LIGNOFIBRILS ON THE EXTERNAL CELL WALL
SURFACE OF CULTURED PLANT CELLS

GARY G. LEPPARD, J. ROSS COLVIN, DYSON ROSE, and S. M. MARTIN

From the Biochemistry Laboratory and the Division of Biology, National Research Council of Canada, Ottawa, and the Département de Biologie, Université Laval, Québec, P. Q., Canada

ABSTRACT
Small strands and bundles of strands extend from the outside surface of suspension-cultured cells of *Daucus*, *Ipomoea*, and *Phaseolus* into the medium. This fibrous cell coat is present in all samples from various growth stages but appears to increase in quantity in the order *Ipomoea* < *Phaseolus* < *Daucus*. The bundles are often many microns in length and display great variation in frequency, size, and form. Identification of the composition of the strands and bundles as lignin is consistent with the following observations: alkaline nitrobenzene oxidation of the strands to compounds which resemble monomers of wood lignin; resistance of the strands to pronase, trypsin, pectinase, and lipase; strong irreversible adsorption of heavy metals; deposition of silver granules by treatment with silver nitrate-hexamine reagent; extraction of the bundles with aqueous dioxane (Björkman procedure); presence in quantity of a structured form of Klason lignin; and existence of material giving a positive test with the Wiesner reagent. Large individual strands (lignofibrils) from *Phaseolus* show the form of a flat ribbon with very thin branches at irregular intervals. This form does not vary with preparatory techniques, although its electron opacity does. Intercellular spaces display considerable structure and sometimes contain sheets of fibrillar material merging with both the middle lamella between the cells and the surface bundles facing the medium. These sheets are probably another form of association of the lignofibrils. It is suggested that natural fibrous lignin may be a much commoner component of plant tissue than suspected hitherto.

INTRODUCTION
The external surface of cell walls of plant cells grown in culture has not been studied extensively with the electron microscope (Halperin, 1969), but there is an increasing interest in the growth of plant cells in suspension culture which requires a knowledge of cell-cell and cell-milieu interactions (Steward, 1970; Halperin, 1969). Clearly, embryogenesis in plant cell cultures is dependent upon the formation of cell contacts, cellular aggregates, and “intercellular glue,” concepts well developed in the literature of animal embryology (Curtis, 1967). Again, the secretion of high molecular weight polymers into liquid media by cultured cells (Aspinall et al., 1969; Olson et al., 1969) presents the major problem of how a distinct structural barrier, the cell wall, permits the exit of large molecules. The various aspects of transport in this barrier are rarely considered in the literature despite the known presence of enzymes in the wall (Knox and Heslop-Harrison, 1970; Newcomb, 1963) and examples of fibrillar, polymeric material deposited external to existing walls (Probine, 1965; Beer and Setterfield, 1958). From a third point of view, research on cell fusion with
plant species (Power et al., 1970) would be facilitated by a greater knowledge of the mechanism of enzyme penetration of walls, because present methods are limited to those species which can withstand enzymatic wall digestion (Keller et al., 1970). Finally, future studies of the penetration of cultured plant cells by pathogens (Cocking, 1970; Braun and Lipetz, 1966) will require a better knowledge of the cell surface encountered by the pathogen. Thus a better understanding of the cell wall of plants and especially its external surface is necessary.

This paper describes the form and general composition of a newly recognized component of the external cell wall surface of cultured cells of Phaseolus vulgaris var. red kidney bean, Ipomoea sp., and Daucus carota. It also suggests that this component may be of considerable importance in ordinary plant tissues.

**MATERIALS AND METHODS**

**Cell Culture**

All three cell lines were isolated by the technique of Veliky and Martin (1970). The isolated cells were cultured in a "V" fermenter using basal medium 67-V (Veliky and Martin, 1970) which is a modification of the PRL-4-C medium of Gamborg (1966) used to study aromatic acid metabolism. The growth stages examined from each of the cultures were as follows: Phaseolus, from a freshly inoculated culture and from a culture in exponential growth; Ipomoea, from a culture entering exponential growth and from one in full exponential growth; Daucus, from a freshly inoculated culture.

**Microscopy**

Cells and cell clumps taken directly from culture were fixed for 1 hr at room temperature in 6% glutaraldehyde at pH 7 in 0.01 M phosphate buffer containing 0.4 M sucrose (Sabatini et al., 1963). They were then washed in buffered sucrose solution, followed by buffer solution, and finally postfixed for 3.5 hr at 0°C. Postfixation was either by 1% osmium tetroxide in 0.05 M phosphate buffer at pH 6.8 or by a filtered, saturated solution of ruthenium red (British Drug Houses, Ltd., Poole, Dorset, England) in 0.05 M phosphate buffer at pH 6.8 containing 1% osmium tetroxide. Fixatives were prepared immediately before use. After postfixation the samples were washed with cold buffer and dehydrated by methanol, methanol-propylene oxide, and propylene oxide.

Embedment was in Epon (Luft, 1961). Sections were cut on a Porter-Blum MT-2 ultramicrotome with a diamond knife and collected on gold grids. All sections except those treated with the silver reagent were poststained for 60 min with uranyl acetate (Watson, 1958) followed by lead citrate (Reynolds, 1963) for 30 min. Observations and photographs were made with a Philips 200 electron microscope at 40 kv.

**General Cytochemistry**

The silver nitrate-hexamine technique of Swift (1968) with the precautions of Thompson and Colvin (1970) was used for the localization of disulfide or aldehyde groups on unstained pale-gold sections mounted directly on gold grids without a supporting film. The technique was also used for sections which previously had been oxidized by 1% aqueous periodic acid for 20 min at room temperature. All grids were subsequently rinsed for 30 min in distilled water (Rambourg and Leblond, 1967). For this phase of the study, all work was done on glutaraldehyde-fixed material without postfixation and then repeated on samples which had also received a postfixation in the osmium-ruthenium solution.

**Enzyme Cytochemistry**

Unfixed cells and cell clumps of Phaseolus were digested in separate experiments by the enzymes pronase, trypsin, pectinase, and lipase. Immediately after digestion the cells were centrifuged, the enzyme solution was decanted, and the pellet was washed three times with buffer solution and fixed as described above. Comparisons were made between cells which had been digested by enzyme solutions and those which were treated for the same time, under the same conditions, by an enzyme solution which had been heated for 20 min in a boiling water bath. The activity of the native enzyme solutions and the inactivity of the heated enzyme solutions was verified before each experiment with appropriate substrates.

In each experiment 1 g of wet cells was added to 10 ml of enzyme preparation. The cells were digested by 0.5% pronase (Streptomyces griseus, protease B grade, Calbiochem, Los Angeles, Calif.) for 1 hr at 37°C in 0.05 M citrate solution at an initial pH of 7.4; by 0.5% trypsin (Worthington Biochemicals Corp., Freehold, N. J.), twice recrystallized, for 3 hr at 37°C in 0.01 M phosphate buffer at pH 7.7; or with 1% pectinase (Nutritional Biochemicals Corp., Cleveland, Ohio) for 24 hr at room temperature (~25°C) in 0.01 M phosphate buffer with an initial pH 5.8. Digestion by lipase (Steapsin, Difco Laboratories, Inc., Detroit, Mich.) was carried out for 1 hr at 37°C with a preparation made up according to the following procedure: 250 mg of the Steapsin sample was suspended in 5 ml of 0.9% NaCl and then centrifuged at 40,000 g for 30 min. 2 ml of the supernatant was diluted to 10 ml with 0.05 M sodium citrate solution at pH 7.2 to give the lipase solution for direct use.
Lignin Cytochemistry

Lignin was prepared from unfixed and glutaraldehyde-fixed Phaseolus cells by the Klason method (Brauns, 1952, p. 55). No difference was observed between the samples derived from fixed and unfixed cells. After preparation of the lignin samples, they were postfixed with the ruthenium-osmium solution and prepared for electron microscopy as described for general microscopy. In addition, Björkman lignin (Björkman, 1956) was extracted from glutaraldehyde-fixed cells of Phaseolus. The residue of cellular material was postfixed with the ruthenium-osmium solution and prepared for electron microscopy as outlined for general microscopy. Paper chromatography of the Björkman lignin fraction was done by the ascending technique using the following solvents: n-butanol-pyridine-water, 10:4:4; n-butanol-acetic acid-water, 10:3:3.

Analytical Chemistry

Cells and cellular aggregates of Ipomoea were broken by sonication and the cell wall fragments were separated from the cytoplasmic residues by repeated differential centrifugation. The cell wall fraction was thoroughly washed in water and examined microscopically for the presence of cytoplasmic residues. A clean sample was analyzed for amino acid composition by the methods of Yaguchi and Perry (1970). A sample of fibrous material (see below) was analyzed similarly.

The fibrous material was separated from cells of Ipomoea and analyzed as follows. The cell suspension in the original medium plus 1% glutaraldehyde was centrifuged for 2 min at full speed in an International clinical centrifuge to sediment the majority of the cells. The decanted supernatant was then centrifuged for 5 min at approximately 7000 g in a Sorvall centrifuge model SS-1, to sediment the remainder of the cells. The supernatant, which had only a trace of Tyndall scattering, was then centrifuged again at full speed (~14,000 g) for 0.5 hr. The supernatant was decanted, and the optically clear gel at the bottom of the tube was retained. This gel was free of cells when examined in the optical microscope and showed only the fibrous material when fixed, postfixed by ruthenium-osmium, and examined in the electron microscope. After washing five times with 0.1 M phosphate buffer (Sorensen's) pH 6.8, the gel was lyophilized and the white fibrous residue was stored over anhydrous calcium chloride.

For examination for the presence of carbohydrates the fibrous residue was partially hydrolyzed by 72% H2SO4 for 0.5 hr at room temperature, the acid was diluted 12 times by the addition of water, and the hydrolysis was completed by heating in a sealed glass capsule for 4 hr at 100°C. After removal of the sulfuric acid by the ion exchange resin Rexyn 203 (Fisher Scientific Co., Pittsburgh, Pa.) and concentration of the neutral supernatant, the sample was examined for the presence of neutral sugars by descending paper chromatography using the solvent, normal butanol-pyridine-water (10:4:4), and alkaline silver nitrate for detection of reducing substances (Trevelyan et al., 1950). Bacterial cellulose was used as a control fibrillar material.

For detection of amino acid residues or nucleic acid bases the fibrillar material was hydrolyzed in 6 n HCl for 16 hr at 100°C in a sealed capsule, the solution was evaporated, and the residue was placed directly on paper chromatograms. For amino acids the developing solvent was normal butanol-acetic acid-water (4:1:1), with ninhydrin as a detector. For the nucleic acid bases the developing solvents were 0.1 M phosphate (pH 6.8)-saturated ammonium sulfate-normal propanol (100:8:2 v/v) and normal butanol-water (85:14 v/v) (Markham and Smith, 1949). The position of the compounds was detected by ultraviolet absorption.

For detection of monomers of lignin or lignin-like compounds, the fibrillar material was hydrolyzed and analyzed by the methods of Stone and Blundell (1951). In addition to the solvents employed by them, we used normal butanol-pyridine-water (10:4:4) as a developing solvent for the hydrolysis products. The position of compounds was detected by ultraviolet absorption and by 2,4-dinitrophenylhydrazine.

RESULTS

Microscopy

Cells of the three species at all culture stages tested showed a distinct fibrous coat on the outside of the wall. This coat was well developed in Phaseolus (Fig. 1) and Daucus (Fig. 2) but weakly developed in Ipomoea (Fig. 3 a; 3 b) where sloughing of this layer from the external portion of the cell wall was more evident (Fig. 3 a; 3 b). In Phaseolus and particularly in Daucus the individual strands of this fibrous coat (lignofibrils; see below) were often associated to form bundles. These bundles often extended many microns from the cell wall surface into the medium and displayed a wide variation in form, arrangement of lignofibrils, and frequency. Many sections presented cell surfaces completely coated with a variable depth of fibers (Fig. 1) while others presented almost no covering whatsoever. In some sections, the cytoplasmic membrane was withdrawn from the inside surface of the wall, probably by plasmolysis during fixation (Figs. 1, 2, 5, 6), but the internal cell damage had no apparent influence on the disposition of the...
lignofibrils outside the cell, as shown by the same general orientation of the fibrils in sections where the damage did not occur (Figs. 3, 8, 10).

Fig. 4 illustrates the ropelike aspect of one form of aggregation of the lignofibrils but there were several others. Where two cell walls touched, the bundles had a tendency to form sheets and to fuse with the material which would be called the middle lamella (Fig. 5) in ordinary plant tissues. These curved sheets often formed pockets in which an empty space (presumably filled with culture medium) was surrounded by segments of a fibrous sheath. When two separate cells of a clump were found in close proximity, the intercellular space was often modified by a merging of the fiber bundles of the two surfaces (Fig. 6). This effect may have been simply the result of compression between the two surfaces, but does represent an interesting form of structural continuity between plant cells. Except for this effect of compression, there were no obvious differences in the disposition of lignofibrils on the outside of single cells and between cells in clumps. On sparsely coated walls, the lignofibrils were usually loosely aggregated (Figs. 1 and 7) or individual, but, when closely aggregated, their bundles had a tendency to be oriented perpendicular to the cell surface.

Individual lignofibrils had a distinctive form which was similar in all three species, although their width varied greatly (some examples from Phaseolus were noticeably wider). This form did not vary with preparatory technique but the electron opacity of the fibril did, being weak with glutaraldehyde fixation alone (Fig. 8) even when followed by uranium and lead poststaining of sections. However, postfixation of cells in the ruthenium-osmium solution followed by poststaining of the sections resulted in a great increase in electron opacity of the fibrils (Fig. 7). Each large lignofibril was a flattened band of variable maximum width, 120 A–200 A. The fiber branched at irregular intervals and the smallest branches had widths which approached the resolution limit for sectioned material (Figs. 1 and 7). Occasionally a fiber appeared to be split longitudinally (Fig. 7). This bifurcation and the flatness of the larger fibers suggests that they may have a fasciculate internal structure, but attempts to demonstrate this directly did not succeed. Nonetheless, the pattern of branching is explicable on that basis.

A series of electron-opaque, roughly circular "dots" was frequently observed to be associated with individual lignofibrils or aggregations of them (Figs. 1, 2, 6, and 7). In addition, vesicular material was sometimes seen among the lignofibrils (Fig. 5). This was probably contaminating material that had become enmeshed among the fibrils. Occasionally, another structure which had the appearance of a granular smear was associated with the fibers (Fig. 7).

Because of their form and position, it was of interest to see if the orientation of the external electron-opaque fibrils had any correlation with that of similar structures within the cell wall. However, such correlations were only general and inexact. The dimensions, electron opacity, granularity, and fibrillar organization of the cell walls of all three species were highly variable. Fig. 9 shows wall bands of different electron opacity which are parallel to the cell surface. A central band usually appeared to be amorphous (Fig. 9a) but sometimes displayed fibers which were perpendicular to the cell surface (Fig. 9b). Fig. 9c illustrates a situation in which there is little or no correlation between the orientation of individual lignofibrils and the orientation of electron-opaque fibrils in the wall. Occasionally, banding patterns were observed which cannot be interpreted by apposition growth (Fig. 10) as conceived by Roelofsen (1965). Thus, at the present time there is no evidence for or

Unless otherwise indicated, all figures were produced from material fixed in glutaraldehyde, postfixed in ruthenium-osmium, and poststained with uranium and lead. The symbol W locates the cell wall. The symbol E locates the exterior milieu. The bar represents 1 μ except when stated otherwise.

**Figure 1** Fibrous cell coat of *Phaseolus*. Loosely aggregated fiber bundles extend many microns into the culture medium from the external surface of the cell wall.

**Figure 2** Fibrous cell coat of *Daucus*. The fibrils extending into the medium are often organized into complex bundles of considerable order in this species. This particular cell surface received additional treatment as part of another study to increase fibril electron opacity. After the postfixation, the cells were soaked for 15 hr in 80% methanol containing salts of lanthanum (1.5%), thallium (1%), and thorium (1%) at room temperature. Also, 0.5% iodine was added to the absolute methanol portion of the dehydration series.
Figure 3. External surface of cell walls of Ipomoea postfixed only in osmium. The weakly developed fibrous cell coat of this species usually appears as a sloughed surface. Note that in Fig. 3 a a fibrous sloughed surface projects into a large intercellular space. In Fig. 3 b there is extensive shedding of the fibrous material from the wall into the culture medium. Because the magnification is low in both photographs in order to show the sloughing clearly, individual lignofibrils are not well resolved but are evident when the photographs are enlarged.
Figure 4  Ropelike aggregate of *Phaseolus* lignofibrils.
FIGURE 5  Fiber bundles projecting into the medium from the junction of two cells of a clump of *Daucus*. Note the sheetlike organization of some bundles and the merging of bundles with the "middle lamella." These cells received the additional treatment outlined for Fig. 2.

FIGURE 6  Intercellular space between cells of a loosely aggregated clump of *Phaseolus*. 

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Figure 7  Loosely aggregated lignofibrils from *Phaseolus* showing some individuals of exceptional thickness at a considerable distance from the external surface of the cell wall (not shown). Note the bifurcations, the dot-like structures, and the "granular smears."

Figure 8  *Phaseolus* lignofibrils without postfixation.
Figure 9  Cell wall banding in *Phaseolus*. (9 a) Typical electron-opaque central band. (9 b) An electron-opaque central band with a fibrous substructure oriented perpendicular to the cell surface and presumably perpendicular to the cellulose microfibrils. (9 c) Central band with a fibrous substructure reflecting the orientation of the external lignofibril bundles.
against correlation of deposition of the external lignofibrils with fibers within the wall.

**Cytochemistry**

The foregoing results from microscopy pose the question of the composition of the external fibers. The following observations help to answer this question.

Hydrolysis of the separated external fibers, followed by chromatographic analysis of samples of 160 µg, showed no detectable neutral reducing sugars (by alkaline silver nitrate, limit of sensitivity 0.1 µg) as a component of the fibrillar material. By using bacterial cellulose as a standard, it was established that the content of any particular neutral reducing sugar in this material must be
Comparison of Amino Acid Composition of Protein (s) from Ipomoea sp. Lignofibrils and Washed Cell Walls

| Component (Protein) | Fibrils (5.3) | Cell walls (5.1) |
|---------------------|--------------|-----------------|
| Aspartic            | 0.64         | 0.44            |
| Glutamic            | 0.33         | 0.30            |
| Glycine             | 0.38         | 0.24            |
| Serine              | 0.38         | 0.49            |
| Phenylalanine       | 0.34         | 0.12            |
| Valine              | 0.31         | 0.22            |
| Threonine           | 0.31         | 0.17            |
| Alanine             | 0.31         | 0.20            |
| Proline             | 0.30         | 0.21            |
| Hydroxyproline      | 0.24         | 1.1             |
| Lysine              | 0.09         | 0.45            |
| Cystine + cysteine  | 0.03         | 0.03            |

Table I

less than 0.1%. Likewise, there were no detectable nucleic acid bases in a portion of 600 μg of the hydrolysate of the external fibers when either of the appropriate developing solvents was used (limit of sensitivity 1 μg). However, preliminary paper chromatography of the hydrolysate for amino acids showed the presence of four faint spots which reacted with ninhydrin. Subsequent quantitative determination of the protein content and the amino acid composition of the fibrils and washed cell wall fragments from Ipomoea gave the results in Table I. These results indicate that, on the basis of their hydroxyproline and lysine content, the separated fibrils cannot be contaminated by more than 20% by cell wall fragments. That the actual contamination is more than an order of magnitude less is shown by the complete absence of neutral reducing sugars in the fibrils. Although it is an assumption as yet, we suggest that the 5% of protein in the lignofibril samples represents material from the medium strongly adsorbed to the strands.

In contrast to the foregoing, chromatographic analysis of the products of the alkaline nitrobenzene oxidation of the separated external fibers gave evidence for compounds which resemble but are not identical with vanillin, p-hydroxybenzaldehyde, or the monomers of spruce wood lignin. The compounds in the hydrolysate of the external fibers did not move at all in petroleum ether saturated with water, in which vanillin migrates, but p-hydroxybenzaldehyde does not, nor in dibutyl ether saturated with water. However, they did migrate in the solvent normal butanol-pyridine-water (10:4:4) in which both vanillin and p-hydroxybenzaldehyde also move with an Rf of approximately 0.85-0.9. In this solvent the hydrolysate from spruce wood lignin shows five compounds, with Rf's in the order 0.05, 0.1, 0.3, 0.6, and 0.9, detectable both by ultraviolet and by 2,4-dinitrophenylhydrazine. In the same solvent, the hydrolysate from the external fibers shows four compounds by ultraviolet with Rf's in the order 0.3, 0.43, 0.5, and 0.9. However, none of these compounds appear to react as readily with 2,4-dinitrophenylhydrazine as those from wood lignin, even when they are at the same concentration, as judged by ultraviolet. Therefore, although it appears that the external fibers are composed of compounds which are similar to those derived from wood lignin, the compounds from the two sources are not identical. This difference is presently under investigation.

The external fibers on the outside of the walls were not digested by pronase, trypsin, lipase, or a pectinase preparation which was in reality a mixture of pectinases, hemicellulases, and cellulase. The fibers from cells treated with each of these enzymes resembled the fibers on the outside of cells which had been treated with boiled enzyme solution, despite the fact that the active enzyme had obviously digested parts of the cells. Fig. 11 is a photograph which is typical of all results from the enzymatic part of the investigation. The external fibers are clearly visible without perceptible attack by the trypsin. This photograph also illustrates a feature of the cell wall found by the morphological studies which is more clearly shown after enzyme digestion. A thin fibrous layer often lines the inner surface of the cell wall, and observations on plasmolysed cells, with or without enzyme digestion, showed a marked similarity of form between large individual fibers of this layer and those of the external surface. In summary, the results of enzymatic digestion were consistent with the conclusion that the external fibers did not contain an appreciable portion of protein, polysaccharides, or lipid polymers.

It is known that the middle lamella contains up to 70% lignin (Neish, 1965), that lignin biosynthesis by cultured cells has been established by tracer studies (Gambar, 1967), and that the outside surface of cultured plant cells has been shown to react positively (White, 1967; Veliky and LaRue, 1967) with the Wiesner reagent (Brauns,
FIGURE 11 Portion of a *Phaseolus* cell after trypsin digestion. Note the similarity between the external lignofibrils and the fibrils lining the inner surface of the cell wall.

FIGURE 12 *Phaseolus* Klason lignin residues. (12 a) Variation in residue substructure. (12 b) Note the residual strands of linear but indefinite form. (12 c) Klason lignin residues having the form of a "collapsed" cell wall.

1952). For these reasons, together with the analytical and enzyme digestion observations, the possibility that the external fibers were composed of lignin was seriously considered, despite the fact that fibrillar lignin has not been described before. Partially extracted cellular material gave a positive reaction with the Wiesner reagent (phloroglucinol-HCl) although color development required several days of exposure to air in comparison with several minutes for wood shavings. Because of the unexplained slow color development the Wiesner reaction could not be used to study lignin localization and extraction in cyto logical preparations. Also, Klason lignin was
isolated from cells of Phaseolus (average content was 30% of the dry weight of cells after extraction in benzene-ethanol, 1:1, for 48 hr). Examination of the Klason lignin showed no surface fibers (Fig. 12 a), although there were many "wispy" strands of indefinite form (Fig. 12 b). Occasionally, the residues of "collapsed" cell walls were observed from which strands of material were in the process of separating (Fig. 12 c), as had been found previously for Ipomoea unextracted cells (Fig. 3 b). Since the Klason method of isolation is known to alter lignin (Neish, 1965) the absence of fibers is not surprising. Nonetheless, all the Klason lignin absorbed the ruthenium-osmium postfixative strongly. In addition, Björkman lignin fractions were extracted from cells of Phaseolus (13% of dry weight), and the extracted cell residues were examined for the presence of the external fibers. Examination of these residues showed only traces of degraded external fibers on the outside of the walls (Fig. 13). Fiber bundles commonly found at the surface of the cells in the morphological studies were not observed in the samples which were extracted for Björkman lignin.

The substances which were extracted by the aqueous dioxane of the Björkman method were concentrated and examined by two-dimensional paper chromatography. As expected, the extract was grossly heterogeneous but many of the substances absorbed in the ultraviolet (2800 A) and gave a pink color with FeCl₃.

Recently, a modification of the Gomori silver nitrate–hexamine reagent has been used by several investigators of plant cell walls for detection of aldehyde groups and disulfide linkages (Pickett-Heaps, 1967 and 1968; Swift, 1968; Thompson and Colvin, 1970). Essentially, this reagent deposits silver granules wherever an aldehyde or disulfide group exists. The technique was applied to sections of Phaseolus cells, both with and without prior oxidation by periodic acid. When sections were oxidized by periodic acid, both the external fibers and the cell walls gave a strong positive reaction for aldehydes and/or disulfide groups (Figs. 14 a and 14 b). Fibrillar sheets in intercellular spaces (Fig. 14 a) and wall lamellae (Fig. 14 b) were displayed clearly by this procedure. When oxidation of the sections by periodic acid was omitted, the cell walls still reacted strongly for aldehyde and/or disulfide groups but the reaction of the external fibrous material was very much less (Fig. 14 c). These results show that the external fibrous material contains a component which can react weakly with the silver reagent without oxidation by periodic acid but which is capable of being oxidized by periodic acid to give many more aldehyde groups. This conclusion is consistent with the presence in lignin of a few residual aldehyde groups and with the presence of other adjacent hydroxyl groups (or potential adjacent hydroxyl groups) which can be oxidized to free aldehydes by periodic acid (Freudenberg and Neish, 1968).

A more extensive description of cultured plant cell surfaces as shown by the silver nitrate–hexamine reagent will be presented elsewhere (Colvin and Leppard, in press).

DISCUSSION

A fibrillar coating has been observed on the external surface of suspension-cultured cells of Ipomoea, Phaseolus, and Daucus. White (1967) has also observed a fibrillar material on the outside of cultured plant cells (Picea glauca) in the light microscope. While these fibrils absorb heavy metals (and presumably the ruthenium salt used here) strongly and the fibers are most easily visualized when postfixed with the ruthenium-osmium solution, they can be observed with osmium postfixation alone or without heavy metal postfixation, if heavy metal poststaining of sections is employed. Therefore, the evidence for the presence of the material itself or for its fibrillar nature is not an artifact produced by postfixation. The fibrils may be single, loosely clumped, or associated into bundles, ropes, or sheets. The fibrils and/or their various forms of aggregation sometimes give rise to structures in the culture medium and intercellular spaces which are quite complex. In addition, they frequently appear as an extension of what would be the middle lamella in plant tissue.

The composition of these fibrils is indicated to be lignin-like by the following evidence. They contain about 5% protein and little or no neutral reducing sugars or nucleic acid bases. They can be oxidized by alkaline nitrobenzene to give compounds which resemble but are not identical with those from wood lignin. They are completely resistant to the action of pronase (a mixture of proteases), trypsin, pectinase (a mixture of polysaccharidases), and lipase. They are partially extracted by the aqueous dioxane method for Björkman lignin, and substances in the extract absorb in the ultraviolet at 2800 A and react with
Remains of *Phaseolus* cells after extraction of Björkman lignin. Note the traces of fibrillar material on the outside surfaces of cell walls and the absence of fiber bundles.

*Figure 14* *Phaseolus* cell surface structure revealed by the silver nitrate-hexamine reagent. (14 a) Sheetlike fiber bundles in an intercellular space after periodate oxidation. (14 b) Wall banding after periodate oxidation. (14 c) Intercellular space, using the silver reagent as a detector of disulfide linkages.

Note the marked reduction in lignofibrils as compared with Figs. 14 a and 14 b.

The fibrils absorb heavy metal cations strongly, which is consistent with the presence of negatively charged acid groups in lignin. The unoxidized fibrils react weakly with the Gomori silver nitrate-hexamine reagent while oxidized fibrils react strongly, facts consistent with the presence of a few residual aldehyde groups and many potential aldehyde groups in lignin. Finally, there is a physical continuity between the fibrous coating and the site of the potential middle lamella,
long recognized as a major region of lignin deposition (Wardrop, 1965). For the above reasons the fibrils are considered to be mostly, if not wholly, lignin and have been called lignofibrils for convenience. This conclusion is consistent with the previous work of White (1967) and Veliky and LaRue (1967) who inferred from histochemical evidence that lignin was a component of extracellular material.

To our knowledge, this is the first description of a natural, fibrillar, lignin-like substance observed with the electron microscope, although White (1967) reached a similar conclusion from observations with the optical microscope. Hitherto, lignin has been considered an amorphous, nonfibrillar component of plant cell walls, except in the semantic sense where it may fill in the longitudinal spaces between the cellulose microfibrils (Frey-Wyssling, 1964). However, the present evidence plus White’s observations is that there are natural fibrils of a lignin-like compound in cultured plant cells, and this fact poses implicitly the question of whether or not these lignofibrils are a special product of plant cells in culture or whether they occur in normal plant tissues. Only more work will answer this question.

Even if natural lignofibrils are restricted to cultured cells, their existence poses the interesting and difficult problem of the mechanism of their initiation and growth. Some photographs suggest that fully formed lignofibrils may be transported across the cell wall, as fibrils of similar appearance are sometimes found lining the inner surface of the cell wall (see Fig. 11) and fibrous material is sometimes found in the wall, oriented perpendicular to the cell surface (see Figs. 9 b and 9 c). However, this evidence is not conclusive.

The use of the ruthenium-osmium fixative for the present investigation was based on recent work of Luft (1966). For the cell-surface coat described here, it appears that the principal virtue of this fixative is to increase the electron opacity rather than to improve preservation. This increase in electron opacity probably is related to the stereochemical aspects of binding of ruthenium red which have been discussed recently by Sterling (1970). Some aspects of the lignofibril coat relate it to the glyocalyx (Bennett, 1969) although it occurs at a considerable distance from the cell membrane and its association with the polysaccharides and proteins of the cell wall has not yet been defined. Other fibrous structures have been observed at cell wall surfaces, and they may be worthy of future investigation. One of these is the “presumed cellulose” fibers projecting from the surface of cultured carrot cells as described by Halperin and Jensen (1967).

Albersheim et al. (1969) have recently placed great emphasis on the role of the cell wall as a barrier between plant cell and pathogen. If the cell coat described here represents a wall surface structure which is normally found in a highly compressed and seemingly amorphous state in intact plant tissue, then suspension-cultured cells afford a unique opportunity for studying pathogen penetration of this barrier. Goodman et al. (1967) stress the interaction of plant and pathogen with regard to intercellular spaces. The structured, intercellular material present in clumps of suspension-cultured cells provides a potentially interesting substance for study of aspects of host-parasite relations. It may help to stimulate the development of microscope techniques which place more emphasis on the cell wall matrix, as opposed to the present technology which maximizes the contrast between microfibril and matrix by deliberately degrading the matrix image.

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REFERENCES

Albersheim, P., T. M. Jones, and P. D. English. 1969. Biochemistry of the cell-wall in relation to infective processes. Ann. Rev. Phytopathol. 7:171.

Aspinall, G. O., J. A. Molloy, and J. W. T. Craig. 1969. Extracellular polysaccharides from suspension-cultured sycamore cells. Can. J. Biochem. 47:1063.

Beer, M., and G. Setterfield. 1958. Fine structure in thickened primary walls of collenchyma cells of celery petioles. Amer. J. Bot. 45:571.

Bennett, H. S. 1969. The cell surface: Components and configurations. In Handbook of Molecular Cytology. A. Lima-de-Faria, editor. North Holland Publishing Co., Amsterdam.
Björkman, A. 1956. Studies on finely divided wood. Extraction of lignin with neutral solvents. *Sovn. Papierw. 59*(13, Pt. 1):477.

Braun, A. C., and J. Lipetz. 1966. The use of tissue culture in phytopathology. *In Cells and Tissues in Culture. E. N. Willmer, editor. Academic Press Inc., London.* 3.

Brauns, F. E. 1952. The Chemistry of Lignin. *Academic Press Inc., New York.*

Cocking, E. C. 1970. Virus uptake, cell-wall regeneration, and virus multiplication in isolated plant protoplasts. *Int. Rev. Cytol. 28*:89.

Colvin, J. R., and G. G. LePard. 1971. The non-uniform distribution of protein in plant cell-walls. *J. Microsc. (Paris).* In press.

Curts, A. S. G. 1967. The Cell Surface: Its Molecular Role in Morphogenesis. *Academic Press Inc., New York.*

Freudentberg, K., and A. C. Neish. 1968. Constitution and biosynthesis of lignin. Molecular Biology, Biochemistry and Biophysics. A. Kleinzeller, G. F. Springer, and H. G. Wittmann, editors. Springer-Verlag, New York Inc., New York. 2.

Frey-Wyssling, A. 1964. Ultraviolet and fluorescence optics of lignified cell walls. In *The Formation of Wood in Forest Trees.* M. H. Zimmermann, editor. *Academic Press Inc., New York.*

Gamborg, O. L. 1966. Aromatic metabolism in plants. 2. Enzymes of the shikimate pathway in suspension cultures of plant cells. *Can. J. Biochem. 44*:791.

Gamborg, O. L. 1967. Aromatic metabolism in plants. 5. The biosynthesis of chlorogenic acid and lignin in potato cell cultures. *Can. J. Biochem. 45*:1451.

Goodman, R. N., Z. Kiraly, and M. Zaitlin. 1967. The Biochemistry and Physiology of Infectious Plant Disease. D. Van Nostrand, Company Inc., Princeton, N.J.

Halperin, W. 1969. Morphogenesis in cell cultures. *Ann. Rev. Plant Physiol. 20*:395.

Halperin, W., and W. A. Jensen. 1967. Ultrastructural changes during growth and embryogenesis in carrot cell cultures. *J. Ultrastruct. Res. 18*:428.

Keller, W. A., B. Harvey, O. L. Gamborg, R. A. Miller, and D. E. Evelhoch. 1970. Plant protoplasts for use in somatic cell hybridization. *Nature (London).* 226:280.

Knox, R. B., and J. Hezlof-Harrabin. 1970. Pollen-wall proteins: localization and enzymic activity. *J. Cell Sci.* 6:1.

Luft, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol. 9*:409.

Luft, J. H. 1966. Fine structure of capillary and endocapillary layer as revealed by ruthenium red. *Fed. Proc. 25*:1773.

Markham, R., and J. D. Smith. 1949. Chromatographic studies of nucleic acids. 1. A technique for the identification and estimation of purine and pyrimidine bases, nucleosides and related substances. *Biochem. J. 45*:294.

Neish, A. C. 1965. Coumarins, phenylpropanes, and lignin. *In Plant Biochemistry. J. Bonner and J. E. Varner, editors. Academic Press Inc., New York.*

Newcomb, E. H. 1963. Cytoplasm-cell wall relationships. *Annu. Rev. Plant Physiol.* 14:43.

Olson, A. C., J. J. Evans, D. P. Frederick, and E. F. Jansen. 1969. Plant suspension culture media macromolecules-peptic substances, protein, and peroxidase. *Plant Physiol.* 44:1594.

Pickett-Heaps, J. D. 1967. Preliminary attempts at ultrastructural polysaccharide localization in root tip cells. *J. Histochem. Cytochem. 15*:442.

Pickett-Heaps, J. D. 1968. Further ultrastructural observations on polysaccharide localization in plant cells. *J. Cell Sci.* 3:55.

Power, J. B., S. E. Cummins, and E. C. Cocking. 1970. Fusion of isolated plant protoplasts. *Nature (London).* 225:1016.

Provine, M. G. 1965. Chemical control of plant cell-wall structure and of cell shape. *Proc. Roy. Soc. London Ser. B.* 161:526.

Rambourg, A., and C. P. Leblond. 1967. Electron microscope observations on the carbohydrate-rich cell coat present at the surface of cells in the rat. *J. Cell Biol.* 32:27.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17:208.

Roelfsema, P. A. 1965. Ultrastructure of the wall in growing cells and its relation to the direction of the growth. *Advat. Bot. Res.* 2:69.

Sabatini, D. D., K. Bensch, and R. J. Barnett. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* 17:19.

Sterling, C. 1970. Crystal-structure of ruthenium red and sterechemistry of its pectic stain. *Amer. J. Bot.* 57:172.

Steward, F. C. 1970. From cultured cells to whole plants: the induction and control of their growth and morphogenesis—The Croonian lecture. 1969. *Proc. Roy. Soc. London Ser. B.* 175:1.

Stone, J. E., and M. J. Blundell. 1951. Rapid micromethod for alkaline nitrobenzene oxidation of lignin and determination of aldehydes. *Anal. Chem.* 23:771.

Swift, J. A. 1968. The electron histochemistry of cystine-containing proteins in thin transverse sections of human hair. *J. Roy. Microsc. Soc.* 88:449.

Thompson, E., and J. R. Colvin. 1970. Electron-cytochemical localization of cystine in plant cell-walls. *J. Microsc.* 91:87.
TREVELYAN, W. E., D. P. PROCTER, and J. S. HARRISON. 1950. Detection of sugars on paper chromatograms. Nature (London). 166:444.

VELIKY, I., and T. A. LARUE. 1967. Changes in soybean root culture induced by Rhizobium japonicum. Naturwissenschaften. 54:96.

VELIKY, I. A., and S. M. MARTIN. 1970. A fermenter for plant cell suspension cultures. Can. J. Microbiol. 16:223.

WARDROP, A. B. 1965. Cellular differentiation in xylem. In Cellular Ultrastructure of Woody Plants. W. A. Cote, Jr., editor. Syracuse University Press. Syracuse. 61.

WATSON, M. L. 1958. Staining of tissue sections for electron microscopy with heavy metals. J. Biophys. Biochem. Cytol. 4:475.

WHITE, P. R. 1967. Some aspects of differentiation in cells of Picea glauca cultivated in vitro. Amer. J. Bot. 54:334.

YAGUCHI, M., and M. B. PERRY. 1970. Ion exchange chromatography of 2-amino-2-deoxy-D-hexoses. Can. J. Biochem. 48:386.