J. and A. B. Hope. 1959. Ionic relations of cells of Chara australis. I. Ion exchange in the cell wall. Aust. J. Biol. Sci. 12:396-411.

Dainty, J., A. B. Hope and C. Denby. 1960. Ionic relations of cells of Chara australis. II. The indiffusible anions of the cell wall. Aust. J. Biol. Sci. 13:267-276.

Delrot, S., M. Faucher, J. L.. Bonnemain and J. Bonmort. 1983. Nycthemeral changes in intracellular and apoplastic sugars in Vicia faba leaves. Physiol. Veg. 21:459-467.

Dempsey, G. P. and S. Bullivant. 1976. A copper block method for freezing noncryoprotected tissue to produce ice-crystal-free regions for electron microscopy. J. Microsc. 106:261-271.

Doman, D. and D. R. Geiger. 1979. Effect of exogenously supplied foliar potassium on phloem loading in Beta vulgaris L. Plant Physiol. 64:528-533.

Drew, M. C. and O. Biddulph. 1971. Effect of metabolic inhibitors and temperature on uptake and translocation of ^{45}Ca and ^{42}K by intact bean plants. Plant Physiol. 48:426-432.

Echlin, P., C. E. Lai and T. L. Hayes. 1982. Low-temperature X-ray microanalysis of the differentiating vascular tissue in root tips of Lemna minor L. J. Microsc. 126:285-306.

Echlin, P. and A. J. Saubermann. 1977. Preparation of biological specimens for X-ray microanalysis. Scanning Electron Microsc. 1977:621-637.

Edwards, A. and D. J. F. Bowling. 1984. An electrophysiological study of the stomatal complex of Tradescantia virginiana. J. Expt. Bot. 35:562-567.

Ehwald, R., D. Kowallick, A. B. Meshcheryakov and V. P. Kholodova. 1980. Sucrose leakage from isolated parenchyma of sugar beet roots. J. Exp. Bot. 31:607-620.

Epstein, E. 1956. Mineral nutrition of plants: mechanisms of uptake and transport. Annu. Rev. Plant Physiol. 7:1-24.

Epstein, E. 1961. The essential role of calcium in selective ion transport by plant cells. Plant Physiol. 36:437-444.

Epstein, E. 1962. Mutual effects of ions in their absorption by plants. Agrochimica 6:293-322.

Epstein, E. 1972. Mineral Nutrition of Plants: Principles and Perspectives. John Wiley and Sons, New York.

Epstein, E. 1976. Kinetics of ion transport and the carrier concept. pp. 70-94. In: U. Luttge and M. G. Pitman (eds.), Transport in Plants II. Encyc. of Plant Physiol., New Series, Vol. 2B. Springer-Verlag, Berlin.

Epstein, E. and J. E. Leggett. 1954. The absorption of alkaline earth cations by barley roots: kinetics and mechanism. Amer. J. Bot. 41:785-791.

Epstein, E., D. W. Rains and O. E. Elzam. 1963. Resolution of dual mechanism of potassium absorption by barley roots. Proc. Natl. Acad. Sci. 49:684-692.

Erwee, M. G. and P. B. Goodwin. 1985. Symplast domains in extrastelar tissues of Egeria densa Planch. Planta 163:9-19.

Erwee, M. G., P. B. Goodwin and A. J. E. Van Bel. 1985. Cell-cell communication in leaves of Commelina cyania and other plants. Plant Cell Environ. 8:173-178.

Esau, K. 1977. Anatomy of Seed Plants. John Wiley and Sons, New York.

Etherton, B. 1968. Vacuolar and cytoplasmic potassium concentrations in pea roots in relation to cell-to-medium electrical potentials. Plant Physiol. 43:838-840.

Evans, H. J. and G. J. Sorger. 1966. Role of mineral elements with emphasis on the univalent cations. Annu. Rev. Plant Physiol. 17:47-76.

Evans, H. J. and R. A. Wildes. 1971. Potassium and its role in enzyme activation. pp. 13-39. In: Potassium in Biochemistry and Physiology. Proc. 8th Cong. International Potash Institute, Bern.

Evert, R. F., C. E. J. Botha and R. J. Mierzwa. 1985. Free-space marker studies on the leaf of Zea mays L. Protoplasma 126:62-73.

Fagerberg, W. R. and G. Culpepper. 1984. A morphometric study of anatomical changes during sunflower leaf development under low light. Bot. Gaz. 145:346-350.

Ferguson, A. R. 1980. Xylem sap from Actinidia chinensis: apparent differences in sap composition arising from the method of collection. Ann. Bot. 46:791-802.

Ferguson, I. B. and E. G. Bollard. 1976. The movement of calcium in woody stems. Ann. Bot. 40:1057-1065.

Fitter, A. H. and R. K. M. Hay. 1981. Environmental Physiology of Plants. Academic Press, New York.

Flowers, T. J. and A. Lauchli. 1983. Sodium versus potassium: substitution and compartmentation. pp. 651-681. In: A. Lauchli and R. L. Bieleski (eds.), Inorganic Plant Nutrition. Encyc. of Plant Physiol., New Series, Vol. 15B. Springer-Verlag, Berlin.

Franke, W. 1967. Mechanisms of foliar penetration of solutions. Annu. Rev. Plant Physiol. 18:281-300.

Franklin, R. E. 1970. Effect of adsorbed cations on phosphorus absorption by various plant species. Agron. J. 62:214-216.

Gaff, D. F. and D. J. Carr. 1961. The quantity of water in the cell wall and its significance. Aust. J. Biol. Sci. 14:299-311.

Gaff, D. F., T. C. Chambers and K. Marcus. 1964. Studies of extrafascicular movement of water in the leaf. Aust. J. Biol Sci. 17:581-586.

Gerson, D. F. and R. J. Poole. 1972. Chloride accumulation by mung bean root tips. Plant Physiol. 50:603-607.

Giaquinta, R. T. 1976. Evidence for phloem loading from the apoplast. Chemical modification of membrane sulfhydryl groups. Plant Physiol. 57:872-875.

Giaquinta, R. T. 1980. Translocation of sucrose and oligosaccharides. pp. 271-320. In: J. Preiss (ed.), The Biochemistry of Plants, Vol. 3. Academic Press, New York.

Giaquinta, R. T. 1983. Phloem loading of sucrose. Annu. Rev. Plant Physiol. 34:347-387.

Glass, A. D. M. 1976. Regulation of potassium absorption in barley roots. An allosteric model. Plant Physiol. 58:33-37.

Glass, A. D. M. 1977. Regulation of K^+ influx in barley roots: evidence for direct control by internal K^+. Aust. J. Plant Physiol. 4:313-318.

Greene, D. W. and M. J. Bukovac. 1971. Factors influencing the penetration of naphthaleneacetamide into leaves of pear (Pyrus communis L.). J. Amer. Soc. Hort. Sci. 96:240-246.

Greenleaf, C. R. J., J. M. Ferrier and J. Dainty. 1980. Free charge is the source of net membrane potential. pp. 427-428. In: R. M. Spanswick, W. J. Lucas and J. Dainty (eds.), Plant Membrane Transport: Current Conceptual Issues. Elsevier, New York.

Greenway, H. and M. G. Pitman. 1965. Potassium retranslocation in seedlings of Hordeum vulgare. Aust. J. Biol. Sci. 18:235-247.

Gunning, B. E. S. and J. S. Pate. 1969. Transfer cells--plant cells with wall ingrowths, specialized in relation to short distance transport of solutes: their occurrence, structure and development. Protoplasma 68:107-133.

Gupta, B. L., T. A. Hall, S. H. P. Maddrell and R. B. Moreton. 1976. Distribution of ions in a fluid-transporting epithelium determined by electron probe X-ray microanalysis. Nature 264:284-287.

Hall, J. L., A. R. Yeo and T. J. Flowers. 1974. Uptake and localization of rubidium in the halophyte Suaeda maritima. Z. Pflanzenphysiol. Bd. 71:200-206.

Hall, S. M. and D. A. Baker. 1972. The chemical composition of Ricinus phloem exudate. Planta 106:131-140.

Hanson, J. B. 1984. The functions of calcium in plant nutrition. pp. 149-208. In: P. B. Tinker and A. Lauchli (eds.), Advances in Plant Nutrition. Praeger, New York.

Harvey, D. M. R., T. J. Flowers and J. L. Hall. 1979. Precipitation procedures for sodium, potassium and chloride localisation in leaf cells of the halophyte Suaeda maritima. J. Microsc. 116:213-226.

Harvey, D. M. R., J. L. Hall and T. J. Flowers. 1976. The use of freeze-substitution in the preparation of plant tissue for ion localization studies. J. Microsc. 107:189-198.

Harvey, D. M. R., J. L. Hall, T. J. Flowers and B. Kent. 1981. Quantitative ion localization within Suaeda maritima leaf mesophyll cells. Planta 151:555-560.

Hawker, J. S., H. Marschner and W. J. S. Downton. 1974. Effects of sodium and potassium on starch synthesis in leaves. Aust. J. Plant Physiol. 1:491-501.

Haynes, R. J. 1980. Ion exchange properties of roots and ionic interactions within the root apoplasm: their role in ion accumulation by plants. Bot. Rev. 46:75-99.

Hayward, H. E. 1967. The Structure of Economic Plants. Macmillan, New York.

Hess, F. D. 1980. Influence of specimen topography on microanalysis. pp. 241-262. In: M. A. Hayat (ed.), X-Ray Microanalysis in Biology. University Park Press, Baltimore.

Hoch, H. C., C. Pratt and G. A. Marx. 1980. Subepidermal air spaces: basis for the phenotypic expression of the Argenteum mutant of Pisum. Amer. J. Bot. 27:905-911.

Hocking, P. J. and J. S. Pate. 1978. Accumulation and distribution of mineral elements in annual lupins Lupinus albus and Lupinus angustifolius L. Aust. J. Agric. Res. 29:267-280.

Hodges, T. K. 1976. ATPases associated with membranes of plant cells. pp. 260-283. In: U. Luttge and M. G. Pitman (eds.), Transport in plants II. Encyc. of Plant Physiol., New Series, Vol. 2A. Springer-Verlag, Berlin.

Hodson, S. and J. Marshall. 1970. Ultracryotomy: a technique for cutting ultrathin frozen sections of unfixed biological tissues for electron microscopy. J. Microsc. 91:105-117.

Hope, A. B. and P. G. Stevens. 1952. Electric potential differences in bean roots and their relation to salt uptake. Aust. J. Sci. Res. B5:335-343.

Hosokawa, Y. and K. Kiyosawa. 1985. Diurnal Rb^+ transport from roots to laminar pulvinus in Phaseolus vulgaris L. Ann. Bot. 55:195-200.

Hsiao, T. C. 1976. Stomatal ion transport. pp. 195-221. In: U. Luttge and M. G. Pitman (eds.), Transport in Plants II. Encyc. of Plant Physiol., New Series, Vol. 2B. Springer-Verlag, Berlin.

Humble, G. D. and K. Raschke. 1971. Stomatal opening quantitatively related to potassium transport. Evidence from electron probe analysis. Plant Physiol. 48:447-453.

Hutchings, V. M. 1978. Sucrose and proton cotransport in Ricinus cotyledons. II. H^+ efflux and associated K^+ uptake. Planta 138:237-241.

Hylmo, B. 1953. Transpiration and ion absorption. Physiol. Plant. 6:333-405.

Ighe, U. and S. Pettersson. 1974. Metabolism-linked binding of rubidium in the free space of wheat roots and its relation to active uptake. Physiol. Plant. 30:24-29.

Ingram, F. D., M. J. Ingram and C. A. M. Hogben. 1972. Quantitative electron probe analysis of soft biological tissue for electrolytes. J. Histochem. Cytochem. 20:716-718.

Jachetta, J. J., A. P. Appleby and L. Boersma. 1986a. Apoplastic and symplastic pathways of atrazine and glyphosate transport in shoots of seedling sunflower. Plant Physiol. 82:1000-1007.

Jachetta, J. J., A. P. Appleby and L. Boersma. 1986b. Use of the pressure vessel to measure concentrations of solutes in apoplastic and membrane-filtered symplastic sap in sunflower leaves. Plant Physiol. 82:995-999.

Jackson, D. C. and D. G. Edwards. 1966. Cation effects on chloride fluxes and accumulation levels in barley roots. J. Gen. Physiol. 50:225-241.

Jacobson, L., R. J. Hannapel and D. P. Moore. 1958. Non-metabolic uptake of ions by barley roots. Plant Physiol. 33:278-282.

Jacobson, S. L. 1971. A method for extraction of extracellular fluid: use in development of a physiological saline for Venus flytrap. Can. J. Bot. 49:121-127.

Kesseler, H. 1980. On the selective adsorption of cations in the cell wall of the green alga Valonia utricularis. Helg. Meeres. 34:151-158.

Kirk, B. I. and J. B. Hanson, 1973. The stoichiometry of respiration-driven potassium transport in corn mitochondria. Plant Physiol. 51:357-362.

Kirkby, E. A. and M. J. Armstrong. 1980. Nitrate uptake by roots as regulated by nitrate assimilation in the shoot of castor oil plants. Plant Physiol. 65:286-290.

Kiyosawa, K. and H. Tanaka. 1976. Change in potassium distribution in Phaseolus pulvinus during circadian movement of the leaf. Plant Cell Physiol. 17:289-298.

Klepper, B. and M. R. Kaufmann. 1966. Removal of salt from xylem sap by leaves and stems of guttating plants. Plant Physiol. 41:1743-1747.

Kochian, L. U. and W. J. Lucas. 1983. Potassium transport in corn roots. II. The significance of the root periphery. Plant Physiol. 73:208-215.

Kursanov, A. L. 1984. Assimilate Transport in Plants. Elsevier, New York.

Kylin, A. 1957. The apparent free space of Vallisneria leaves. Physiol. Plant. 10:732-740.

Kylin, A. 1960. The apparent free space of green tissues. Physiol. Plant. 13:385-397.

Kylin, A. and B. Hylmo. 1957. Uptake and transport of sulphate in wheat. Active and passive components. Physiol. Plant. 10:467-484.

Laties, G. G. 1959. Active transport of salt into plant tissue. Annu. Rev. Plant Physiol. 10:87-112.

Lauchli, A. 1969. Radioassay for β-emitters in biological materials using Cerenkov radiation. Intl. J. Appl. Rad. Isot. 20:265-270.

Lauchli, A. 1972a. Electron probe analysis. pp. 191-236. In: U. Luttge (ed.), Methods of Microautoradiography and Electron Probe Analysis. Springer-Verlag, Berlin.

Lauchli, A. 1972b. Translocation of inorganic solutes. Annu. Rev. Plant Physiol. 23:197-218.

Lauchli, A. 1976. Apoplasmic transport in tissues. pp. 3-34. In: U. Luttge and M. G. Pitman (eds.), Transport in Plants II. Encyc. of Plant Physiol., New Series, Vol. 2B. Springer-Verlag, Berlin.

Lauchli, A. and E. Epstein. 1970. Transport of potasssium and rubidium in plant roots. The significance of calcium. Plant Physiol. 45:639-641.

Lauchli, A. and R. Pfluger. 1978. Potassium transport through plant cell membranes and metabolic role of potassium in plants. pp. 111-163. In: Potassium Research--Review and Trends. Proc. 11th Cong. International Potash Institute, Bern.

Lauchli, A., A. R. Spurr and R. W. Wittkopp. 1970. Electron probe analysis of freeze-substituted, epoxy resin embedded tissue for ion transport studies in plants. Planta 95:341-350.

Leonard, R. T. 1982. The plasma membrane ATPase of plant cells: cation or proton pump? pp. 633-637. In: A. N. Martonosi (ed.), Membranes and Transport, Vol. 2. Plenum Press, New York.

Leopold, A. C. 1980. Temperature effects on soybean imbibition and leakage. Plant Physiol. 65:1096-1098.

Levitt, J. 1956. The physical nature of transpirational pull. Plant Physiol. 31:248-251.

Levitt, J. 1957. The significance of "Apparent Free Space" (A.F.S.) in ion absorption. Physiol. Plant. 10:882-888.

Lingle, J. C. and O. A. Lorenz. 1969. Potassium nutrition of tomatoes. J. Amer. Soc. Hort. Sci. 94:679-683.

Little, T. M. and F. J. Hills. 1978. Agricultural Experimentation. Design and Analysis. John Wiley and Sons, New York.

Lott, J. N. A., J. S. Greenwood, C. M. Vollmer and M. S. Buttrose. 1978. Energy-dispersive X-ray analysis of phosphorus, potassium, magnesium and calcium in globoid crystals in protein bodies from different regions of Cucurbita maxima embryos. Plant Physiol. 61:984-988.

Luttge, U. 1983. Import and export of mineral nutrients in plant roots. pp. 181-211. In: A. Lauchli and R. L. Bieleski (eds.), Inorganic Plant Nutrition. Encyc. of Plant Physiol., New Series, Vol. 15A. Springer-Verlag, Berlin.

Luttge, U. and N. Higinbotham. 1979. Transport in Plants. Springer-Verlag, Berlin.

Luttge, U. and M. G. Pitman. 1976a. General introduction. Transport processes in leaves. pp. 157-159. In: U. Luttge and M. G. Pitman (eds.), Transport in Plants II. Encyc. of Plant Physiol., New Series, Vol. 2B. Springer-Verlag, Berlin.

Luttge, U. and M. G. Pitman. 1976b. Transport and energy. pp. 251-259. In: U. Luttge and M. G. Pitman (eds.), Transport in Plants II. Encyc. of Plant Physiol., New Series, Vol. 2A. Springer-Velag, Berlin.

Lyons, J. M. and J. K. Raison. 1973. Chilling injury in plants. Annu. Rev. Plant Physiol. 24:445-466.

MacDonald, I. R. 1975. Effect of vacuum infiltration on photosynthetic gas exchange in leaf tissue. Plant Physiol. 56:109-112.

MacDonald, I. R. and A. E. S. Macklon. 1972. Anion absorption by etiolated wheat leaves after vacuum infiltration. Plant Physiol. 49:303-306.

MacNicol, D. K., R. E. Young and J. B. Biale. 1973. Metabolic regulation in the senescing tobacco leaf. Plant Physiol. 51:793-797.

MacRobbie, E. A. C. and J. Dainty. 1958. Ion transport in Nitellopsis obtusa. J. Gen. Physiol. 42:335-353.

Malek, F. and D. A. Baker. 1977. Proton co-transport of sugars in phloem loading. Planta 135:297-299.

Madore, M., J. W. Oross and W. J. Lucas. 1986. Symplastic transport in Ipomea tricolor source leaves. Plant Physiol. 82:432-442.

Madore, M. and J. A. Webb. 1981. Leaf free space analysis and vein loading in Cucurbita pepo. Can. J. Bot. 59:2550-2557.

Markhart, A. H. and A. Lauchli, 1982. The comparison of three freezing methods for electron probe X-ray microanalysis of hydrated barley root tissue. Plant Sci. Letters 25:29-36.

Marschner, H. 1983. General introduction to the mineral nutrition of plants. pp. 5-60. In: A. Lauchli and R. L. Bieleski (eds.), Inorganic Plant Nutrition. Encyc. of Plant Physiol., New Series, Vol. 15A. Springer-Verlag, Berlin.

Marschner, H. and J. V. Possingham. 1975. Effect of K^+ and Na^+ on growth of leaf discs of sugar beet and spinach. Z. Pflanzenphysiol. Bd. 75:6-16.

Marshall, A. T. 1972. Ionic analysis of frozen sections in the electron microscope. Micron 3:99-100.

Marshall, A. T. 1980a. Frozen-hydrated bulk specimens. pp. 167-196. In: M. A. Hayat (ed.), X-Ray Microanalysis in Biology. University Park Press, Baltimore.

Marshall, A. T. 1980b. Frozen-hydrated sections. pp. 197-205. In: M. A. Hayat (ed.), X-Ray Microanalysis in Biology. University Park Press, Baltimore.

Marshall, A. T. 1980c. Principles and instrumentation. pp. 1-64. In: M. A. Hayat (ed.), X-Ray Microanalysis in Biology. University Park Press, Baltimore.

Marshall, A. T. 1980d. Sections of freeze-substituted specimens. pp. 207-239. In: M. A. Hayat (ed.), X-Ray Microanalysis in Biology. University Park Press, Baltimore.

Marx, G. A. 1982. Argenteum (Arg) mutant of Pisum. Genetic control and breeding behavior. J. Hered. 73:413-420.

Mecklenburg, R. A., H. B. Tukey, Jr., and J. V. Morgan. 1966. A mechanism for the leaching of calcium from foliage. Plant Physiol. 41:610-613.

Mengel, K. and W. W. Arneke. 1982. Effect of potassium on the water potential, the pressure potential, the osmotic potential and cell elongation in leaves of Phaseolus vulgaris. Physiol. Plant. 54: 402-408.

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.

Mengel, K. and M. Helal. 1967. The effect of the exchangeable Ca^{2+} of young barley roots on the flux of K^+ and phosphate--an interpretation of the Viets effect. Z. Pflanzenphysiol. Bd. 57:223-234.

Mengel, K. and E. A. Kirkby. 1982. Principles of Plant Nutrition. International Potash Institute, Bern.

Mengel, K. and R. Pfluger. 1972. The release of potassium and sodium from young excised roots of Zea mays under various efflux conditions. Plant Physiol. 49:16-19.

Mengel, K. and M. Viro. 1974. Effect of potassium supply on the transport of photosynthates to the fruits of tomatoes (Lycopersicon esculentum). Physiol. Plant. 30:295-300.

Mitchell, P. 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev. 41:445-502.

Mondal, M. H. 1975. Effects of gibberellic acid, calcium, kinetin and ethylene on growth and cell wall composition of pea epicotyls. Plant Physiol. 56:622-625.

Moorby, J. and R. T. Besford, 1983. Mineral nutrition and growth. pp. 481-527. In: A. Lauchli and R. L. Bieleski (eds.), Inorganic Plant Nutrition. Encyc. of Plant Physiol., New Series, Vol. 15B. Springer-Verlag, Berlin.

Morgan, A. J. 1979. Non-freezing techniques of preparing biological specimens for electron microprobe X-ray microanalysis. Scanning Electron Microsc. 1979:635-648.

Morgan, A. J. 1980. Preparation of specimens. Changes in chemical integrity. pp. 65-165. In: M. A. Hayat (ed.), X-Ray Microanalysis in Biology. University Park Press, Baltimore.

Morrod, R. S. 1974. A new method for measuring the permeability of plant cell membranes using epidermis-free leaf discs. J. Exp. Bot. 25:521-533.

Morvan, C., M. Demarty, A. Monnier, R. Muqbill and M. Thellier. 1980. Titration of isolated cell walls of Lemna minor L. pp. 423-426. In: R. M. Spanswick, W. J. Lucas and J. Dainty (eds.), Plant Membrane Transport: Current Conceptual Issues. Elsevier, New York.

Muhlethaler, K. 1967. Ultrastructure and formation of plant cell walls. Annu. Rev. Plant Physiol. 18:1-24.

Nagahashi, G., W. W. Thomson and R. T. Leonard. 1974. The casparian strip as a barrier to the movement of lanthanum in corn roots. Science 183:670-671.

Nakajima, N., H. Morikawa, S. Igarashi and M. Senda. 1981. Differential effect of calcium and magnesium on mechanical properties of pea stem cell walls. Plant Cell Physiol. 22:1305-1315.

Neumann, P. M. and Z. Stein. 1983. Xylem transport and the regulation of leaf metabolism. What's New Plant Physiol. 14:33-36.

Nobel, P. S. 1975. Chloroplasts. pp. 101-124. In: D. A. Baker and J. L. Hall (eds.), Ion Transport in Plant Cells and Tissues. Elsevier, Amsterdam.

Northcote, D. H. 1972. Chemistry of the plant cell wall. Annu. Rev. Plant Physiol. 23:113-132.

O'Brien, T. D. and D. J. Carr. 1970. A suberized layer in the cell walls of the bundle sheath of grasses. Aust. J. Biol Sci. 23:275-287.

Oertli, J. J. 1968. Extracellular salt accumulation, a possible mechanism of salt injury in plants. Agrochim. 12:461-469.

Osmond, C. B. 1968. Ion absorption in Atriplex leaf tissue. I. Absorption by mesophyll cells. Aust. J. Biol. Sci. 21:1119-1130.

O'Toole, J. C., K. Treharne, M. Turnipseed, K. Crookston and J. Ozbun. 1980. Effect of potassium nutrition on leaf anatomy and net photosynthesis of Phaseolus vulgaris L. New Phytol. 84:623-630.

Pallaghy, C. K. 1973. Electron probe microanalysis of potassium and chloride in freeze-substituted leaf sections of Zea mays. Aust. J. Biol. Sci. 26:1015-1034.

Pate, J. S. and B. E. S. Gunning. 1969. Perivascular transfer cells in angiosperm leaves: a taxonomic and morphological survey. Protoplasma 68:135-156.

Peel, A. J. 1963. The movement of ions from the xylem solution into the sieve tubes of willow. J. Exp. Bot. 14:438-447.

Peel, A. J. and S. Rogers. 1982. Stimulation of sugar loading into sieve elements of willow by potassium salts. Planta 154:94-96.

Penny, M. G. and D. J. F. Bowling. 1974. A study of potassium gradients in the epidermis of intact leaves of Commelina communis L. in relation to stomatal opening. Planta 119:17-25.

Penny, M. G., K. G. Moore and P. H. Lovell. 1976. Effects of potassium deficiency on cotyledon photosynthesis and seedling development in Cucumis sativus L. Ann. Bot. 40:981-991.

Peoples, T. R. and D. W. Koch. 1979. Role of potassium in carbon dioxide assimilation in Medicago sativa L. Plant Physiol. 63:878-881.

Persson, J. 1969. Labile-bound sulphates in wheat roots: localization, nature and possible connection to the active absorption mechanism. Physiol. Plant. 22:959-976.

Peterson, T. A., E. S. Swanson and R. J. Hull. 1986. Use of lanthanum to trace apoplastic solute transport in intact plants. J. Exp. Bot. 37:807-822.

Pettersson, S. 1966. Active and passive components of sulphate uptake in sunflower plants. Physiol. Plant. 19:459-492.

Pierce, W. S. and N. Higinbotham. 1970. Compartments and fluxes of K^+, Na^+ and Cl^- in Avena coleoptile cells. Plant Physiol. 46:666-673.

Pitman, M. G. 1963. The determination of the salt relations of the cytoplasmic phase in cells of beetroot tissue. Aust. J. Biol. Sci. 16:647-668.

Pitman, M. G. 1965a. Ion exchange and diffusion in roots of Hordeum vulgare. Aust. J. Biol. Sci. 18:541-546.

Pitman, M. G. 1965b. The location of the Donnan free space in discs of beetroot tissue. Aust. J. Biol. Sci. 18:547-553.

Pitman, M. G. 1969. Adaptation of barley roots to low oxygen supply and its relation to potassium and sodium uptake. Plant Physiol. 44:1233-1240.

Pitman, M. G. 1975. Whole plants. pp. 267-308. In: D. A. Baker and J. L. Hall (eds.), Ion Transport in Plant Cells and Tissues. Elsevier, New York.

Pitman, M. G., A. Lauchli and R. Stelzer. 1981. Ion distribution in roots of barley seedlings measured by electron probe X-ray microanalysis. Plant Physiol. 68:673-679.

Pitman, M. G., U. Luttge, D. Kramer and E. Ball. 1974a. Free space characteristics of barley leaf slices. Aust. J. Plant Physiol. 1:65-75.

Pitman, M. G., U. Luttge, A. Lauchli and E. Ball. 1974b. Ion uptake to slices of barley leaves and regulation of K^+ content in cells of the leaves. Z. Pflanzenphysiol. Bd. 72:75-88.

Poole, R. J. 1978. Energy coupling for membrane transport. Annu. Rev. Plant Physiol. 29:437-460.

Preston, R.D. 1979. Polysaccharide conformation and cell wall function. Annu. Rev. Plant Physiol. 30:55-78.

Priestley, J. H. 1929. Cell growth and cell division in the shoot of the flowering plant. New Phytol. 28:54-81.

Protter, M. H. and C. B. Morrey, Jr. 1967. Calculus for College Students. Addison-Wesley, Reading, Mass.

Rains, D. W., W. E. Schmidt and E. Epstein. 1964. Absorption of cations by roots. Effects of hydrogen ions and essential role of calcium. Plant Physiol. 39:274-278.

Raschke, K. and M. P. Fellows. 1971. Stomatal movement in Zea mays: shuttle of potassium and chloride between guard cells and subsidiary cells. Planta 101:296-316.

Robards, A. W. and M. E. Robb. 1974. The entry of ions and molecules into roots: an investigation using electron-opaque tracers. Planta 120:1-12.

Robertson, R. N. 1951. Mechanism of absorption and transport of inorganic nutrients in plants. Annu. Rev. Plant Physiol. 2:1-25.

Robinson, J. B. 1971. Salinity and the whole plant. pp. 193-206. In: T. Talsma and J. R. Philip (eds.), Salinity and Water Use. John Wiley and Sons, New York.

Robinson, J. B., and F. A. Smith. 1970. Chloride influx into Citrus leaf slices. Aust. J. Biol. Sci. 23:953-958.

Robson, A. D. and M. G. Pitman. 1983. Interactions between nutrients in higher plants. pp. 147-180. In: A. Lauchli and R. L. Bieleski (eds.), Inorganic Plant Nutrition. Encyc. of Plant Physiol, New Series, Vol. 15A. Springer-Verlag, Berlin.

Satter, R. L. and A. W. Galston. 1971. Phytochrome-controlled nyctinasty in Albizzia librium. III. Interaction between endogenous rhythm and phytochrome in control of potassium flux and leaflet movement. Plant Physiol. 48:740-746.

Satter, R. L., R. C. Garber, L. Khairallah and Y. Cheng. 1982. Elemental analysis of freeze-dried thin sections of Samanea motor organs: barriers to ion diffusion through the apoplast. J. Cell. Biol. 95:893-902.

Scholander, P. F., E. D. Bradstreet, H. T. Hammel and E. A. Hemmingsen. 1966. Sap concentrations in halophytes and some other plants. Plant Physiol. 41:529-532.

Scott, L. I. and J.H. Priestley. 1928. The root as an absorbing organ. I. A reconsideration of the entry of water and salts in the absorbing region. New Phytol. 27:125-174.

Seveus, L. 1978. Preparation of biological material for X-ray microanalysis of diffusible elements. J. Microsc. 112:269-279.

Seveus, L. 1980. Cryoultramicrotomy as a preparation method for X-ray microanalysis. Scanning Electron Microsc. 1980:101-170.

Shone, M. G. T. and A. V. Flood. 1985. Measurement of free space and sorption of large molecules by cereal roots. Plant Cell Environ. 8:309-315.

Smith, F. A. and A. L. Fox. 1975. The free space of Citrus leaf slices. Aust. J. Plant Physiol. 2:441-446.

Smith, F. A. and J. A. Raven. 1976. H^+ transport and regulation of cell pH. pp. 317-346. In: U. Luttge and M. G. Pitman (eds.), Transport in Plants II. Encyc. of Plant Physiol., New Series, Vol. 2A. Springer-Verlag, Berlin.

Smith, F. A. and J. B. Robinson. 1971. Sodium and potassium influx in Citrus leaf slices. Aust. J. Biol. Sci. 24:861-871.

Smith, R. C. and E. Epstein. 1964a. Ion absorption by shoot tissue: kinetics of potassium and rubidium absorption by corn leaf tissue. Plant Physiol. 39:992-996.

Smith, R. C. and E. Epstein. 1964b. Ion absorption by shoot tissue: technique and first findings with excised leaf tissue of corn. Plant Physiol. 39:338-341.

Sommarin, M., T. Lundborg and A. Kylin. 1985. Comparison of K, Mg ATPases in purified plasmalemma from wheat and oat. Substrate specificities and effects of pH, temperature and inhibitors. Physiol. Plant. 65:27-32.

Sotera, J. J., M. F. Bancroft, S. B. Smith, Jr., and T. L. Corum. 1981. Atomic Absorption Methods Manual, Vol. 2. Flameless Operations. Instrumentation Laboratory, Andover, Mass.

Sotera, J. J. and R. L. Stux. 1979. Atomic Absorption Methods Manual, Vol. 1. Standard Conditions for Flame Operation. Instrumentation Laboratory, Andover, Mass.

Spurr, A. R. 1980. Applications of X-ray microanalysis in botany. Scanning Electron Microsc. 1980:535-564.

Steel, R. G. and J. H. Torrie. 1980. Principles and Procedures of Statistics. McGraw-Hill, New York.

Steineck, O. and H. E. Haeder. 1978. The effect of potassium on growth and yield components of plants. pp. 165-187. In: Potassium Research--Review and Trends. Proc. 11th Cong. International Potash Institute, Bern.

Stiles, W. and A. D. Skelding. 1940. The salt relations of plant tissues. II. The absorption of manganese salts by storage tissue. Ann. Bot. 4:673-700.

Suelter, C. H. 1970. Enzymes activated by monovalent cations. Science 168:789-795.

Sutcliffe, J. F. 1976. Regulation in the whole plant. pp. 394-417. In: U. Luttge and M. G. Pitman (eds.), Transport in Plants II. Encyc. of Plant Physiol., New Series, Vol. 2B. Springer-Verlag, Berlin.

Thompson, W. W., K. A. Platt and N. Campbell. 1973. The use of lanthanum to delineate the apoplastic continuum in plants. Cytobios 8:57-62.

Ting, I. W. 1982. Plant Physiology. Addison-Wesley, Reading, Mass.

Turrell, F. M. 1936. The area of the internal exposed surface of dicotyledon leaves. Amer. J. Bot. 23:255-264.

Tyree, M. T. and H. Richter. 1982. Alternate methods of analyzing water potential isotherms: some cautions and clarifications. II. Curvilinearity in water potential isotherms. Can J. Bot. 60:911-916.

Vakhmistrov, D. B. 1967. On the function of the apparent free space in plant roots. A study of the absorbing power of epidermal and cortical cells in barley roots. Sov. Plant Physiol. 14:103-107.

Van Iren, F. and P. B. Van der Sluijs. 1980. Symplasmic and apoplasmic radial ion transport in plant roots. Planta 148:130-137.

Van Steveninck, R. F. M. 1962. Potassium fluxes in red beet tissue during its "lag phase." Physiol. Plant. 15:211-215.

Van Steveninck, R. F. M. 1976. Cellular differentiation, aging, and ion transport. pp. 343-371. In: U. Luttge and M. G. Pitman (eds.), Transport in Plants II. Encyc. of Plant Physiol., New Series, Vol 2B. Springer-Verlag, Berlin.

Van Steveninck, R. F. M. and A. R. F. Chenoweth. 1972. Ultrastructural localization of ions. I. Effect of high external sodium chloride concentration on the apparent distribution of chloride in leaf parenchyma cells of barley seedlings. Aust. J. Biol. Sci. 25:499-516.

Van Steveninck, R. F. M. and M. E. Van Steveninck. 1978. Ion localization. pp. 187-233. In: J. L. Hall (ed.), Electron Microscopy and Cytochemistry of Plant Cells. Elsevier, Amsterdam.

Viets, F. 1944. Calcium and other polyvalent cations as accelerators of ion accumulation by excised barley roots. Plant Physiol. 19:466-480.

Vreugdenhil, D. 1985. Source-to-sink gradient of potassium in the phloem. Planta 163:238-240.

Walker, N. A. and M. G. Pitman. 1976. Measurements of fluxes across membranes. pp. 93-126. In: U. Luttge and M. G. Pitman (eds.), Transport in Plants II. Encyc. of Plant Physiol., New Series, Vol. 2A. Springer-Verlag, Berlin.

Wallace, A. 1968. Retranslocation of ^{86}Rb, ^{137}Cs and K to new leaf growth in bush beans. Plant Soil 29:184-188.

Waughman, G. J. and D. J. Bellamy. 1981. Movement of cations in some plant species prior to leaf senescence. Ann. Bot. 47:141-145.

Weatherley, P. E. 1970. Some aspects of water relations. pp. 171-206. In: R. D. Preston (ed.), Advances in Botanical Research, Vol. 3. Academic Press, New York.

Widders, I. E. and O. A. Lorenz. 1979. Tomato root development as related to potassium nutrition. J. Amer. Soc. Hort. Sci. 104:216-220.

Widders, I. E. and O. A. Lorenz. 1982a. Ontogenetic changes in potassium transport in xylem of tomato. Physiol. Plant. 56:458-464.

Widders, I. E. and O. A. Lorenz. 1982b. Potassium nutrition during tomato plant development. J. Amer. Soc. Hort. Sci. 107:960-964.

Widders, I. E. and O. A. Lorenz. 1983a. Effect of leaf age on potassium efflux and net flux in tomato leaf slices. Ann. Bot. 52:499-506.

Widders, I. E. and O. A. Lorenz. 1983b. Effects of leaf age and position on the shoot axis on potassium absorption by tomato leaf slices. Ann. Bot, 52:489-498.

Winter, H. 1961. The uptake of cations by Vallisneria leaves. Acta Bot. Neerl. 10:341-392.

Zimmerman, U. 1978. Physics of turgor and osmoregulation. Annu. Rev. Plant Physiol. 29:121-148.