

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

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[Manuscript received 29 November 1971]

Abstract

An ultrastructural method (Komnick and Bierther 1969) was used to show the cellular distribution of chloride in the mesophyll cells of barley seedlings which were raised on half-strength Hoagland solution in the presence or absence of 100 mM NaCl.

The foliage of salt-stressed seedlings contained twice as much Cl^- as control plants and showed a considerably greater amount of AgCl deposited in the tissue, especially in the plasmodesmata and in the cytoplasmic compartment. Owing to a non-random distribution of deposits the method was unsatisfactory for a quantitative assessment of Cl^- in the vacuolar compartment, but allowed a relatively accurate estimate of Cl^- concentration of chloroplasts which confirmed Larkum's (1968) finding that chloroplasts constitute a compartment of high Cl^- concentration (about 300 mM Cl^-). In addition it was shown that this Cl^- is mainly associated with the grana lamellae.

The distribution and nature of the deposits in the chloroplasts depended on whether the foliage was exposed to a light or dark period immediately before fixation. It is feasible that these differences in Cl^- distribution could result from changes in charge distribution of macromolecular components.

It is indicated that the principal pathway of Cl^- from cell to cell is through the plasmodesmata but special areas in the cell wall are possibly also involved in the passage of Cl^- .

A critical evaluation of the method is given, taking into account diffusion artifacts, size of deposits, and the possibility of Ag^+ reduction by argentophilic cellular components.

I. INTRODUCTION

Attempts to understand the mechanism of salt transport in plants depend on knowledge of the cellular distribution of the ions under study. A number of studies at the cellular level try to distinguish between transport at the vacuolar membrane or tonoplast and the outer membrane or plasmalemma (Welch and Epstein 1969; Cram and Laties 1971; MacRobbie 1971), and it has been suggested that membrane-bound cytoplasmic vesicles and the membrane system of the endoplasmic reticulum may play a role in the transport of ions from the external solution to the vacuole (Jackman and Van Steveninck 1967; MacRobbie 1969, 1970).

Studies on chloroplasts and mitochondria *in vitro* have shown that these organelles are capable of accumulating ions using energy derived from respiratory or

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light-induced processes (Packer, Murakami, and Mehard 1970). Numerous cases have been documented in which ion accumulation is shown to be intimately related to energy transducing processes (Robertson 1968), but the precise role of mitochondria and chloroplasts in the intact cell has not been ascertained due to lack of information about the actual distribution of ions in these organelles and in other cytoplasmic components. Some noteworthy attempts to determine the *in vivo* ionic status of chloroplasts have been made by Larkum (1968) and Larkum and Hill (1970) by using a rapid, non-aqueous technique for isolation of chloroplasts from freeze-dried leaf segments. Chloroplasts were shown to constitute a compartment of high salt concentrations. Further analysis showed especially that concentrations of K^+ and Cl^- were high relative to those determined for the cytoplasmic phase. However, in order to be able to assess the success of this indirect approach it is necessary to show that the chloroplast and cytoplasmic concentrations differ by a considerable margin. It is obvious that a direct method of measuring the ion gradients *in vivo* would be preferable.

As a result of a growing awareness that primary processes of energy transfer and conservation involve ion movements across membranes (Mitchell 1966; Greville 1969; Packer 1970) coupled with the idea of functional usage of characteristic regions of permeability in these membranes, it has become important to develop techniques for the ultrastructural localization of ions. It might be possible then to directly link processes of energy transfer with changes in ion distribution associated with the membrane systems within an organelle.

The histochemical localization of Cl^- using silver acetate as a reagent (Komnick 1962) was attempted using barley seedlings with large differences in Cl^- contents of the leaf tissue. Komnick and Bierther (1969) indicated in a critical review that the ultrastructural detection of Cl^- by precipitation as $AgCl$ has found widespread use in animal tissues. But, apparently only one previous application on plant tissue (salt glands in *Limonium*) seems to have been recorded by Ziegler and Lüttge (1967), who used $KMnO_4$ as a fixative for a period of 7 hr, a fixation technique which now may be regarded as unsatisfactory.

This work is intended as a preliminary account as there are some doubts about the validity and quantitative uses of the technique. These include consideration of possible diffusion artifacts and the occurrence of non-specific metallic silver deposits due to reduction by argentophilic compounds, e.g. mucopolysaccharides. According to Komnick and Bierther (1969) these effects can be avoided by direct fixation in OsO_4 , a strong oxidizing agent. However, plant cells may require further investigation because of the different nature of some cellular components, e.g. cell walls, starch grains, lipid bodies, etc.

II. METHODS AND MATERIALS

(a) Growth of Seedlings

Seeds of barley (*Hordeum vulgare*) were sterilized by treatment with 5% Dairychlor for 5 min, and placed in Petri dishes on sterile filter paper moistened with deionized water. At 3 days uniform seedlings were selected and transferred under sterile conditions to Pyrex tubes (8 in. long, 1 in. diam.) where they were supported on wire loops immediately above the surface of 25 ml

half-strength sterile Hoagland's solution, with or without 100 mM NaCl. The tubes were stoppered with cotton-wool to keep transpirational effects to a minimum, and placed in a Sherer growth cabinet providing a 16-hr photoperiod at a temperature of $26 \pm 2^\circ\text{C}$ (day) and $18 \pm 2^\circ\text{C}$ (night), and a light intensity of approximately 9150 lux. After 7 days of growth, by which time the secondary leaf had emerged, seedlings were harvested to determine length and fresh weight of shoots and roots. Shoots were digested in dilute HNO_3 for determination of the concentrations of K^+ and Na^+ by flame-photometry (EEL) and Cl^- by a potentiometric method (Furman and Low 1935).

(b) *Electron Microscopy*

All samples were taken from within a 1-cm region half-way along the length of the primary leaves.

Pieces of tissue 1 mm^2 were fixed in 2% OsO_4 in 0.1M cacodylate-acetate (pH 7.2) for 1 hr at 0°C , either in the presence or absence of 0.5% silver acetate. This was followed by three brief rinses in small volumes of 0.1M cacodylate acetate (pH 7.2), and dehydration in an acetone series and propylene oxide before embedding in Araldite. Tissue pieces were also fixed in 4% glutaraldehyde in 0.1M cacodylate acetate (pH 7.2) for 3 hr at 0°C , rinsed in buffer solution + 10% sucrose, followed by post-fixation with 2% OsO_4 in 0.1M cacodylate at room temperature before rinsing with water. The above procedure of dehydration and embedding was followed. Uniform "silver" sections were cut with an LKB Ultratome and were examined unstained to allow for detection and resolution of finely divided silver deposits. Alternatively, for the enhancement of ultrastructural detail, sections were stained with lead citrate according to Reynolds (1963), before viewing in a Siemens I or IA microscope at 40 or 60 kV.

(c) *Estimation of Chloride Concentration from Electron Micrographs*

Approximate concentrations of Cl^- in the chloroplast were estimated from the electron micrographs as follows. The size and shape of chloroplast AgCl deposits was uniform enough in any preparation to assume each deposit to approximate a sphere of, for example, 25 nm diameter, which would represent a volume of $8.2 \times 10^{-6}\text{ }\mu\text{m}^3$. The equivalents of Cl^- represented by one grain of AgCl is given by the product of the volume \times density of AgCl ($= 5.46\text{ g/ml}$) divided by the molecular weight of AgCl ($= 143$). For the above particle size this would be equal to $(8.2 \times 10^{-6} \times 10^{-12} \times 5.46)/143$ which equals 0.32×10^{-6} p-equiv. Assuming sections to be 80 nm thick the area of a square of side $1\text{ }\mu\text{m}$ on the micrograph would represent a volume of $0.08\text{ }\mu\text{m}^3$, which would represent a concentration of $0.32 \times 10^{-6} \times (10^{15}/0.08) = 4 \times 10^{-3}\text{M}$ or 4 mM.

Grain counts were normally made on two random areas of $1\text{ }\mu\text{m}^2$ on each of three or four micrographs and the results averaged for each treatment, i.e. in presence or absence of 100 mM NaCl, and light or dark treatment immediately before fixation.

III. RESULTS

After 7 days growth the seedlings were sampled for ion content. By this stage the presence of 100 mM NaCl caused the growth of the shoots to be inhibited by 30% while root growth was little affected (Table 1). Table 1 also shows that both Na^+ and Cl^- contents of the shoots had doubled as a result of the high NaCl concentration in the medium, while the K^+ content of the NaCl-treated seedlings was reduced by 26.5% compared to the control seedling. It appears then that the NaCl-treated seedlings were growing under a condition of moderate stress (Greenway 1965) and that this condition induced significant differences in Na^+ and Cl^- contents.

It seemed feasible that a period in light or dark immediately before fixation might induce significant changes in the distribution of Cl^- , particularly with respect

to the chloroplasts. Hence, after 7 days all seedlings were transferred to darkness for 12 hr. Subsequently, one batch was exposed to $2\frac{1}{2}$ hr of light at 9150 lux while the remaining seedlings were kept in the dark until the tissue was in contact with the fixative.

TABLE 1

ION CONCENTRATIONS OF SHOOTS AND GROWTH OF 10-DAY BARLEY SEEDLINGS RAISED ON HALF-STRENGTH HOAGLAND SOLUTION IN THE PRESENCE OR ABSENCE OF 100 mM NaCl

	Shoot length (cm)	Shoot fresh weight (g)	Root fresh weight (g)	Ion concn. (m-equiv./kg fresh wt.)		
				Na ⁺	K ⁺	Cl ⁻
Control	15.8	0.20	0.084	75	94	8
+NaCl	11.3	0.14	0.080	158	69	18

(a) *Seedlings Grown in the Dark in presence of NaCl*

Generally, leaf parenchyma cells consist of a large central vacuole and a relatively small amount of cytoplasm, the bulk of which is taken up by chloroplasts (Fig. 1). The chloride precipitation method is found to be of very little value for the purpose of detecting Cl⁻ in the vacuole. Deposits are absent from most vacuolar sections, but occasionally a cross-section will reveal a large amount of deposit (Fig. 1). This non-random distribution pattern might be due to deposits being swept to one side of the vacuole probably by the penetrating embedding medium. Since many hundreds of sections can be cut in traversing one leaf parenchyma cell the relatively low occurrence of dense aggregates deposited in the vacuole is easily understood. It is also clear that this type of irregular distribution would defy any attempt to assess these deposits quantitatively.

The distribution of AgCl precipitate in the cytoplasmic phase follows a more regular pattern, although deposits usually occur as clumped aggregates close to the tonoplast and in the space between plasmalemma and cell wall (Fig. 2). Prominent deposits occur in most of the plasmodesmata (Fig. 4), while occasionally the cell wall may exhibit distinct areas of very dense deposit of $\frac{1}{2}$ –1 μ m in width (Figs. 1, 5, and 6). An apparent excess of this deposit seems to be swept in one direction, presumably between cytoplasm and wall. Except for those regions next to intercellular spaces where walls of neighbouring cells join (Fig. 3) very little deposit or none at all occurs elsewhere in the cell wall.

The chloroplasts always showed the presence of relatively intensive but well-dispersed deposits of grains of uniform size (Fig. 6). However, when a distinction between lamellar and non-lamellar regions was possible (Fig. 7), it became evident that most of the larger grains were associated with the grana lamellae, while the stroma region showed fewer smaller grains. It was calculated (see Section II) that this AgCl would represent a concentration of 350 mM Cl⁻ for the boxed area with grana and stroma lamellae and 65 mM Cl⁻ for the area without lamellae (Fig. 7).

Fig. 2.—Detail of cytoplasm. Deposits occur predominantly near the tonoplast and between the cell wall and plasmalemma. Note signs of melting of silver deposits (small arrows). When viewing under an intense beam, deposits show a rapid transition from an angular to a rounded shape leaving small vacant areas not penetrated by embedding medium. $\times 30,000$.

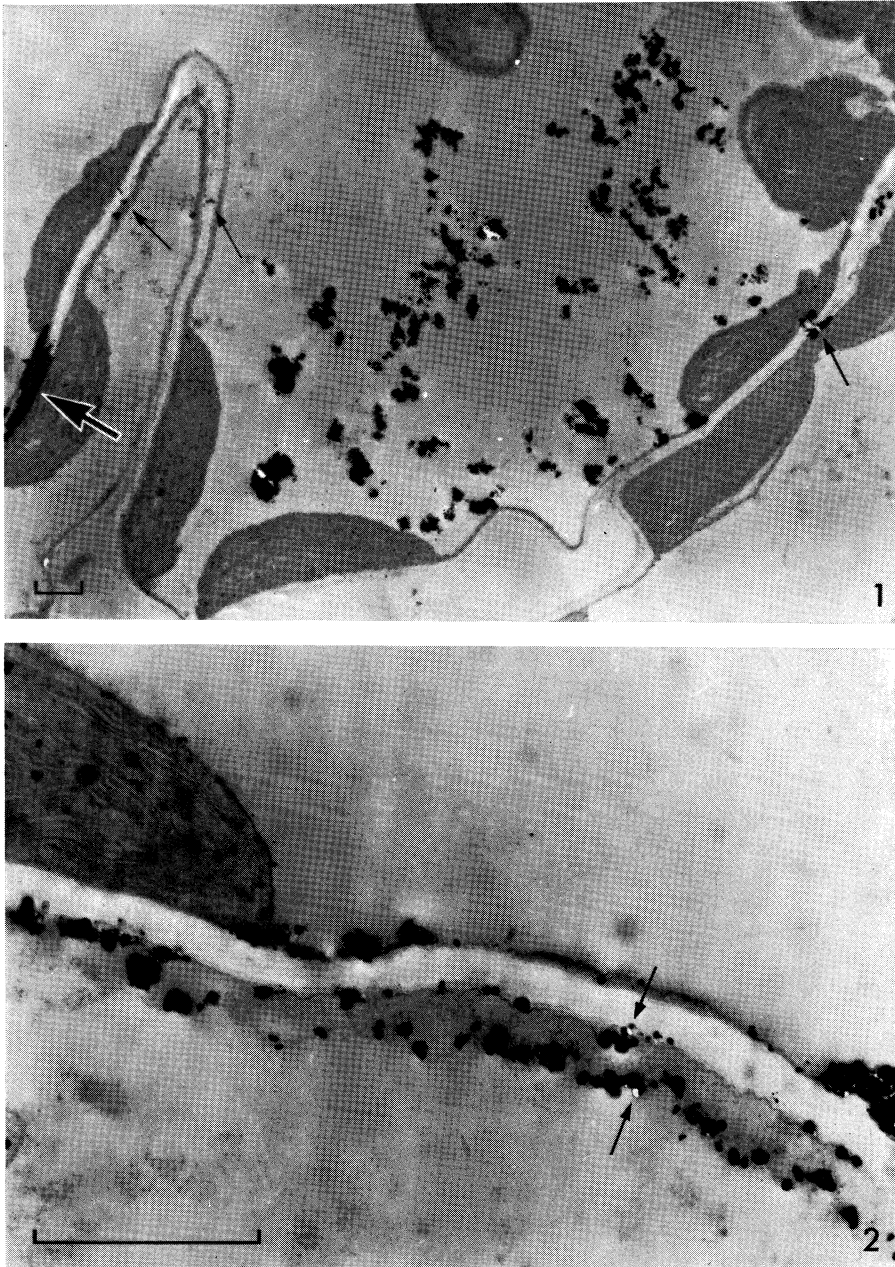


Fig. 1.—A portion of a typical leaf parenchyma cells with large central vacuole and thin layer of cytoplasm. Plasmodesmata indicated by small arrows. Note cell wall region with heavy deposit (larger arrow). $\times 6,400$. [On all figures, the length of the bar represents $1\text{ }\mu\text{m}$, and all except Figures 13 and 14 were taken from sections which were not stained. In Figures 1–7, seedlings were grown in the dark in the presence of NaCl, and in Figure 8 in the dark without NaCl. In Figures 9–12, 15, and 16 seedlings were grown in the light with NaCl and in Figures 13 and 14 they were grown in the light without NaCl.]

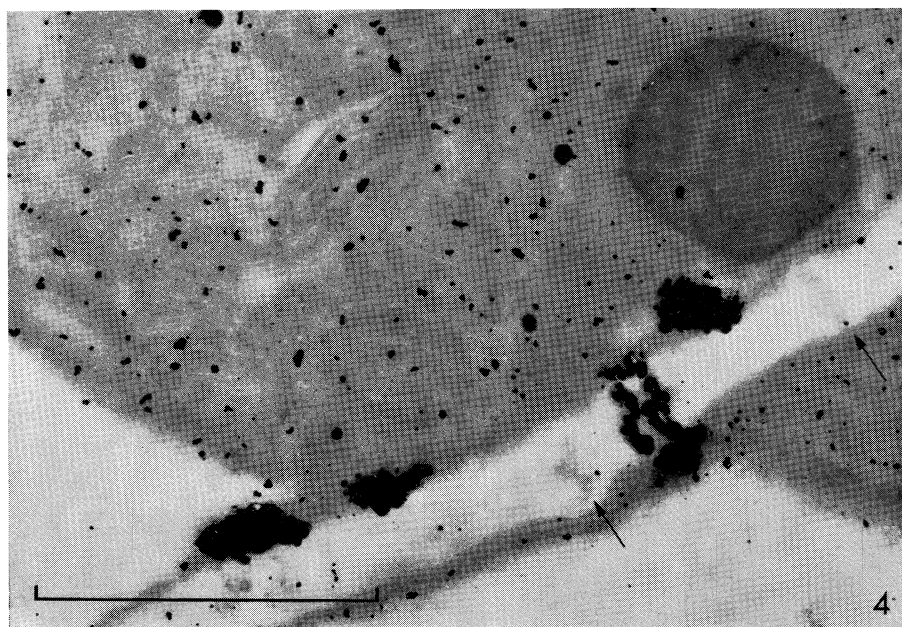
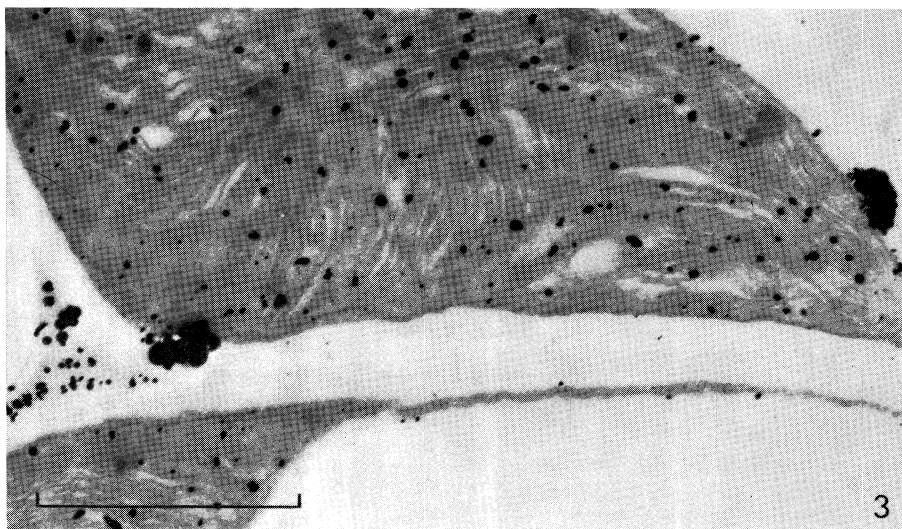


Fig. 3.—Note presence of silver deposits on the cell wall region adjoining an intercellular space. $\times 35,000$.

Fig. 4.—Detail of silver deposits in plasmodesmata. Two of the plasmodesmata shown did not contain a deposit (arrows). $\times 45,600$.

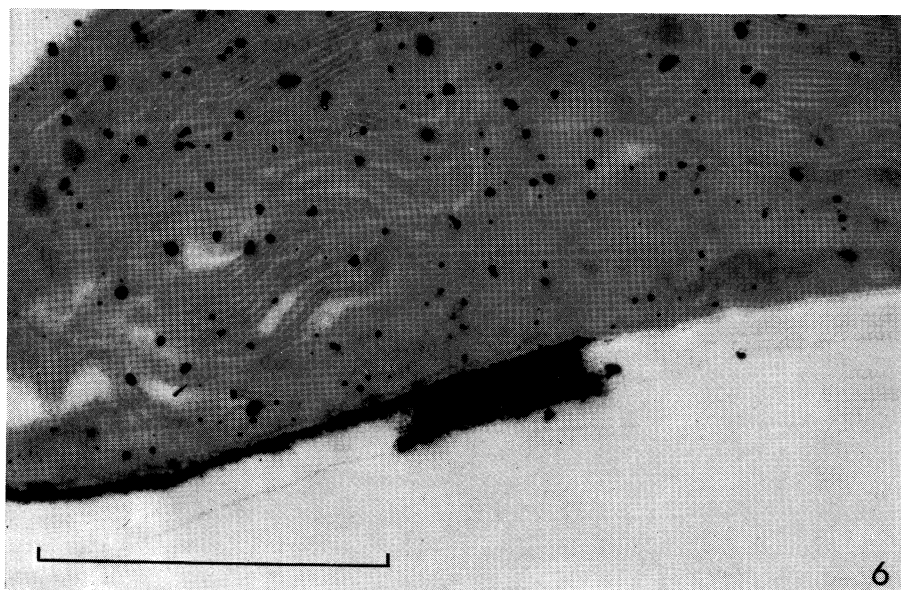
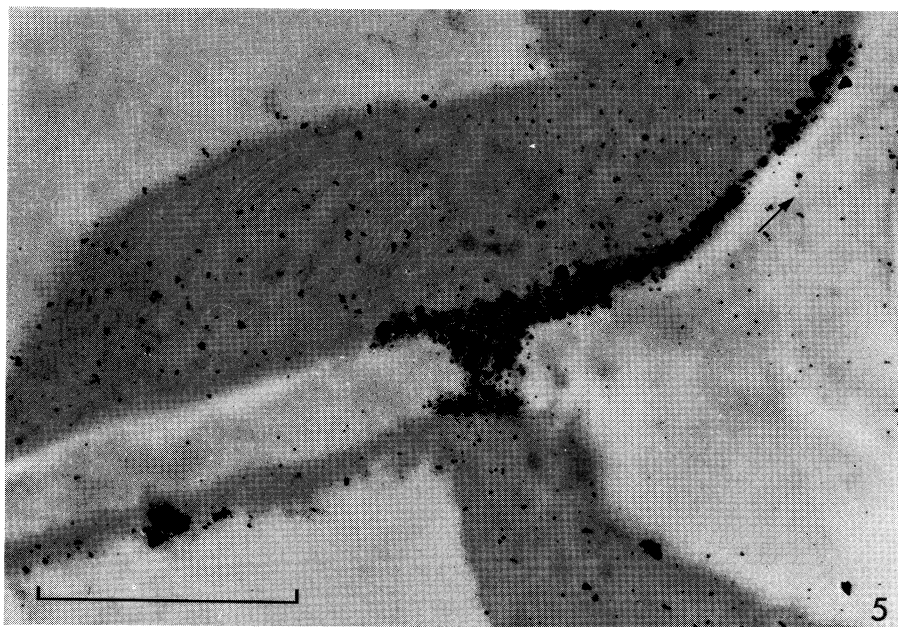


Fig. 5.—Detail of wall region with heavy deposit. Note the shape of deposit between wall and cytoplasm suggesting the movement of material in direction of arrow. $\times 34,500$.

Fig. 6.—Detail of another wall region with heavy deposit. Again a suggestion of movement in one direction is apparent. $\times 46,600$.

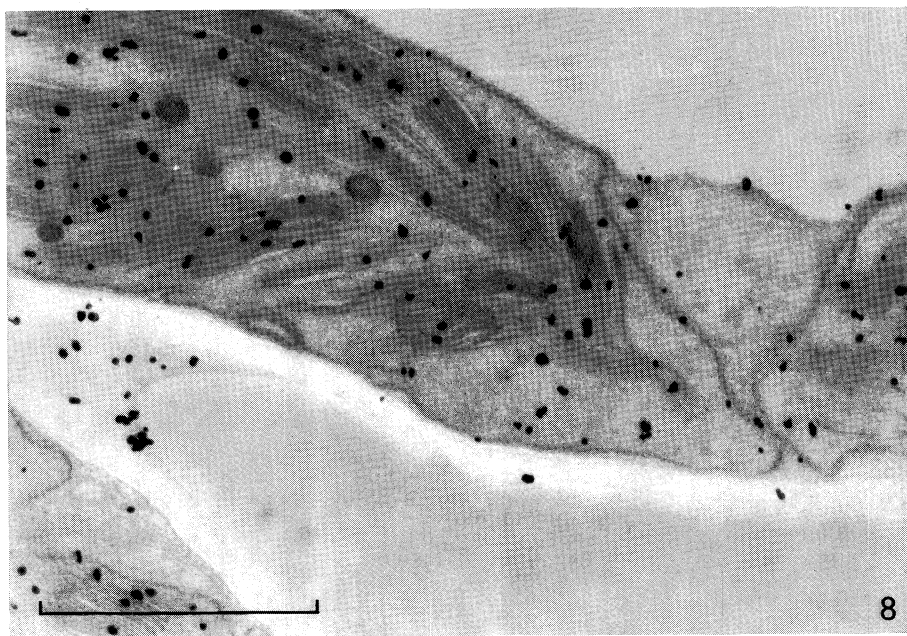
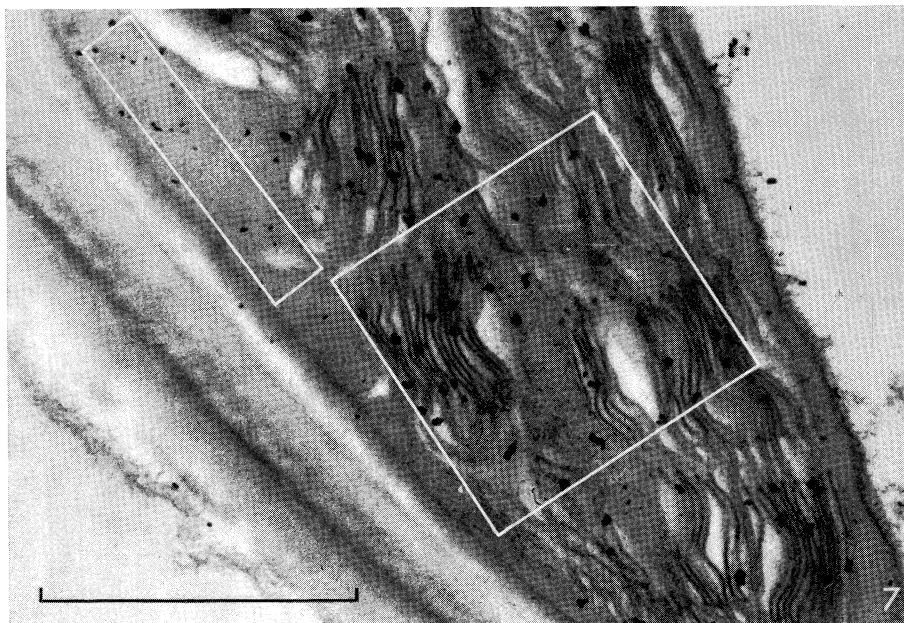


Fig. 7.—Detail of deposits in chloroplast. Note their angular shape. The larger deposits are mainly associated with the grana lamellae. The deposits enclosed in the boxed areas of grana lamellae and of the stroma represent concentrations of 350 mM Cl^- and 65 mM Cl^- , respectively. $\times 41,600$.

Fig. 8.—Deposits are prominent inside the chloroplasts, but no large deposits occurred in the cytoplasm or plasmodesmata. $\times 36,800$.

(b) Seedlings Grown in the Dark without NaCl

Although the number of deposits found in chloroplasts did not visually appear to be much different from that in NaCl-treated seedlings (Fig. 8), systematic counts of randomly chosen areas indicated a significantly lower Cl^- content in the chloroplasts of seedlings without added NaCl (Table 2). The larger clumps of deposits observed in

TABLE 2
CONCENTRATION OF CHLORIDE IN CHLOROPLASTS CALCULATED
ON THE BASIS OF SIZE AND NUMBER OF AgCl DEPOSITS IN THIN
SECTIONS (ASSUMED TO BE $0.08 \mu\text{m}$ THICK) OF MESOPHYLL
CELLS OF THE PRIMARY LEAF OF BARLEY SEEDLINGS
 n = number of $1 \mu\text{m}^2$ areas counted

Conditions of experiment	Cl^- concn. \pm S.E.M. (mm)	n
No NaCl, dark	171 ± 4.0	9
NaCl present, dark	333 ± 8.7	9
No NaCl, light	167 ± 2.1	11
NaCl present, light	$147 \pm 2.0 + 238 \pm 5.0^*$	$7 + 4^*$

* Additional concentration based on the area with accumulated deposit such as shown in Figures 11 and 12.

the seedlings grown in presence of NaCl were practically absent from the cytoplasm when NaCl was deleted. Deposits were also absent from the plasmodesmata, but present in the cell wall near the intercellular spaces where walls of neighbouring cells join (Fig. 8).

(c) Seedlings Grown in the Light in presence of NaCl

In this treatment the plasmodesmata were always prominent because of the large amount of silver deposit inside them (Figs. 9 and 10). In addition, in almost every instance large aggregates of deposits were observed in the cytoplasm adjacent to the plasmodesmata. Other large deposits which were apparently not associated with plasmodesmata occurred in the cytoplasm (Fig. 11).

A special feature was the fine dispersal of silver deposits especially in the chloroplasts. Some confined regions of the chloroplast showed a particularly high density of deposits (Figs. 11 and 12). In the chloroplast shown in Figure 12 it was calculated that the high-density area represented an approximate concentration of 240 mm Cl^- if the deposit was equally distributed over the chloroplast, while the finely dispersed deposit would represent an additional concentration of 130 mm Cl^- . The total approximate concentration of 370 mm Cl^- is not greatly different from that determined for the chloroplast in Figure 7 (dark + NaCl).

(d) Seedlings Grown in the Light without NaCl

In this treatment deposits were practically absent from the plasmodesmata (Fig. 13). Cytoplasmic deposits were relatively sparse; large aggregates of deposits as observed in the seedlings grown in the presence of NaCl were absent. Generally, the

chloroplasts showed less deposit than the latter seedlings, and again chloroplasts which exhibited a distinct non-lamellar area showed that the deposits were predominantly associated with grana lamellae. Calculated concentrations of the boxed areas of Figure 14 were 150 and 196 mM Cl^- for the lamellar area and 29 mM for the stroma area without lamellae.

(e) *Removal of Cl^- after Fixation in OsO_4*

Preliminary experiments were carried out in which leaf tissue of seedlings which had been grown in NaCl and fixed for 2 hr in OsO_4 was rinsed in relatively large volumes of 0.1M cacodylate acetate buffer overnight in order to remove any unbound Cl^- from the tissue. The tissue was then placed in 0.5% silver acetate in 0.1M cacodylate acetate (pH 7.2) in the presence or absence of OsO_4 for 2 hr and subsequently embedded and sectioned by the standard procedure already described.

Figure 15 shows that in the absence of OsO_4 large amounts of deposit occur even though Cl^- is presumed to be absent. The deposits, however, are of a different nature and do not have the angular appearance of the deposits obtained when Cl^- is still present in the tissue (e.g. Figs. 7 and 14). Also, the deposits often display a halo of finely divided silver grains around them.

When OsO_4 was present in the post-treatment with silver acetate deposits were practically absent in the chloroplasts, but occasionally large aggregates of silver occurred, e.g. in the nucleus (cf. Fig. 16).

IV. DISCUSSION

The first histochemical precipitation of chloride by means of AgNO_3 dates back more than 100 years (see MacCallum 1905), but the first use of silver lactate or silver acetate was made by Komnick (1962). Komnick and Bierther (1969) provided evidence of the authenticity of the AgCl deposits by means of electron diffraction patterns and the inclusion of certain diffusion experiments with NaCl-containing gelatin blocks. They concluded that interpretation is especially difficult when extracellular precipitates are present and while intracellular deposits absent or scarce, and gave the following reasons:

- (1) diffusion of Cl^- outwards will result in precipitation on earlier formed crystals;
- (2) intercellular Cl^- may be present but the concentration may be too low to produce AgCl or very small AgCl deposits may dissolve during subsequent steps;
- (3) tendency of already existing crystals to grow at the expense of smaller crystals.

It is important to keep these considerations in mind when attempting to make a quantitative assessment of the amount of deposit present or even when attempting to find an explanation for the big variation in size of deposits in the cytoplasm or in the stroma or grana region of the chloroplasts.

It is pertinent to consider the detection limit when a 0.5% solution (30 mM) of silver acetate is used. The solubility product, $K_{\text{AgCl}} = 0.2 \times 10^{-10}$ at 2°C , hence Cl^- precipitation should commence at concentrations above $7 \times 10^{-7}\text{M}$. The tissue

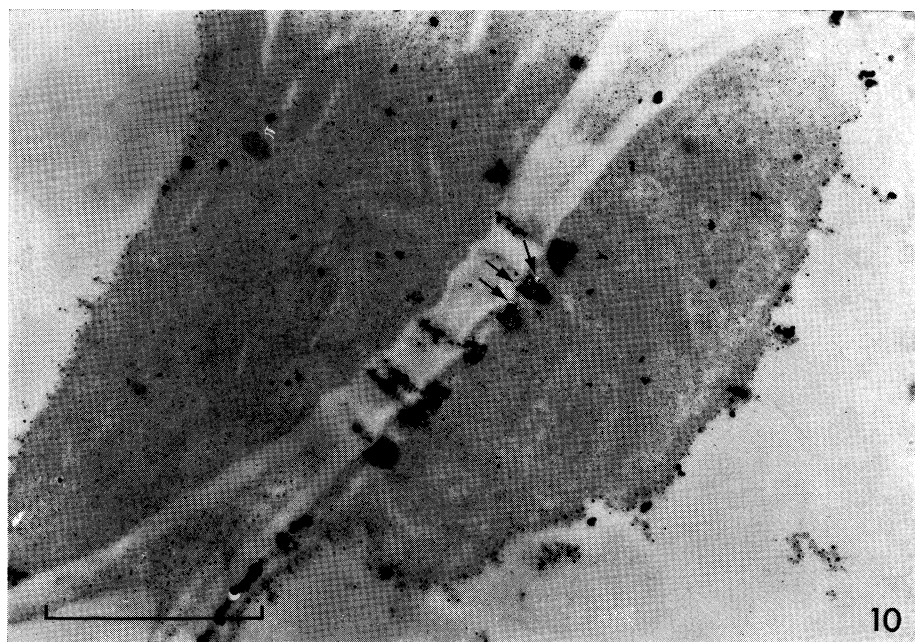
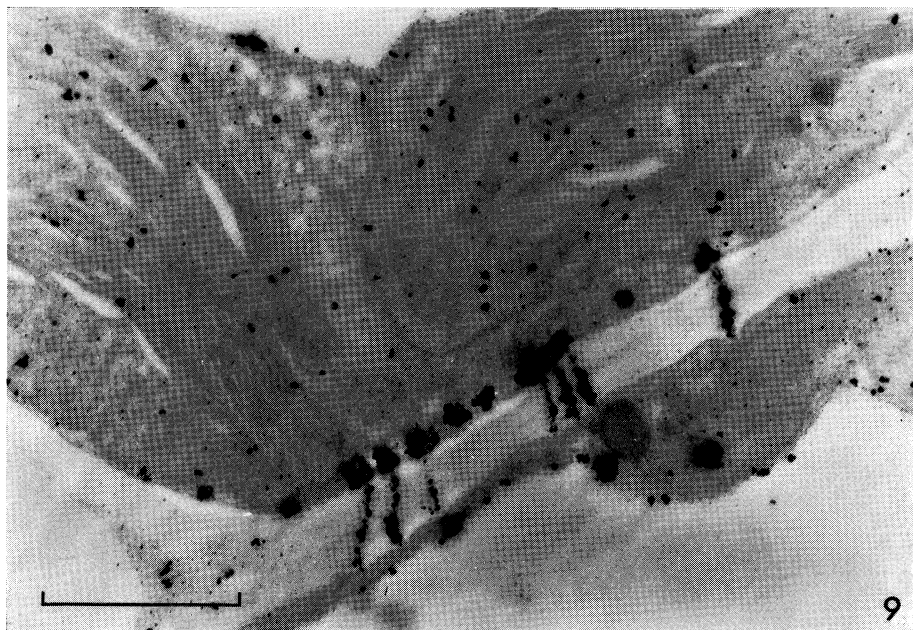


Fig. 9.—Plasmodesmata are prominent because of heavy deposits inside them and nearby in the cytoplasm. $\times 25,600$.

Fig. 10.—Plasmodesmata with deposits; some of these melted due to intensity of the electron beam (arrows). Generally, the deposits were more finely dispersed than in plants kept for a period in the dark immediately before fixation. $\times 28,800$.

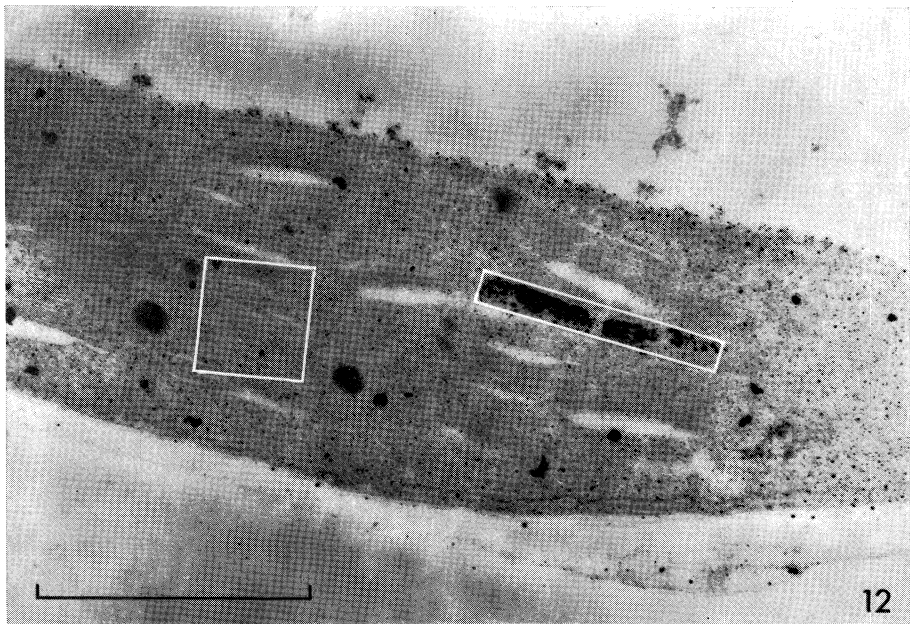
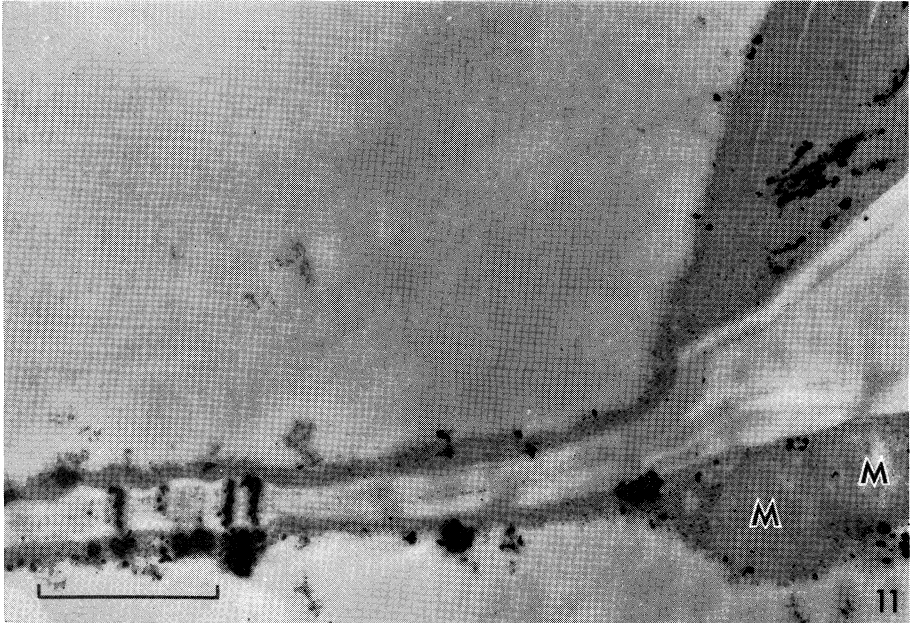


Fig. 11.—Some large deposits are present in the cytoplasm apparently not in direct contact with plasmodesmata. Deposits in the chloroplast show a region of high density; these were rather common in light-treated plants. Mitochondria (*M*) show a lower density of deposit than the surrounding cytoplasm. $\times 23,400$.

Fig. 12.—High-density region in chloroplast. This region represents an approximate concentration of 240 mM Cl^- for the whole of the chloroplast. The other boxed area represents a concentration of approximately 130 mM Cl^- . $\times 36,100$.

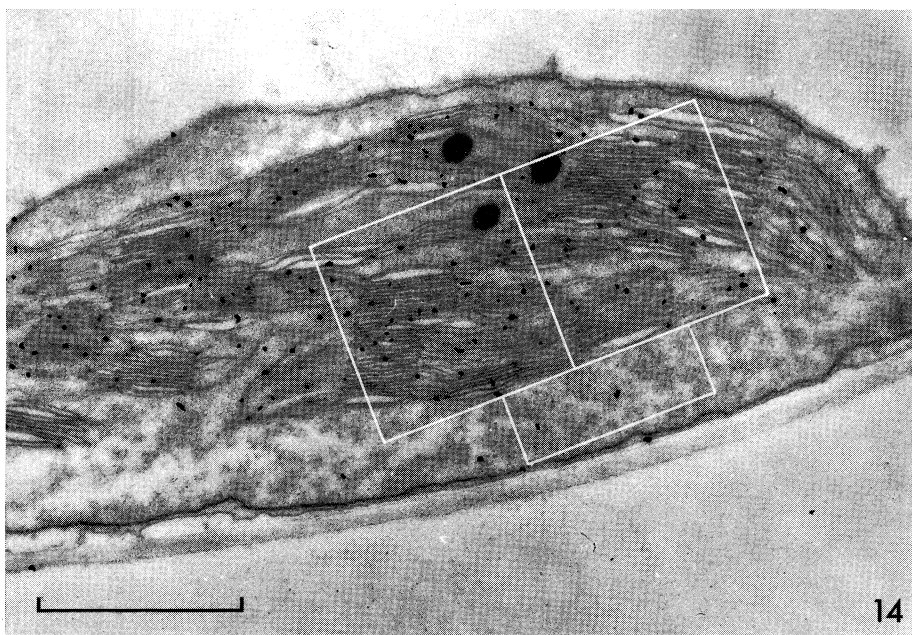
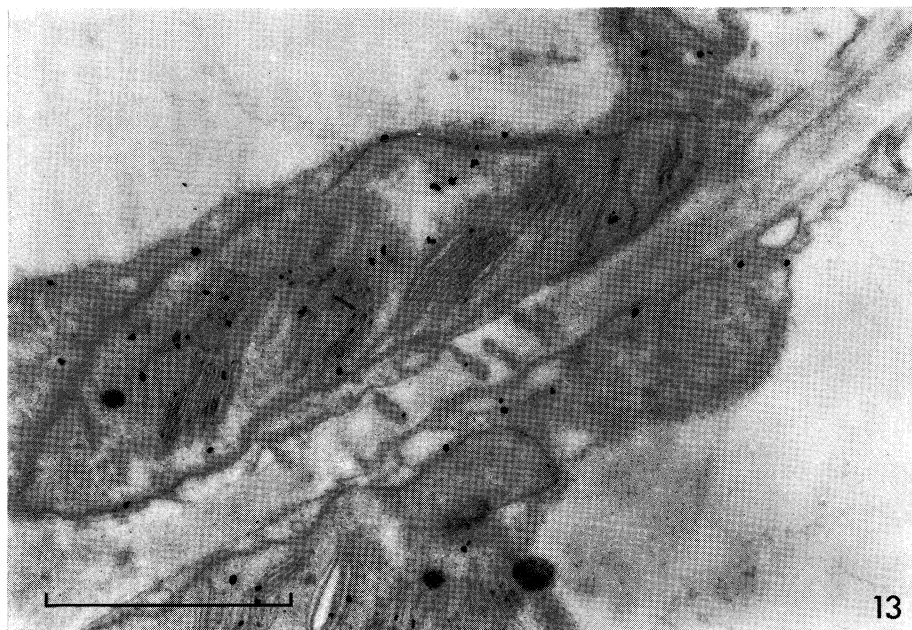


Fig. 13.—Plasmodesmata containing very little or no deposit. Lead stained. $\times 32,400$.

Fig. 14.—Detail of chloroplast. Deposits in the boxed area of stroma represent a concentration of 29 mM Cl^- while the two areas of grana lamellae represent concentrations of 150 and 196 mM Cl^- respectively. Lead stained. $\times 27,000$.

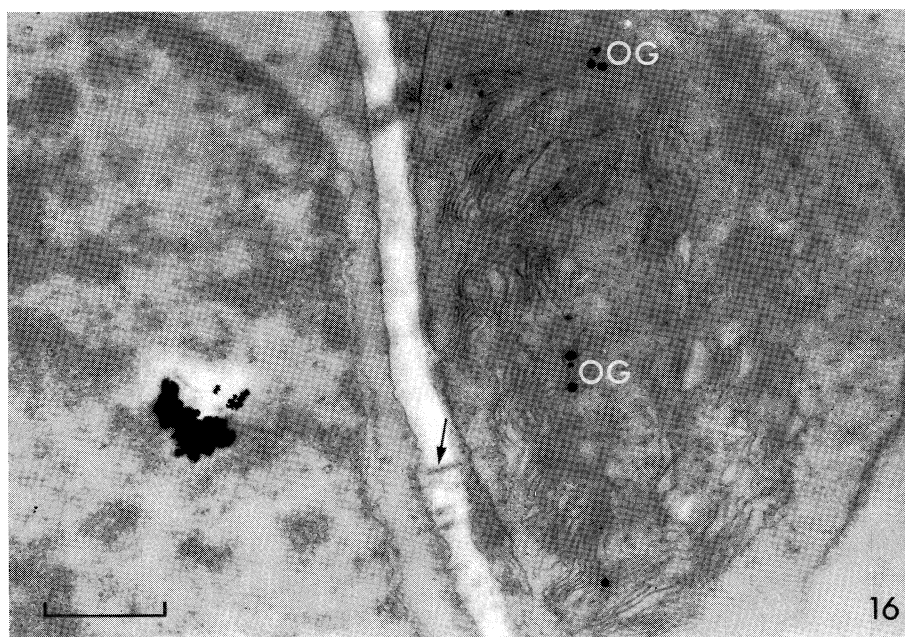
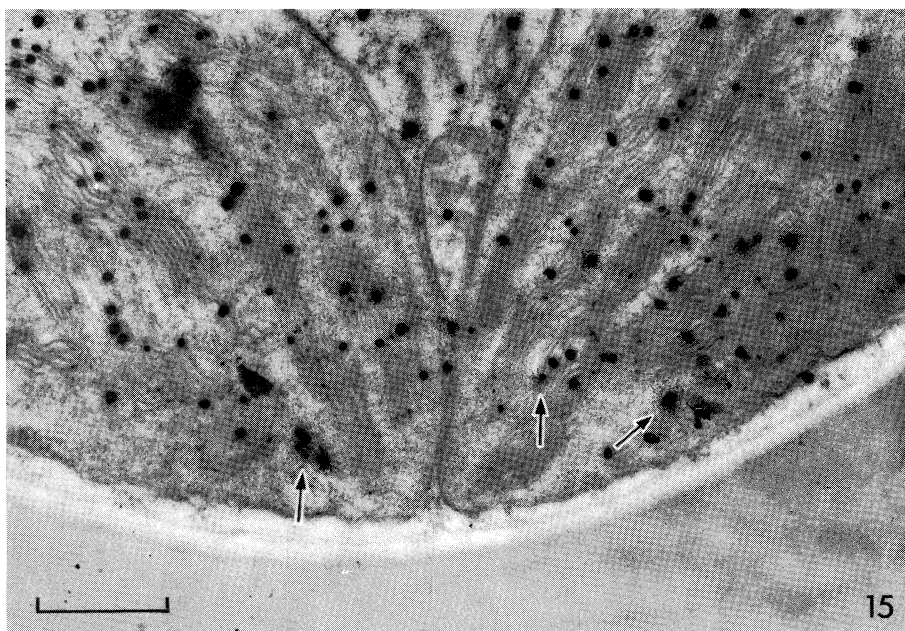


Fig. 15.—Tissue fixed in OsO_4 , rinsed in buffer overnight, and subsequently placed in 0.5% silver acetate in 0.1M cacodylate acetate buffer for 2 hr. Silver deposits do not have angular shape and often display a halo of small silver grains (arrows). $\times 17,100$.

Fig. 16.—Tissue fixed in OsO_4 , rinsed in buffer overnight, and subsequently exposed to 0.5% silver acetate in 0.1M cacodylate acetate + 2% OsO_4 for 2 hr. Silver deposits were practically absent in chloroplasts plasmodesmata (arrow), but occasionally large aggregates of Ag were found in random distribution. This particular one in the nucleus. OG, osmiophilic globules. $\times 15,300$.

concentration was 8×10^{-3} and 18×10^{-3} Cl^- equivalents per kilogram fresh weight in control and NaCl-treated seedlings respectively. Hence the method should be quite adequate to detect the Cl^- present. Relatively large diffusion paths are involved, for instance, in the formation of the two only silver deposits present in the boxed area of matrix in the chloroplast shown in Figure 14. They represent a concentration of $29 \times 10^{-3} \text{M}$ and it is evident that a random distribution of these relatively large deposits in a section thickness of $0.08 \mu\text{m}$ would prevent us from making any meaningful quantitative assessment of concentrations lower than $30 \times 10^{-3} \text{M}$ Cl^- . However, in preparations of mangrove, *Aegiceras corniculatum* (Van Steveninck, unpublished data), it was possible to detect grain sizes of 5 nm diameter, which represent approximately 1/200th the volume of the grain sizes shown in Figure 13. It is obvious that a quantitative assessment based on 400 grains per unit volume would be of much greater accuracy than one based on 2 grains per unit volume. On the other hand, losses of AgCl due to subsequent steps of specimen preparation (rinses in buffer, dehydration, etc.) would be more likely to cause significant losses of AgCl when the deposits are distributed as very small grains. Komnick and Bierther (1969) claimed that if a 1 mm^3 block of tissue was rinsed in 6 ml water at room temperature, this would remove from the tissue all AgCl, equivalent to a concentration of $56 \times 10^{-3} \text{M}$ Cl^- . We rinsed the tissue, about 50 pieces, which is equivalent to 10 mm^3 , in less than 0.6 ml of buffer solution, very briefly, at 2°C ($K_{\text{AgCl}} = 0.2 \times 10^{-10}$ at 2°C , which is one-seventh of its value at 25°C), which represents 1/700th of the solubilizing power given in Komnick and Bierther's example. Hence, it is unlikely that a measurable quantity of AgCl has been removed from the tissue during this brief rinse. However, it is possible that the finest deposits of less than 5 nm diameter are removed by these subsequent steps. A 5 nm deposit represents 2.56×10^{-9} p-equiv., which is only 1550 AgCl molecules.

At present it should be possible to resolve deposits five times smaller again, i.e. of 1 nm diameter. Thus it should be possible to study mechanisms where clusters of the order of 10 Cl^- ions were involved. The feasibility of this is controlled by K_{AgCl} and the ionic densities required in order for AgCl molecules to interact to form a core of deposit. At $7 \times 10^{-7} \text{M}$, Cl^- ions in random distribution are at an average distance of 135 nm from each other, hence at this concentration considerable diffusion is necessary before sufficient interaction can occur to form a AgCl deposit. Chloride electrostatically associated with a carrier would most likely be present at a much greater density and therefore it seems ultimately feasible to pinpoint Cl^- at a carrier site with the minimum of diffusional effects. It should be worth while investigating whether briefest possible fixation, dehydration, and embedding periods would reduce diffusion effects, e.g. AgCl crystal growth at the cost of the smallest AgCl nuclei. Abbreviated methods of specimen preparation have been published recently (Bain and Gove 1971) and appear to give a quality of preservation equal to conventional methods.

In connection with some of the localized large deposits in cell wall, cytoplasm, and chloroplasts, it is also instructive to consider the other limit, that is the assumption that all space is taken up by AgCl. This would represent 5560 g/l or 39M. The 0.5% silver acetate reagent used is equivalent to a 30 mM solution and hence this would presume a solution/tissue ratio of more than 1000 during the exposure of the tissue to the reagent solution (actual solution/tissue ratio was approximately 60). Of

course, the deposits are strictly localized and constitute a small portion of the tissue volume, but the above thousand-fold ratio does indicate that their existence must largely depend on diffusion. It appears that at present the extreme limits, i.e. the lower detection limit and detection of very high localized concentrations, present situations which are unrealistic and beyond the scope of the present technique.

Another uncertainty presents itself in that reduction of Ag^+ may result in the formation of metallic silver deposits. According to Komnick and Bierther (1969) the simultaneous presence of OsO_4 , a strong oxidant, during the fixation period required for Cl^- localization would prevent the formation of metallic silver deposits by normally recognized argentophilic structures. Our preliminary experiments on tissue which was fixed for 2 hr and rinsed in buffer overnight to remove all chloride and then exposed to either silver acetate alone or silver acetate and OsO_4 combined showed that Komnick's claim proved to be substantially correct for plant tissues. Silver acetate in the absence of OsO_4 gave deposits which were much heavier than any shown in this paper, especially in the chloroplasts, while in the presence of OsO_4 , deposits were practically absent, randomly dispersed, and could have been due to insufficient rinsing. Further work is in progress to determine the nature of these random deposits.

Arguments in favour of the contention that the deposits are indeed due to AgCl could be summarized as follows:

- (1) Significantly larger amounts of silver deposits were observed in seedlings grown under high-salt conditions especially in the plasmodesmata and cytoplasmic phase. This observation was substantiated by the fact that salt-grown seedlings contained twice as much Cl^- as control seedlings.
- (2) The concentrations of Cl^- calculated for the chloroplasts based on the size of the deposits were in close agreement with values determined by Larkum (1968) and Larkum and Hill (1970).
- (3) Deposits, especially the larger ones in the plasmodesmata, frequently showed evidence of melting in the electron beam (Figs. 2 and 10, arrows). AgCl melts at a much lower temperature than metallic silver (melting points 455 and 960.5°C respectively).

On the other hand the following arguments may be used against the reliability of the method:

- (1) Unequivocal evidence by means of an electron diffraction pattern that the deposits indeed consist of AgCl has not been obtained.
- (2) The possibility of argentophilic compounds, insufficient rinsing, light-induced reduction of silver which may cause unspecific deposits to be present and therefore make any work of a quantitative nature less reliable.
- (3) The large diffusion paths which must be involved when considering that one grain of 250 nm diameter represents a Cl^- concentration of 4 mM in a volume of $0.08\text{ }\mu\text{m}^3$ (see Section II).

It now remains to consider the distribution of the deposits and determine their physiological significance. Firstly, the principal pathway of Cl^- from cell to cell appears to be through the plasmodesmata. This observation favours the concept of transport through the symplasm (Arisz 1956; Tyree 1970). Deposits elsewhere in the cell wall were practically absent except for some small areas near the intercellular

spaces which appeared to contain a different, more electron-dense component, possibly of a proteinaceous character which could harbour a relatively high density of positive charges at normal pH.

Occasionally, certain areas which showed an extremely high density of deposit were present in the cell wall and in the absence of confirmation that this deposit consists of AgCl we are at this stage somewhat hesitant about a possible interpretation. Spear, Barr, and Barr (1969) and Smith (1970) have reported on the presence of distinct acid and alkaline surface areas on cell walls of *Nitella* and *Chara*, respectively. These alternating bands of acid and base formation especially occurred in the light and these authors have suggested that Cl^- influx occurs only, or largely, in the acid-extruding areas. It is possible that we may have detected a similar phenomenon of cell wall areas with a specific function. These wall areas showed no evidence of being structurally different from the rest of the wall. On the other hand, the nature of the deposits inside the cell suggest that these deposits might not be due to Cl^- but could result from a secretion of substances of a highly argentophilic nature, e.g. acid mucopolysaccharides.

In mature leaf cells, the cytoplasmic phase is mainly taken up by chloroplasts. The rest of the cytoplasm, a very small volume relative to the total size of the cell, contains very little endoplasmic reticulum (ER). There was little evidence of silver deposits in any cytoplasmic vesicles or cisternae of the ER and hence we have not obtained any evidence of a special involvement of the ER in Cl^- transport as suggested by MacRobbie (1969, 1970). However, Larkum's finding that chloroplasts constitute a phase of high Cl^- concentration was confirmed. In addition, we were able to show that the region of grana lamellae contained a much higher Cl^- concentration than the stroma region. Hence, it appears that the high Cl^- content of the chloroplasts is maintained through the association of this Cl^- with the lamellar structure. As no limiting membrane exists between the lamellae and the stroma it appears that the uneven Cl^- distribution might result from the presence of charges on macromolecular components of the membrane system, resulting in a Donnan system. This charge distribution on the macromolecular components appears to alter significantly during the exposure of the leaves to light. The marked alteration in distribution and grain size of deposits caused by exposure of leaves to light immediately prior to fixation suggests that these charges are indicative of light-induced changes in charge distribution determined by macromolecular changes (conformational changes, oxidation-reduction, proton movements).

Obviously, further work is necessary, but at this stage it appears that the method of ultrastructural localization of ions might provide some useful information with respect to the gross morphological aspects of the mechanism of ion transport, e.g. involvement of vesicles, endoplasmic reticulum, pinocytosis. When the method can be refined so that small groups of the order of 10 Cl^- ions can be resolved, it might be possible to contribute to molecular aspects of ion transfer, e.g. conformational changes, carriers, etc.

V. ACKNOWLEDGMENTS

The authors thank Mr. J. V. Hardy for use of facilities of the Electron Microscope Centre, University of Queensland, and Miss W. D. Armstrong for her capable assistance. The work was supported by the Australian Research Grants Committee.

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