STUDIES OF EXTRAFAVFASCICULAR MOVEMENT OF WATER IN THE LEAF

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Summary

An extrafascicular pathway of water movement is examined and evidence is presented from electron-microscope observations on the distribution of gold colloids which confirms Strugger’s observations of a mass flow of water through the cell walls. This appears to be confined to microcapillaries in some regions of the cell walls of Helxine leaves.

I. INTRODUCTION

The transpirational flow of water from the xylem to the evaporative surface has long been regarded by many workers as an osmotic flow of water from one protoplast to the next. This view has been opposed by Strugger (1949), who concluded from observations on the movement of fluorescent dyes that a mass flow of water occurred along the cell walls. Other literature supporting Strugger’s conclusion has been discussed elsewhere (Gaff and Carr 1961). The lack of general acceptance of his views led Strugger and Peveling (1961) to use the electron microscope to study the resulting distribution of colloidal gold and silver particles which had been taken up by plant tissues. They reported the accumulation of the particles in the cortical cell walls of transpiring shoots whose cut ends were immersed in sols. This appears to be strong evidence for a mass flow of water in the wall, as the particle sizes (50–100 Å) were not much less than the diameter of the interfibrillar spaces in the wall of approximately 100 Å (Frey-Wyssling 1953). The resistance to diffusional movement of the particles under these circumstances must be very great, yet the illustrations published by Strugger and Peveling show that after only 3 hr the wall becomes thickly packed with a dense granular material interpreted by them as gold.

Their experiments, however, have certain shortcomings. The study was confined to the stem cortex—no further information is given to location of the region studied, or its proximity to the xylem and to the cut end of the stem. Moreover only one species was investigated—Helxine soleirolii, a prostrate mesophytic plant which grows in moist shady situations. The very density of the electron-opaque areas in published electron micrographs leads one to suspect an artefact; for in a section 200 Å thick, particles dispersed at the concentration of the sols used by Strugger and Peveling (0·12 g/100 ml—Peveling, personal communication) would cover only 0·04% of the area of the cell wall.† The density of the apparent gold particles would imply a surprisingly large accumulation. Further reason for ques-

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‡ For a 50 Å particle, the ratio of cross-sectional area to mass = 15·5 × 10^4 cm^2/g. Since the mass of gold in an area y (Å^2) of a section 200 Å thick is 24y × 10^{-26} g, the area of gold in y (Å^2) is 24y × 10^{-26} × 15·5 × 10^4 cm^2 = 37y × 10^{-21} cm^2, i.e. 0·037% of the area.

tioning the identity of the electron-opaque areas comes from their statement that similar areas occurred occasionally in control plants in water. Also, the poor fixation of the cytoplasm evident in Strugger's electron micrographs increases the suspicion that artefacts could arise in the wall.

In view of this an attempt was made to repeat this type of experiment and extend the observations to the leaf tissue of a number of species including Helxine and to establish the identity of the electron-dense areas reported by Strugger and Peveling.

II. MATERIALS AND METHODS

(a) Preparation of Gold Sols

Gold sols were prepared by reducing a dilute solution of chlorauric acid with phosphorus. An ether solution of phosphorus (1 ml, one-fifth of concentration at saturation) was added slowly to a mixture of 2.5 ml of chlorauric acid (0.6%), 3 ml of potassium carbonate (0.72N), and 120 ml of distilled water. The mixture was left for 10 min at room temperature and was then heated to boiling until it became bright red and cooled in an ice-bath. The sol was dialysed for 24 hr against distilled water to remove dissolved salts, then reduced in volume with a Rinco rotating evacuator until a concentration of 0.12% was reached. The concentration was checked by weighing the dried residue of a known volume of sol. The colloidal particles produced by this procedure were approximately 40 Å in diameter. Electron micrographs (e.g. Plate 2, Fig. 2) were taken of droplets of the gold sol which had been dried on to carbon-coated grids (procedure as in Valentine and Bradfield 1954) and these were used to measure particle sizes. The distribution of size groups is plotted in the histogram (Plate 2, Fig. 3).

(b) Treatment of Plants with Gold Sols during Transpiration

A range of species which included both mesomorphs and xeromorphs was used. These included Beta vulgaris L. cv. Mangel (small mature leaves close to inflorescence), Australina muelleri Wedd. (mature leaves), Helxine soleirolii Req. (leafy shoot), and Eucalyptus globulus Labill. (mature leaves of previous season's growth). In each case a control was placed in water under the same environmental conditions as the plants treated with gold. In a third treatment for each experiment eosin dye was used to indicate macroscopically how far the transpiration stream had moved. The petioles, shoots, or leaves were cut under water and the cut ends submerged into gold sols in small tubes 7 mm in diameter by 2.5 cm. The dimensions of the tube were necessitated by the small volumes of gold sol available. In the case of the juvenile leaves of E. globulus, only the apical 2 cm of the leaves were employed, the midrib below it was trimmed to simulate a petiole and entirely submerged in the sol.

In order to maintain constant reproducible conditions, the leaves were allowed to transpire in a growth room which was illuminated with banks of fluorescent tubes, the light intensity on the plants being 500 f.c. (Weston meter). The spectral composition ranged from 3000 to 7000 Å with a maximum at 5600 Å. Compared with overcast daylight, the red wavelengths predominated (30% more than in day-
light) over the blue. The room was maintained at 25°C with a relative humidity of approximately 85%.

The tubes and shoots were weighed at the outset and periodically during the experiment to provide a rough measure of the transpiration rate. Tubes of water (without plant tissue) were also weighed to correct for evaporation from the surface of the sol.

(c) Electron Microscopy

When dye uptake was visible in the eosin-treated plant, narrow strips of leaf tissue 1 by 3–5 mm from both the gold-treated and control plants were fixed in either 2% osmium tetroxide fixative buffered to pH 7.4 with acetate-veronal and containing 4·3% sucrose, 0·01% CaCl₂, and 0·01% MgCl₂, or in 2% buffered osmium tetroxide containing 0·5% MgCl₂ at 2°C for 25 min, allowing it to come to room temperature for up to 15 min. Fixation was followed by dehydration with at least 5 min in each of 10% steps of ethanol. The specimens were infiltrated for 24 hr in three changes of monomer mixture [25 : 75 v/v methyl methacrylate–butyl methacrylate, containing 2% benzoyl peroxide as a catalyst] and this was followed by further infiltration in partially prepolymerized monomer for at least 24 hr at 2–4°C and final polymerization was at 60°C. Sections were cut on a Porter–Blum microtome, using glass knives, and examined with a Siemens’ Elmiskop I electron microscope at 80 kV.

III. Results and Discussion

No gold particles were detected in the initial experiments with Australina, Beta, and Eucalyptus. The leaves were allowed to transpire for 4 hr during which water loss was approximately 75, 35, and 45% of the fresh weight for the three species respectively. In the eosin-treated specimens, the dye was visible in the smallest veins of Australina at 3 hr, and at 23 hr in the other species. Little penetration of particles larger than 100 Å had been anticipated, this being the generally accepted width of the interfibrillar spaces. Although the transpiration rates were not high, they were sufficient for several replacements of the capillary water in the cell wall, if water flow was restricted to this pathway.

The results up to this stage gave no support to those of Strugger and Peveling. Further doubt was cast on their conclusions by the observation of dark-spotted areas in walls of the lead-stained B. vulgaris controls, the appearance of which resembled the electron micrographs of Strugger and Peveling. In view of these considerations, a closer examination was conducted using Helixina soleirolii, the plant used by these authors. At 3, 6, and 26 hr the lowest leaf remaining on the treated shoot was removed for electron-microscopic investigation; the transpiration at these times amounted to approximately 30, 30, and 65% of the shoot fresh weight. (The lower transpiration rates at 6 hr if real may arise from blockage of the pathways for water movement by the gold colloid.) Eosin-treated shoots exhibited dye penetration into the veinlets of the two lowest leaves at 3 hr, and into all the leaves by 6 hr. By 26 hr the eosin had killed the shoot.

Electroni-opaque areas similar to those reported by Strugger and Peveling were found in the outer wall of the epidermal cells, and these were quite densely packed
even in the leaf treated for only 3 hr (Plate 1, Figs. 1 and 2). An attempt was made to establish the presence of gold in these areas by means of electron-diffraction studies. While diffraction rings characteristic of gold were obtained for dried films of the original sol, the background pattern from the leaf section, essentially that of amorphous carbon compounds, was so marked that any diffraction due to gold at this concentration would have been easily obscured. However, higher-resolution micrographs of the dark areas of the epidermal cell walls (Plate 2, Fig. 1) demonstrated that they were not diffuse (as might be expected for an adsorption of electron-dense ions) but were composed of discrete particles which show a size distribution (Plate 2, Fig. 3a) closely similar to those of the gold sol (Plate 2, Fig. 3b), although the proportion of particles greater than 50 Å is reduced. This is reasonably conclusive evidence that the particles were in fact from the applied gold sol. Their distribution in the tissue lends weight to this conclusion. Within the wall they were unevenly distributed, whereas negative adsorption sites are more evenly spread across the wall, with a marked accumulation in the middle lamella (Albersheim and Killias 1963). Also the particles were very dense in the cuticle where presumably negative charges are rare. In the tissue as a whole the particulate regions were evident only at or near the evaporative surfaces where the sol would be concentrated, i.e. outer epidermal walls just beneath the cuticle (suggestive of a high cuticular transpiration in this species), and to a lesser extent at the walls lining intercellular spaces (Plate 1, Fig. 1). These intercellular spaces presumably connect with the stomatal chambers. Dark walls (i.e. electron-dense areas) in the xylem region were apparently not particulate and were indistinguishable from the dark areas in the control (Plate 1, Fig. 3).

The fact that the particles were not filtered off in the walls close to the xylem but were carried through to the epidermis indicates a surprisingly facile transport through the wall in channels (presumably much greater than 40 Å) which intercommunicate freely. The apparent absence of particles from the intervening wall is to be expected, for here the sol would not be concentrated by evaporation, and on the basis of the earlier calculations, the gold would appear as only 0·037% of the area of the channel. There was no evidence of gold uptake into the cytoplasm or cell vacuole. This may well be due to the particle size (40 Å diameter) being too large to pass passively through the cell membranes.

In epidermal walls the areas containing the particles were patchily distributed and within these areas the particles themselves were restricted to isolated patches 300–400 Å wide in the outer part of the wall, and 200–300 Å in the anticlinal walls. Presumably these areas represented the broader capillary spaces which were freely continuous with the xylem. Their apparent width would be exaggerated in the micrographs by the depth of the capillary. To the authors' knowledge, the only report of spaces of this order is that by Dainty and Hope (1961) who estimated that the pores in jute fibres have diameters of 50–200 Å compared to 50–60 Å in the walls of Chara.

Counts made of the number of particles per unit area in 25 capillaries gave a mean value of 6·7 per 10⁵ Å². The cross-sectional area of a 41 Å particle (= mean particle diameter) is 1320 Å²; therefore the gold constituted (6·7 × 1320 × 10⁻⁵ × 10²%) = 8·8% of the capillary area of the electron micrograph. Since for a 200 Å depth of section a value of only 0·037% would be expected at the concentration of the applied
sol, the fluid in the capillary has been concentrated $8 \cdot 8/0.037 = 240$ times.* Some idea of the speed of flow in the wall can be gained from counts of the number of particles which have presumably passed up the anticlinal wall. Scoring for a distance 2.5 $\mu$ either side of the anticlinal walls in the section shown in Plate 2, Figure 1, an estimated total of 6640 particles are in the outer wall and the cuticle. The volume of 0.12% (w/v) sol containing this number of particles is $3 \times 10^{-12}$ ml. The 200 A deep cross-sectional area of the anticlinal wall $= 1.5 \times 10^6$ A²,* and the capillary space $= 12%$* of this area (value based on area of the outer wall constituted by capillary spaces in electron micrographs).

For the volume of $3 \times 10^{-12}$ ml to pass through this area in about half an hour (it seems unlikely from the eosin-treated shoot that the gold sol had reached the leaf epidermis before 2 ½ hr) the flow rate must have been:

$$2 \times (3.5 \times 10^{-12} \times 10^{24})/(0.18 \times 10^6) \, \text{Å/hr} \approx 4 \, \text{mm/hr}.$$  

The density of gold in Plate 1, Figure 2, implies a much greater flow rate in this particular wall. The flow rate possibly varies greatly from one position to another; many epidermal walls from neighbouring areas showed no gold accumulation.

The effects of fixation, dehydration, and embedding of the leaves on the distribution of gold is not known. The danger of a general elution exists. Gold has apparently been washed from the lumina of the xylem vessels but there is no indication of the extent of elution from the cell walls. We feel that it is unlikely that the failure to detect gold in the earlier trials was due to its elution from the cell walls; there is no reason to assume a radically different wall structure in the different species. Why only one species should give a positive result with this technique remains at present unexplained.

IV. CONCLUSIONS

In sections of Helxine leaves which were allowed to transpire water from a gold sol, electron-opaque areas were identified as gold particles. These accumulated on the transpiring surfaces—i.e. the epidermis, and to a lesser degree mesophyll walls neighbouring the intercellular spaces. The conduction of the particles appeared to be restricted to numerous discrete areas of the wall which were presumed to be capillary spaces of approximately 300 A diameter. The rate of flow in an anticlinal wall of the epidermis was estimated at 4 mm/hr.

So far experiments with gold sols have successfully demonstrated mass flow in the walls of only one species (Helxine). This is sufficient, however, to support the validity of Strugger’s earlier observations with fluorescent dyes, the movement of which indicated water movement in the walls of H. soleirolii, Tradescantia albiflora, Parietaria judaica, Ranunculus ficaria, and Elodea densa (Strugger 1938-39, 1949).

V. REFERENCES


* These values must, of course, only be regarded as approximations.
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Explanation of Plates 1 and 2

Reference numbers refer to negatives stored at the Electron Microscope Unit, Botany School,
University of Melbourne. Sections illustrated were fixed in 2% osmium tetroxide in acetate-
veronal buffer, pH 7.4, and containing 4.3% sucrose, 0.01% CaCl₂, and 0.01% MgCl₂

Plate 1

Fig. 1.—Leaf epidermal section from gold-treated Helxine shoot (after 3 hr treatment) showing
clusters of gold colloid particles (each cluster appears as a single mass at this
magnification) in the outer epidermal wall (EpCW) and a rather denser aggregation
of particles along the cell wall-cuticle interface (c). Virtually no gold is visible in the
mesophyll walls but some particles appear to line the intercellular space (IS). No. 3793.
2 × 10,000 = × 20,000.

Fig. 2.—As in Figure 1 but showing a region in which a very dense aggregation of gold colloids is
present. Significantly the cytoplasm (Cy) completely lacks any gold. No. 4030. 2 × 10,000
= × 20,000.

Fig. 3.—Leaf section from a control shoot of Helxine showing the epidermal cells (Ep) which in
nature are large and vesicular. The anticlinal walls have become folded during prepara-
tion. The cell walls (OW) and cuticle (c) completely lack any gold particles. No. 2998.
2 × 10,000 = × 20,000.

Plate 2

Fig. 1.—Section of gold-treated leaf of Helxine after treatment. At this high magnification the
dark patches in the epidermal cell wall are resolvable into aggregations of discrete
particles, the size distribution of which is similar to that of the colloidal gold particles
(see Fig. 3 of this plate). No. 4028. 6 × 10,000 = × 60,000.

Fig. 2.—Electron micrograph of dried droplet of gold sol as used in the Helxine experiments.
It is considered that aggregations of these particles have resulted in the dense clumps
to be seen in Figure 1 of this plate. No. 3994. 1.5 × 40,000 = × 60,000.

Fig. 3.—Frequency histograms showing the size distribution of gold particles. Those from the
wall were measured from fields such as that shown on Figure 1 of this plate, those of the
sol from Figure 2.
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