THE DEVELOPMENT OF THE SECONDARY WALL
OF THE XYLEM IN ACER PSEUDOPLATANUS

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ABSTRACT
The development of the spirally thickened xylem element from a cambium initial of sycamore Acer pseudoplatanus has been traced by means of electron microscopy. The narrow elongated cambial initial undergoes considerable expansion in all dimensions. The cytoplasm at this stage is distributed in a thin skin between the cell wall and a large vacuole. No correlation has been observed between the distribution of any organelle and the pattern of the eventual thickenings. After the sites of thickening deposition have become apparent, the most conspicuous feature of the cell is the proliferation of Golgi bodies and vesicles. It is suggested that the material of the developing thickenings stems from direct apposition of the material in the Golgi vesicles. After glutaraldehyde fixation, microtubules (200 to 220 A in diameter) are seen to be sited in specific relation to the thickenings, the orientation of the tubules mirroring that of the fibrils seen in the thickenings. Possible reasons for absence of an observable pattern in the expanded but relatively undifferentiated cell are given, and the possible roles of the Golgi apparatus and microtubules in the thickening production are discussed.

INTRODUCTION
Thornber and Northcote (13) have traced in detail the nature of the polysaccharides laid down during secondary thickening in sycamore. The present report deals with the ultrastructure involved in such a process in the young stems of the same species in which at maturity the bulk of the xylem has spiral or annular thickenings. Hepler and Newcomb (5), studying regenerating Coleus xylem, have micrographs of a fairly late stage in this process, and Porter (10) has a picture of a similar development in onion root tip. Other than these papers, little work has been published on this topic.

The development of the patterned thickenings on the vessel wall seems to offer an ideal system for investigating the cellular basis for a structural pattern and for studying the means whereby the cell produces the large amount of wall material subsequent to cell expansion.

MATERIAL AND METHODS
The fixatives employed were: 1. Potassium permanganate (2 per cent) unbuffered, containing sodium, potassium, and calcium chlorides to a final total concentration of 0.4 M. Dehydration in alcohol series; and 2. Glutaraldehyde (12 per cent) in sucrose (10 per cent) followed by osmium tetroxide (1 per cent) in veronal buffer in sucrose (10 per cent) also. Dehydration in acetone, 15-minute “stain” with potassium permanganate (1 per cent) in 100 per cent acetone.

Segments (1 to 2 mm) of year-old stems of Acer pseudoplatanus (approximately 2 mm in diameter) were cut into the above fixatives in which they were left 1 hour, and then dehydrated, embedded in Araldite, and sectioned, with diamond knives, on a mechanical advance microtome. Sections were picked up on copper grids with a carbon supporting film and were stained with uranyl acetate (saturated solution in 50
RESULTS

The development of a spirally thickened xylem element has been well established with the light microscope.

The narrow cylinder of the cambial initial enlarges greatly in all dimensions and becomes completely vacuolate, save for a thin skin of cytoplasm two or three times as thick as the primary wall. Such a cell can be readily distinguished from xylem parenchyma by its far greater size, degree of vacuolation, and simplicity of the organelles contained within it. In the longitudinal sections of the cell, no indications of any regularity in the distribution of organelles or density variation in the cytoplasm have been observed which bear any relation to the spacing of the spiral or annular thickenings in the mature wall as seen in Figs. 1 and 2. Sinnott and Bloch (12), Barkley (1), and, earlier, Cruger (2) found that with the optical microscope a pattern in the cytoplasm could be seen similar in every respect to that shown by the fully developed thickening of the wall and able to be demonstrated in the plasmolysed cytoplasm of cells which had no signs of deposition on the wall. This disparity is discussed below.

Early and middle developmental stages are seen in Figs. 1 and 2. The locations of the final thickenings are defined by a darkening of the area immediately adjacent to the middle lamella (Fig. 1). Between this and the plasmalemma there is a "halo" of electron-transparent material. Hepler and Newcomb (5), studying xylem development in regenerating Coleus stem, consider that this darkening, which increases progressively, is due to lignin formation and/or its deposition, localised by reaction with the permanganate and greatly enhanced by lead staining. Certainly in sycamore, this darkening is found solely in those areas of wall in which lignin would be expected to occur. The areas can be seen to be fibrillar in nature in the figures, and this agrees with the concept that the lignin is deposited around the cellulose microfibrils, which usually display little contrast. Depending on the plane of section, most walls show a fibrillar texture if stained with uranyl acetate followed by lead hydroxide. This is true after fixation with formaldehyde, glutaraldehyde, or permanganate, but only the last fixative produces the darkening of the lignified areas. Further development of the thickenings occurs by an increase in size of both dark and light areas, the dark area gradually encroaching on and finally excluding this light "halo," when the final stage is reached.

The halo material, which is outside the plasmalemma, is exactly similar in electron opacity to the material enclosed in the large Golgi vesicles in the cytoplasm. This proliferation of Golgi bodies and vesicles is the outstanding feature of the developing cell (Figs. 2, 3, 5, 6, and 10). This is particularly apparent if the Golgi bodies of a developing xylem cell are compared with those of an adjacent parenchyma cell (Fig. 6). At this stage of development, the occasional plastids are simple sacs with no lamellar system inside and the mitochondria are small and numerous (Fig. 10).

The plasmalemma around the halo may be smooth, but at the edge of the halo bulges are frequently seen which seem, by their size, to have come from a Golgi vesicle and suggest that material

Abbreviations

cw, cell wall.
er, endoplasmic reticulum.
gg, Golgi apparatus.
gv, Golgi vesicle.
h, "halo" area of developing spiral thickening.
xp, xylem parenchyma.
m, mitochondrion.
p, plastid.
pl, plasmalemma.
s, spiral or annular thickening.
t, microtubule.
v, vacuole.

All figures are of longitudinal sections.

FIGURE 1 Earliest stage of thickening development observed. Note dark fibrillar areas in the thickenings. Permanganate fixation. X 8100.

FIGURE 2 Cell at the middle stage of thickening development. Note sites of vesicle apposition at arrows. Mature thickenings and degenerating organelles can be seen in the cell at the right of the micrograph. Permanganate fixation. X 14,000.
is being added by apposition. Figs. 2 and 3 show examples of this.

The endoplasmic reticulum is frequently observed to end abruptly at the edge of a developing thickening, and there is some indication that it may be continuous with the plasmalemma (Fig. 4). Porter (10) shows a micrograph which demonstrates the occurrence of the endoplasmic reticulum between each thickening of a protoxylem vessel of onion root tip. He tentatively suggests that there may be some connection between the two patterns. In sycamore this regularity has been observed only once and, even in this case, the endoplasmic reticulum was also present above the thickenings, as well as between them. In the developing xylem cell the endoplasmic reticulum often appears as reticulate profiles (Figs. 3 and 6).

After glutaraldehyde fixation the microtubules (approximately 200 A in diameter) described by Ledbetter and Porter (7) can be seen, and these are found in close relation to the developing thickenings. In sections through these peripheral areas, the microtubules can be seen to pass above the halo material. Fig. 7 shows an oblique section leaving the cell and entering the wall in such a way that the plasmalemma is not well defined and there is an overlap, within the section, of tubules and halo material. When the plane of the cell section is more radial rather than tangential, the tubules are rarely seen in longitudinal section, but can be seen in the cytoplasm immediately above the plasmalemma in transverse section (Figs. 4 and 8). This would indicate that they occur solely in the peripheral location of the cell, as already found by Ledbetter and Porter (7). That the system of microtubules is restricted to the areas immediately above the thickenings can be seen in Figs. 4, 7, and 8. Fig. 9 is a section very close to a wall of a developing xylem vessel and shows the microtubules running solely in bands above the thickenings, most of which are out of the plane of the section. The tubules are rarely found in the xylem parenchyma, though they are frequent in phloem tissue which is producing wall material.

There does seem to be a correlation between wall growth and frequency of tubule occurrence. No association of the microtubules with any other organelle has been observed.

Microtubules have been seen in association with developing cell plates in this tissue, although nuclear division stages have not been observed. These tubules are similar in appearance to those described above, but are smaller in diameter (180 to 200 A). This again is in agreement with Ledbetter and Porter's findings (7).

A section of a mature vessel is shown in Fig. 2. The breakdown of the organelles is rapid after the thickenings have reached their final dimensions. The cytoplasm thins, and the nucleus breaks down as the mitochondria, Golgi bodies, and the endoplasmic reticulum, which have become gradually fewer, disintegrate and disappear (Fig. 2).

DISCUSSION

The problem of relating the spiral or annular thickenings on the walls of the xylem elements to some pattern in the expanded, but as yet relatively undifferentiated, cell remains unresolved. Sinnott and Bloch (12), Barkley (1), and Cruger (2) have all demonstrated such patterns in the cytoplasm of cells destined to become xylem elements, using the light microscope. Hepler and Newcomb (5) presume this pattern to be due to a collection of organelles such as Golgi bodies, the endoplasmic reticulum, and mitochondria in cytoplasmic streams above the locus of the final deposition. No such distribution of organelles, even of microtubules, has here been observed. This may be due to one of four main causes. Firstly, the thinness of the sections means that even slight discontinuities in the cytoplasmic pattern could result in any one longitudinal section's lacking sufficient regularity of elements to be recognisable as such. It is hoped to resolve this difficulty by the use of serial sections. Secondly, the fixatives and techniques used here could be too insensitive to allow the detection or preservation of a pattern which may even be apparent only at a molecular level. However, Heslop-
Harrison (6), investigating pollen grain wall growth, and Esau et al. (3), studying phloem sieve plate development, have shown that an exact correspondence of elements of the endoplasmic reticulum with a pattern in the wall is maintained using permanganate fixation and epoxy resin embedding. Glutaraldehyde is generally accepted to be a better fixative than permanganate, since it preserves far more of the cellular structure; so it is unlikely that the procedures used in this work would have destroyed or displaced these organelles. Thirdly, the pattern may be too diffuse or too small to detect with present techniques. If, for example, the pattern were determined by a difference in the hydration state of the cytoplasm, it is doubtful whether this would be visible with thin sections unless the differences were considerable. Lastly, there is the possibility that the cytoplasmic pattern is transient, and that the times chosen for tissue fixation have been unfortunate.

That cellular organelles are concerned directly with the synthesis of material laid down in the spiral thickenings, there seems little reason to doubt. The evidence involving the Golgi apparatus in the final stage of wall precursor formation and transfer to site of deposition is clearly indicated by the evidence presented here. Hepler and Newcomb (5) suggest that this is probable, but they have no direct pictorial evidence. However, a similar involvement of Golgi vesicles in the development of the cell wall has been shown by Whaley and Mollenhauer (15) and Sievers (11). The three main constituents of the spiral thickening are cellulose microfibrils and hemicellulose and lignin as amorphous, interspersed packing. If the darkly stained areas seen in the sections of the xylem wall after the permanganate-lead treatment are assumed to indicate the presence of lignin, then the fibrillar synthesis and lignification proceed concurrently from the inception of the thickening, which is at an earlier stage in wall development than is usually considered. Since the dark fibrillar area encroaches upon, and finally replaces the halo of electron-transparent material under the plasma-lemma, then it seems reasonable to assume that this halo represents precursor material for one or all three of the polymer classes cited as constituents of the final thickening. It has been shown that cellulose microfibrils can be formed extracellularly by bacteria, either from a diffusible amorphous oligosaccharide precursor of some 200 to 220 units (9) or from a glucose-lipid compound (14). The process is probably enzymic, and it is quite possible that there are enzymes or enzyme systems present in the halo area. However, since the cellulose microfibrils are usually oriented in a definite manner which in the secondary cell wall is similar to that of the microtubules, these may be involved in either the synthesis or the orientation of the cellulose. They appear to be located solely above the thickenings, being rarely seen elsewhere in these cells. The tubules could actually be cellulose fibrils, being spun into the cell wall from some unknown site in the cytoplasm; or they could act as extrusion moulds down the centre of which the precursor material is funnelled, oriented, and polymerised; or they may be purely a cytoskeletal system. Frey-Wyssling (4) has pointed out that fibrillar isotropy cannot be produced by stress forces unless there is some resistance against which the force is acting. When the fibrils in a cell wall are laid down, they cannot at the same time produce the stress forces along which they are oriented. The microtubules always found, as yet, parallel to the microfibrillar direction, could play this cytoskeletal function, rather than a synthetic role.

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**Figure 5** Middle stage of xylem development. Note the numerous Golgi bodies with their associated vesicles. Glutaraldehyde fixation. × 34,000.

**Figure 6** Middle stage of xylem development. Compare the form of the Golgi bodies in the developing xylem vessel with those in the xylem parenchyma below. Permanganate fixation. × 10,000.
REFERENCES


Figure 7 Middle stage of xylem development. Semi-surface section of a xylem thickening showing the microtubules restricted to the area of the developing thickening. Glutaraldehyde fixation. × 64,000.

Figure 8 Middle stage of xylem development. Transverse section of the microtubules. The plasmalemma is clear and the tubules again are restricted to the area of the thickening. Glutaraldehyde fixation. × 83,000.
FIGURE 9  Middle stage of xylem development. Surface section of the developing thickenings which run between aa and bb. Note the restriction of tubules to the areas immediately above the thickenings. The three dark crystals at the upper right are staining artefacts. Glutaraldehyde fixation. × 68,000.

FIGURE 10  Middle stage of xylem development. Note the simplicity of the internal organisation of the plastids and the large vesicles associated with the Golgi bodies. Permanganate fixation. × 88,000.