ELECTRON PROBE MICROANALYSIS OF POTASSIUM AND CHLORIDE IN FREEZE-SUBSTITUTED LEAF SECTIONS OF *ZEA MAYS*

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Abstract

Small sections of leaves were floated on distilled water under either light or dark conditions, and were freeze-substituted in a 1% solution of osmium tetroxide in acetone at −78°C followed by embedding in an epoxy resin. Approximately 1-μm-thick sections were cut using a dry diamond knife and examined by scanning transmission electron microscopy. The relative concentrations of potassium and chloride in subcellular compartments were determined using an energy dispersive X-ray analyser. The concentration of sodium in the leaf (1·7 m-equiv/kg of wet tissue) was too low to be detected by this method. The spatial resolution of this technique was sufficient to distinguish between concentrations in the chloroplasts, cytoplasm, vacuole, and nuclei. The concentration of chloride in stomata and some other epidermal cells was very much higher than in either mesophyll or bundle sheath cells. The potassium concentration in some vascular cells was at least two- to threefold higher than that in mesophyll or bundle sheath cells. The Cl : K ratio in mesophyll and bundle sheath cells resembled that in the solution (0·10) used for growing the plants. The concentration of chloride in the "free" cytoplasm of mesophyll cells was always very low. Significant differences were found in the "ion" relations of mesophyll and bundle sheath cells. Whereas the ratio of potassium concentration between the vacuole and chloroplasts of mesophyll cells was high (1·19) in the light and low (0·65) in the dark, the opposite was true for bundle sheath cells—0·65 and 0·86 respectively. The ratio of potassium concentration between the vacuoles of mesophyll and those of bundle sheath cells was 1·48 in the light, but only 0·76 in the dark. These concentration gradients are discussed in relation to a possible transfer of organic acid salts of potassium between these two cell types.

I. INTRODUCTION

Localization of elements in individual cells or layers of cells in tissues by electron probe analysis has been successfully applied in a number of recent investigations using both animal (Tousimis 1969; Gehring *et al.* 1971; Sutfin *et al.* 1971; Ingram *et al.* 1972) and plant tissues (Läuchli 1967; Läuchli and Lüttge 1968; Sawhney and Zelitch 1969; Läuchli *et al.* 1970, 1971; Humble and Raschke 1971; Kaufman *et al.* 1971; Raschke and Fellows 1971; Satter and Galston 1971; Soni *et al.* 1972). With this technique, specific elements can be identified from their characteristic X-ray emission spectra (Sutfin and Ogilvie 1970; Russ 1972), thus avoiding the possibility of misinterpretation which seems to be a feature of methods based on the use of ion-specific stains (Weavers 1971; van Steveninck *et al.* 1973).

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Preparation of biological tissues for electron probe analysis may involve freeze-drying (e.g. Raschke and Fellows 1971; Ingram *et al.* 1972), cryosectioning (e.g. Läuchli and Lüttge 1968; Tousimis 1969; Gehring *et al.* 1971; Waisel and Eshel 1971), or freeze-substitution (Läuchli *et al.* 1970; Fisher and Housley 1972). Ultra-cryomicrotomy (Appleton 1972), although perhaps potentially the most useful technique, nevertheless requires either prior fixation of the tissue with glutaraldehyde which leads to a loss of ions (Tousimis 1969), or pretreatment of the tissue with cryoprotective agents such as glycerol or dimethyl sulphoxide to reduce ice crystal growth (Moor *et al.* 1961; Mazur 1969). The latter method involves either growing the material in a concentrated solution of the cryoprotectant or infiltrating the tissue with it prior to snap-freezing the tissue, both of which are untenable from a physiological standpoint in most situations. Even if cryoprotectants were used, the uneven growth of ice crystals in highly vacuolated plant cells would present a formidable problem for cryosectioning (Gehring and Doerge, personal communication).

Although freeze-drying may be applicable under certain conditions, it is known to distort tissue (Bieleski 1966; Trip and Gorham 1967), particularly at the subcellular level.

Läuchli *et al.* (1970) reported a method of freeze-substitution suitable for embedding plant tissues in an epoxy resin. The chief attraction of this technique is that relatively thin sections (1–0·5 μm) can be analysed. Tousimis (1969) demonstrated that under optimum conditions a spatial resolution of 0·2 μm may be achieved. For valid results, the technique of freeze-substitution requires the greatest care with regard to anhydrous procedures (Fisher 1972; Fisher and Housley 1972).

This paper reports a preliminary study of the "ion" relations of mesophyll and bundle sheath cells in leaf sections of *Zea mays*, using the method of freeze-substitution (Rebhun 1965; Hereward and Northcote 1972) for sample preparation. *Z. mays* is one of a group of plants having the so-called C_4-dicarboxylic acid photosynthetic pathway (Hatch and Slack 1970) in which the bundle sheath chloroplasts differ from the mesophyll chloroplasts in being deficient in photosystem II (Polya and Osmond 1972). Similar studies, but employing $^{36}Cl^-$ tracer techniques, have been made by Lüttge (1971) and Lüttge *et al.* (1971). This paper describes a technique suitable for the measurement of endogenous levels of "ions" in a variety of cellular compartments of the leaf without having to immerse leaf sections in radioactively labelled solutions (MacRobbie 1971). The intention of these investigations is to study ultimately the ion relations of intact leaves.

II. Methods

(*a*) *Materials and Pretreatment of Tissue*

Z. mays (hybrid DS-28, purchased from Gippsland and Northern Co. Ltd.) was grown in the glasshouse in Hoagland solution (Table 1) supported by a mixture of sand and peat moss.

For the experiments the fifth leaves, counted in sequence of emergence, were used when the plant was about 3 weeks old. Leaves were kept in a beaker of distilled water for 20 min prior to use. An area measuring 10 by 5 mm was then cut from a region approximately halfway along the length of the leaf and immersed in distilled water. Immersion of leaf cells in distilled water (Pallaghy 1971) or in electrolyte solutions (Osmond 1968; Rains 1968) does not appear to impair their physiological behaviour. A large number of small sections (2 by 0·5 mm) were quickly cut with a razor blade and randomly distributed into two Petri dishes of distilled water. One lot was placed into a light cabinet

(12,900 lux, G.E.C. 400-W MBFR/U mercury lamps) while the other was kept under dark conditions. Temperature was maintained at 22±0·5°C.

TABLE 1

CONCENTRATIONS OF IONS IN CULTURE MEDIUM AND IN UNWASHED LEAF TISSUE OF *Z. MAYS**

Ion	Concn. in Hoagland's solution (m-equiv/l)	Concn. in leaf tissue (m-equiv/kg fresh wt.)	Ion	Concn. in Hoagland's solution (m-equiv/l)	Concn. in leaf tissue (m-equiv/kg fresh wt.)
Mg^{2+}	4·0	—	Fe^{3+} (EDTA complex)	0·037	—
Ca^{2+}	8·0	low	$H_2PO_4^-$	1·1	—
K^+	6·0	155	SO_4^{2-}	4·0	1·4
Na^+	0·61	1·7	NO_3^-	14·1	—
NH_4^+	1·1	—	Cl^-	0·60	42

* The midrib of the leaf was excluded from analysis. Analyses were kindly performed by Water Science Laboratories Pty. Ltd., Melbourne.

(*b*) *Preparation of Tissue*

(i) *General*

For freeze-substitution the sample is rapidly frozen and the ice slowly replaced by an anhydrous solvent at low temperature followed by low-temperature infiltration of the tissue with an appropriate embedding medium. Osmium tetroxide (1%) is sometimes included in the anhydrous solvent to improve clarity of the sections when viewed by transmission electron microscopy and to effect partial fixation of the tissue at low temperature (Rebhun 1965; Hereward and Northcote 1972). All handling of the sample must be carried out under the most stringent anhydrous conditions (using activated molecular sieve—Union Carbide, type 3A PLTS) right up to the point of introduction of the sample into the vacuum chamber of the scanning electron microscope. Fisher (1972), Fisher and Housley (1972), and De Filippis and Pallaghy (1973) have shown that migration of water-soluble substances will occur if the samples are not maintained in an anhydrous state for each step of the preparation procedure. The molecular sieve used in the present study was always activated by heating in a Pyrex tube, under vacuum, over a bunsen flame.

During this study the following additional problems related to the embedding of the tissue were encountered.

(1) The Spurr's medium (Spurr 1969), and particularly the flexibilizer DER 736 (Polysciences), used in some of this work was found to contain a high concentration of chlorine. This was eliminated by using a modification of Spurr's formula as follows:

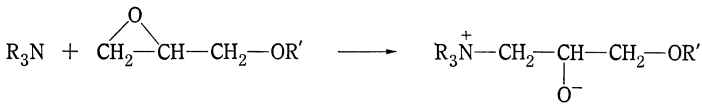
Ingredient	Weight	Ingredient	Weight
Vinylcyclohexene dioxide (VCD)	10	Dibutyl phthalate	12
Nonenyl succinic anhydride	26	DY 064 (CIBA)	1·6

This was preferable to using Epon (Ingram *et al.* 1972), since the low viscosity feature of Spurr's formula could be retained.

(2) For flattening of the sections cut on the dry knife, methyl cyclohexane was occasionally helpful. Chloroform vapour could not be used since it is readily absorbed by the section, giving rise to a high level of chlorine contamination in the sample.

(3) Incorporation of acid anhydrides in the embedding medium leads to the formation of free water as a product of polymerization during curing (Brydson 1969). The concentration of this internally generated water is unknown, but it may cause some redistribution of ions in the freeze-substituted tissue. In an attempt to overcome this problem, Al_2O_3 (Brockman basic, grade II) or pellets of molecular sieve were always also added to the resin mix.

(4) The tertiary amines, which are often used as catalysts for curing epoxy resins, polymerize with glycidyl ethers as follows:



This ion will then open up a new epoxy group, generating another ion and so on. Since R′ also terminates in an epoxy ring these reactions will occur at both ends of the molecule (Brydson 1969). The ions of the tissue therefore become embedded in a Donnan matrix (Overbeek 1956) as the resin cures. This may promote some migration of ions over short distances, especially if some water is also generated (see 3 above). We should therefore proceed with caution when attempting to localize ions at the ultrastructural level.

(ii) *Freezing of Fresh Tissue*

An effective method was devised for totally submerging the tissue in the cold bath with a minimum of delay, while avoiding injury to the tissue from contact with forceps. A section measuring 2 by 0·5 mm was picked up by surface tension on to the tip of a stainless steel needle and excess water was carefully blotted away. The needle was then quickly plunged full-length into anhydrous acetone near its freezing point (−94°C) and immediately withdrawn, leaving the instantly frozen section of tissue deep in the cold acetone. Upon shaking the sealed vial, the tissue buried itself into the large amount of activated molecular sieve which had been previously added together with OsO_4 (1% by weight).

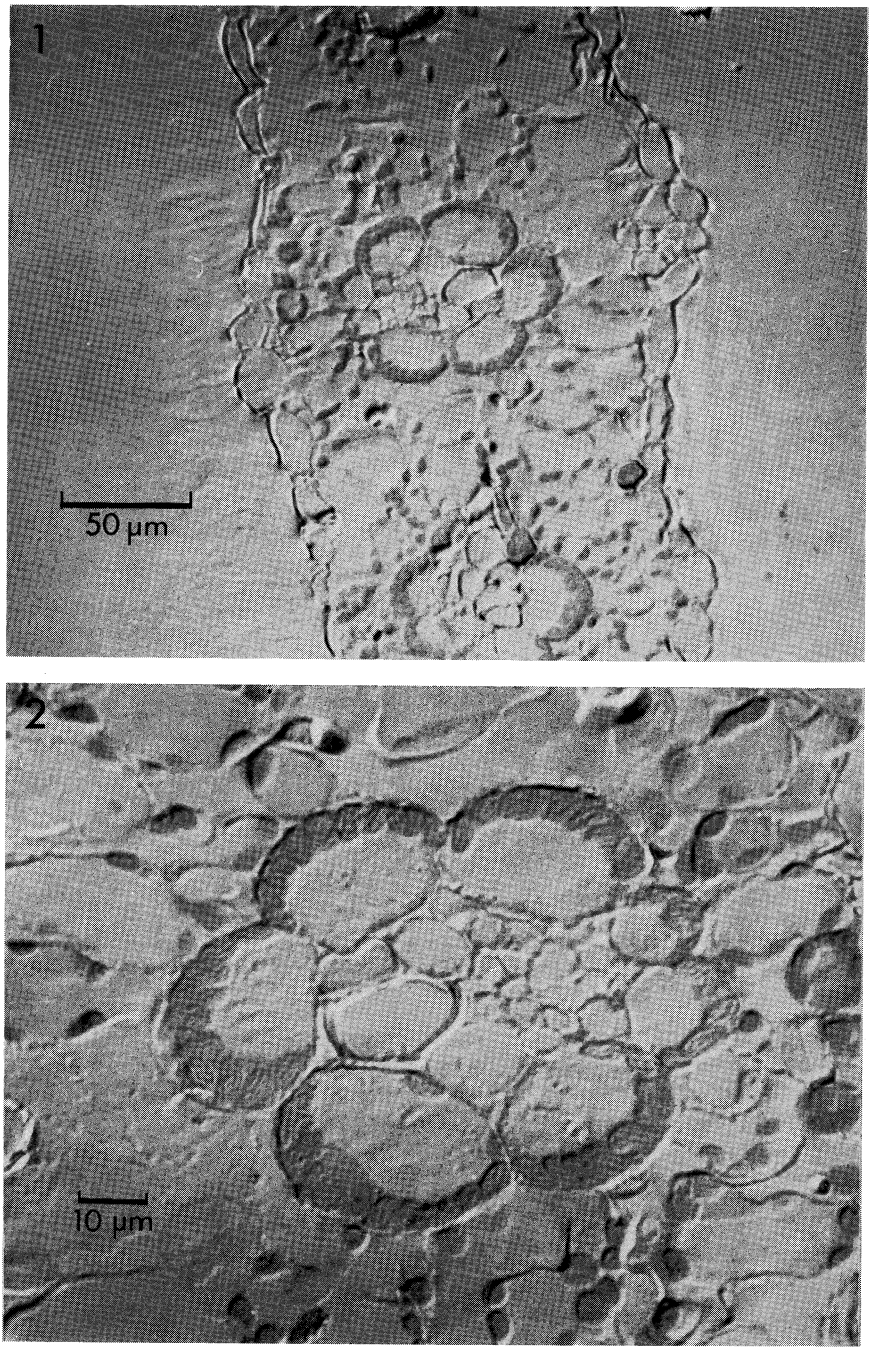
(iii) *Freeze-substitution and Embedding*

The vial was transferred into a Dewar flask containing a slush bath of acetone and dry ice at −78°C, and placed into a refrigerated box held at −48°C. The temperature of the vial was held near −78°C for 2 or 3 days, but was then allowed to rise slowly to −48°C over the next 5 days. It was assumed that this period was sufficient for complete freeze-substitution (Pease 1964; Fisher and Housley 1972; Hereward and Northcote 1972). In this work the tissue was freeze-substituted in acetone rather than in ether (Läuchli *et al.* 1971), which has a lower flash point.

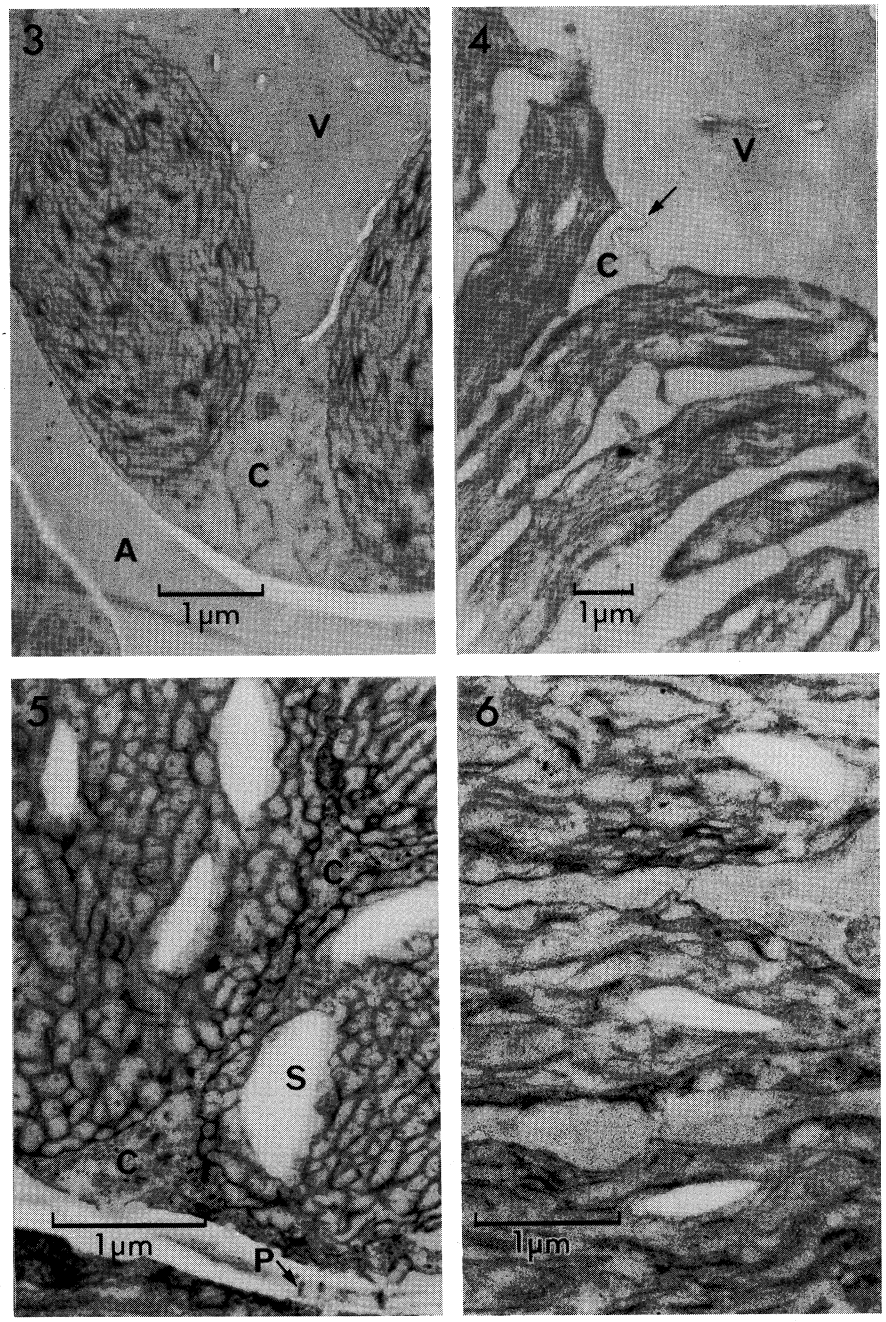
When freeze-substitution was completed, the vial was transferred into a dry box and allowed to warm up to −10°C over a period of 4 hr. The OsO_4 was eluted from the tissue with two washes of cold anhydrous acetone (−10°C) over a period of 1 hr and the tissue was then allowed to warm to 0°C. The samples were then infiltrated with modified Spurr's medium at this temperature over a period of approximately 30 hr. The resin, and all mixtures thereof, had been previously dried for 3 days with activated molecular sieve. The embedded samples were then transferred into capsules containing 0·5 g of Al_2O_3 and placed into a sealed glass jar half-filled with activated molecular sieve. The jar was then transferred from the dry box into an oven (60°C) and cured for 18 hr. The cured samples were stored over silica gel in desiccators.

(*c*) *Preparation of Sections*

Sections were cut using a dry diamond knife. The dry sections were picked up using a finely pointed stick or eyelash and spread on to a dry Parlodion film stretched across a narrow hole of a graphite sample holder as recommended by Gehring and Doerge (personal communication). The mounted sections were stored in desiccators and analysed the same day. Several methods were attempted to remove wrinkles from the dry sections. Best results were obtained by coating the Parlodion film with a thin coating of dry diffusion pump oil before mounting the sections. This allowed sections to spread slightly when placed under the electron beam. The improvement, however, was only marginal.



Figs. 1 and 2.—Section (at different magnifications) of freeze-substituted leaf tissue of *Z. mays* embedded in epoxy resin and photographed using Nomarski interference phase contrast.



Figs. 3–6.—Electron micrographs of chloroplasts in freeze-substituted tissue. Sections were post-stained in uranyl acetate and lead citrate. *V*, vacuole; *C*, cytoplasm; *A*, air space; *P*, plasmodesmata; *S*, starch grains. *3*, Section across mesophyll cell. *4*, View of bundle sheath chloroplasts after light-pretreatment. Although the tonoplast (arrow) is evident in thin sections, it was not apparent in the thicker (1–1·5 μm) sections used for electron probe analysis. *5*, Swollen bundle sheath cell chloroplasts in dark-pretreated tissue. *6*, Flattened bundle sheath chloroplasts in light-pretreated tissue.

(*d*) *Electron Probe Microanalysis*

The samples were examined in a Jeolco-JSMU3 scanning electron microscope fitted with a Nuclear Diodes energy-dispersive X-ray analyser (EDAX) which had a detector window thickness of 7 μm and spectral resolution of 178 eV (Sutfin and Ogilvie 1970; Russ 1972). Sections were viewed and analysed with the instrument in transmission mode using magnification factors ranging between 3000 and 8000.

Spot analyses were performed at 25 keV using a take-off angle of 45° and a beam current of 2·5 nA (Sutfin and Ogilvie 1970; Russ 1972). Errors arising from backscatter electrons were minimized by employing a carbon sample holder provided with a narrow bore for passage of the electron beam. It was found that whereas thick sections sustained severe burn marks in the region of the applied beam, thin sections (1–1·5 μm) showed remarkably little damage, especially on line profile analyses.

Condensation of diffusion pump oil on to the beryllium window of the X-ray detector was kept to a minimum by occasionally wiping the window with tissue paper soaked in methanol. If this is not regularly checked, the window thickness of the detector effectively increases and discriminates in sensitivity against sodium and magnesium (Russ 1971). Astigmatism could not always be satisfactorily corrected in our instrument and varied greatly in magnitude from day to day. This was due to extraneous electrical interference.

(*e*) *Calibration of X-ray Detector*

There are several satisfactory methods for calibrating electron probe analysers. The use of small crystals as reported by Humble and Raschke (1971) has been successfully used for the analysis of non-aqueously isolated chloroplasts (De Filippis and Pallaghy 1973), but was not appropriate to the present investigation. Nor was the technique of embedding the tissue in an albumin matrix of known electrolyte concentrations (Gehring *et al.* 1971; Ingram *et al.* 1972) generally applicable, although a modification of it was used in a test case as described below.

As already mentioned, Spurr's medium contains a large amount of chlorine, but an insignificant amount of potassium. This situation was used to advantage by embedding freeze-substituted leaf sections in Spurr's medium and comparing the chlorine X-ray emission (by spot analysis) of an area external to the tissue with that for potassium within the tissue in the same section, thus avoiding the necessity to measure section thicknesses. Using this technique and making the assumption that the X-ray detector is equally sensitive to potassium and chlorine (De Filippis and Pallaghy 1973), the following results were obtained for a leaf section (2 by 0·5 mm) floated on distilled water for 2 hr in darkness:

> The average peak heights in the X-ray spectra for potassium in chloroplasts and for chlorine in epoxy resin were 1550 and 3530 counts per 400 s respectively. Chemical analysis of cured Spurr's resin showed a chlorine content of 410 m-equiv/1000 cm^3 of resin (Australian Microanalytical Service, CSIRO, University of Melbourne). The concentration of potassium in freeze-substituted chloroplasts was therefore approximately equal to 180 m-equiv/1000 cm^3 of chloroplast volume. The potassium content of the vacuoles of mesophyll cells was approximately 93 m-equiv/1000 cm^3 of vacuole. This calibration technique is similar to the one used by Gehring *et al.* (1971) and Ingram *et al.* (1972), but is unfortunately not as useful since it masks the relatively low amounts of chloride in the tissue, and was not employed in further experiments for this reason.

(*f*) *Electron Microscopy*

Thin sections of freeze-substituted tissue were cut and collected on water. The sections were stretched out with chloroform vapour, mounted on Formvar-coated grids, post-stained with uranyl acetate and lead citrate in the usual fashion, and examined with a Siemens Elmiskop 1A electron microscope. Blocks of freeze-substituted tissue that had been exposed to moisture in this way were not used for electron probe analysis.

III. Results

(*a*) *Preservation of Structure*

Figure 1 shows part of a cross-section of a freeze-substituted leaf of *Z. mays* and demonstrates the feasibility of measuring the relative concentration of elements in both epidermal and other cells of the same section. This is usually not possible when using cryosectioning, as thinly cut sections become fragmented and vary in thickness within each section. The preservation of structure is good and compares well with that obtained for other tissues by Fisher (1972). The difference in structure between the chloroplasts of mesophyll and those of bundle sheath cells is well demonstrated at higher magnification by Nomarski interference phase contrast (Fig. 2).

Although the fine structure of the chloroplasts is somewhat disrupted (Figs. 3–6), it compares reasonably well with conventionally fixed and embedded material (Bishop *et al.* 1971; Laetsch 1971). Grana are evident in the mesophyll chloroplasts and the thylakoid membranes can be easily distinguished (Fig. 7). The chloroplasts

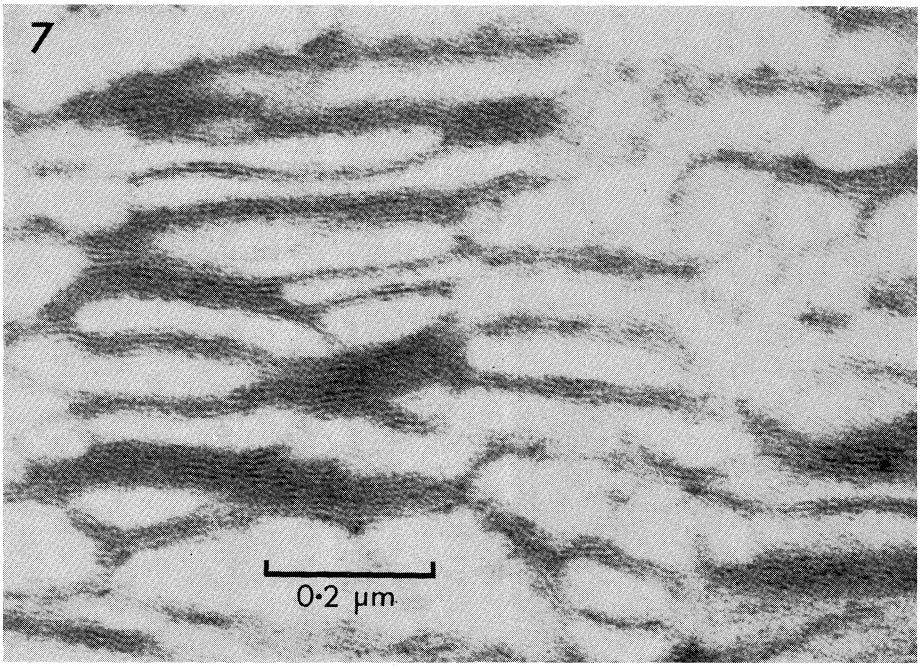


Fig. 7.—Granal thylakoids from a freeze-substituted mesophyll chloroplast. The section was stained with uranyl acetate and lead citrate.

of mesophyll cells are ellipsoids (Fig. 3), resembling those of *Elodea* (De Filippis 1972), and show only a slight reduction of their minor axes on exposure of the leaf to light. Bundle sheath chloroplasts, on the other hand, swell considerably in the dark and come to occupy almost the whole of the cytoplasmic volume (Fig. 5), making it difficult to distinguish between individual chloroplasts. In the light they flatten in a spectacular manner (Figs. 4 and 6). Therefore, although precise measurements were not made, it would appear that under the conditions of illumination used

here the volume of bundle sheath chloroplasts is markedly light-dependent, whereas that of mesophyll chloroplasts is only slightly affected by illumination. These observations are consistent with those of De Filippis (1972) who examined glutaraldehyde-fixed leaf sections of identically grown material.

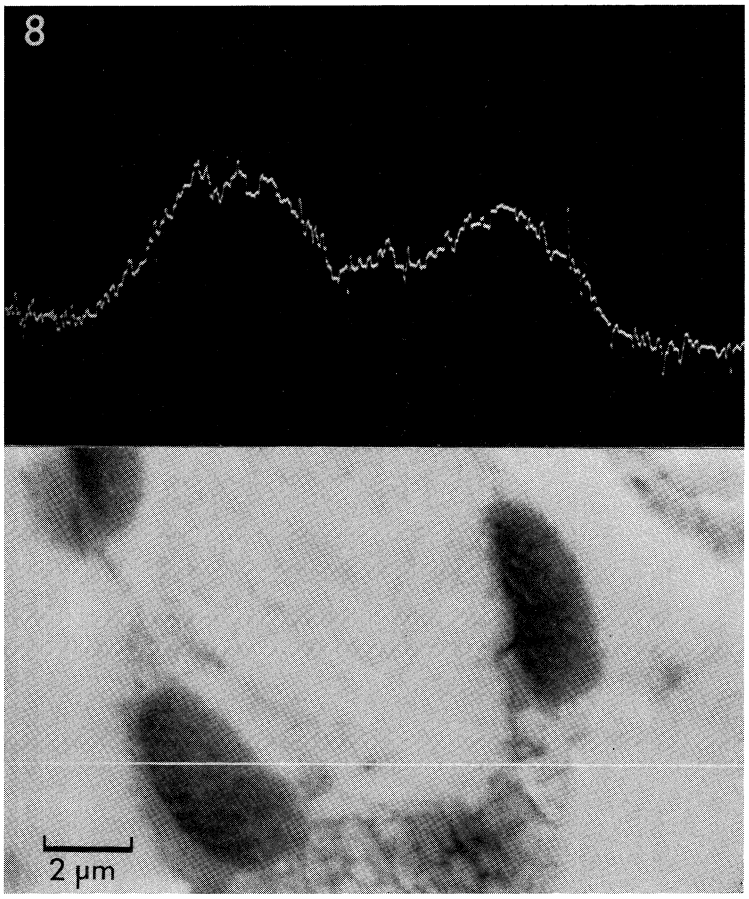
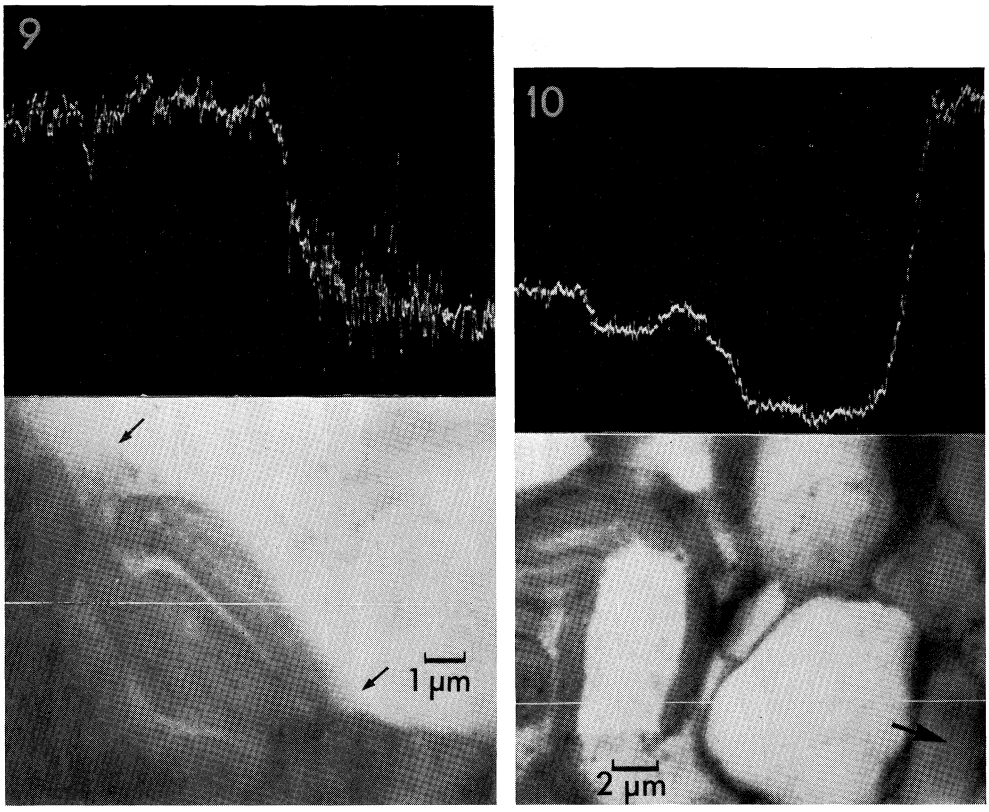


Fig. 8.—Potassium concentration profile across a light-pretreated mesophyll cell in freeze-substituted tissue. The ordinate on the oscilloscope trace shows the count rates of K_α X-ray emission for potassium on an arbitrary scale for a line scan of 250 s duration. Stretch marks are evident in this section. The sections (approx. 1 μm thick) were viewed using transmission scanning electron microscopy at 25 keV and a beam current of 2·5 nA.

(*b*) *Electron Probe Microanalysis*

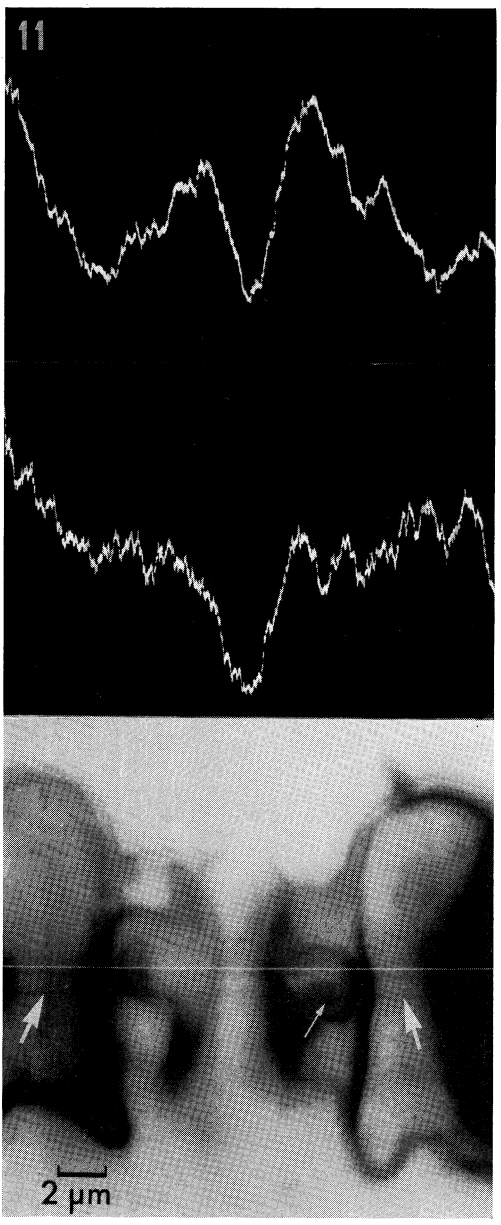
Figures 8–12 show profiles of potassium concentration across a variety of cells and cellular compartments. The ordinates of the concentration profiles record the count rates of the potassium X-ray emission as the electron beam traverses across the specimen. Since line scans were usually performed over 250–500 s, some image drift was unavoidable in most cases.

Figure 8 illustrates that it is possible to measure the relative concentrations of potassium in the various cellular compartments. In this particular example, stretching resulting from sectioning of the sample is quite apparent.



Figs. 9 and 10.—Potassium concentration profiles across various cellular compartments. *9*, Light-pretreated bundle sheath cell, where the concentration of potassium in the chloroplasts was almost twice that observed in the vacuole. The arrows show the probable location of the tonoplast. *10*, The line scan traverses a bundle sheath cell, a large empty xylem vessel, and a smaller "xylem" vessel (arrow). The concentration of potassium in electron-opaque vascular cells is very much higher than in either mesophyll or bundle sheath cells. Section thicknesses were approximately 1 μm.

A notable feature observed throughout this work is the abundance of chloride in epidermal cells, including the bulbous ends of guard cells of open stomata (Fig. 11), and its relative absence within mesophyll and bundle sheath cells. It is interesting that analysis of the same guard cells in one of the serial sections, but across the jaws of the guard cells (as defined by Raschke 1970), showed no detectable chloride, whereas potassium concentrations were relatively high (Fig. 12). This is consistent with some of the observations of Raschke and Fellows (1971) on guard cells in freeze-dried epidermal strips of leaves of *Z. mays*. Their Figure 11 indicates a much higher K : Cl ratio in the tubular than in the inflated spherical parts of light-pretreated guard cells. The same figure also shows very marked variations in the K : Cl ratios for different regions within one of the subsidiary cells. These results suggest differences



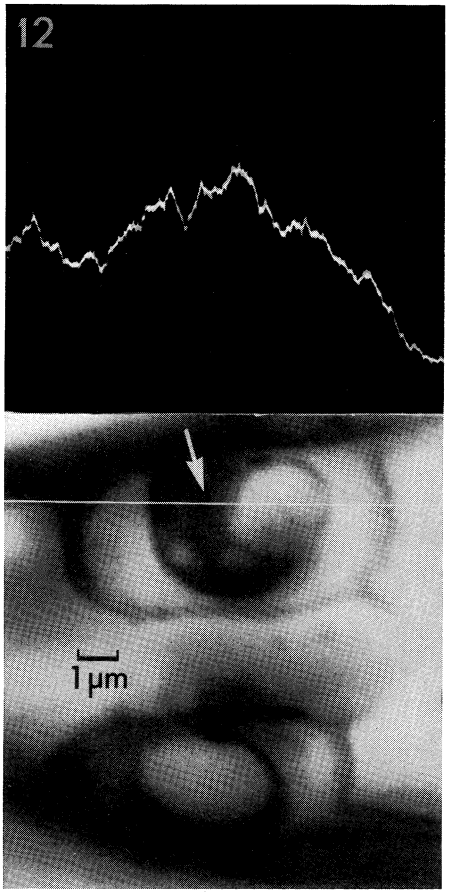


Fig. 11.—Concentration profiles for chloride (upper trace) and potassium (lower trace) across an open stoma near the bulbous ends of the guard cells. The scale on the ordinate of the oscilloscope traces, although arbitrary, was the same for both elements. The subsidiary cell to the left of the guard cell contains a high concentration of chlorine, as also do the guard cells. The large arrows point to a burn trace of a previous line scan analysis not shown here. The small arrow shows the burn resulting from an extended spot analysis of a guard cell chloroplast when using a beam current of 2·5 nA at 25 keV. The section thickness was approximately 1·5 μm.

Fig. 12.—Potassium concentration profile across the jaws of an open stoma. Although this is one of the serial sections of the same stoma shown in Figure 11, there was no detectable chloride in the guard cell. The arrow points to a burn mark resulting from an extended spot analysis of a guard cell "nucleus".

in the compartmentation of the chloride and of organic acid salts of potassium. This is further supported by the data of Humble and Raschke (1971) which suggest that in guard cells of *Vicia faba* the K : Cl ratio in the nucleus may differ markedly from that in the vacuole.

The data indicate a high concentration gradient of both potassium and chloride from certain cells of conducting vessels to the bundle sheath and mesophyll cells. Similar gradients for potassium alone have been previously reported by Läuchli (1967). Very large xylem vessels, which are not electron-dense and appear empty (Fig. 10), are devoid of potassium, perhaps because they were cut during sectioning.

TABLE 2

RELATIVE CONCENTRATIONS OF POTASSIUM AND CHLORIDE IN A VARIETY OF CELLULAR COMPARTMENTS AS ESTIMATED FROM THE PEAK HEIGHTS OF X-RAY EMISSION SPECTRA OBTAINED BY SPOT ANALYSIS

Leaf sections of *Z. mays* were pretreated by floating for 2 hr on distilled water in the light or in darkness. Although it is valid to compare concentrations across the table for either light or dark treatment, it is not possible to make similar comparisons between light and dark samples in absolute terms since the thickness of the sections varied. Each value represents the results obtained from 40 cells. In obtaining the values for vascular cells empty cells were ignored. Standard errors of the means are shown in parentheses

Illumination	Element	Bundle sheath cells		Mesophyll cells			Vascular cells, vacuole
		Chloroplast	Vacuole	Chloroplast	Cytoplasm	Vacuole	
Light	K	1	0·65 (0·03)	0·81 (0·03)	0·65 (0·02)	0·96 (0·04)	2·5 (0·3)
Light	Cl	0·03 (0·006)	0·036 (0·01)	0·015 (0·004)	0·013 (0·004)	0·029 (0·004)	0·42 (0·1)
Dark	K	1	0·86 (0·04)	1 (0·03)	0·80 (0·02)	0·65 (0·02)	2·0 (0·2)
Dark	Cl	0·06 (0·01)	0·12 (0·02)	0·10 (0·02)	0·10 (0·02)	0·08 (0·02)	0

The data in Table 2 summarize the results obtained by spot analysis of various cellular compartments of light- and dark-pretreated leaf sections which had been floated for 2 hr on distilled water. The relative count rates for potassium and chloride were estimated from the corresponding peak heights in the X-ray spectrum after subtraction of background as judged by eye. De Filippis and Pallaghy (1973) reported that the calibration curves for chloride and potassium were almost linear for X-ray spectra analysed on the basis of peak heights. The range of count rates observed for bundle sheath chloroplasts in the light ranged from 800 to 1300 counts/400 s and depended on section thickness. Calibration of count rates in absolute terms was not attempted in this preliminary work except in a test case as mentioned in Section II(*g*). The data were obtained by analysing alternately a chloroplast, a vacuole, etc. and estimating the ratio of count rates for each relative to the count rate in a bundle sheath chloroplast, before moving on to another section or part of the same section. By using this method, variation in absolute count rates due to variations in section thicknesses could be ignored.

A disadvantage of this method, of course, is that one could not make a quantitative comparison between light- and dark-treated samples because of the large variations in the absolute count rates between different sections. The Casley-Smith (1972) technique for measuring section thickness might perhaps overcome this difficulty, but was not attempted in this investigation. The ratios of the count rates, all measured relative to the bundle sheath chloroplasts, were averaged and the standard errors of the mean calculated.

Table 3 shows an unusual situation where high concentrations of chloride were detected in both bundle sheath and mesophyll cells immediately adjacent to the vascular bundle. The proximity of both cells to the vascular bundle may be a significant factor in this case.

TABLE 3

SPOT ANALYSES OF THREE NEIGHBOURING CELLS IN THE SAME SECTION

This example, taken from a leaf section pretreated for 2 hr in the dark, is atypical since such high concentrations of Cl were not usually observed in these cells. The count rates for K (scintillations/400 s) are similar to those normally observed

Cell	Element	Count rate (scintillations/400 s) in:			
		Chloroplast	Nucleus	Cytoplasm	Vacuole
Bundle sheath cell	K	1640	1480	1320	960
	Cl	200	320	40	800
Mesophyll cell in contact with bundle sheath cell	K	1320	—	960	805
	Cl	40	—	40	330
Mesophyll cell separated from bundle sheath cell by air space	K	1620	1765	1355	800
	Cl	20	20	20	20

Although the beam diameter used for spot analysis was approximately 15–20 nm, the effective area excited by the electron beam is probably 1 μm in diameter (Russ 1972). Figures 11 and 12 show typical burn marks resulting from spot analysis of guard cells. The beam current used in these experiments was 2·5 nA at 25 keV.

IV. DISCUSSION

(*a*) *Validity of Procedures Used*

In spite of the imperfections of the technique of freeze-substitution used here [see Section II(*b*)], there is fairly clear evidence that potassium and chloride "ions" remain localized, at least at a cellular level. Although mitochondrial membranes are destroyed in the process, this method seems to be a useful approach for the study of compartmentation of ions in higher plant cells (MacRobbie 1971). The damage to organelles may be due to the absence of suitable fixatives in these preparations or possibly because the freezing rate of the wet tissue was not sufficiently rapid. Liquid Freon 22 is normally used to freeze biological material (Moor *et al.* 1961) because of its low melting point (−146°C) and high thermal conductivity. However, pre-

liminary studies with the light microscope showed that leaf sections frozen in acetone (−94°C) were indistinguishable from those frozen in liquid Freon 22; acetone was therefore chosen for convenience. Fisher (1972) and Läuchli *et al.* (1970) used an isopentane–cyclohexane mixture (−170°C), but the results obtained by them are in no way superior to those reported here. It is possible that the use of liquid Freon 22 may lead to some improvement. The use of solvents other than acetone or application of low temperature curing of resins, such as methacrylate with u.v. radiation (Pease 1964), may help to preserve mitochondria and is being investigated at present.

In a recent paper, Spurr (1972) also reported the absence of recognizable mitochondria in sections of freeze-substituted tomato leaf tissue. Spurr (1972) used diethyl ether for freeze-substitution, but was unable to retain ions in the sections without including cation-complexing agents such as benzamide in the freeze-substitution fluid. The method reported in this paper does not require the use of such additives. Examination of freeze-substituted epoxy-embedded leaf sections of *Mesembryanthemum crystallinum* showed high concentrations of potassium, sodium, and chloride in the chloroplasts (Pallaghy, unpublished data). Although van Haareveld and Cowell (cited by Pease 1964, p. 78) found that sodium chloride was somewhat soluble in cold acetone (−50°C), De Filippis and Pallaghy (1973) were able to retain high concentrations of potassium, sodium, and chloride in chloroplasts of *Elodea densa* isolated under non-aqueous conditions using acetone. We believe that the incorporation of molecular sieve at all stages of treatment may have lowered the solubility of these salts in the media to a very low level.

In the present investigation the concentration of potassium in mesophyll chloroplasts in freeze-substituted tissue was approximately 180 m-equiv/l, whereas that in the mesophyll vacuole was approximately 93 m-equiv/l. These values are reasonably consistent with those found by chemical analysis of intact leaf tissue (Table 1) and confirm the observation of Stocking and Ongun (1962) on the distribution of potassium between the chloroplasts and vacuole of mesophyll cells in bean leaves. The difference in K : Cl ratios between intact leaf tissue and freeze-dried leaf sections (pretreated by floating on distilled water, Fig. 13) can probably be attributed to a leaching of chloride from sections floated on distilled water, or to the loss of chloride from cut xylem elements where it otherwise appears to accumulate (Fig. 10, Table 2).

(*b*) *Distribution of Ions in Leaf Sections*

Considering the low chloride concentration in the culture solution, the guard cells, subsidiary cells, and some other epidermal cells appear to have a high affinity for chloride consistent with the observations of Raschke and Fellows (1971) who used intact leaves of *Z. mays*.

Except for isolated instances (Table 3), the chloride level of bundle sheath and mesophyll cells was very low (Table 2). By using the calibrations discussed in Section II(*g*), together with the data in Tables 2 and 3, approximate estimates can be made of the potassium and chloride levels in these cells. The concentration of potassium in bundle sheath cell chloroplasts in the dark may be as high as 200 m-equiv/l of chloroplast volume, while that of chloride is only 12 m-equiv/l. In light-treated samples the discrimination between potassium and chloride appears to be even greater (Table 2). It is striking, however, that the Cl : K ratios for bundle sheath cell vacuoles (0·14),

mesophyll cell chloroplasts (0·10), mesophyll cell cytoplasm (0·12), and mesophyll cell vacuoles (0·12) reflect very closely the Cl : K ratio of the solution (0·10) in which the plants were grown.

Sodium could not be detected in either freeze-substituted or in freeze-dried (Fig. 13) preparations of leaf sections. Chemical analysis of intact leaves of *Z. mays* (Table 1) showed that the K : Na ratio of the intact leaves was 91 as compared with 10 in the Hoagland solution (Table 1).

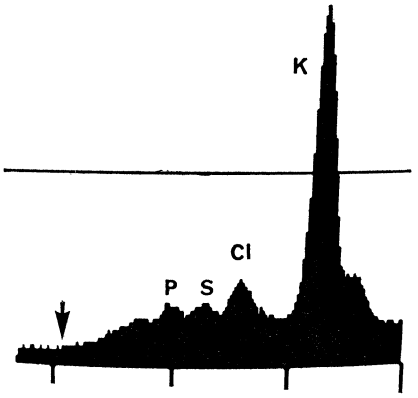


Fig. 13.—X-ray emission spectrum obtained by analysing a 50 by 50-μm area of a freeze-dried leaf section. Sodium, which should peak at 1·04 keV (K_β radiation) if present, could not be detected (arrow). The electron beam current was 2·5 nA at 25 keV.

Some cells of the vascular bundle have very high concentrations of potassium (Table 3), and also of chloride in light-treated leaf sections. The apparent absence of chloride in such cells in dark samples suggests that this ion was either leached out of these cells or that it was absorbed from vascular cells by the mesophyll and bundle sheath cells via a dark-stimulated transport mechanism. The latter suggestion would be consistent with the higher Cl : K ratios in the tissue of dark-treated samples, and the fact that dark-induced swelling of chloroplasts in *Elodea densa* causes a marked increase in their chloride content (De Filippis and Pallaghy 1973). Large xylem vessels appeared to be empty (Fig. 10), but it is possible that their ion content may also be high in intact tissue. This would account for the discrepancy between the K : Cl ratios measured in intact leaves (Table 1) and those found by X-ray analysis for leaf sections floated on distilled water.

The very high concentration of potassium found in some cells of the vascular bundle (Table 2) is similar to the situation described for root tissue by Läuchli *et al.* (1971). This large supply of potassium could be readily tapped by leaf cells and could simultaneously provide a sink for excess H^+ ions in the leaf. The problem of providing a "balance sheet" for H^+ ions in leaf tissue has been previously discussed by Raven and Smith (1973).

(*c*) *Compartmentation of Potassium and Chloride in Cells*

The major compartments (i.e. chloroplasts, cytoplasm, and vacuole) can be readily distinguished in mesophyll cells. Unfortunately, it is very difficult to locate the cytoplasm in bundle sheath cells because of the very tight packing of chloroplasts, especially in dark-treated samples (Fig. 5). This would not be a problem if sections were thinner and perfectly flat, but we could not achieve this in view of the poor cutting qualities of our modified Spurr's medium [Section II(*b*)] when cut with a

dry knife. Nuclei, although often evident (Table 3), were not studied in this investigation.

There are two points worth noting with regard to the "ion" content of the cytoplasm in mesophyll cells. Firstly, the relative concentration (count rates) of potassium between the chloroplasts and cytoplasm appears to be the same for light- and dark-treated leaf sections. Secondly, if we take note of the example provided in Table 3, where one is dealing with more significant amounts of chloride, the values show that whereas the concentration of potassium in the cytoplasm is reasonably high, that of chloride is very low when compared with that in the chloroplast and in the vacuole. A similar distribution of ions has previously been described for the giant cells of members of the Characeae, where the chloride concentration of the streaming cytoplasm can be estimated either by direct chemical analysis (Kishimoto and Tazawa 1965; Larkum 1968) or by specific ion electrodes (Coster 1966).

(*d*) *Differences in Ion Relations between Bundle Sheath and Mesophyll Cells*

Although the data for chloride distribution between bundle sheath and mesophyll cells are not statistically very significant (Table 2), they are nevertheless in general agreement with the results of Schöch *et al.* (cited by Lüttge 1971) who found that after uptake of $^{36}Cl^-$ by leaf strips of *Z. mays*, the label was equally distributed between mesophyll and bundle sheath cells.

Investigations of the effect of light on chloride uptake by leaf slices of three C_4 species and three C_3 species have shown that light always stimulated uptake in C_4 plants (Lüttge 1971; Lüttge *et al.* 1971). In C_3 plants light sometimes stimulated and sometimes inhibited chloride uptake. Since it is well established that stomata of *Vicia faba* (a C_3 plant) have a very low Cl : K ratio (Humble and Raschke 1971) whereas those of *Z. mays* (a C_4 plant) have a much higher Cl : K ratio (Raschke and Fellows 1971), it would be interesting to know in the above experiments the proportion of the light-stimulated chloride uptake which could be accounted for by uptake into the cells (stomata) of the epidermis. Figure 11 shows that in the leaf slices used under our conditions, chloride was mostly in the epidermal cells, and also in some cells of the vascular bundle in light-treated tissue (Table 2), but there was very little in either mesophyll or bundle sheath cells where the Cl : K ratio was in fact higher in the dark than in the light. The explanation suggested here for the observed difference between light-stimulated chloride uptake by C_3 and C_4 plants (Lüttge 1971; Lüttge *et al.* 1971) is further supported by the observations of Schöch *et al.* (cited by Lüttge 1971) who demonstrated that isolated bundle sheath tissue of *Z. mays* has a low capacity to accumulate chloride as compared with intact 0·5-mm-wide leaf strips.

The trends evident in the compartmentation of potassium in the various cells (Table 2) were also apparent in each section analysed. Figure 14(*a*) shows the effect of illumination upon the ratio of potassium concentration between the vacuole and chloroplasts for bundle sheath and mesophyll cells. These values suggest that the direction of ion fluxes in these two chloroplast types were oppositely affected by light. It is known that bundle sheath chloroplasts are deficient in photosystem II (Polya and Osmond 1972). Other explanations are also possible—there is evidence suggesting

that during light-induced shrinkage of chloroplasts in leaves of *Elodea*, the concentration of ions in the chloroplasts may rise even though there is a net efflux of ions from chloroplasts (De Filippis and Pallaghy 1973). If we assume that light induces a similar net efflux of ions from both bundle sheath and mesophyll chloroplasts, we might expect the concentration of ions to decrease in mesophyll chloroplasts on the basis of their small reduction in volume upon illumination. In bundle sheath chloroplasts which shrink considerably in light, however, the concentration of ions may remain steady or perhaps even rise as in the case of *Elodea*.

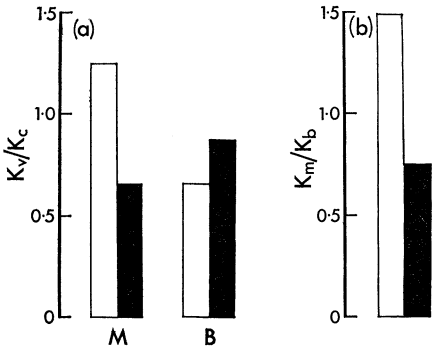


Fig. 14.—Effect of light on the ratio of potassium concentration (*a*) in vacuoles to that in chloroplasts (K_v/K_c), shown separately for mesophyll (*M*) and bundle sheath cells (*B*), and (*b*) in the vacuoles of mesophyll to that in the vacuoles of bundle sheath cells (K_m/K_b).
■ Dark pretreatment.
□ Light pretreatment.

The possibility cannot be ignored of a light-dependent shuttle of potassium ions between the mesophyll and bundle sheath cells. A light (or carbon dioxide, in the case of stomata) driven shuttle of potassium ions has been proven to occur in several plant systems (Pallaghy 1971; Raschke and Fellows 1971; Satter and Galston 1971). Moreover, the generally accepted scheme for the C_4 dicarboxylic acid pathway for photosynthesis requires the transfer of malate (or aspartate) from mesophyll to bundle sheath cells, and that of pyruvate from bundle sheath to mesophyll cells (Osmond 1971). These transfers of organic acids would presumably involve their potassium salts. Electron probe analysis of both freeze-substituted and freeze-dried preparations (Fig. 13) has demonstrated the absence of a high concentration of chloride in leaf sections of *Z. mays* floated on distilled water. Therefore, although bicarbonate, dihydrogen phosphate, or other similar ions (but not sulphate—see Table 1) may act as counter ions for potassium, it is likely to be present in the form of the salt of an organic acid.

The effect of light in changing the direction of the concentration gradient of potassium between the vacuoles of mesophyll and bundle sheath cells [Fig. 14(*b*)] suggests that there may indeed be a shuttle of potassium between these cells. The shuttle may simply involve the diffusion of potassium malate or potassium pyruvate along their respective concentration gradients (Osmond 1971). The pathway of this transfer is obscure, but may involve plasmodesmata (Osmond 1971) which are well preserved in freeze-substituted preparations (Fig. 5). *Z. mays* has proven to be excellent material for these studies and more detailed investigations using intact leaves should not prove difficult as the leaves are sufficiently thin to be frozen quickly for freeze-substitution.

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