A POSSIBLE FUNCTION OF MICROTUBULES
SUGGESTED BY THEIR ABNORMAL
DISTRIBUTION IN RUBBERY WOOD

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SUMMARY
A difference in the distribution of microtubules in the peripheral cytoplasm of developing fibres has been observed between normal apple wood and that infected with 'rubbery wood' disease. In normal wood, where microtubules are abundant, the fibre wall is lignified, compact in texture and of limited thickness. In rubbery wood, where there are fewer microtubules, lignification is not complete in the fibre wall, which has a coarse, loose texture, and is abnormally thick. The whole stem is highly flexible. The pit membrane, which is un lignified in both normal and rubbery wood, has no microtubules adjacent to the plasmalemma.

It is suggested that microtubules are concerned in channelling into the wall substances, such as lignin precursors, which are essential for certain steps in the polymerization of the amorphous component of the wall.

INTRODUCTION
The histological characteristics of 'rubbery wood' disease of apple trees were first described by Beakbane & Thompson (1945). At that time they considered the infection to be due to a virus, although more recently Beakbane, Mishra, Posnette & Slater (1971) have reported finding mycoplasma-like organisms in the phloem. The disease causes the wood in the scions of some varieties, notably Lord Lambourne, to become highly extensible, giving a feel of rubberiness to the branches. The abnormal flexibility of the stems is associated with lack of lignification of many of the xylem fibres and vessels, shown by the failure of fresh wood to stain with phloroglucinol and hydrochloric acid. This symptom usually appears after the first year's growth in shoots and is particularly marked in the summer wood. In the incompletely lignified areas the walls of the vessels and fibres give the usual staining reactions of cellulose. The vessels have irregularly shaped walls which, in some cases, may have collapsed altogether. The xylem fibres are more or less circular in transverse section with abnormally thick walls surrounding very narrow lumina.

Anatomically, rubbery wood is similar to reaction wood but the abnormal features occur around the whole circumference of bent rubbery shoots, whereas they are usually confined to the upper side in bent healthy shoots. Rubbery wood also differs chemically from reaction wood (Sondheimer & Simpson, 1962).

Nelmes & Preston (1968) investigated the ultrastructure of both normal and rubbery apple wood. They found that the normal steepening of the microfibrillar helix around the fibre walls as the fibre lengthens from one annual ring to the next is suppressed in rubbery wood. This potentially confers upon the rubbery wood a low tensile
strength and high extensibility. By itself this would not account for the flexibility of rubbery wood, since normally the turns of the helix are linked by the lignin polymerized between and attached to them, perhaps through the hemicelluloses, and therefore the part played by the abnormal lignin metabolism must be considered.

It appears that the virus interferes in the metabolic methylation pattern of lignin and that monomers with an abnormally large number of unmethylated phenolic groups are present (Sondheimer & Simpson, 1962). In addition, analyses carried out by Scurfield & Bland (1963) on a number of samples showed that the lignin content of rubbery wood was consistently lower than that of normal wood and that it was less firmly attached to the polysaccharide framework than in normal wood. If this is correct then rubbery wood owes its flexibility to the failure of the matrix material to hold securely together the turns of the potentially more extensible microfibril helices.

Since the structure of the mature wall is the result of the activity of the cytoplasm during differentiation from cambium to xylem, the modifications induced by the virus provide a tool for elucidating further the role of various cytoplasmic organelles involved in normal wall metabolism.

MATERIAL AND METHODS.

Three-year-old, upright stems were collected at intervals throughout the growing season from both normal and rubbery trees of apple var. Lord Lambourne. The fixative (3 % glutaraldehyde in 0.02 M phosphate (K+) buffer at pH 7.0) was taken out to the collection site in ice-packed containers and the bark removed from short lengths of freshly cut 3-year-old twigs while these were immersed in fixative. If the complete cambium was required, the outer part only of the bark was pared off, rather than peeling the bark as a whole. These lengths of twig were fixed for 2 h at 4 °C, after which thin slices, which included cambium and developing xylem, were cut from the exposed surface and transferred to fresh fixative for a further 2 days. The tissue was then washed in several (at least 3) changes of 0.02 M phosphate buffer for 8 h and postfixed in 0.02 M phosphate-buffered osmium tetroxide overnight. This was followed by standard procedures for ethanol dehydration, infiltration and embedding in TAAB Embedding Resin, carried out over a period of 5 days. All tissues and reagents were kept at 4 °C until the first change of absolute ethanol, after which they were allowed to reach room temperature. The final embedding was done in flat polyethylene troughs using fresh resin. Polymerization was at 60 °C for 48 h. Sections were cut at approximately 90 nm using a diamond knife and stained with uranyl acetate and lead citrate. All micrographs were taken on a Philips EM 200 electron microscope.

Optical sections were cut at 7 μm on a sledge microtome and stained with safranin and fast green. Light-microscope photographs were taken on a Zeiss-Ultraphot II.

Surface replicas were prepared by a simple 2-stage technique using cellulose acetate sheet for the primary replica.

RESULTS

Electron micrographs of ultrathin sections through the developing xylem of apple wood show that, in many respects, the fine structure closely resembles that described by previous workers for this tissue in other species (Cronshaw, 1964, 1965; Cronshaw & Bouck, 1965; Esau, Cheadle & Gill, 1966a, b; Robards, 1968; Wardrop, 1964; Wooding & Northcote, 1964). However, when normal and rubbery wood are compared there is a noticeable difference in the number of cortical microtubules present in the developing fibres.
Function of microtubules

In normal fibres, microtubules are abundant where the wall is undergoing secondary thickening. They form a layer, sometimes two or three deep, in the peripheral cytoplasm just inside the plasmalemma, which is often much convoluted (Fig. 1). In transverse section the microtubules show the typical appearance, first described by Ledbetter & Porter (1963), of a number of darkly staining subunits arranged in a cylinder with external diameter approximately 25 nm and internal diameter approximately 10 nm.

Although microtubules are most numerous at the stage when the S2 layer of the fibre wall is being formed they are also present near the very young xylem wall and in sections of late wood collected in October. These late wood fibres have compact walls of limited thickness and the large lumen still has living contents.

In the pits of normal fibres large numbers of microtubules occur adjacent to the plasmalemma of the border (Fig. 2). Where the border has been cut tangentially the microtubules appear to run around the perimeter of the pit aperture in a manner similar to that observed by Robards & Humpherson in Salix fragilis (1967). However, in the region adjacent to the pit membrane, although there are numerous ribosomes, microtubules appear to be absent. This is the only place in normal fibres where they have not been observed.

Bamber (1961), using safranin and fast green to differentiate between lignified and non-lignified tissue, showed that the pit membrane of various eucalypts and softwoods was un lignified. Wardrop (1964), on the basis of potassium permanganate stained ultrathin sections, also considered this probable. To find whether this is true also for apple wood, radial longitudinal sections were cut at 7 μm and stained with safranin and fast green, as described by Bamber. The membranes of the parenchyma pits, the ray parenchyma to vessel pits and the vessel-to-vessel pits were differentiated green in contrast to the unpitted wall which was stained red. The pit membranes in apple would therefore appear to be non-lignified, i.e. in small, specific areas, which are devoid of microtubules, the middle lamella and the primary wall, elsewhere the most heavily lignified layers, have no lignin.

In rubbery wood some microtubules are present, particularly in the spring wood, but they are not nearly as numerous as in normal wood. The difference is most marked in fibres where the S2 is being laid down (Fig. 3), that is, at the stage at which the layer which gives the wood its most important structural characteristics is being formed. If this micrograph is compared with those shown in Figs. 1 and 2 it can be seen that the microtubules are far less abundant and may even be absent altogether from some regions of the wall. The plasmalemma over most of the wall is much less convoluted than in normal fibres at this stage, although occasionally it gives the appearance of having proliferated to such an extent that it has formed numerous overlying folds.

In sections of late wood the wall is abnormally thick and appears coarse in texture, with the microfibrils lying separately from each other (Fig. 4). In the majority of fibres the lumen is very small and contains only moribund remnants of protoplasm. In the occasional fibre which still has living contents very few microtubules are visible.

The pits in rubbery wood fibres are similar in general structure to those in normal
wood but with fewer microtubules associated with the border, and the border itself tends to have a thicker, blunter outline in cross-section (Fig. 5) compared with the more tapered appearance in normal wood. Again, microtubules are absent from the pit membrane but frequently this is lined with abundant ribosomes.

DISCUSSION

Microtubules in plant cells were first described by Ledbetter & Porter (1963) and have since been found in a variety of tissues where glutaraldehyde fixation has been employed. There has been considerable speculation as to their function. The situation regarding them has been reviewed by Mühlethaler (1967) and Newcomb (1969). Their frequent occurrence in the peripheral cytoplasm of cells actively engaged in wall formation has led to the belief that they are, in some way, involved in this process.

The orientation of microtubules frequently reflects that of the microfibrils in the adjacent cell wall. This has led a number of authors (e.g. Ledbetter & Porter, 1963; Cronshaw, 1967; Wooding & Northcote, 1964) to consider, rather tentatively, whether microtubules might play a role in the synthesis or orientation of microfibrils, but the idea has now been generally abandoned in favour of the 'ordered-granule' hypothesis, first presented in formal terms in 1963 (Preston, 1964). More recently, Murmanis (1971) suggested that microtubules provide structural support for particles at the plasmalemma/cell-wall interface and also states that an amorphous substance has been occasionally observed extruding from the outer ends of microtubules. McManus & Roth (1965) also suggest that microtubules may be concerned with the piping of fluids. The interpretation of microtubule function put forward in the present work is based on consideration of their occurrence and distribution in relation to the structural and pathological features of normal and rubbery apple wood.

Microtubules are present in large numbers in the peripheral cytoplasm of developing fibres of normal wood. They appear to be intimately associated with a highly convoluted plasmalemma at a stage when wall material is being rapidly laid down, suggesting that both structures are involved in very active wall synthesis. The fibre wall can be shown to stain with standard reagents for lignin, it is rigid and in electron micrographs appears compact in texture and of limited thickness. In fibres from rubbery wood microtubules are fewer and the plasmalemma is flatter. Where microtubules are very sparse, as in the late wood, the wall is abnormally thick and of loose texture. Staining reactions of sections cut from portions of the stem adjacent to those used for ultrathin sectioning show the late wood walls to be incompletely lignified. In both normal and rubbery wood, microtubules are absent from the cytoplasm adjacent to the un lignified pit membrane and there is evidence that the pit membrane in hardwoods tends to have a lower density and looser texture than non-pitted areas of the wall (Schmid, 1964). The absence of microtubules therefore appears to be associated with abnormal wall structure. Malformed thickening of the cell wall associated with the absence of microtubules has also been observed in the xylem of root tips and coleoptiles of wheat after treatment with colchicine (Pickett-Heaps, 1967).
Function of microtubules

Abnormal wall structure could be due to defects in either the cellulose or the amorphous phase. There is no indication that, in rubbery wood, the ability to synthesize cellulose is impaired. The un lignified walls of the late wood stain with fast green and microfibrils can be seen in surface replicas (Fig. 6). According to Scurfield & Bland (1963) the percentage of cellulose in rubbery wood, as determined by chemical methods, was the same as in normal wood.

It therefore seems probable that in a normal wall microtubules play some role in the synthesis of the amorphous phase. Much of the amorphous material, including pectins and hemicelluloses, is secreted into the wall by Golgi vesicles (Cronshaw, 1965, 1967; Northcote & Pickett-Heaps, 1966; Srivastava & O’Brien, 1966; Whaley & Mollenhauer, 1963) and there is some evidence (Pickett-Heaps & Northcote, 1966) that microtubules play a role in vesicle alignment at cell-plate formation. Robards (1968) suggested that this function may be continued throughout wall development, although in apple wood groups of vesicles can be seen, apparently passing from Golgi bodies towards the wall, in regions such as the pit membrane, where there are no microtubules.

In apple wood there seems to be a definite link between the presence of microtubules during development and the subsequent lignification of the wall, even though in other species microtubules have been observed in locations where the wall remains un lignified. However, microtubules are commonly associated with the formation of secondary walls, bands of thickening, etc., and similar situations where strength and rigidity are provided by the wall. That their absence in rubbery wood is associated with an abnormally flexible, loose-textured wall, where the microfibrils can be seen lying separately from each other, leads to the suggestion that the function of microtubules is concerned with channelling into the wall substances which are essential for certain steps in the polymerization of the amorphous component of the wall and the bonding of one microfibril to another. The exact nature of these substances remains to be investigated but in apple wood it seems probable that lignin precursors are involved.

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REFERENCES

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ABBREVIATIONS ON PLATES

l lumen
p plasmalemma
pm pit membrane
to wall
pb pit border
Function of microtubules

Figs. 1 and 2. For legend see p. 748.
Fig. 1. Normal wood. This shows a small part of the wall (s) and cortex of a developing fibre. The plasmalemma (p) is highly convoluted and appears to be in a very active state. Microtubules form a multiple layer in the cortical zone. × 132 000.

Fig. 2. Normal wood. Bordered pit showing microtubules adjacent to the pit border (pb) and traversing the perimeter of the pit aperture but absent from the zone near the pit membrane (pm). The pit membrane is lined with abundant ribosomes. × 55 200.

Fig. 3. Rubbery wood. Transverse section of a developing fibre. The plasmalemma is much flatter than in normal wood except for localized regions (arrow) where it appears to have proliferated to the extent of forming overlying folds. Microtubules are more sparsely distributed than in normal wood. × 30 000.

Fig. 4. Rubbery wood. Transverse section of a late-wood fibre. The lumen (l) is small and contains only remnants of protoplasmic material. The wall appears coarse in texture with the microfibrils lying separately from each other. × 46 000.
Fig. 5. Rubbery wood. The borders of this pit are thicker and blunter than those seen in normal wood in Fig. 2. There are comparatively few microtubules adjacent to the border and, as in normal wood, they are absent from the pit membrane. × 30,000.

Fig. 6. Rubbery wood. Surface replica of late-wood fibre wall. The microfibrils are clearly visible and well oriented. × 23,700.