Planta (Berl.) 120, 1—12 (1974)
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The Entry of Ions and Molecules into Roots: an Investigation Using Electron-Opaque Tracers

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Received June 15, 1974

Summary. Various solutions containing ions or molecules which may be visualized in the electron microscope have been presented to barley (*Hordeum vulgare* L.) roots. Large molecules such as gold sol particles (< 6.0 nm) or ferritin (approx. 12.0 nm) were not found to be taken into the cytoplasm of any cells. Very dilute solutions of uranyl acetate or lanthanum nitrate resulted in the presence of typical electron-opaque crystals in the cortical apoplasm, as well as in cytoplasmic vesicles of cortical and some endodermal cells, but not in the cytoplasm or cell walls of stellar cells. Colloidal lanthanum hydroxide, however, while also impeded by the Casparian band, accumulated in vesicles in endodermal cells, and also penetrated into the stele.

These results support the concept that different pathways exist for the movement of water and different ions across barley roots. They also indicate the relevance of the Casparian bands, the suberin lamellae, the formation of endocytotic vesicles, and the plasmodesmata, in studies on water and ion uptake.

Introduction

The entry of molecules, ions and water into higher plant roots, and subsequent translocation into the conducting elements of the xylem, is a physiological problem that remains incompletely solved. Physiologists have, at best, carried out uptake experiments using whole roots or root segments, but in either case it has not been possible to relate experimentally determined rates of uptake to relevant structural features. The first stage of uptake is generally conceded to be diffusion from the external solution into the free space (cell walls and intercellular spaces) of the root cortex. Thereafter, the centripetally moving material encounters the barrier of a cylindrical sheath of cells—the endodermis. In barley these cells have a relatively prolonged process of cell wall development: starting from thin-walled, undifferentiated cells immediately behind the meristem; then progressing through the development of Casparian bands on the transverse and longitudinal radial walls; and finally entering the extended period of 'tertiary' wall development which produces much greater thickening on the inner half of the cell

wall than the outer (Robards *et al.*, 1973). It has long been considered that the Casparian bands act as effective barriers to apoplastic transport, and that all inward movement of solutes must proceed through the endodermal cells (symplastic pathway) rather than through the free space of their walls (apoplastic pathway). Further, the unthickened endodermal cells, often found among those with thick cell walls, have usually been referred to as 'passage cells', so implying a physiological function. Recent studies (Robards *et al.*, 1973) show that, at least in barley roots, passage cells are temporary phenomena: all endodermal cells 40 cm or more distal from the root apex possess suberinized, and heavily thickened, walls. Such considerations as these therefore make it imperative that rates of uptake are measured from small segments of the root (as, for example, done in the experiments of Russell and Sanderson, 1967), and that these rates are related to changes in structural features along the length of the root. It has been shown (Clarkson *et al.*, 1968) that rates of uptake for different ions and water vary along the length of barley roots and, also, that the pattern of variation can be different for different ions. Therefore it is important that structural variability should be related to the uptake of a specific solute, or water.

Our present experiments have been concerned with direct observations of electron-opaque marker molecules at different points along barley roots. The aim has been to establish how changing structure, particularly in the endodermis, affects the distribution of the marker. Two main systems have been used, both of which have already been established as suitable for this type of investigation: firstly a very dilute solution of uranyl acetate which is revealed in the electron microscope as typical needle-shaped crystals of a uranyl complex that has been produced at the binding sites by, among other reactions, precipitation with phosphate contained in the fixative solutions (Wheeler and Hanchey, 1971; Robards and Robb, 1972); and, secondly, a colloidal lanthanum hydroxide solution (Revel and Karnovsky, 1967). A simple solution of lanthanum nitrate has also been used as a comparison with the colloidal form of the element. The iron-protein complex, ferritin, and gold-sol preparations have been employed to investigate the peneration of relatively large molecules into the root free space.

Materials and Methods

Seeds of barley (*Hordeum vulgare*, cv Proctor and cv Midas) were soaked in aerated distilled water for 6 h and placed to germinate at 20° C in the dark on moist filter paper in a petri dish. After 2–3 days, germinated seeds were transferred to 10% culture solution (Russell and Sanderson, 1967) and grown for 1 week in a controlled environment of 16 h daylength; 1000 ft. candles illumination; air temperature 20° C; and growth solution temperature 12–15° C. Seedlings were then transplanted into individual jars containing full strength culture solution, and grown under the same conditions, with weekly changes of culture solution, until required.

Attached roots of 2–4 week old plants were treated with one of the following solutions:

1. 10^{-3} (1.25 h) or 10^{-4} M (22 h) aqueous uranyl acetate. In some experiments specific areas of the root were labelled by sealing 3.5 mm long segments of intact roots into plastic tubes in an experimental tank (Russell and Sanderson, 1967).
2. Colloidal lanthanum hydroxide prepared by the method of Revel and Karnovsky (1967) for 3, 5, or 18 h.
3. Aqueous lanthanum nitrate at 4.5×10^{-2} M for 6 h or at 10^{-5} M for 5 and 18 h.
4. 0.5% aqueous ferritin (Calbiochem) for 21 h.
5. Colloidal gold prepared by the method of Feldherr (1962, 1965) for 41 h (4.6 nm $\pm$ 0.063 diameter particles); and colloidal gold coated with polyvinylpyrrolidone (PVP) (5.8 nm $\pm$ 0.069 diameter particles) for 23 h.

After treatment with one of the above solutions, roots were thoroughly rinsed in distilled water; segments were excised from various parts of the root, fixed for 6 h in 1.5% glutaraldehyde buffered to pH 7.2–7.3 in 0.05 M sodium phosphate, washed in 6 changes of 0.15 M buffer over 20 h and were then treated either with 1.0% osmium tetroxide buffered to pH 7.2–7.3 in 0.1 M phosphate at 4° C for 5 h, or were passed straight on to the dehydration stage. Dehydration was either through an acetone series and propylene oxide into an Araldite/Epikote embedding medium (Mollenhauer, 1964) or by diffusion dehydration into the same resin or into the low viscosity epoxy resin recommended by Spurr (1969).

Thick (1–2 μm) or thin (silver) sections were cut using an LKB Ultrotome III ultramicrotome. Thick sections were heat-fixed to slides and stained with toluidine blue. Thin sections were mounted on copper coated or uncoated grids and were examined without staining in AEI EM6B and Hitachi HS8 electron microscopes.

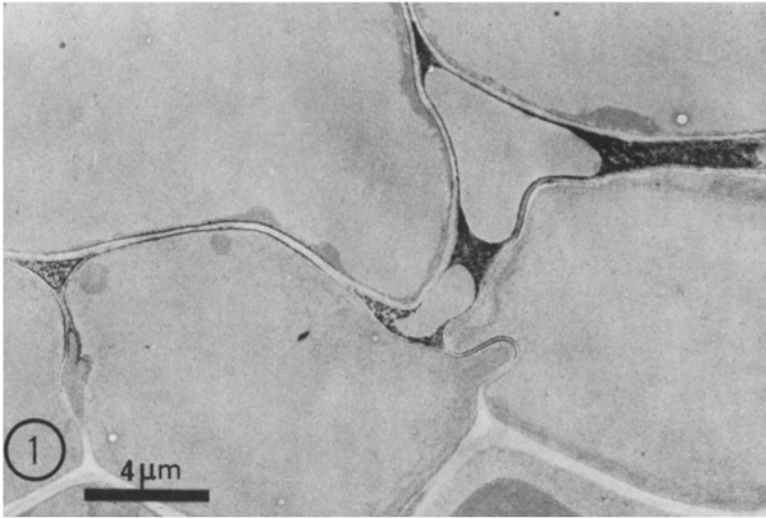
Osmium was removed from sections, when required, by floating coated grids bearing sections on a 10% aqueous solution of hydrogen peroxide for 1 h at 60° C followed by rinsing with distilled water.

Results

Gold sol particles, whether coated with PVP or not, never penetrated far into any part of the roots. Usually the intercellular spaces of the outer two or three layers of cortical cells contained particles while, less frequently, a few particles penetrated as far as the outer surface of the endodermal cells. No particles were ever identified as such within cells.

Ferritin uptake was very similar to that of the gold sol particles except that the iron-protein complex appeared to penetrate as far as the endodermis with greater ease. Ferritin was never unequivocally identified within cells (and it is considered that positive identification would have been possible). It has often been noticed, both in this study, as well as in previous work (Robards *et al.*, 1973), that the vacuole of endodermal cells contains material which has an opaque, finely granular appearance after processing for electron microscopy. Considerable attention was given to determining whether this material ever included particles of ferritin, but no such inclusions were ever identified.

Uranyl acetate has been used in a number of experiments concerning uptake of ions in cereal roots (Wheeler and Hanchey, 1971; Robards and Robb, 1972; Easton and Hanchey, 1972; Wheeler and Baker, 1973). In brief, the typical needle-like crystals are found to be bound in spaces



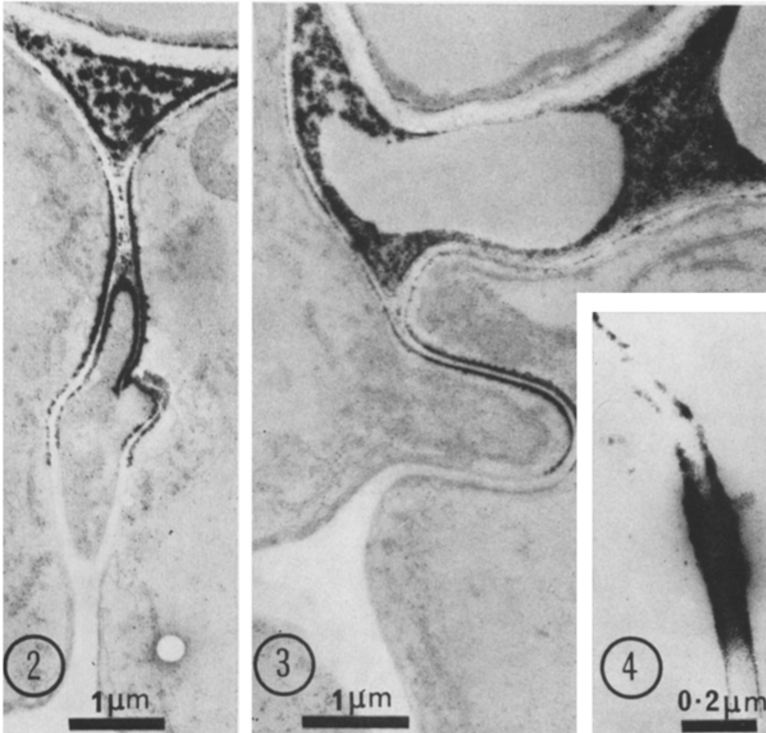


Fig. 1. Transverse section (*TS*) of cortical and endodermal cells of a root treated with 10^{-5} M lanthanum nitrate for 18 h. The apoplasm (margins of cell walls and intercellular spaces) up to the Casparian band are clearly heavily labelled with the opaque tracer. This pattern is closely similar to that obtained using uranyl acetate. × 4000. (1 cm behind root tip)

Figs. 2 and 3. Detail from Fig. 1. × 12000 and × 14000

Fig. 4. As Figs. 1–3, but showing the accumulation of La^{3+} in the cell wall immediately external to the Casparian band. × 50000

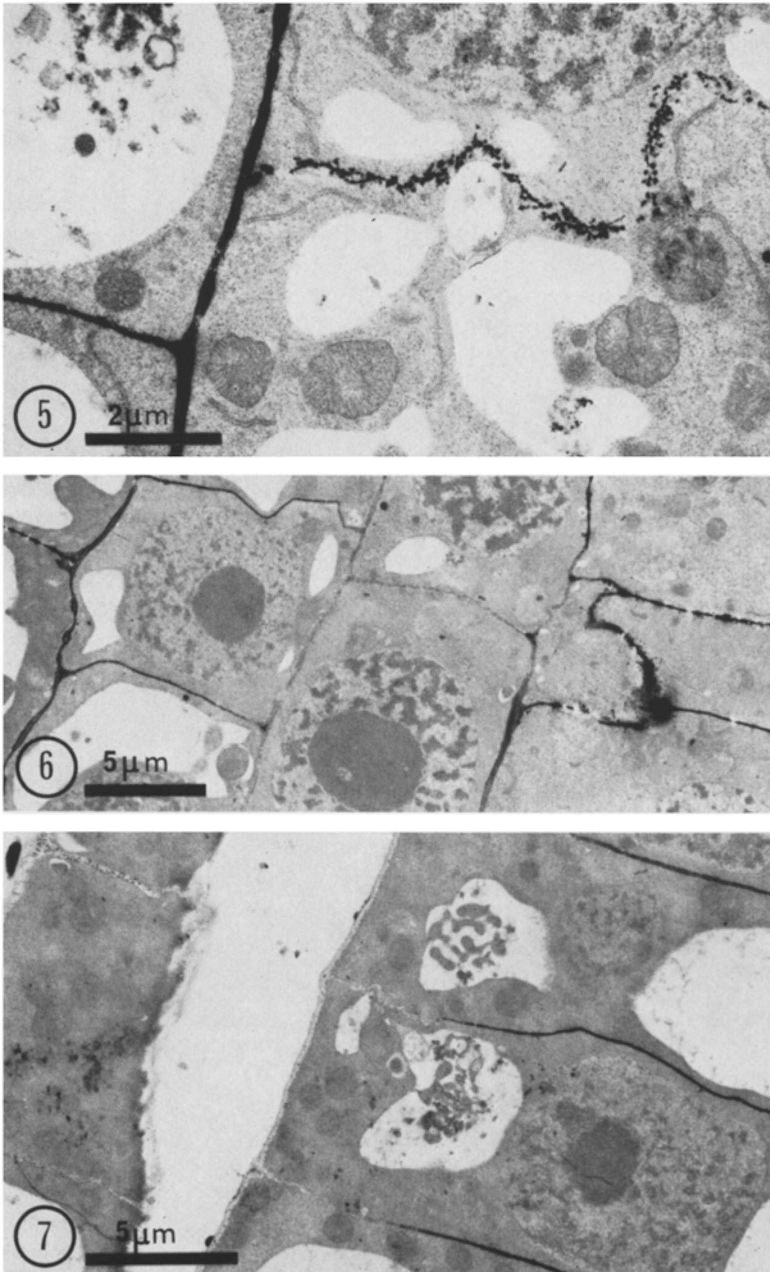


Fig. 5. TS through meristematic zone of a root treated with colloidal lanthanum hydroxide for 18 h. The cell walls have become heavily labelled, as have the vesicles of the developing cell plate. × 9000

Figs. 6 and 7. As Fig. 5, but demonstrating the variation in the pattern of binding in different cell walls and different parts of the same cell wall. × 3200 and × 4000

related to the apoplasm (intercellular spaces; junction of plasmalemma and cell wall). When the crystals are found within cells, then they are always isolated by inclusion within membrane bound vesicles. Root cap cells and cortical cells quite frequently have such vesicles; the uranyl complex has also been seen in the endodermis, so indicating the possibility of an endocytotic uptake in these cells (Robards and Robb, 1972). The Casparian bands, and later the suberin lamellae of the State II endodermal cells, appear to act as effective barriers to the centripetal movement of the uranyl salts, and no bound crystals are found within the stele even when the complex is present in endodermal cells (Wheeler and Hanchey, 1971; Robards and Robb, 1972).

Lanthanum nitrate behaved in an essentially similar manner to uranyl acetate, but the opaque material was of a finer, particulate, form than the needle-shaped uranyl complex crystals (Figs. 1–4). The insoluble lanthanum complex accumulated heavily in the intercellular spaces external to the endodermal cells (Figs. 2, 3) and, often, particularly heavily in the cell wall abutting the outer extremity of the Casparian band. (Fig. 4).

Colloidal lanthanum produced the most interesting results of the present study when fed to barley roots. In the meristematic region of the root the uptake pattern, and binding, closely followed that already described for uranyl acetate (Robards and Robb, 1972). Young, newly-formed cell walls accumulated the tracer molecules in high concentration (Figs. 5–7); often these walls showed a gradient of lanthanum deposition in a transverse direction (Figs. 6, 7), while walls of adjacent cells were markedly less heavily stained (Fig. 7). A striking and consistent phenomenon, that has also been reported in uranyl treated material, was the heavy labelling of vesicles that were aggregating to form the cell plate of dividing cells (Fig. 5). In some cases vacuolar inclusions were seen,

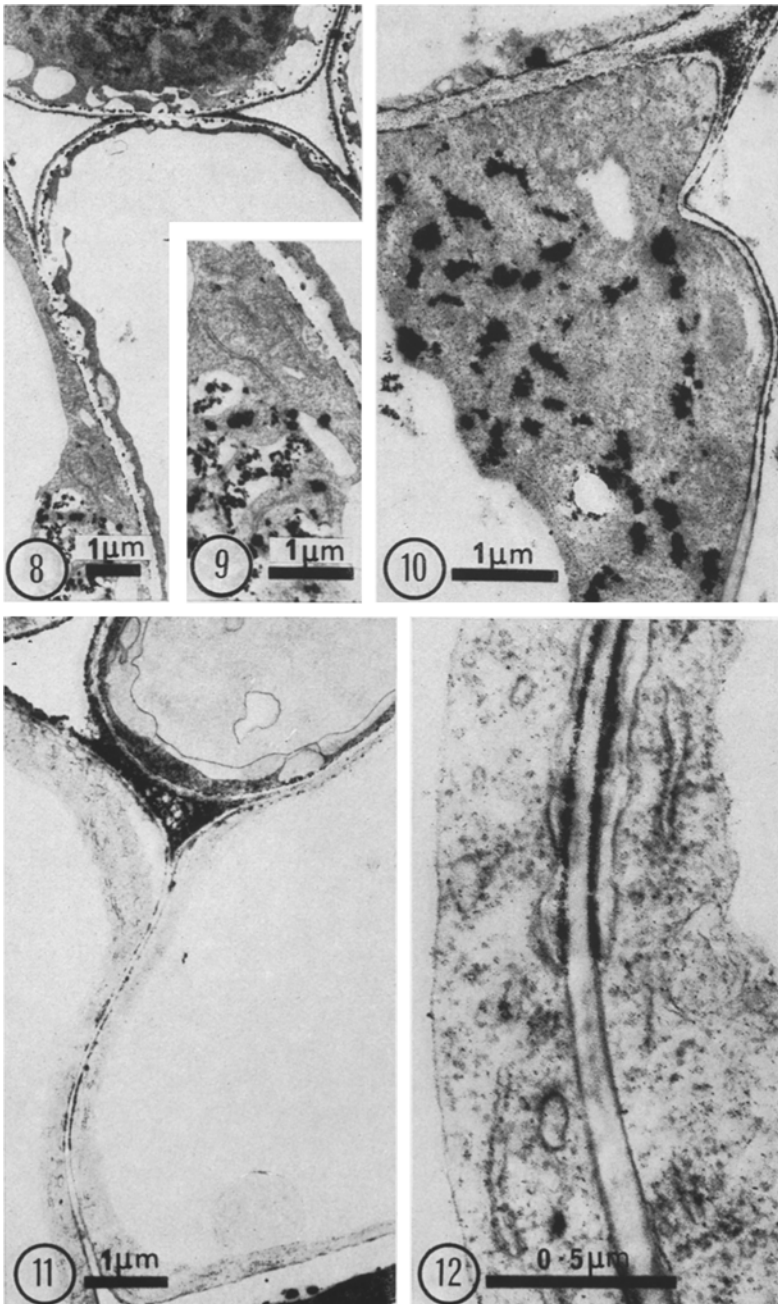
Fig. 8. TS cortical cells of a root treated for 18 h with colloidal lanthanum hydroxide. The lanthanum is seen in the apoplasm, and also in vesicles within the cytoplasm. of the cortical cells. Vesiculation of the plasmalemma along the cell walls is apparent. × 7000. (3 mm behind root tip)

Fig. 9. Detail of cytoplasmic vesicles from Fig. 8. × 11000

Fig. 10. Lanthanum hydroxide within the cytoplasm of endodermal cells. The cytoplasm is intact, and the tracer appears to be located within membrane bound spaces. The Casparian band is at the lower right of the micrograph. × 14000. (1 cm behind root typ)

Fig. 11. The pattern of lanthanum hydroxide distribution in cortical cell walls and the radial wall of the endodermis. × 11000. (1 cm behind root tip)

Fig. 12. Detail from Fig. 11 demonstrating the complete tight junction formed by the apposition of the Casparian band and the plasmalemma. × 50000



Figs. 8—12

as well as other indications of incipient cytoplasmic autolysis. However, while treated roots did not continue with elongative growth, they did not show any obvious signs of degeneration when grown in normal culture solution for 1 or 2 weeks after treatment. Further, the intact plant remained healthy and continued to grow. In the more mature parts of the root (7 mm from the tip), the by now typical response was seen, with the opaque tracer accumulating in the apoplasm up to, but not beyond, the outer border of the Casparian band (Figs. 11, 12). In some cortical cells and endodermal cells the opaque material was seen located within cellular membrane systems, possibly the endoplasmic reticulum/vacuole complex. (Figs. 8–10). (The opaque material was confirmed to be the lanthanum tracer by X-ray energy dispersive analysis using an AEI EMMA 4). One particular feature distinguished the appearance of the lanthanum complex from the distribution obtained with uranyl acetate or lanthanum nitrate: some opaque material was seen within the stele in lanthanum treated roots; this was never the case with uranyl acetate or lanthanum nitrate. Despite considerable efforts to establish whether the penetration of the lanthanum hydroxide into the stele represented an artifact caused, for example, by the breakage of walls in adjacent cells, but not those seen within the same observed section, there seems little doubt that the finding is a valid one and that the lanthanum colloid did penetrate into the stele and become bound. It is apparent (Fig. 12) that the Casparian band, as usual, has totally blocked the passage of the marker, and the radial walls immediately internal to this zone were free, or almost totally so, of the lanthanum.

Discussion

No evidence at all was obtained from this study that relatively large particles, ferritin or gold sol, were able to penetrate into the cytoplasm of living root cells. To this extent, these results are in agreement with those of Barton (1964) who studied the uptake of ferritin in *Phaseolus*. We have no reason for expecting the uptake of such particles into the cytoplasm, and, in any case, such an activity would presumably have to take place through some endocytotic event (for example, see Mayo and Cocking, 1969). That this was not observed in cells which would be considered important in translocatory processes is significant. However, the vacuole of endodermal cells was frequently seen to contain deposits of granular, electron-opaque material. Whether, in some cases, this material represented degraded ferritin molecules remains open to conjecture pending further experiments but, among other things, it does appear that the endodermal vacuole may act as a 'filter' across the centripetally flowing symplastic pathway. The roots examined by Barton were sectioned at 6 and 10 mm from the apex; that is, just behind the meristem. The roots in this study were sampled over a much wider area.

The results obtained using lanthanum nitrate or uranyl acetate were roughly comparable, in that the ions of both heavy elements became bound and locatable within the apoplasm of the cortical and endodermal cells up to, but not beyond, the Casparian bands of the endodermal cells. Subsequently the suberin lamellae of the State II endodermal cells (Robards *et al.*, 1973) curtailed the inward movement. Vesicles containing the tracer complexes were found in meristematic cells and cortical cells and, to a lesser extent, in endodermal cells. However, movement from the endodermis into the stele appeared to be blocked. Thus, although the lanthanum and uranyl ions had apparently penetrated the symplast, they did not appear to be transported within it.

Although it was not the primary area of interest during this study, it is relevant to draw attention to the pattern of binding seen in the cells of the meristem in barley roots. The cell plate and young cell walls became extremely heavily impregnated with electron-opaque material after either lanthanum nitrate or uranyl acetate treatment. Further, vesicles which were about to fuse with the young cell plate, but had not yet done so, also became well labelled. In such cells the solutes clearly penetrated the symplasm and ions became bound. It appears that such binding was possibly preferentially located at the sites of polyanionic polysaccharides.

In a recent paper, Nagahashi, Thomson and Leonard (1974) supplied young corn (*Zea mays*) roots with a 1% solution of lanthanum nitrate. They observed that "lanthanum deposits were found only in cell walls and on the outside of the plasma membrane of epidermal, cortical, and endodermal cells up to the Casparian strip." They also stated that "The cation La^{3+} does not penetrate cell membranes." While the main conclusion—that La^{3+} presented as $La(NO_3)_3$ apparently does not penetrate to the stele—is in accord with our own observations, the assumption that the ion does not penetrate cell membranes is open to dispute. Our results showed the accumulation of both UO_2^{2+} and La^{3+} in cytoplasmic vesicles in cortical and endodermal cells and, particularly, in meristematic cells (which were not considered in the paper by Nagahashi *et al.*). Further, the concentration of lanthanum nitrate used by Nagahashi *et al.* (approx. 3×10^{-2} M) is extremely high: Clarkson (1965), for example, found that 10^{-4} M La^{3+} at pH 4.4 completely and irreversibly inhibited cell division in the root meristem of *Allium cepa*, thus indicating the effect of the ion *within* the cells. While the effect of $La(NO_3)_3$ may not be so damaging on non-dividing cells, the use of the salt at such high concentrations (1000 times greater than that used in most of our experiments) leads to inevitable difficulties in interpreting any cytoplasmic and/or energy dependent processes associated with the tracer. Indeed, it may well be that highly toxic concentrations of such elements serve only to demonstrate the free-space (apoplasm) of root cells external to the endodermis. In the case of lanthanum nitrate,

there also exists the possibility that colloidal lanthanum hydroxide will be formed in the cortical apoplasm, and that it is this opaque tracer that is visualized in the electron microscope. A distinction must be made between the absence of opaque complexes from the cytoplasm of some cells in the mature roots and the heavy deposition in the youngest cells. The alternatives are either that ions may be excluded from the cytoplasm, or that they enter but do not become bound. The former proposition appears most probable: the markers are clearly observable within cells which have become labelled; and it is doubtful whether, having entered a cell, all traces of the solutes would be washed out again before some precipitation had occurred at non-specific sites during fixation—a phenomenon that was not observed.

The case of the lanthanum hydroxide colloid is less clear than where true solutions were used. Even after most stringent precautions had been taken, some of the tracer material appeared to penetrate into the stele where it became bound. Lanthanum hydroxide has been widely used in uptake experiments prior to electron microscopy (Gunning and Pate, 1969; Revel and Karnovsky, 1967; Rowley and Flynn, 1971), and it must be assumed that the material is capable of being moved from the apoplasm, into the cells, and thence symplastically via the plasmodesmata into the symplasm and/or apoplasm of the stele.

The results presented in this paper once again draw attention to two features of barley endodermal cell structure that are of crucial importance in determining the pathways and patterns of translocation within the root: firstly, the barrier across the apoplast constituted by the Casparian band and, later, the more extensive limitation of the suberin lamella; secondly, the presence of plasmodesmata through all walls of endodermal cells. The concept that development of the suberin lamella effectively blocks all further centripetal flow is now well established to be a false one (Clarkson *et al.*, 1971). However, the suberin lamella obviously does act as an apparently total restraint upon the passage of solutes from the apoplasm into the endodermal symplasm. Therefore, inward flow can only occur by symplastic means: which must involve plasmodesmata. Resulting from this conclusion, it is pertinent to consider the means whereby substances might move from either cortical symplasm (S) or cortical apoplasm (A) into the endodermal symplast. In State I cells alternatives are: i) by transport across membranes ($A \rightarrow S$); ii) by formation of endocytotic vesicles ($A \rightarrow S$); or iii) via plasmodesmata from cortical cells ($S \rightarrow S$). At the State II stage, the opportunities are confined to the latter case ($S \rightarrow S$ via plasmodesmata). It is to be expected that water and many ions pass across the membranes of State I endodermal cells by 'normal' processes well documented in the physiological literature (Anderson, 1972; Epstein, 1973). However, there are increasing indications that some cells, including endodermal cells, have the capacity to move material into their cytoplasmic compartments by endocytotic

processes. Baker and Hall (1973) have recently considered such events and have given evidence for their importance. The results obtained in the present work (as well as those from earlier sources: Wheeler and Hanchey, 1971; Robards and Robb, 1972) all illustrate the likelihood of endocytosis being important in at least some apoplast→symplast movements. It would be difficult to overstress the limiting and controlling role of the endodermis in the A→S transition and, therefore, it is of particular interest to note that lanthanum hydroxide tracer appeared within membrane-bound vesicles in the endodermal cells. The fact that some tracer was also found within the stele implies that it could move through plasmodesmata, and it is also clear that uptake, in common with uranyl acetate (Robards and Robb, 1972), could be by endocytotic means.

The importance of the endodermal cells lends increased significance to any reports of attempts to use tracers for following the course of water or ion uptake across roots. However, there are technical difficulties and objections, particularly in respect of the possible toxic effects of the solutions being used. Tanton and Crowdy (1972) have used a method of feeding plant roots with a lead chelate and then precipitating the lead as lead sulphide with H_2S. Among other things, they conclude: "lead precipitate was absent from the protoplasm of the cortical cells but was clearly visible in several of the endodermal cells (Plates 1E and 3C)." This is a significant statement, the more so because the authors assume that the pattern of lead sulphide deposition will mirror the actual pathway of water uptake: a debatable assumption. However, observation of the Figures cited shows that 1E does not illustrate endodermal cells, and 3C has clearly lost the bulk of its cytoplasm and merely has lead sulphide on the inner cell wall surface (a common result with the tracers in our own experiments when processing damage had occurred). Therefore it is not possible to assess whether the lead chelate actually penetrated the living endodermal cells.

Although the results of electron-opaque labelling experiments need to be interpreted with extreme caution, it does seem clear that a consistent pattern of movement is obtained. This supports the idea that: i) the Casparian band blocks apoplastic flow between the cortex and the stele; ii) the suberin lamella acts as a total barrier to direct interchange between the cortical and endodermal apoplastic compartments; iii) cortical and, possibly, endodermal cells have the capacity to take up water-carried ions and molecules by the formation of endocytotic vesicles; iv) plasmodesmata must be effective in allowing flow between otherwise mutually inaccessible symplastic compartments.

This work was carried out under a grant from the Science Research Council. We are indebted to Dr. D. T. Clarkson (A. R. C. Letcombe Laboratory) for his continuing help and advice with this work.

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