

# An Electron Microscopic Investigation into the Effect of EDTA on Plant Cell Wall\*

By SHIMON KLEIN, Ph.D., and B. GINZBURG, Ph.D.

(From the Department of Botany, The Hebrew University, Jerusalem)

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## ABSTRACT

To study the effect of EDTA on cell wall structure and the reversal of this effect by uranyl ion, thin sections of pea root tips were examined in the electron microscope. EDTA is known to facilitate separation of the cells in root tips. When sections of fixed and embedded EDTA-treated roots are floated on a uranyl-acetate solution, a loose network is revealed that would seem to be cellulose. Incorporation of uranyl into the roots, if it occurs prior to fixation, brings about recementation of the cells. After such treatment, a marginal darker area and a median brighter one can be observed in the wall, and the whole structure appears more compact again. Comparison of the results of the various treatments suggests that cellulose-cementing material is dispersed throughout the entire wall, and that its distribution parallels that of cellulose.

## INTRODUCTION

According to generally accepted concepts, the young cell wall contains pectins, gums, waxes, and proteins, in addition to cellulose. In structural research, especially in electron microscopy, emphasis has been placed on the arrangement of cellulose strands which have for the most part been investigated after the elimination of the other cell wall components. The investigation of the role and location of non-cellulose wall material may well be important since, by virtue of their ability to cement cells together, to mention one function, these substances determine to a large extent the properties of the cell wall (1). Non-cellulose wall materials are also widely held to play an important part in the regulation of cell growth.

In his studies of the cementing property of the cell wall, Ginzburg presented evidence (2, 3) for the existence of a protein-gel structure, cross-linked by metal cations, that acts as the cementing substance between cells. This evidence was based on the modification by various treatments of the action of chelates on cell separation.

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Ginzburg suggested that some of the cationic linkages involved in cementation of cells consist of heavy metal ions in addition to the  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$  ions known to be present (4). EDTA (ethylenediaminetetracetic acid), by chelating these metals, causes weakening of the cementing material and allows easy separation of the cells. By allowing EDTA-treated roots to take up metal ion, the EDTA effect can be reversed; *i. e.*, the cells are again bound together.

The aim of the present work was to study the location of the "cell-cementing" substance in cell wall. To this end, the changes in cell wall structure effected by the chelating agent EDTA were observed in the electron microscope. Since the action of EDTA can be reversed by treatment with metal ions, the sites of the "recementing" material can be investigated by utilizing the "staining" properties of such ions.

In the present work uranium was used to reverse EDTA effects. Ions of this heavy metal possess the same ability to recement cells as that previously demonstrated (3) for Ca, Mg, Cu, Fe, Mn, and Zn. The uranyl ion in uranium acetate also has been shown to be a good "electron stain" (5). In order to make use solely of the contrast-increasing properties of uranium, thin sections of EDTA-treated roots, after they were fixed and

embedded, as described by Watson (5), were floated on concentrated uranyl-acetate solution, in which case the metal's ability to recement cells, and hence to introduce structural changes, was precluded.

The results of these experiments indicate that the structure of cell wall becomes loosened after EDTA treatment, and that the distribution of "cell-cementing" substance(s) throughout the whole wall is similar to that of cellulose.

The term "cell wall" is used here to indicate the entire structure between two adjacent protoplasts. This region is therefore identical with that called "membrane pectocellulosique" by Buvat (6), or "wall" by Lund *et al.* (7), etc.

#### Methods and Materials

Alaska pea seeds were set in Petri dishes and germinated in the dark at 25°C. Roots of 2-day old seedlings were cut approximately 2 mm. above the tips. The tips were then divided into groups for various treatments; all of them were fixed for 60 to 90 minutes in the cold, in a veronal-buffered OsO<sub>4</sub> solution of pH 7.4 (8). The treatments applied were:

1. Root tips were fixed immediately, without additional treatment ("untreated roots").
2. Prior to fixation, the root tips were immersed in a 0.1 M EDTA solution at pH 8.0 at 30°C. for 24 hours ("EDTA-treated roots").
3. Same as 2. above, but followed by immersion in a saturated uranyl-acetate solution for 1 hour and rinsed before fixation ("EDTA-uranyl-treated roots").

The fixed tissues were dehydrated in graded alcohols in the cold and embedded in a butyl-methyl methacrylate mixture 1:4. Thin sections were cut with glass knives on a Porter-Blum microtome and examined in an RCA EMU 3C microscope at 100 kv. under low-beam intensity. After the first examination, the sections were floated on a concentrated uranyl-acetate solution for 2 minutes, dried, and then examined again in the microscope.

#### RESULTS

*Untreated Roots.*—In untreated roots, contrast in the cell walls is low and does not reveal details of wall structure (Fig. 1). In general, the outer edges of the wall appear denser than the adjacent inner areas, while sometimes a diffuse darker line appears in the middle of the wall. Contrast in the wall can be increased by using very fresh fixative.

*Untreated Roots, Sections Stained with Uranyl.*—Figs. 2 and 3 show that uranyl accumulates primarily in the cell wall area which now, in contrast to cell walls in unstained sections, appears much

darker than the protoplasm. Although staining was sometimes so strong that the cell walls appeared completely black, staining density varied considerably even in different areas of the same grid. In less densely stained sections, the uranyl seems to be spread in the form of a more or less uninterrupted network over the whole cell wall area, occasionally with ordered lines appearing on the margins of the wall. The uranyl is evidently only loosely bound by the cell wall after this short treatment, since it can be washed out easily by merely allowing the stained grid to float upside-down on a water surface. (For this reason, electron photomicrographs here of sections floated on uranyl solution show unwashed or very quickly rinsed grids.)

*EDTA-Treated Roots.*—Again, in these EDTA-treated roots, which were fixed in OsO<sub>4</sub> without additional "electron staining," contrast in the cell wall is very low (Fig. 4). Occasionally, however, very fine, mostly longitudinal lines can be seen. In pictures of lower magnification, which show a larger number of cells, the walls are seen to be of a looser structure than in untreated roots. As has been noted before, cells of such treated roots can be separated easily by slight pressure.

*EDTA-Treated Roots, Sections Stained with Uranyl.*—Uranyl dramatically increases the contrast in the cell wall and so accentuates the effects of EDTA on wall structure (Figs. 5, 6). In place of the more or less evenly dense network visible in untreated sections (Figs. 3, 4), what appear are accretions of strands forming a loose network. In most of the sections examined, the strands fill the cell wall area evenly. Sometimes however, a higher concentration of parallel strands occurs at the margins of the cells, while the center is occupied by what appears to be a network. The pictures recall the electron micrographs of Setterfield and Bailey (9), which show the configuration of cellulose after other wall components have been removed. Presumably, uranyl reveals the configuration of cellulose because the uranyl is bound (albeit loosely) by it.

*EDTA-Uranyl-Treated Roots.*—While the cells of roots treated with EDTA could easily be separated from each other by light tapping, this was no longer possible if the roots were subsequently, but before fixing and embedding, submerged for a reasonable time in uranyl solution. Evidently a recementation of the cells had occurred. Sections of the roots treated in this way differ in appearance in the electron micrograph from sections of un-

treated roots and from those treated with EDTA alone. Most frequently, two distinct layers can be distinguished in the wall—a dense outer one, and a less dense central part. No strands can be found, nor any network such as that seen in EDTA-treated roots. Since the background is identical in both the marginal and central areas, the darker appearance of the marginal areas may be attributed to a granular inclusion (Fig. 7).

Occasionally, additional structures with very high electron-scattering properties could be seen in part of the light median area. These dense regions seem most frequently to be connected to the intercellular triangular spaces between the cells and to extend for various distances between the cells, sometimes even to the extent of connecting two intercellular triangular spaces.

In many cases, intercellular spaces appear empty. Sometimes the triangular spaces between cells are filled with strongly electron-scattering material usually forming irregular networks. However, due to the alterations of protoplasmic material after the treatments, it is impossible to determine with accuracy whether these triangular spaces are actually intercellular or whether they represent cytoplasmic extensions. Since this report deals exclusively with cell wall, these structures will not be discussed.

#### DISCUSSION

Light microscopic and chemical investigations show the cambial cell walls to be composed largely of cellulose and polyuronides (10). Other microscopic work on very young meristematic cell walls indicating the presence of cellulose-frame work embedded in non-cellulose material, has been summarized by Frey-Wyssling (11) and more recently by Northcote (12). In most electron microscopic investigations on thin sections however, in order to show the configuration of cellulose fibers, it was necessary to remove the intercellulose substance together with the embedding material, as in the studies by Setterfield and Bailey (9). Asunmaa (13), using thallium ethylate as an "electron stain" for delignified wood, employed methods that probably removed intercellular material, and investigated embedded sections.

As Setterfield and Bailey point out (9), the removal of intercellulose material causes a noticeable loosening of the cell wall, giving it an exaggerated thickness. Since the aim of the present work was specifically to study the effect of EDTA on the loosening of cell wall structure, it was not

possible to use this method, and it was thought preferable to use intact thin sections, despite the anticipated lack of contrast in the cell wall. Even floating the sections of untreated roots on uranyl solution, despite the resulting dense coloration of the wall, did not reveal sufficient detail of internal cell wall structure. Since strands and a more or less irregular network appeared only after EDTA treatment, it would seem that some specific activity of EDTA produced this appearance. Furthermore, since EDTA is known for its high chelating power, it can be assumed that this activity consists in the removal of metal cations from the cell wall, thus loosening the entire structure. The lines that appear after floating sections of EDTA-treated roots on uranyl solution, as noted before resemble the arrangement of cellulose fibers in loosened cell walls shown by Setterfield and Bailey (9). Although there is no direct evidence by which to identify the lines observed in the present study as cellulose fibers, one may assume them to be one and the same and not to represent intercellulose material; especially since uranyl also "stains" thin sections of pure cellulose (Whitman filter paper No. 1) Floating the grids on uranyl solution increased the contrast in thin sections of such material in approximately the same degree as it did in root material.

It is unlikely that EDTA would act directly on cellulose, since cellulose does not bind metals tightly. It is far more probable that EDTA loosens the cellulose network by acting on the intercellulose material. The fact that chelation of metals by EDTA not only loosens the cellulose strands (Figs. 5, 6), but also results in ready separability of cells, suggests that the cellulose network may be bound by the same cement that binds cells. This material seems to exist throughout the entire thickness of the cell wall, since photomicrographs do not indicate any specific localization of "cell-cementing" substance.

The disappearance of clearly distinguishable "cellulose strands" after EDTA treatment plus recementation with uranyl ion shows once more that the loosening of cell wall structure is due to removal of metal ions. (Even after floating such treated sections on uranyl solution, strands could not be seen.) Furthermore, the appearance of the sections of recemented cells allows a conclusion as to the sites of recementing by metal ions. The fact that darker and brighter areas appear only after recementation by metal ions and the fact that metals tend to be strong electron-scatterers,

make it plausible that the sites of the metal incorporation are in fact indicated by the darker areas. In contrast to uranyl staining by floating, in which case binding is weak (as loss of staining after very brief rinsing implies), uranyl incorporated into the root before fixation remains there despite the lengthy fixing, washing, and dehydration schedule. The evident stronger binding forces operating in the latter instance could be taken as indicative of site specificity.

According to Setterfield and Bailey (9), cellulose fibers are most densely packed in the marginal part of the cell wall, although they may be present throughout the whole thickness of the wall. It is of interest to note that uranyl incorporation occurs mainly in these marginal areas. Thus, the material that is able to bind metal ions tightly has the same distribution in the wall as cellulose. This material may consist of pectins and/or proteins, both of which substances would be able to bind metal strongly. This intercellulose material may be an important site of action of 3-indole-acetic acid, which is known to possess chelating properties (14, 15). Many changes or transformations occurring in the cell wall during growth and development may possibly be connected with the functions of these intercellulose substances, not the least important of which would be their ability to strengthen or loosen cell wall structure.

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#### EXPLANATION OF PLATES

The micrographs show transverse sections through cell walls in the roots of etiolated 10-day old pea seedlings. Thin sections were taken 1 mm. above the root tips and micrographed without removing the embedding medium.

CW = Cell wall.

I = intercellular space.

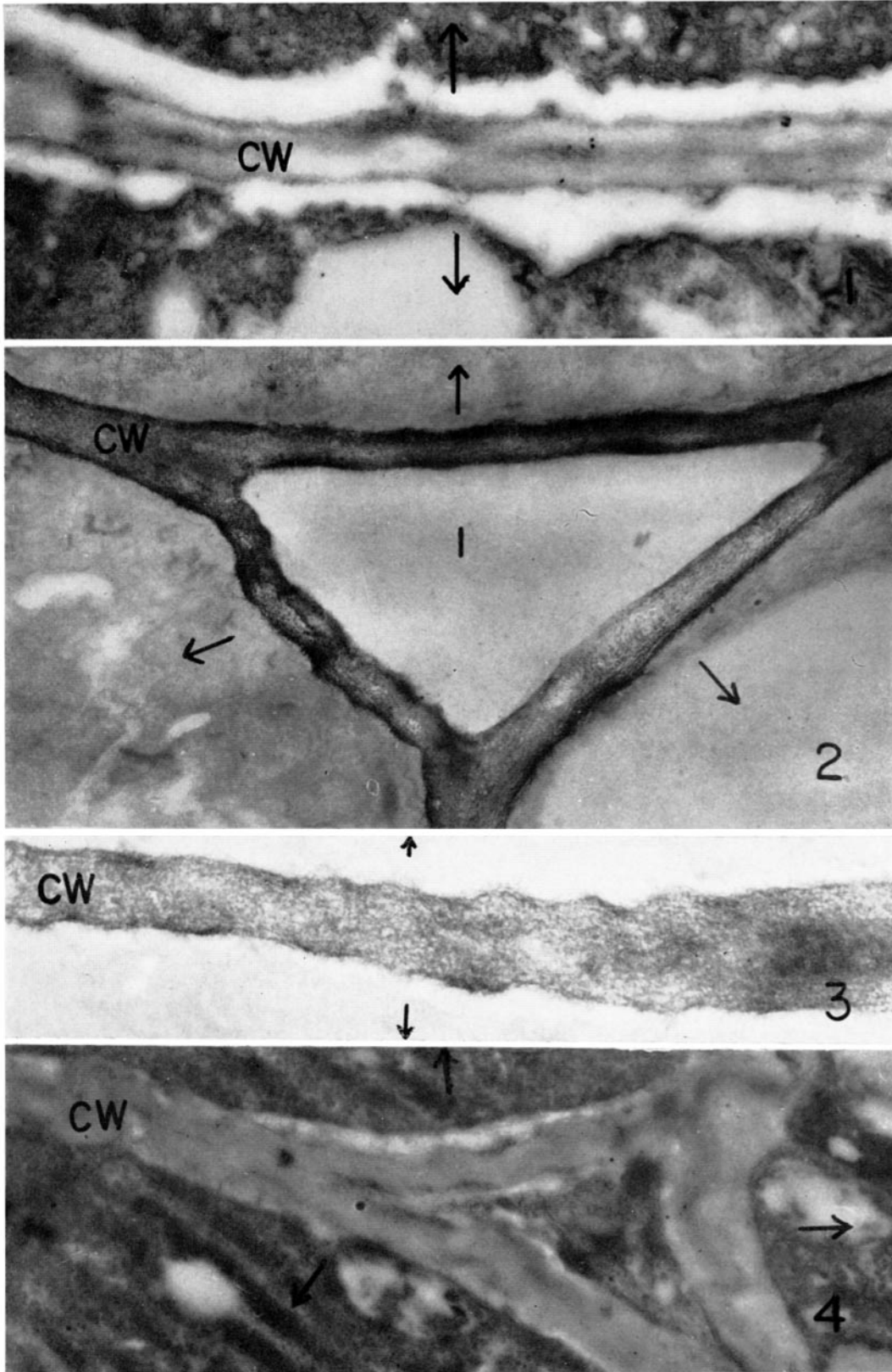
The arrows point towards the center of each cell.

#### PLATE 169

FIG. 1. Micrographs of OsO<sub>4</sub>-fixed specimens, which were sectioned without additional treatment. × 53,000.

FIGS. 2 and 3. Micrographs of OsO<sub>4</sub>-fixed specimens after the sections had been floated on an uranyl-acetate solution. Contrast between wall and plasma is strongly increased. Fig. 2. × 34,000; Fig. 3. × 73,000.

FIG. 4. Cell walls in EDTA-treated roots. The roots were fixed in OsO<sub>4</sub>, embedded, sectioned, and micrographed without additional treatment. × 34,000.

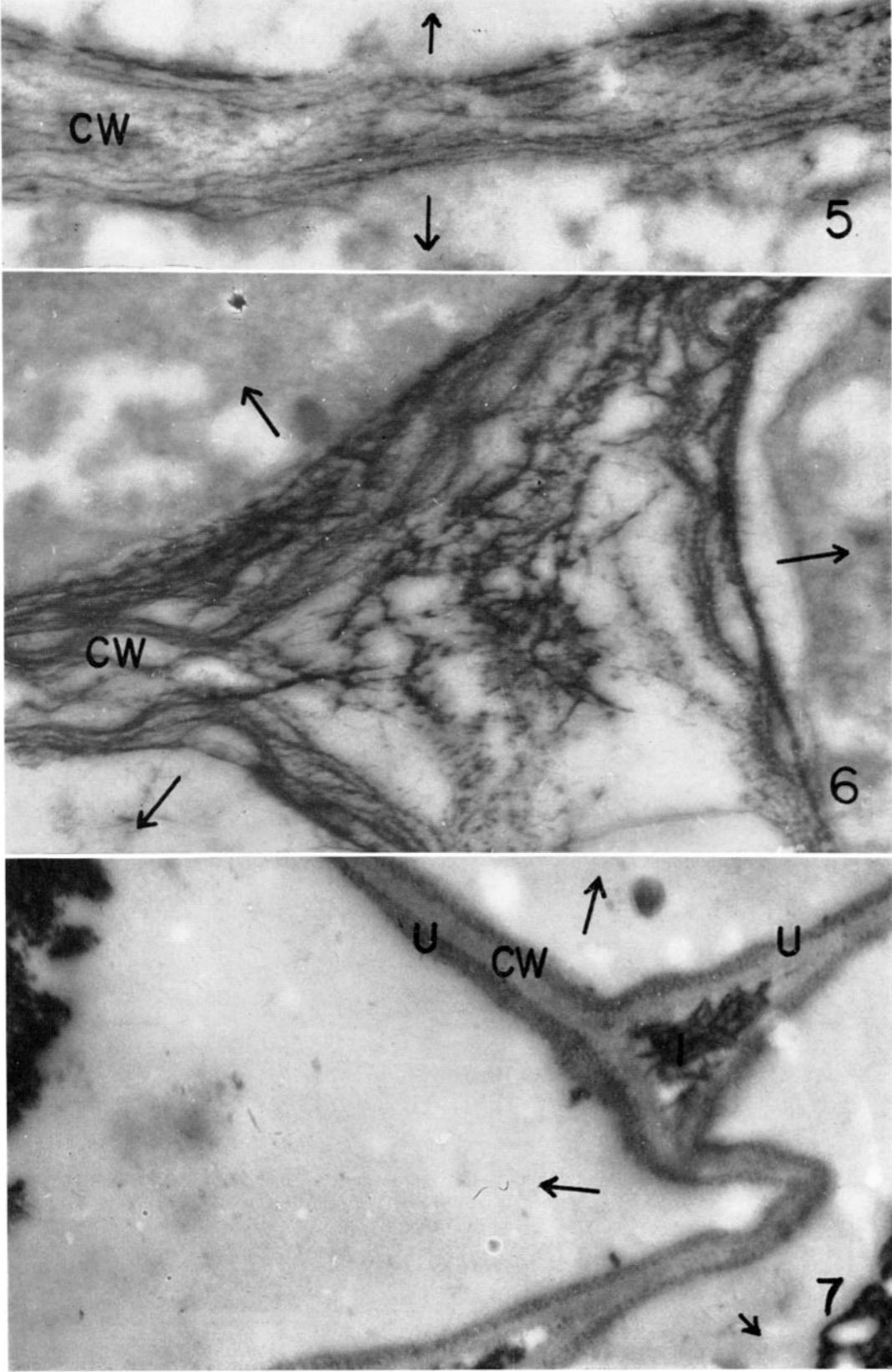


(Klein and Ginzburg: Effect of EDTA on plant cell wall)

PLATE 170

FIGS. 5 and 6. Cell walls in EDTA-treated roots. Embedded sections of specimens fixed in  $\text{OsO}_4$  were floated on a uranyl-acetate solution. The effect of EDTA in loosening the cellulose strands is visible, owing to the electron-stain. Fig. 5.  $\times 47,000$ ; Fig. 6.  $\times 39,000$ .

FIG. 7. Cell walls in roots which were treated with EDTA and immersed in a uranyl-acetate solution prior to  $\text{OsO}_4$  fixation. Uranyl ions cause a recementation of both cells and cell walls. Single strands are no longer visible in the sections. The uranyl is probably located principally in the darker area (*U*).  $\times 28,000$ .



(Klein and Ginzburg: Effect of EDTA on plant cell wall)