FINE STRUCTURE OF THE MIDDLE LAMELLA OF AGGREGATES OF PLANT CELLS IN SUSPENSION CULTURE

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INTRODUCTION

Up to the present, light microscopy has shown the middle lamella between plant cells as an amorphous, intercellular substance (Esau, 1965). Furthermore, this concept of middle-lamellar structure has been strengthened by the few available micrographs from electron microscopy. The micrographs seem to have been chosen to emphasize cell wall microfibrils rather than to show the components of the surrounding matrix. Hence, an
image of an amorphous middle lamella often appears as an addendum to the major study. This image may be valid for certain cases, but the generalized amorphous aspect is certainly open to question. It was decided to examine the fine structure of the material between adjacent cells of small cell-aggregates in culture because cultured cells have been so useful for studies of morphogenesis (Steward, 1970), and a staining technique has just been developed which greatly facilitates examination of their intercellular material (Leppard et al., 1971). There may be a semantic problem as to whether or not this layer of material should be called a middle lamella, but, since its location, function, and frequently its appearance correspond in all respects to the present loose use of the term (Esau, 1965), "middle lamella" will be employed here. This note reports a study of the middle lamella of aggregates of suspension-cultured cells of Daucus carota, Ipomoea sp., and Phaseolus vulgaris, var. red kidney bean, by electron microscopy. In contrast to previous studies, heavy metals were employed to emphasize the intercellular material rather than cell wall microfibrils.

MATERIALS AND METHODS

The three cell lines were isolated by the technique of Veliky and Martin (1970). Isolated cells were cultured in a "V" fermenter using the basal medium 67-V of Veliky and Martin (1970) which is a modification of the PRL-4-C medium of Gamborg (1966). The Daucus cell study, which constitutes the major portion of the work, was done with cells from a freshly inoculated culture.

Cells and cell clumps taken directly from culture were fixed in 6% glutaraldehyde at pH 7 in 0.01 M phosphate buffer containing 0.4 M sucrose for 1 hr at room temperature (Sabatini et al., 1963). They were then washed in buffered sucrose solution, washed in buffer solution, and finally postfixed for 3.5 hr at 0°C. Postfixation was done either with 1% osmium tetroxide in 0.05 M phosphate buffer at pH 6.8 or with a filtered, saturated solution of ruthenium red (British Drug Houses, Ltd., Poole, Dorset, England; Ru₄(OH)₂Cl₄•7NH₄•3H₂O) in 0.10 M phosphate buffer at pH 6.8 to which was added, immediately before use, an equal volume of unbuffered 2% osmium tetroxide. After postfixation, some samples were washed with cold buffer and dehydrated in a progressive methanol series, then a methanol-propylene oxide series, then three changes in pure propylene oxide with a total dehydration time of 1 day. Other samples were washed in distilled water and dehydrated as above with the following alterations: (a) the 20% methanol step was prolonged from 10 min to 15.5 hr at room temperature, and to this solution was added, before use, the following heavy metal salts in the proportions indicated—1.5% lanthanum nitrate, 1% thallous acetate, and 1% thorium nitrate; (b) the above step was followed by a 10 min wash in 20% methanol before proceeding to 33%; (c) the first and second of the three changes of pure methanol, for 20 min each, contained 0.5% I by weight.

The samples were embedded 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 used for cytochemistry were poststained for 60 min in uranyl acetate (Watson, 1958) followed by lead citrate (Reynolds, 1963) for 30 min. Examinations were made with a Philips EM-200 at 40 kv.

The silver nitrate-hexamine technique of Colvin and Leppard (1971) was used for the localization of disulfide and/or aldehyde groups on pale-gold sections mounted directly on gold grids without a supporting film. The same technique was also used for sections which had been previously oxidized by 1% aqueous periodic acid for 20 min at room temperature. All grids were subsequently rinsed thoroughly.

Abbreviations

W, wall G, granules
F, fibers L, lacuna
V, vesicles M, membrane
R, rods

Unless otherwise indicated, all dimension markers represent 0.5 μ and the material received a post-fixation in ruthenium-osmium.

Figure 1 Cross-section of the middle lamella of Daucus, stained to show highly oriented fibers.

Figure 2 Vesicles and electron-opaque fibers in the middle lamella of Daucus. Note also the vesicles in the space between the wall and the cytoplasmic membrane.

Figure 3 Electron-transparent rods in the Daucus middle lamella which are outlined by the negative stain. This material received the preembedding heavy-atom stain treatment.
for 30 min in distilled water (Rambourg and Leblond, 1967). For this phase of the study, cells which had not been postfixed or otherwise treated with heavy metals were also used for comparative purposes.

RESULTS

Cross-sections of cells from all species showed that the outside surface of the cells had a fibrillar coat where the surface of the cell was in direct contact with the medium (Leppard et al., 1971). Wherever two cell surfaces were in contact in the clump, their fibrillar outer coats merged to form an intercellular layer (see below) which had the same appearance in the light microscope as the middle lamella between ordinary plant cells. This intercellular layer had the same appearance irrespective of whether it seemed to be between two cells pressed together or two cells which may have divided recently. In the electron microscope this middle lamella was not amorphous and often showed considerable structure. Of the three species studied, *Daucus*, which showed the most differentiation, is described. Among the readily classifiable structural forms were electron-opaque fibers (Fig. 1), vesicles (Fig. 2), electron-transparent rods (Fig. 3), myelin-like figures (Fig. 4), electron-opaque granules (Fig. 5), and lacunae or gaps (Fig. 6). Valid quantitative estimates of the frequency of each of these forms could not be made because of morphological variability between cell clumps.

Each of the above forms was investigated in some detail. First, the physical continuity between the layer of electron-opaque fibers on the external surface of the wall next to the culture medium and the layer of electron-opaque fibers between two cells was established repeatedly. The dispersed fibers outside the wall gradually become more and more tightly compressed until they form a dense layer between the cells in which the individual fibers are scarcely visible. This dense layer has the same appearance as a normal middle lamella between cells of ordinary tissues. Occasionally, however, when three cells met at an intercellular space an anomaly was found both by the heavy metal stain and by silver deposition which could not be explained so simply (Fig. 7 a, Fig. 7 b). This anomaly was a succession of layers of wall material and of electron-opaque fibers which cannot be explained simply by compression of two adjacent cells.

Electron-transparent rods or fibers were observed in the sections when they were outlined by stain (Fig. 3, Fig. 8). These rods varied in width from 50 A to 80 A and were always disposed parallel to the cell surface. Nothing is known about their composition.

Rounded vesicles of variable diameter were repeatedly observed in the middle lamellae and the intercellular spaces (Fig. 2). Vesicles were never observed in the cell wall itself. At high magnification, flattened vesicles were resolved to show triple-layered membranes with a thickness of about 90 A and some evidence of a granular substructure (Fig. 9). Plasmodesmata and membrane-bound cytoplasmic protrusions into walls were rarely encountered.

The middle lamella sometimes contained small masses of apparently amorphous, electron-opaque material which, at high magnification, could be resolved into complex forms which included smaller amorphous bodies, membranes, and myelin-like figures. The significance of these masses is not clear but they were more abundant when a necrotic cell was nearby in a clump.

Electron-opaque granules, two to four times smaller than the ribosomes seen in the same section, were observed repeatedly in the middle lamella (Fig. 5). Occasionally, only granules and fibers that approached the resolution limit for sectioned material were found between two adjacent cells (Fig. 2, lower right corner). It is possible that these granules are cross-sections of the electron-opaque fibers referred to earlier.

Finally, gaps in the middle lamella (lacunae) between cells were seen occasionally (Fig. 6). These gaps were often fringed by electron-opaque fibers with a diameter which approached the resolution limit (Fig. 6). At present, one cannot decide whether the gaps are an artifact of preparation (i.e., shrinkage) or existed in the native tissue.

DISCUSSION

Clearly, the middle lamella of some suspension-cultured plant cells is not amorphous but contains many elements of structure. It is possible that some of these elements, such as part of the vesicular material and the myelin-like figures, may have been introduced adventitiously from autolysis of other cells during development of the clump (Fig. 4). It is certain, however, that another part of the vesicular material (Fig. 2 and Fig. 9), the electron-opaque fibers and the electron-transparent rods, are an inherent part of the middle lamella which
FIGURE 4  Myelin-like figures, membranes, and electron-opaque bodies in the middle lamella of *Daucus*. This material received the preembedding heavy-atom stain treatment.
FIGURE 5 A cross-section of a middle lamella of *Phaseolus* which illustrates the granular structure which is sometimes observed. Note the difference in size between ribosomes and granules. Postfixation was done in osmium.

FIGURE 6 Lacunae or gaps, of unexplained origin, in the middle lamella of *Daucus*.
Figure 7a  Cross-section of the middle lamella of *Phaseolus* which shows an example of a typical discontinuity. Note the anomalous alteration of layers of electron-opaque fibers and wall material.

Figure 7b  A discontinuity in *Phaseolus* outlined by silver deposition. Note the general resemblance to Fig. 7a.
FIGURE 8 An example of electron-transparent fibers which are outlined by negative staining. These fibers border a lacuna in the middle lamella of *Daucus*.

FIGURE 9 An example of a flattened vesicle which is occasionally observed in the middle lamella of *Daucus*.

FIGURE 10 A cross-section of two cell walls of *Daucus*, which illustrates how the electron-opaque fibers of the cell coat merge gradually into the middle lamella between cells. This material received the pre-embedding heavy-atom stain treatment.
has not been reported before, except for some vesicles (Halperin and Jensen, 1967).

Before speculating upon several aspects of these structures, it is necessary to stress that, although the heavy metal staining techniques reveal the order more clearly, they do not produce it. The ruthenium-osmium postfixation adapted from Luft (1966) does not "create" the electron-opaque fibers which are faintly discernible with only glutaraldehyde fixation (Leppard et al., 1971). In addition, no evidence was observed for any special preservation of the cell-surface material by the stain or for any rearrangement of the surface by it. The molecular basis for enhancement of contrast of these fibers by ruthenium-osmium is not known but it may be similar (not necessarily identical) to that described by Sterling (1970) for ruthenium red staining of pectin. The use of a preembedment treatment with lanthanum, thallium, thorium, and iodine was based solely on the knowledge that these four heavy atoms are sometimes adsorbed strongly to biological material, thereby increasing contrast. At the present time, so little is known about the specificity of adsorption of these ions that speculation about particular sites of retention is not justified.

Nothing is yet known precisely about the mode of biosynthesis or the composition of the electron-opaque fibers although all present information is consistent with the assumption that they are lignin-like (Leppard et al., 1971). Further work is necessary. Even less is known about the composition of the electron-transparent rods. The rods may be a truly amorphous substance which is compressed by the electron-opaque fibers into a rod-like shape, receiving its apparent linear form from spatial restrictions (Frey-Wyssling, 1964).

The unexpected complexity of the middle lamella of suspension-cultured cells suggests several problems with respect to more usual plant tissues. It is quite possible that the structures observed in the middle lamella of cultured cells are the result of a response to abnormal conditions and have no counterpart in ordinary plant tissues. On the other hand, it is equally possible (indeed probable) that the structures reported here may exist in the middle lamellae of ordinary plant cells but are badly obscured. In cultured cells, the middle lamella is less compressed than in most plant tissues and therefore details may be observed more easily. If the cells were strongly compressed as they are in normal tissues, the fibers and/or granules might be unobservable. The effect of compression is illustrated by Fig. 10.

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REFERENCES

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

Eisau, K. 1965. Plant Anatomy. John Wiley and Sons Inc., New York. 2nd edition.

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. 153.

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

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

Leppard, G. G., J. R. Colvin, D. Rose, and S. M. Martin. 1971. Lignofibrils on the external cell wall surface of cultured plant cells. J. Cell Biol. 50:63.

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 endodermal layer as revealed by ruthenium red. Fed. Proc. 25:1773.

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.

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 stereochemistry 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. Ser. B.* 175:1.

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

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