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The Transpiration Stream in the Leaf Apoplast: Water and Solutes

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The transpiration stream in the leaf apoplast: water and solutes

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SUMMARY

Flow of the transpiration stream in the lumen apoplast of the xylem appears hydrodynamically orthodox in being approximately described by the Hagen–Poiseuille Law, and by Murray's Law for branching pipes. Flow may be followed in the major (supply) veins by labelling the stream with dye solutions. Progress of the dye in the stream into the minor (distribution) veins is obscured by surrounding tissues. Observations of the spread of fluorescent tracers from these veins in living leaves gave results that have been seriously misinterpreted to present a false view of the cell wall apoplast. Microscopy of the stabilized water-soluble fluorescent tracers moving out of the minor veins has revealed that: (i) the dye is separated from the water by filtration through cell membranes, and the water moves through the symplast; and (ii) the dye diffuses in the cell wall apoplast at rates 1/100 to 1/10 000 the rate of diffusion in water. As a consequence of (i), high concentrations of dye build up at sites called sumps. In grasses these sumps may be in the intercellular spaces outside the xylem. In dicotyledons these sumps are within the small tracheary elements. In fact, flow in the lumen apoplast is flow through leaky tubes, and is inadequately described by the Hagen–Poiseuille Law. Leaky tubes have a critical radius, below which (for a given pressure gradient) flow cannot occur. As a consequence of this, a wedge of xylem made up of vessels of different radii acts as a unit to concentrate dye tracers in a sump at its apex. Sumps may also be formed by evaporation of the water in the stream, especially at leaf margins. Investigations with the cryo-analytical scanning electron microscope of the natural ions of the transpiration stream reveal high concentrations of K, Cl, P and Ca in the stream in all the sizes of vessel and vein of sunflower leaves. These high concentrations appear to be produced, not by the mechanisms responsible for the formation of sumps of dyes, but by some other processes, probably occurring in the stem. The absence of sump formation by ions at the places where dyes form sumps is probably due to the more rapid penetration of the ions through the cell membranes. Reasons for the discrepancy between these measurements of salt concentrations in the stream and those obtained from sap expressed from leaves by pressure vessels are discussed. Implications of these facts for the design and interpretation of experiments with leaves are presented.

1. FLOW IN THE LUMEN APOPLAST

Start with the simple experiment of putting a cut leaf in coloured water and watching the spread of colour. It will move rapidly (minutes) all over the leaf, colouring the major veins. This, of itself, is quite informative. As the Hagen–Poiseuille Law says that volume flow in tracheary elements varies as $(\text{radius})^4$ and velocity as $(\text{radius})^2$, the rapid progress of the dye reveals the existence, in all the major veins, of at least one tracheary element of large radius. In a grass leaf these major (lateral) veins are parallel and fairly widely spaced, and the colour runs to the tip of the leaf in these before it has moved sideways into the minor (intermediate and small) veins that lie between them (Rouschal 1941; Altus & Canny 1985a). This slow sideways spread through transverse veins into the minor veins implies, by the same reasoning, that the tracheary elements in the minor veins have radii smaller than those in the major veins. In wheat, the radii of vessels in the lateral veins were 10 to 20 μm, and in the intermediates, 4 to 5 μm (Altus *et al.* 1985).

In a broad leaf of a dicotyledon the vein system is more elaborate, but the conclusions are the same. There are about five sizes of vein from the midvein (V1) to the smallest (V5), and the colour spreads all over the leaf to the margins in the three largest vein types. As in the grass leaf, at this stage the regions between the major veins are not coloured, and progress into the V4 and V5 veins takes half an hour or more. Again we deduce the presence of vessels of large radius in veins V1 to V3, and of small radius in V4 and V5. Generalizing a step further, in both types of leaf the vein network comprises a supply network of veins with large vessels which carries the stream all over the leaf quickly, and a distribution network of veins with small vessels which disperses the stream among the mesophyll cells locally and slowly.

The dispersal of a stream into the ramifications of a branching pipeline has been of more interest to animal

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Table 1. *Test of Murray's Law for branching pipes in the vein system of sunflower leaves*
(Data of Wang (1985), Tables 5 & 7, and Canny (1990*a*), Fig. 3.)

	column			
vein class	A (vein number per cm^2)	B (vessel number per vein)	C (vessel radius r/μm)	D[a] (Σr^3)
1	1	4	12.5	7812
1	1	6	10	6000
2	2.0	6	8.5	7369
3	6.3	2	8	6451
4	33.3	1	6	7192
5	820	1	2	6560

[a] $D = A.B.C^3$.

physiologists studying blood circulation than to plant physiologists considering the transpiration stream. There is a law which governs the relative sizes of parent and daughter pipes, called after its discoverer, Murray's Law (Murray 1926; La Barbera 1990). This states that for bulk laminar flow of a Newtonian fluid, at any branch point the radius of the parent vessel (r_0) cubed is equal to the sum of the cubes of the radii of the daughter vessels (r_1, r_2, . . .). That is,

$$r_0^3 = r_1^3 + r_2^3 + \ldots + r_n^3.$$

A more useful form of Murray's Law is the corollary that, at any level in the hierarchy of pipes, the sum of the cubes of the radii should be a constant (Murray 1926; Sherman 1981). In this form it may be tested without much trouble in the vein networks of leaves. Some measurements are available for sunflower leaves that allow a test of the law.

Wang (1985, Table 5) gives measurements of the numbers of veins of each of the five sizes in a square centimetre of leaf. Those for V2 to V5 are collected in column A of table 1 of the present paper. For the midvein (V1) Wang's data have a large variance, so the V1 data are taken from figure 3 of Canny (1990*a*). In this figure there are two sets of measurements at different places on the proximal part of the midvein, giving the numbers of large vessels and their radii, and both these are included (table 1, columns A, B and C). To complete the data for column B and C for V2 to V5, the more adequate sampling from Table 7 of Wang (1985) is used. The products $A.B.C^3$ ($=\Sigma r^3$) are calculated in column D. In accord with Murray's Law, the sums of the cubes of the radii are remarkably constant. Thus the vein system appears to be relatively orthodox from the hydrodynamic viewpoint, though we shall see later that these simple descriptions are inadequate.

We lost track of the stream of coloured water at the point where it left the major veins and entered the minor veins, and this experiment cannot help us much further. The smallest veins are hidden by mesophyll and bundle sheath cells, and the progress of the colour is obscured. The next important technical advance was made by Strugger (1938, 1939*a*,*b*, 1940) who fed leaves with fluorescent dyes, and examined them alive with the newly available epi-fluorescence microscope. With the paths of both the exciting and emitted light undistorted by tissues, he was able to resolve fine details of fluorescence on the leaf surface, and in a cell or two below. He introduced three new dyes as tracers: berberin sulphate, pyrene trisulphonate, and sulphorhodamine G. He tested them for mobility in analogues of cell wall material, such as gelatine and filter paper, and showed that the last two dyes, being negatively charged, travelled almost as fast as water in his test systems. The images he recorded from the leaves transpiring in the dye were precise and consistent, though he still could not see the fine veins within the mesophyll, even in the thin leaf of *Helxine*. But above these veins he saw cell walls that showed progressively brighter fluorescence, the anticlinal walls of the upper mesophyll and epidermal cells, and the walls of the guard cells. The cells themselves remained dark. He interpreted these images as evidence that the transpiration stream beyond the lumen apoplast of the xylem flowed in the cell wall apoplast to the sites of evaporation near the stomata. He envisaged the cell wall as an open, spongy matrix that permitted flow of solvent and solutes, so that each protoplast was bathed in an extension of the transpiration stream, and able to exchange solutes with it. He called this interpretation the 'extended cohesion theory' (Strugger 1943). If this theory is accepted, it would follow that at the terminus of the stream where water evaporated, solute should be deposited. After a leaf had transpired for a long time in a dye solution, one would expect to find accumulations of dye near the stomata, either on the guard cells or on the walls of the cavity below. This was never observed. And the puzzling absence of accumulated solutes was never explained.

This view of the cell wall as an open matrix and flow space was adopted by the leading expert of the time on cell wall structure, Frey-Wyssling, who coined the term 'holopermeable' to describe it (Frey-Wyssling 1976). Knowledge of Strugger's work spread rather slowly in the English-speaking world, partly because of the interruption of the supply of German journals by the war, and partly because it was written in German. But by the early 1960s the idea of the cell wall as a flow space with little restriction of solute movement was widespread and firmly held.

This paradigm led to a series of other concepts

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about the wall apoplast: an apoplastic solution that might be washed out by a stream of water perfused through the walls, and its solutes measured; an apoplastic transport space in the continuum of cell walls which was available to transfer solutes (e.g. sucrose from mesophyll cells to phloem); flow assistance of such transport in the cell wall apoplast of the root cortex, where incoming water might carry soil solutes inward towards the xylem, and in the wall apoplast of leaves where the outgoing water could carry solutes to and around all the cells of the leaf. These concepts are still widely held today (e.g. Sutcliffe 1986). They spread from Strugger's observations of fluorochromes in the leaf, and have persisted for fifty years.

2. ABSENCE OF FLOW IN THE WALL APOPLAST

(*a*) *False deductions from movement of tracers*

The history of the lone protest by Hülsbruch (1944, 1954, 1956) that this interpretation of the images was wrong, has been recounted in Canny (1990*a*). Here it is sufficient to stress the point she tried to make about tracer molecules in flowing solutions. This protest was that the following two assumptions were both invalid: (i) where the flow goes it carries tracer with it; and (ii) where the tracer is found, the flow has carried it. She was more concerned about the error in the second assumption, which should be constantly remembered. The very fact of introducing to a local space a distinct molecular species that can be differentiated from others around it implies a gradient of concentration of this species away from the space. If diffusion of the molecule is possible it will proceed out from the space. Put another way, if there is a flow space containing the tracer surrounded by a diffusion space with no flow, the tracer will enter the diffusion space even though the flow does not. This applies even to labelled water in unlabelled water. Hülsbruch was right, but no-one heeded her.

(*b*) *Diffusion and sumps*

The next advance in technique, which permitted observation of the fluorescent tracers in the transpiration stream at the scale of the fine veins, and as they left the xylem apoplast flow space and entered the wall apoplast, was a technique for the retention of water soluble compounds *in situ* during microscopical preparation. It relied on rapid freezing, anhydrous freeze-substitution, embedding and sectioning, and was introduced by Altus & Canny (1985*b*), applied initially to keeping ^{14}C-sucrose in place for autoradiography. The transpiration stream carrying sulphorhodamine in all the veins of wheat leaves was stabilized and measured by Canny (1988). The results were surprising, and quite inconsistent with the prevailing paradigm. The highest concentration of tracer (most intense fluorescence) was found, not in the vessels of the veins where the stream arrived, nor near to the stomata where the stream was presumed to evaporate, but in the walls between the two sheaths that surround the veins, the mestome and parenchyma sheaths. More surprising still, deposits of solid crystalline sulphorhodamine were found in the intercellular spaces between these two sheaths (Canny 1990*a*, figure 7A).

Somehow, the dye had become concentrated at this point many times above the level in the fed solution. Evaporative separation of the solute from the water was impossible so deep within the tissue, so remote from the dry air at the stomata. The only possible explanation was that the dye had been separated by osmotic filtration as the water entered the symplast through the cell membranes of the parenchyma sheath, and had accumulated in the intercellular spaces outside the cell walls. Sulphorhodamine is only sparingly soluble in water, so concentration to a level above the saturation point can occur relatively easily. In fact, Rouschal (1941) had observed granular deposits of the same dye in the same place in his experiments with flow in leaves of grasses and sedges. So here is an illustration of error in the first assumption about tracers, a situation where water has flowed and tracer has not followed it. A similar separation of lanthanum from the stream at the endodermis of roots is discussed by Clarkson in this volume.

If the water stream has freed itself from the tracer dye, how can we follow it further? And what is happening to the tracer? The first question has at present no answer. The answer to the second question can be gained by following the progress of the tracer away from its peak concentration through the wall apoplast in successive freeze-substituted samples. Fluorescence images of the dye distribution at successive times show that its progress through the cell walls of the fibres of the vein extensions, to the upper and lower epidermises and along the epidermal periclinal walls, is slow. It takes about 90 min to spread through the space between two veins in a wheat leaf (250 μm), coming from both veins (Canny 1990*a*,*b*). Further, its rate of spread is not linear with time, but decreases the further it goes. The advance follows the kinetics of diffusion

$$\text{distance} \propto \sqrt{\text{time}}.$$

Measurements of these times and distances allow the estimation of values for the diffusivity of the dye in the wall apoplast, and gave values in the wheat leaf 1/100 to 1/10 000 the value for diffusivity in water (Canny 1990*b*). So here is an example of an error in the second assumption about tracers: the tracer in the cell wall apoplast is not evidence of flow there. It has arrived by diffusion at a rate far slower than it would have done by flow.

The slow diffusive advance of sulphorhodamine in the cell walls cannot be ascribed to adsorption on an ion-exchange bed, like the advance of labelled calcium ions up a vessel exchanging with calcium bound to the xylem cell walls. Sulphorhodamine is negatively charged. Strugger selected it as an apoplastic tracer because it moved almost as fast as water through his chromatographic test systems (Strugger 1939*a*); and Canny (1988) showed that its binding to wheat leaf

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cell walls and other cellular materials was very weak (about 40% above the concentration in the bathing solution). It is a large ion, and it is not claimed that it will diffuse as fast as a small nutrient ion, but the kinetics of its advance are those of diffusion (distance $\propto \sqrt{\text{time}}$), not of chromatographic exchange in a fluid flowing through a matrix (distance $\propto$ time).

To emphasize the point that the spread of the tracer into the wall apoplast cannot be flow-assisted, consider the residence time of the water in leaves. A vigorously transpiring leaf loses its own mass of water every 10 to 20 min. If all the water in the leaf (in walls, cytoplasm and vacuoles) is equally mobile, then it is replaced with this rapidity. If tracer moved in the stream of this water it would move from a vein to the nearest stoma in a time of the same order. In the model proposed by Strugger, the cell wall water is more mobile than the cellular water, and, as it has a much smaller volume than the static cellular water, progress of the tracer through the walls to a stoma would be a matter of seconds. Experiment shows that the transit time is more like an hour. Moreover, the diffusive spread in the cell walls is independent of transpiration, and occurs at the same rate whether the leaf is transpiring or not (Schlafke 1958). The somewhat puzzling fact, that in experiments of the Strugger type, accumulations of fluorochrome are not found at the evaporation sites near stomata, finds a clear explanation.

Aston & Jones (1976) claimed to have measured accumulations of a tracer solute on transpiring wall surfaces of guard cells and other cells close to open stomata, but they were deluding themselves. They used silicic acid as a tracer, fed to the roots of oat plants in water culture, and estimated silicon in cell walls of the leaves by X-ray microanalysis after freeze-substitution and resin embedding and sectioning. The silicic acid tracer was fed for 3 to 18 h. In 3 h, leaf water would have been replaced about nine times, and in 18 h, about 50 times. If the silicon is left behind by the water at evaporative surfaces there should have been a large excess of it there, compared with the concentration arriving in the xylem vessels. What they observed (their Table 1 and Fig. 1) was, in fact, highest silicon concentration in the walls of the xylem vessels. The guard cell and other walls near the stomatal pore showed a maximum of 70% of the xylem value, and mostly 20 to 40% of it. They did not measure the silicon content of the walls of the parenchyma sheath–mestome sheath boundary where the highest accumulation of tracer would be expected. Their measurements, including the insensitivity of the silicon content to time between 3 and 18 h, are fully consistent with diffusion of silicic acid in the cell walls from a sump in that position. Terminal evaporative sumps near stomata have still not been demonstrated.

To summarize the conclusions up to this point.

1. Solutes in the transpiration stream may become highly concentrated at local sites because they are left behind when water enters the symplast. Such local high concentrations were termed 'sumps' (Canny 1990*a*), and the word will be used in this sense here. We must reckon with the possibility that sumps may be formed from the native solutes of the stream, and that such sumps may be important in salt-tissue interactions.

2. The cell wall apoplast in leaves is not an open, spongy, holopermeable flow space, but a tight, low-permeability diffusion space, where solutes diffuse at a small fraction of their rate in water.

(*c*) *Sumps in dicotyledon leaves; flumes*

The evidence discussed so far has been gained from measurements with wheat leaves. What is the progress of the dye in the V4 and V5 veins of dicotyledon leaves? The same techniques of freezing and anhydrous processing, applied to soybean leaves with sulphorhodamine in their transpiration stream, showed that the sumps formed in the finest veins (V4 and V5) (Canny 1990*c*). The fluorescence intensity was much higher in these veins than in the V3 and larger veins from which they arose and from which they drew the labelled stream, and again sump formation proceeded to the deposition of solid sulphorhodamine. And, in contrast to the wheat sumps, the sumps of soybean were within the tracheary elements, not outside them (figure 1). The separation of the water into the symplast apparently occurred at the cell membranes of the bundle sheath cells where they were in contact with the transpiration stream through the pits in the walls of the small tracheary elements. The general term for such a site of entry of water to the symplast is a 'flume' (Canny 1990*a*). The site of a flume can be revealed by the presence of a sump. In this mode the tracer is acting not to show where the stream is going, but where it has changed its milieu from apoplast to symplast.

Also in contrast to wheat leaf venation, the net venation of soybean is not fully connected (closed). The finest (V5) veins often end blindly in the mesophyll, and it was at these blind endings that the sumps piled up after 40 min of dye feeding (figure 1*c*) (Canny 1990*b*). Earlier, after about 20 min of dye feeding, sumps were found further back on the V5 and V4 veins, proximal to the terminations (figure 1*b*). This showed that there were flumes all over the xylem of these fine veins. Progress of the fluorescent dye solution (less concentrated than the solid deposits of the dye) was very slow into the V5 termini. Remember that the dye reached the nearest V3 vein (distant say 2 mm) in 5 min (figure 1*a*). After 20 min it was in the V4 and V5 veins forming sumps there, but had not yet reached the end of the V5 veins (figure 1*b*). To travel the final 50 μm or so to the terminus took a further 20 min. This sluggish forward movement was not just a consequence of the Hagen–Poiseuille Law in vessels of small radius, but was due also to radial loss of water through the flumes.

The sumps of dye in the tracheary elements of fine veins of soybean leaves are, as in the grasses, the sources of diffusion in the wall apoplast. Dye spreads into the cell walls of the bundle sheath cells and from there in the mesophyll walls and eventually in the epidermal walls. This advance is shown diagrammatically in figure 2. Again the spread is slow and obeys

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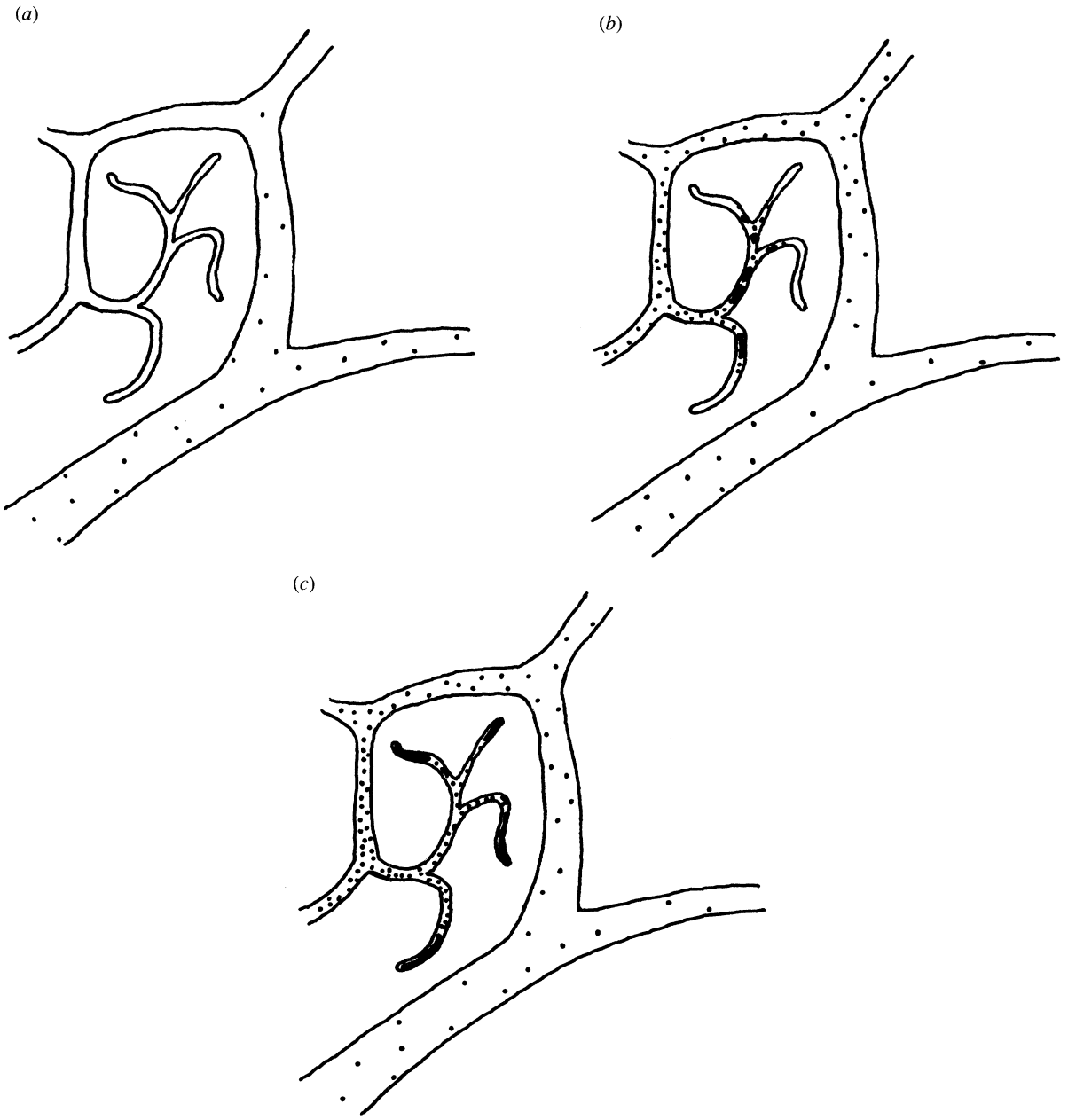

Figure 1. Diagrams of ultimate branches of tracheary elements in a transpiring soybean leaf carrying tracer solute (dots, density proportional to concentration) at successive times from the introduction of the tracer to the cut petiole. (*a*) After 5 min; (*b*) after 20 min; (*c*) after 40 min.

diffusion kinetics. Again the measured diffusivities are in the range 1/100 to 1/10 000 the value in water. At the stage of dye accumulation reached in figure 1*c*, the fluorescence had barely reached the upper epidermis. Only a few anticlinal epidermal walls directly above the sumps in the veins could be seen to fluoresce. In sclerophyllous leaves, the wall diffusivity was at least another order of magnitude less.

3. TRACHEARY ELEMENTS AS LEAKY PIPES

(*a*) *Critical radius*

The slow advance of dye solution into the V5 veins (figure 1*b*) brings us squarely to confront the fact that xylem elements are not the ideal pipes for which the Hagen–Poiseuille and Murray's Laws were formulated. They are leaky pipes. Altus *et al.* (1985) had called attention to this fact in their model of the operation of the vascular network in wheat leaves. Their plot of $(\text{velocity})^2$ versus vessel radius was a straight line, as the Hagen–Poiseuille Law requires, but the line did not pass through the origin (Altus *et al.* 1985, Fig. 6). Zero velocity was found in vessels of 6 μm radius or less. They interpreted this as due to the radial flow (leakiness), and modelled flow into the network as balanced by radial losses from it. The magnitude of the departure from the ideal flow caused by leaks depends on the radius. Since forward flow $\propto (\text{radius})^4$ and leaks are through the surface ($\propto r$), for large radii the leaks are insignificant compared with the forward flow. But as the radius is reduced,

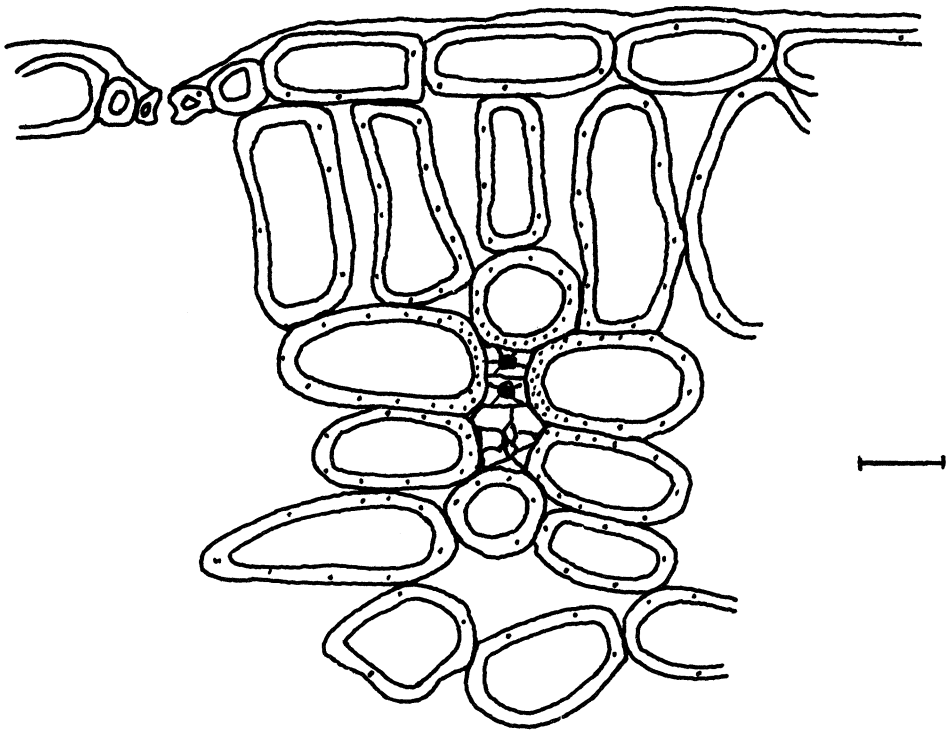

Figure 2. Diagram of diffusion of solute (dots, density proportional to concentration) in the cell wall apoplast of a dicotyledon leaf, 30 min after it has accumulated at high concentration (a sump) inside the tracheary elements of a fine vein. (See text.) Bar = 10 μm.

flow is attenuated more rapidly than leaks, and for a given gradient of pressure, a critical radius is reached at which there is no forward flow. All the fluid entering the pipe leaks out through the surface. The details of these equations and the consequences for the functioning of xylem have been treated by Canny (1991). For our consideration of the leaf apoplast one of these consequences is recalled.

(*b*) *Sump formation in the xylem wedge*

Vascular strands commonly contain blocks of tracheary elements of graded size, produced by the differentiation of successively larger elements along a radius of an organ. A wedge of adjacent elements is formed with the smallest, earliest element at the apex. In stems and petioles the apex lies towards the centre, in the leaf veins, towards the adaxial surface. All the elements in the wedge are leaky tubes, and, depending on the gradient of tension, some of them may have radii smaller than the critical radius, and so carry no axial flow, only radial flow (leaks). Their leaks are supplied by radial flow from their leaky neighbours. Considered as a whole, this wedge has leaks all over its surface, and axial flow only in its larger elements. Since the leaks are supplying water to flumes in the surrounding symplast, solutes that cannot cross the cell membranes will accumulate in the apex of the wedge. They arrive with the stream in the larger elements, and become trapped in the small elements (figure 3). This is another mechanism by which sumps

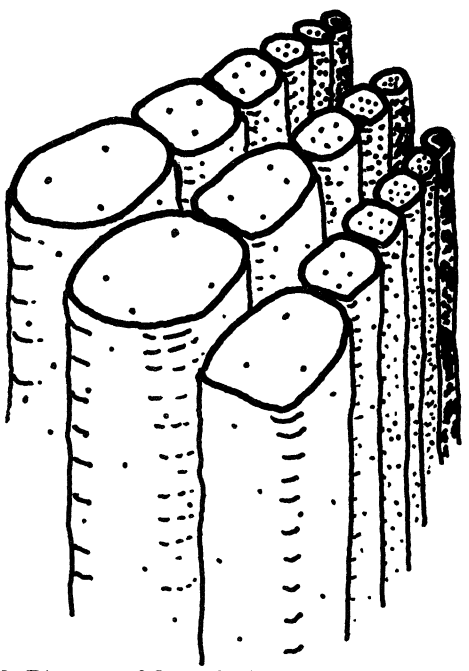

Figure 3. Diagram of flow of solution in a wedge of xylem. Solute concentration is represented by the density of dots. Axial flow occurs in the larger vessels. Water leaks from all the vessels at a uniform rate per unit surface, and enters the cell membranes of the neighbouring parenchyma cells. Small vessels have no axial, only radial flow, fed from their larger neighbours. The solute (presumed unable to enter the cell membranes) accumulates at the apex of the wedge where forward flow is reduced in relation to the leaks (see text).

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may be formed, and such apices of xylem wedges are places to look for them. These, like the sumps in the two other locations identified, the fine veins of dicotyledon leaves and the parenchyma sheaths of grass leaves, will act both as sources generating diffusion in the wall apoplast, and as substrate concentrations for specific carrier molecules in cell membranes. Also, it might be anticipated that xylem-feeding aphids (see Press & Whittaker, this volume) would find their way to sumps to feed on enriched sap there. Finding their stylets in such locations would accord with the line of argument developed here.

4. EVAPORATIVE SUMPS; EXCRETION

The most obvious mechanism of concentrating solutes of the transpiration stream to form a sump, is by the evaporative separation of water. Considering that a leaf is a machine for evaporating water, it is surprising that such evaporative sumps are not more obvious and widespread. On Strugger's model, as has been said, every stoma would be expected to nurture an evaporative sump. The one place where such sumps have been located is along the margins of grass leaves that have a large vessel in the fimbrial vein. The maize leaf is a striking example (Canny 1990*a*) where dye from the transpiration stream accumulated at the margin, inside the very large vessel of the fimbrial vein. This vessel lies, unprotected on its upper side by sheath cells, in the air space. Guttation from the transpiration stream under positive pressure collects as drops on the surface at the leaf margin, driven out of the large vessel into the space, and on to the leaf surface, and must flush away solutes that have collected in the sump within this vessel. Sugarcane appears to have a similar excretion mechanism: Evans (1942) injected acid fuchsin solution into stems of plants in the field. Next day the leaves of plants that had not been injected but growing close to the injected plant were spattered with the dye. The dye had been exuded from the margins of the leaves of the injected plant during the night. Solutes accumulating at evaporative sumps, being destined probably for excretion, may offer no attraction to xylem-feeding aphids.

5. NATURAL SOLUTES OF THE STREAM

(*a*) *Ions of the xylem sap*

All the evidence so far has been provided by exogenous tracers in the transpiration stream. It is time to ask to what extent the processes revealed by the tracers are operating on the natural solutes of the stream. The evidence about them is that which can be gained by X-ray analysis of elements in bulk-frozen tissues in the scanning electron microscope. (See Leigh & Tomos, this volume.) The relevant elements that are susceptible to such analysis are Na, Mg, P, S, Cl, K and Ca. The questions of interest are: whether we can find sumps of any of these in the V5 veins, at the apex of xylem wedges, at the parenchyma sheath of grass veins, or in marginal veins; whether we can measure diffusivities of them in the wall apoplast; what is the steady state distribution of them in the leaf apoplast, and how do their concentrations change in space and time when the elements are fed into the stream? Only a small beginning has been made in exploring these questions.

The transpiration stream comes from the root as a dilute solution whose main cation is K, the concentration of which in the xylem sap is 0.5 to 12 mM (Robson & Pitman 1983). The highest value is close to the lower limit of detection in the cryo-analytical SEM for bulk frozen tissue. An orthodox view of the progress of the K in the stream would be that as it travelled through the stem xylem, some might be absorbed into the xylem parenchyma. What remains would be distributed through the leaf veins, transferred from xylem to phloem, and returned by translocation to growing tissue at the stem apex (Sutcliffe 1986). Other solutes might behave similarly, but Ca, being immobile in the phloem, would remain in the leaves, probably sequestered as the oxalate.

(*b*) *Ions of the leaf lumen apoplast*

The observations to be described were made mostly on the leaves of *Helianthus annuus*. The sap expressed under pressure from the root system of this species has been analysed by Gollan *et al.* (1992), who record: K^+ 4.4 mM; Ca^{2+} 0.7 mM; Na^+ 0.06 mM; NO_3^- 7.3 mM; PO_4^{3-} 0.6 mM; Cl^- 0.4 mM; SO_4^{2-} 0.4 mM. The sap also contained about 200 mM amino acids, mostly glutamine. Nitrogen as nitrate or amino acid is at present inaccessible to analysis in the SEM, but the others may be measured. These values will serve as the presumed input to the base of the stem. At the levels given, none of these ions would be detected by the cryo-analytical SEM. Finding them in the xylem sap would imply some kind of sump formation.

It is worth recalling how the macro samples of xylem sap are obtained on which such analyses are based. Root xylem can be sampled when there is positive pressure in the xylem (naturally, or induced in a pressure vessel as in Gollan *et al.* (1992)), by collecting the bleeding sap from the cut proximal end of a root. Stem xylem sap can often be sucked or blown from the lumens of xylem in a cut segment. Leaf xylem sap is obtained only by the application of pressure to gas surrounding the lamina while the cut base is outside the pressure chamber. We shall return later to the question of whether saps obtained in these ways are likely to be representative of the saps present in the xylem. As a first simple conclusion it is obvious that such extractions will select the contents of vessels of large radius in preference to the contents of small vessels ($\propto r^4$).

(*c*) *Ions of the minor veins. Sumps?*

For a start let us look at the cryo-SEM analyses of tracheary elements in the V5 veins of sunflower leaves. The methods of preparation, analysis and calibration have been detailed in Canny & Huang (1993). Briefly, small pieces of laminae of fresh leaves were frozen in

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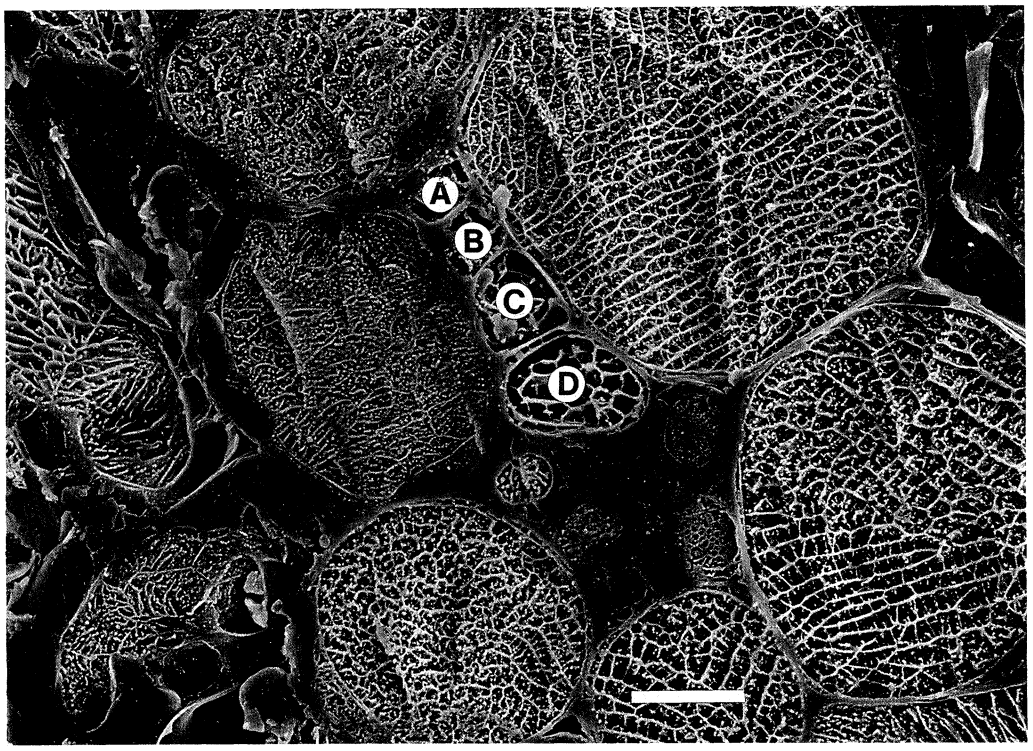

Figure 4. Planed face of frozen leaf of *Helianthus* showing V5 vein. The contents of the cells labelled A to D were analysed in the cryo-analytical SEM to give the elemental concentrations listed in table 2. Bar = 10 μm.

Table 2. *Measured concentrations of elements in xylem sap and parenchyma vacuoles of V5 vein of* Helianthus *leaf shown in figure 4*

(te, tracheary element; x par, xylem parenchyma cell; —, below detection limit, *ca.* 12 mM.)

		concentration/mM			
label	cell type	P	Cl	K	Ca
A	te	28	—	108	53
B	te	32	—	111	58
C	te	33	21	103	34
D	x par	104	—	287	23

N_2-slush, planed at −80°C to give a flat face, very lightly etched in the vacuum of the microscope to reveal cell outlines, coated with Al, and observed and analysed while still frozen at −170°C. The resolution of the analyses can be as small as 1 μm^2, but was adjusted to average heterogeneities over the area of the tracheary element lumens, cell vacuoles, etc. Let us begin with the questions: (i) are there enhanced concentrations of ions in the tracheary elements of V5 veins compared with the V1 to V4 vein; and (ii) are the concentrations in the smaller vessels higher than those in the larger vessels, as predicted in § 3*b*?

A planed face of a V5 vein of a sunflower leaf is shown in figure 4, and the analyses of the contents of the three tracheary elements and the xylem parenchyma cell are given in table 2. The concentrations of K, P and Ca in the tracheary elements were very much higher than those given by Gollan *et al.* (1992) for these ions leaving the root (K, 24 times; Ca, 69 times; P, 52 times). These levels were about a third of the levels of K and P found in the vacuole of the xylem parenchyma cell adjacent, but Ca in this cell was low. The enhancement above the concentrations found in the root seems to support the idea of sump formation in the fine veins. Note, however, that the smallest element (A) did not have conspicuously more of the ions than the other two, suggesting that the wedge mechanism had not been operating.

A broader comparison with the contents of neighbouring cells is shown for a V5 vein of *Nicotiana* in figure 5 and table 3. Again the levels of K, P and Ca were much higher than in root sap; the xylem and phloem parenchyma cells were surprisingly rich in Ca; the bundle sheath cell had low Ca. The chloride levels were below the limit of detection.

Turning to the next largest vein of *Helianthus*, a V4 vein is shown in figure 6, and the elemental contents in table 4. The levels were similar to those in the V5

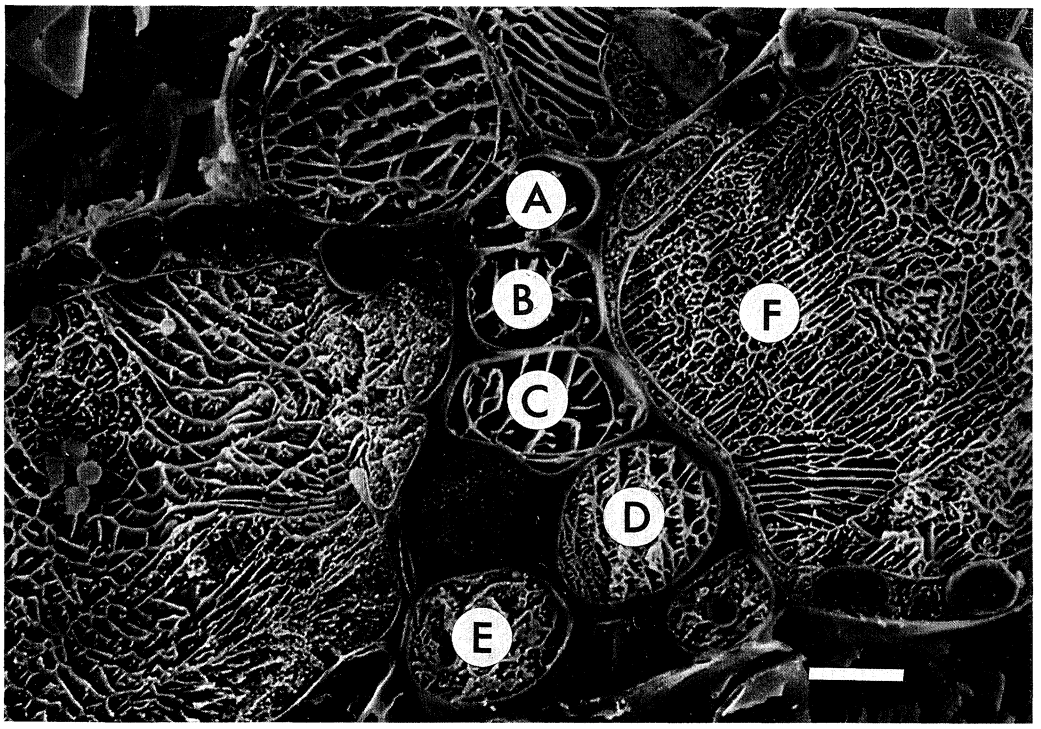

Figure 5. Planed face of frozen leaf of *Nicotiana* showing V5 vein. The contents of the cells labelled A to F were analysed in the cryo-analytical SEM to give the elemental concentrations listed in table 3. Bar = 5 μm.

Table 3. *Measured concentrations of elements in xylem sap and neighbouring cell vacuoles of V5 vein of* Nicotiana *leaf shown in figure 5*

(te, tracheary element; bs, bundle sheath cell; ph par, phloem parenchyma cell; —, below detection limit, *ca.* 12 mM.)

		concentration/mM			
label	cell type	P	Cl	K	Ca
A	te	31	—	36	80
B	te	24	—	32	73
C	x par or te?	98	—	64	66
D	ph. par	22	—	389	35
E	ph. par	47	—	152	42
F	bs	78	—	44	—

veins of tables 2 and 3, and the small tracheary element (C) showed no enhancement of solutes above the larger ones.

(*d*) *Ions of the major veins*

Any complacency that the high levels of ions in the fine veins can be explained by analogy with the formation there of sumps of exogenous dye was shattered when we measured the contents of the stream in the larger veins. Figure 7 shows part of the xylem of the midvein (V1) of a *Helianthus* leaf midway along the lamina, and the analyses for the marked tracheary elements are given in table 5. The K concentrations were mostly around 200 mM, or 45 times the concentration in the root sap. Chloride has shown up above the detection limit. The cell H is surely part of some other population, though it looks like an orthodox tracheary element. Its very high K and high Cl are characteristic of developing tracheary elements in *Helianthus*, and in spite of its heavily thickened secondary wall it was probably still alive. A clearer example of an immature tracheary element is shown in figure 8 and table 6, cell K. Again this cell stood out from the mature elements (A to J) by its higher K and Cl.

In none of these samples of xylem having tracheary elements of diverse radii, is there any evidence that the small tracheary elements contain higher concentrations of the ions than the larger elements. The answer to question (ii) of § 5*c* seems to be that the enhancement of concentration at the apex of a wedge

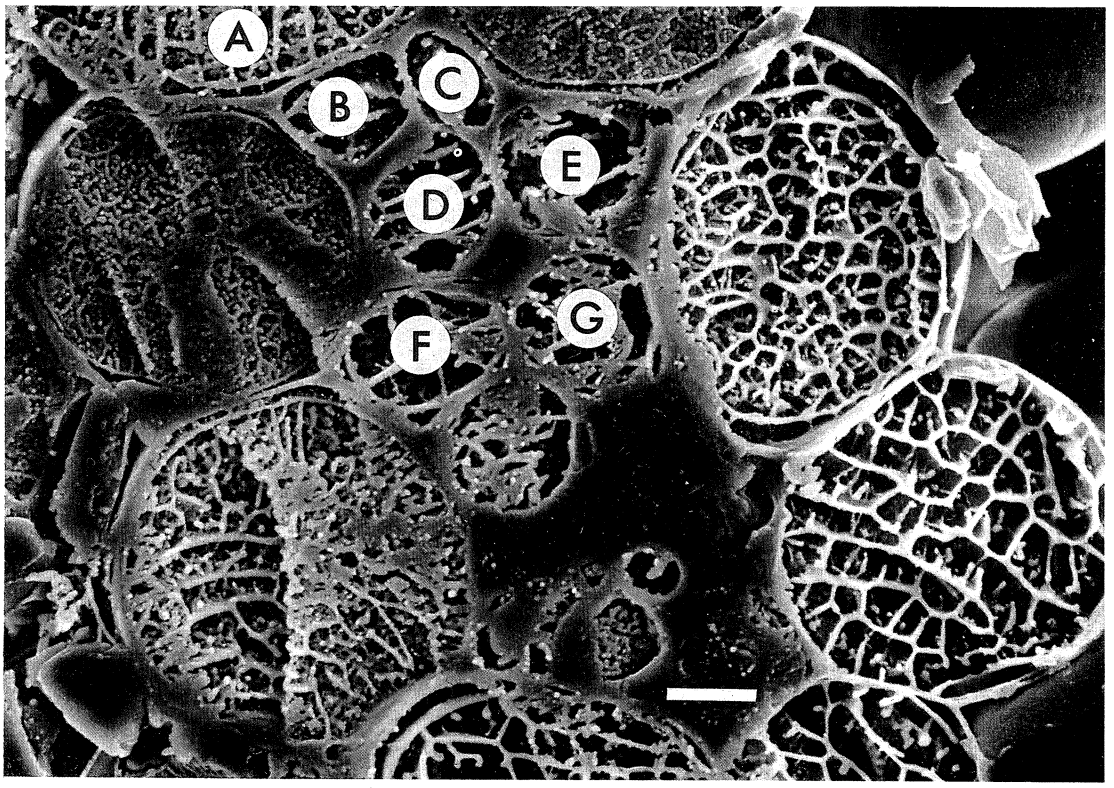

Figure 6. Planed face of frozen leaf of *Helianthus* showing V4 vein. The contents of the cells labelled A to G were analysed in the cryo-analytical SEM to give the elemental concentrations listed in table 4. Bar = 20 μm.

Table 4. *Measured concentrations of elements in xylem sap and parenchyma vacuoles of V4 vein of* Helianthus *leaf shown in figure 6*

(te, tracheary element; bs, bundle sheath cell; x par, xylem parenchyma cell; —, below detection limit, *ca.* 12 mM.)

		concentration/mM			
label	cell type	P	Cl	K	Ca
A	bs	74	—	133	—
B	te	41	—	133	—
C	te	31	—	71	39
D	te	26	13	63	29
E	te	29	—	63	31
F	x par	28	—	155	—
G	x par	40	—	160	19

postulated in § 3*b* has not been operating in these leaf veins for these ions, or has been overridden by some other process such as uptake of the ions into living cells, or diffusive dispersal.

The high salt concentrations of the lumen apoplast were not, then, confined to the small veins, but extended back down the system of vein branches right to the main trunk, the midvein. Though the data are not shown, the same high salts were found in the vessels of the petiole. Rather than these high concentrations being produced by sump formulation in the leaves, it seems likely that the sap coming from the stem xylem is probably high in salts, and this possibility is now being investigated.

The excess of total cations over total anions in tables 2–6 is constantly observed, not only in the xylem sap, but also in the vacuoles of the cells. The balancing anion(s) are not being detected, which may mean that they are organic acids or nitrate. The X-ray detector is capable of analysing nitrogen, but in the frozen preparations the nitrogen peak is obscured by the massive peak from oxygen in the water. The data presented by Leigh & Tomos in this volume suggest that there may be much nitrate present.

These analyses are representative of many leaves studied. However, high salt concentrations were not found universally in the tracheary elements. Some leaves had almost undetectable (i.e. less than 10 mM)

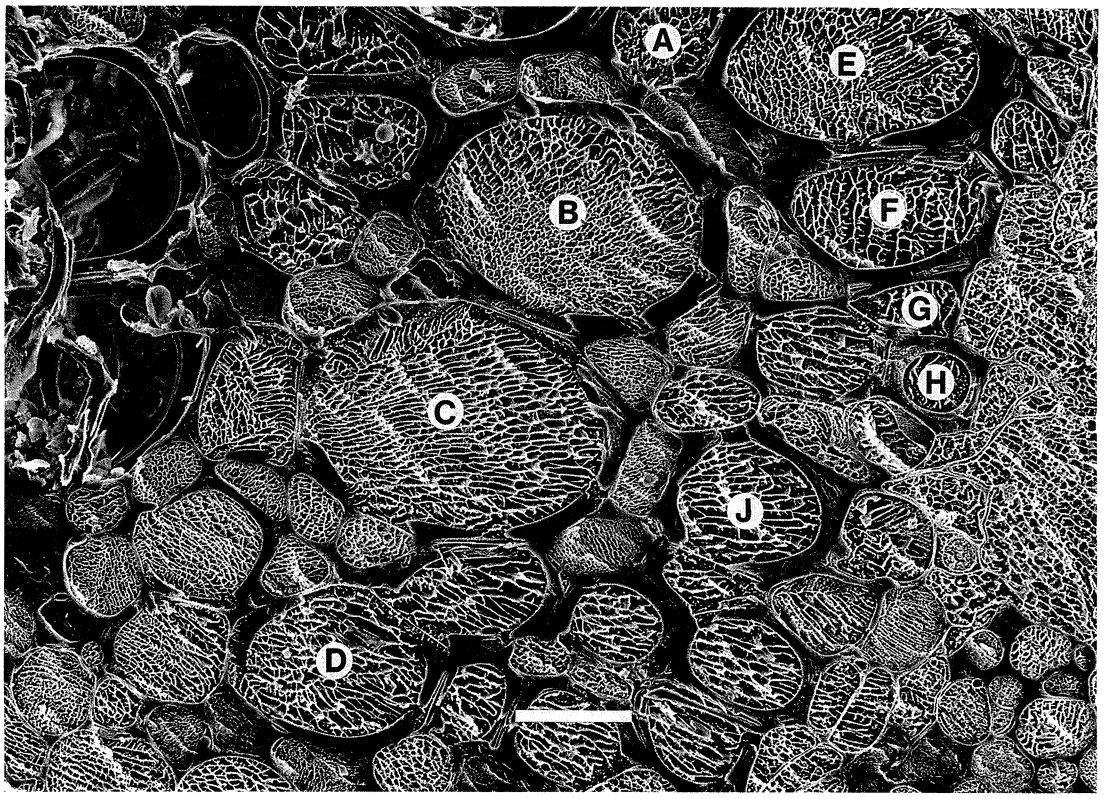

Figure 7. Planed face of frozen leaf of *Helianthus* showing V1 vein. The contents of the cells labelled A to J were analysed in the cryo-analytical SEM to give the elemental concentrations listed in table 5. Bar = 20 μm.

Table 5. *Measured concentrations of elements in xylem sap of V1 vein of* Helianthus *leaf shown in figure 7*

(te, tracheary element; —, below detection limit, *ca.* 12 mM.)

		concentration/mM			
label	cell type	P	Cl	K	Ca
A	te	62	24	146	20
B	te	91	17	188	19
C	te	97	18	199	13
D	te	97	17	194	—
E	te	93	17	198	30
F	te	80	—	197	10
G	te	85	22	177	—
H	te?	—	107	358	20
J	te	100	22	208	23

concentrations of these elements present in the transpiration stream. At present not enough data have been collected to relate these differences to the developmental age of the leaves or to the time of day when samples were collected, or to the environmental conditions of the transpiring plant.

(*e*) *Comparisons with composition of pressure-expressed sap*

The measurements of ion concentration in the transpiration stream of leaves in §§ 5*c*,*d* are not consistent with values measured on sap expressed from leaves by pressure chambers. For example, Jachetta, *et al.* (1986) collected sap from sunflower leaves with small increments of pressure (0.02 to 0.04 MPa above the balance pressure), and give a graph (their Fig. 3) of the osmolality of successive sap samples at increasing steps of balance pressure. At each step, 8 μl of sap was collected and measured. The graph shows an initial plateau at 20 mOsm kg^{-1}. As the pressure was increased, the solute content fell to a second plateau at 8 mOsm kg^{-1}, and at higher pressures, fell further. They interpret these drops in concentration as due to the progressive dilution of tracheary element sap with water forced out of living cells through the cell

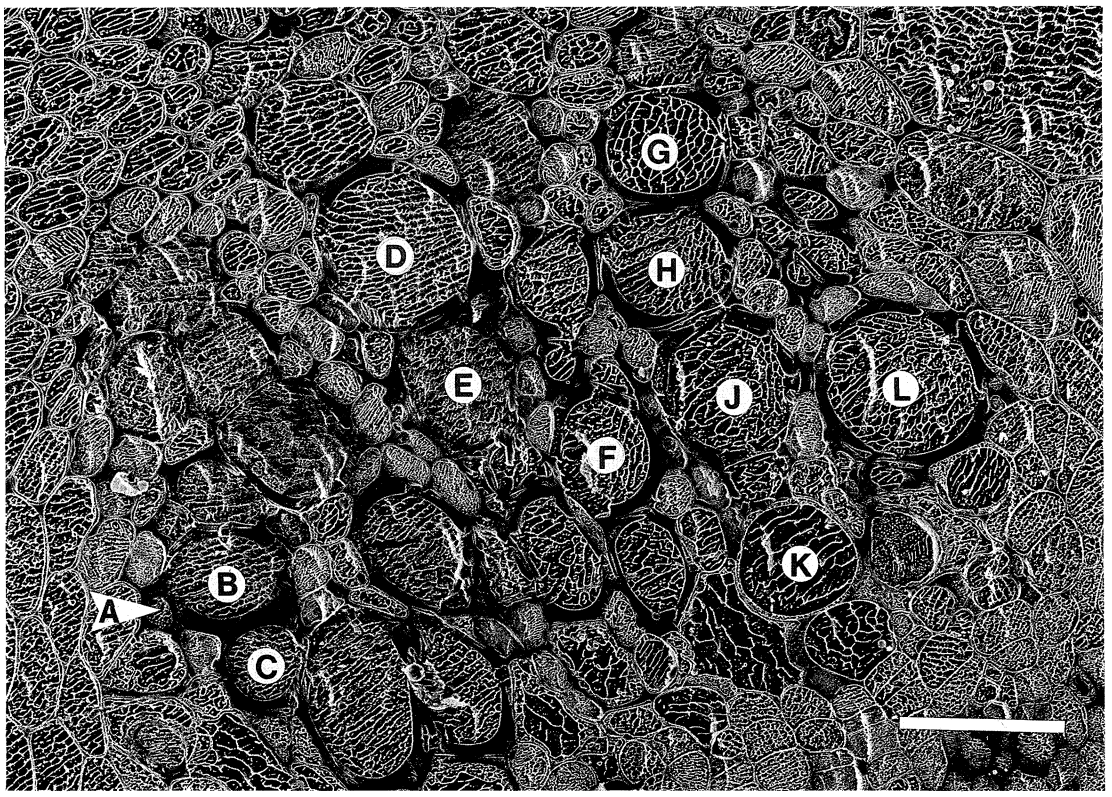

Figure 8. Planed face of frozen leaf of *Helianthus* showing V1 vein. The contents of the cells labelled A to L were analysed in the cryo-analytical SEM to give the elemental concentrations listed in table 6. Bar = 50 μm.

Table 6. *Measured concentrations of elements in xylem sap of V1 vein of* Helianthus *leaf shown in figure 8*

(te, tracheary element; dev te, developing tracheary element; —, below detection limit, *ca.* 12 mM)

		concentration/mM			
label	cell type	P	Cl	K	Ca
A	te	32	19	145	14
B	te	28	20	182	—
C	te	27	35	171	13
D	te	49	20	143	29
E	te	32	18	111	—
F	te	27	32	137	13
G	te	39	18	146	23
H	te	31	22	130	20
J	te	31	18	126	21
K	dev.te	36	94	295	6
L	te	36	19	150	22

membranes, and conclude that the native sap had a concentration equivalent to the first plateau (20 mOsm kg^{-1}). This would be about a tenth of the measured concentrations in §§ 5*c*,*d*. Besides the obvious question of whether the freezing-analytical procedure induces massive artefacts, it is worth asking how representative of the tracheary element contents the pressure-expressed solution is likely to be. Jachetta *et al.* (1986) give some consideration to this, and conclude that the first fraction of sap obtained with the pressure steps just above the balance pressure 'contains solutes from the petiole and midrib of the leaf.' This phrase does not necessarily imply that the concentration of these solutes is the same as it was before the leaf was cut. Their discussion includes the assumption of a mobile apoplastic solution in the cell walls, an assumption that I have been at some pains to show is wrong.

At the balance pressure, the volume of water in the leaf xylem is presumably the same as it was in the intact leaf, and the concentration of solutes in the xylem is likely to be close to that in the intact leaf. Any increase in pressure (overpressure) that extrudes sap from the cut petiole must be pushing water from

somewhere into the xylem, and so diluting the solution wherever that water enters. If this additional water enters the xylem from parenchyma cells at the ends of the fine veins (call this distal dilution) and pushes the contents back down the branch tree of V5, V4, etc. to V1 veins, then the successive samples in Jachetta *et al.*'s (1986) graph will indeed represent first the natural sap solution, and later a dilution of it. However, there seems to be no reason why the overpressure should not force water out of parenchyma cells anywhere distal to the cut in the petiole, and dilute the solution nearer to the collection site. Indeed, since the large vessels of the V1 and V2 veins offer much less resistance to flow ($\propto r^4$), and are closer to the collection site, it seems very probable that the extra water would enter there, diluting the samples at small overpressures (this may be called 'proximal dilution'). Each successive sample collected by Jachetta *et al.* (1986) was 8 µl. From the data in tables 5 and 7 of Wang (1985) I calculate that the total volume of all the xylem elements in the five vein sizes of a 70 cm^2 sunflower leaf is about 8 µl. Of this, 3 µl is in the V1 vein, and 3 µl in the V2 veins. Suppose, as a contrast to the distal dilution, that water enters by a lower resistance path in the proximal 1/4 volume of the V1 vein. This would be an extreme opposite to the distal dilution. The volume of V1 xylem it enters is 3/4 µl. So if 8 µl enters this proximal region, it dilutes the xylem contents by a factor: $8 \div 3/4 \approx 11$ times. Then the first 8 µl sample measured by Jachetta *et al.* (1986) would have had a concentration in the intact leaf of $11 \times 20 = 220$ mOsm kg^{-1}. This is roughly comparable with the concentrations that might be estimated from tables 2–6 with the addition of NO_3^- or some organic anion to balance the K^+ unmatched by PO_4^{3-} and SO_4^{2-}. The high values found by the analytical SEM for salt concentrations in the xylem sap should not then be dismissed as too improbable to be taken seriously. The possibilities of artefact in the expressed-sap samples are far from negligible.

(*f*) *Absence of sumps in the leaf veins. Chutes*

The two mechanisms identified for the formation of osmotic-filtration sumps of dyes in dicotyledon leaves, water separation at flumes around tracheary elements of the finest veins (§ 2*c*), and accumulation at the apices of xylem wedges (§ 3*b*), appear not to be operating on the ions measured in §§ 5*c*, *d*. The ion concentrations of the stream are enhanced about 50-fold above those in the root, but the enhancement appears to occur elsewhere, and may be by a different process. Since the two mechanisms do operate for dyes that penetrate the cell membranes only slowly (Canny 1990*b*, 1991), we may conclude that the membranes of cells surrounding the leaf xylem are fairly permeable to these ions. Jachetta *et al.* (1986) reached a similar conclusion on the evidence that the salt concentration of the pressure-expressed sap fell to very low levels when the stream was held static in a non-transpiring leaf.

The site where a solute crossed cell membranes to enter the symplast, together with the flux of the solute at that site, was termed a chute (Canny 1990*a*), and graded as weak or strong according to the magnitude of the flux. The evidence of the measurements presented suggests strong chutes for K, P, Cl and Ca on the bundle sheath membranes of veins of all sizes. Experimental manipulation of the contents of the stream, followed by analyses of the vessel contents over time should elucidate the relative strengths of the chutes of these ions, and of other solutes introduced to the xylem, such as heavy metals.

6. CONCLUSIONS

By way of summarizing the argument and evidence, and to emphasize the changed point of view they compel, I present a series of cautionary statements about the stream in the apoplast of leaves.

1. Be wary about interpreting the results of tracer experiments in terms of flow. Tracers may diffuse into spaces where there is no flow, and flow may proceed into spaces where the tracers do not follow.

2. There is no cell wall apoplast solution in the sense of a fluid that can be perfused out of the wall. Solutes of the wall apoplast can be exchanged only slowly by diffusion, at rates much less than diffusion in water. These cautions should be remembered in planning any procedures for extracting the apoplast solutes, or for applying extraneous solutes through the apoplast.

3. There is no cell wall apoplast flow space available for transport. Since transport there is by diffusion, its rate will be very strongly dependent on distance.

4. Natural solutes in the transpiration stream probably travel beyond the xylem in the symplast. There, they can be regulated, sorted and directed in complex patterns.

5. Exogenous solutes in the transpiration stream probably travel beyond the xylem in the apoplast by diffusion. There, their movement will be slow and subject mainly to variations in diffusivity of different zones of the cell walls.

6. As a consequence of 4 and 5, be wary about interpreting the distribution of exogenous tracers as representing the distribution of natural solutes.

7. The lumen apoplast may often contain large and variable concentrations (200–300 mOsm kg^{-1}) of natural solutes which may affect leaf water potential and cell turgor.

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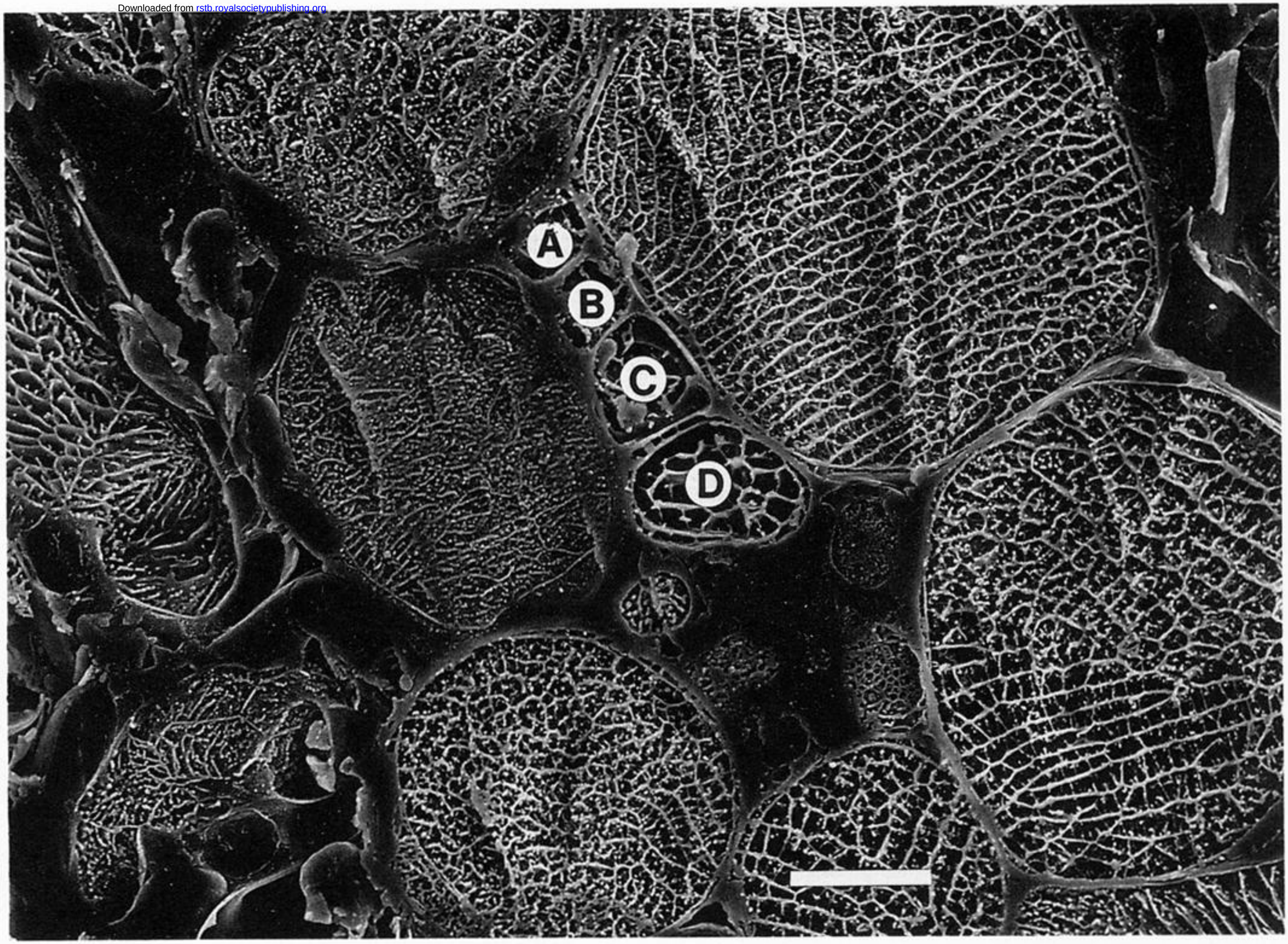

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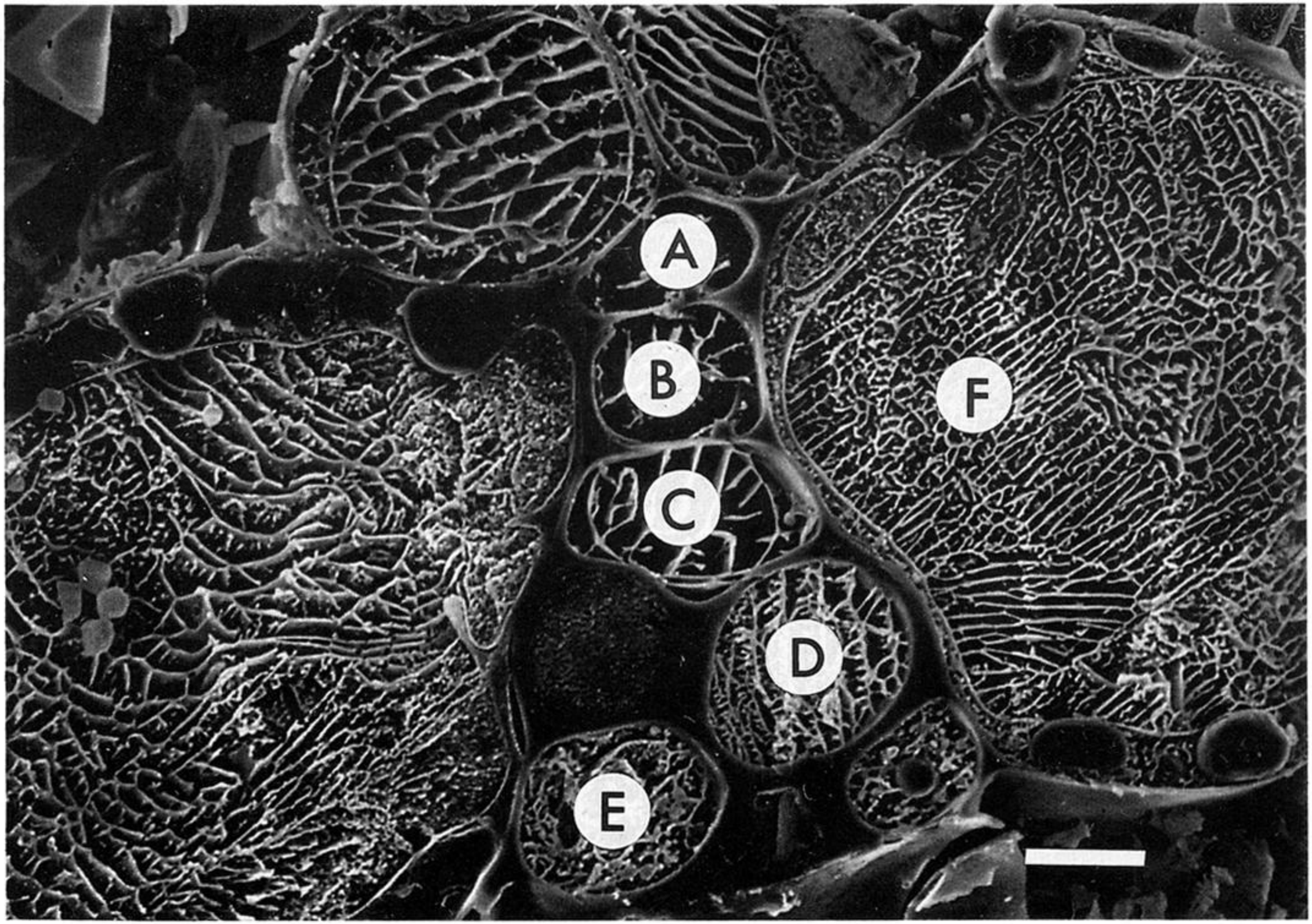

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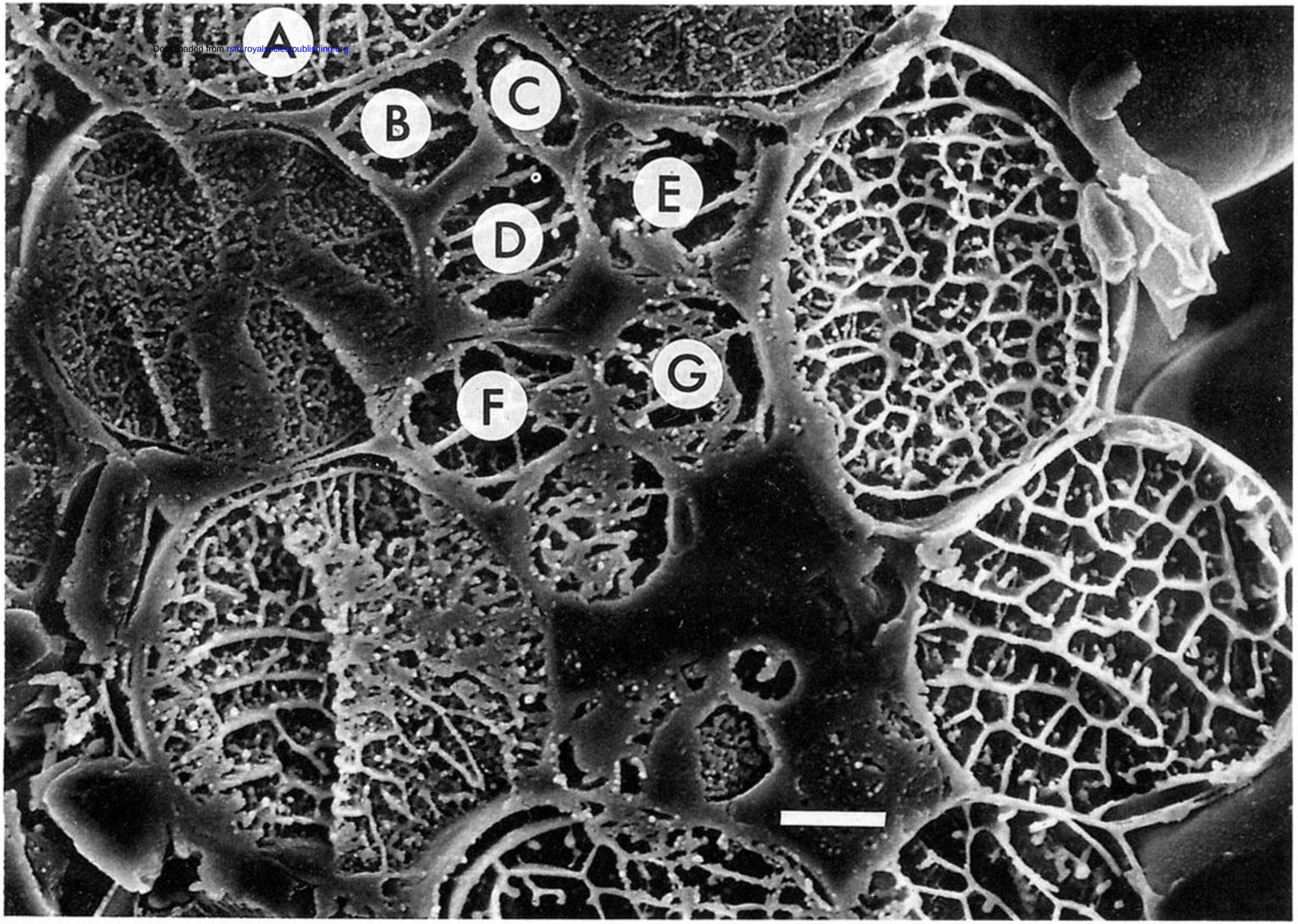
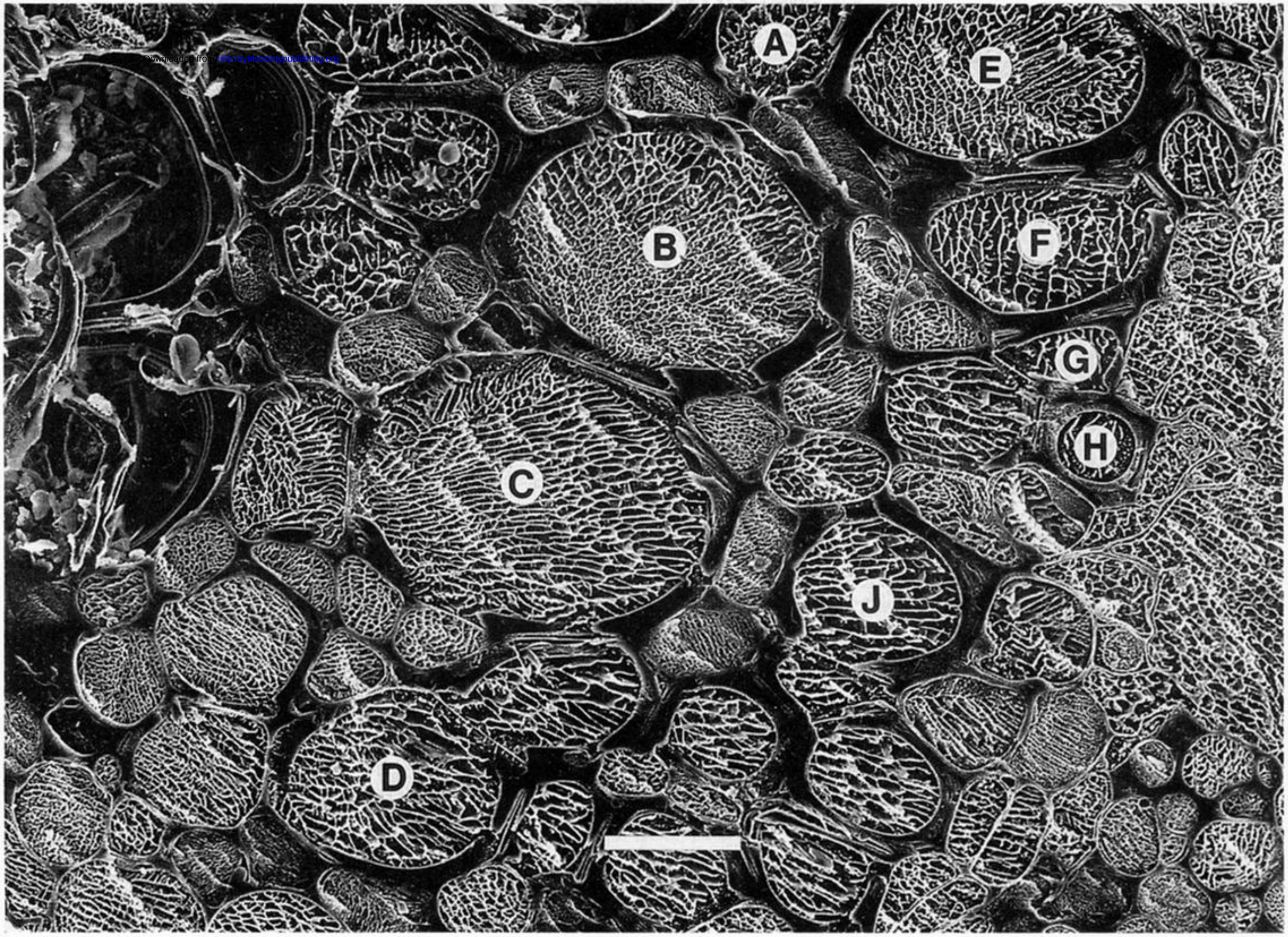
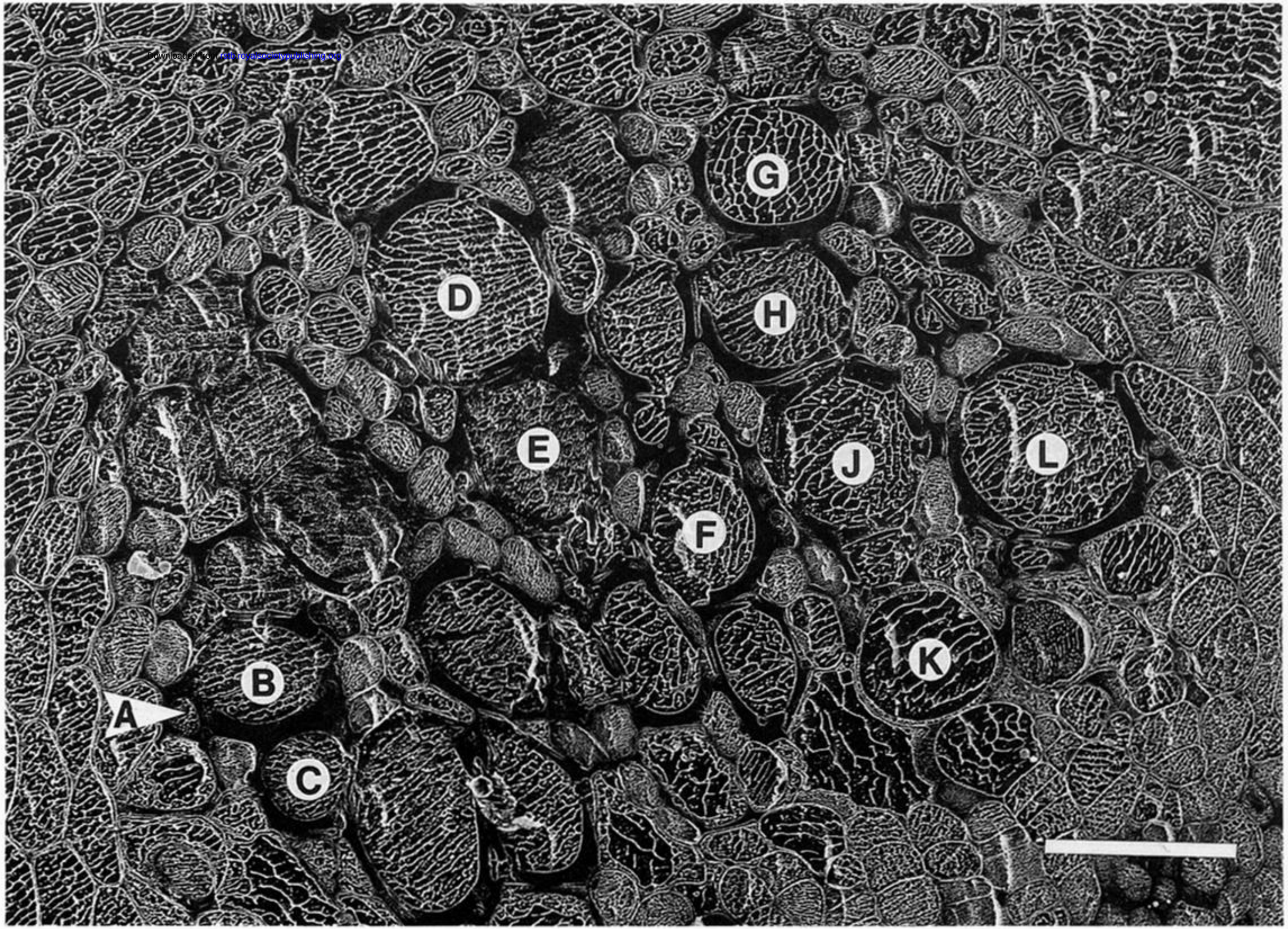


gure 6. Planed face of frozen leaf of *Helianthus* showing V4 vein. The contents of the cells labelled A to G were analysed in the cryo-analytical SEM to give the elemental concentrations listed in table 4. Bar = 20 μm.



gure 7. Planed face of frozen leaf of *Helianthus* showing V1 vein. The contents of the cells labelled A to J were analysed in the cryo-analytical SEM to give the elemental concentrations listed in table 5. Bar = 20 μm.



gure 8. Planed face of frozen leaf of *Helianthus* showing V1 vein. The contents of the cells labelled A to L were analysed in the cryo-analytical SEM to give the elemental concentrations listed in table 6. Bar = 50 μm.

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