

COAGULANT AND NON-COAGULANT FIXATION OF PLANT CELLS

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Abstract

The appearance of cells from the meristem of the root tip of *Phaseolus vulgaris* after fixation in a variety of coagulant and non-coagulant fixatives is described and illustrated by correlated light and electron microscopy. The action of these same fixatives and some of their components upon the living cells of the petiolar hairs of *Heracleum mantegazzianum* is then described. Glutaraldehyde emerged as an excellent fixative for general use from these studies and further tests show that it will stabilize Hecht's threads in plasmolysed onion epidermis against breakage during dehydration. However, the formation of rounded cytoplasmic vacuoles from a pre-existing set of canalicular vacuoles and transformations of the cell membrane and tonoplasts were noted during these studies and none of the fixatives tested prevent the formation of these artefacts.

I. INTRODUCTION

Tissues fixed in glutaraldehyde-osmium tetroxide and embedded in epoxy resins have become the mainstay of investigations into the fine structure of plant tissues. Although these procedures are used increasingly for light microscopy it is still common to find papers in which coagulant fixatives (formalin-acetic acid-alcohol; chromic acid-acetic acid-alcohol; Bouin's) and paraffin wax techniques have been used to prepare specimens for light microscopy while "parallel" investigations have been carried out using glutaraldehyde-fixed epoxy-embedded sections for electron microscopy.

In recent years many authors have stressed the value of correlating light and electron microscopy using plant tissues fixed in non-coagulant fixatives and embedded in plastics. A method was described in 1968 (Feder and O'Brien) which enabled biologists to obtain very much improved images from non-coagulant fixatives for light microscopy. However, there appears to be no comparative study by modern methods of the fine structure of plant tissues fixed in coagulant or non-coagulant fixatives. There were some early attempts to examine the structure of coagulant-fixed plant cells embedded in polymethacrylate but these yielded totally unsatisfactory images that cannot be evaluated (Rozsa and Wyckoff 1951).

The absence of such a comparative study is no doubt due in part to the fact that the complete assemblage of useful technology which we now take so much for

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granted is relatively recent. Important examples are lead citrate staining of sections (Reynolds 1963), low viscosity epoxy resins (Spurr 1969) which are particularly useful for infiltration of such dense objects as cell walls, and the modern ultramicrotomes and diamond knives that have made sectioning of otherwise impossibly difficult plant cells almost a matter of routine (see, for example, Zee and O'Brien 1970).

In this paper we have set out to do three things. Firstly, we have prepared correlated light and electron microscopic images of bean root-tip cells fixed in a variety of coagulant and non-coagulant fixatives and embedded by a standard modern procedure in Spurr's (1969) resin. Secondly, to try to understand the basis of the destruction wrought by the coagulants, their effects (and those of some of their components) were compared with non-coagulants upon living cells of the petiolar hairs of *Heracleum mantegazzianum*. Glutaraldehyde (or glutaraldehyde-OsO₄) emerged from both of these studies as the fixative of choice.

Thirdly, in order to test just how good a fixative glutaraldehyde really is we have used it to fix plasmolysed onion epidermal cells and shown by phase-contrast light microscopy that it will preserve Hecht's threads against dehydration in methyl cellosolve. However, in the course of the studies of onion epidermis and *H. mantegazzianum* hairs, we have noted transformations which appear to be responses to injury on both the cell membrane and vacuolar membrane, transformations that are *not* prevented by glutaraldehyde.

II. MATERIALS AND METHODS

(a) *Light and Electron Microscopy of Root-tip Cells*

Lateral roots 2–3 mm long of young bean seedlings (*Phaseolus vulgaris* L.) were fixed according to one of the following schedules:

- (1) 1.5% potassium dichromate in distilled water at room temperature for 16 hr.
- (2) 2% OsO₄ in 0.025M phosphate buffer, pH 6.8, at room temperature for 16 hr.
- (3) 3% glutaraldehyde (diluted from 50% Fisher Biological Grade) in 0.025M phosphate buffer, pH 6.8, room temperature, for 16 hr.
- (4) As for (3), followed by five rinses in buffer and 1% OsO₄ for 1 hr at room temperature.
- (5) As for (3), followed by five rinses in buffer and 1.5% potassium dichromate for 1 hr at room temperature.
- (6) 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.025M phosphate buffer, pH 7.2, for 16 hr at room temperature.
- (7) 10% acrolein (redistilled before dilution) in 0.025M phosphate buffer, pH 6.8, for 16 hr at 0°C.
- (8) Formalin-acetic acid-alcohol (FAA), 16 hr at room temperature (Sass 1958).
- (9) Chromic acid-acetic acid-alcohol (CRAF III), 16 hr at room temperature (see Sass 1958).
- (10) Boiling water, 5 min
- (11) Cold water, brought to the boil with the tissue immersed.
- (12) Acetic acid-alcohol (1 : 3; Clarke's fluid), 16 hr at room temperature.

Treatments (1)–(7) and (9), (10), and (11) were rinsed in two changes of 0.025M phosphate buffer at 0°C and dehydrated by two changes of methyl cellosolve before transfer to ethanol. Treatments (8) and (12) were dehydrated gradually with ethanol, commencing at 50%. All specimens were embedded in low-viscosity epoxy resin (Spurr 1969). Resin concentration was increased very

slowly over 2 days to minimize damage. A zone in the meristem about 0.5 mm from the root tip was selected for study. Longitudinal sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and photographed at $\times 21,000$ in the electron microscope. Because the different treatments gave very variable staining results with cationic dyes, sections were cut 0.25 μm thick, dried from water onto glass slides, and treated with ammoniacal silver solution (Goldblatt and Trump 1965) and photographed under phase-contrast. This procedure gave maximum contrast to these thin sections and each of the electron micrographs carries as an inset a light micrograph prepared in this way. Since the image produced by treatment (5) was not recognizably different from that produced by (3), nor (10) from (11), no illustrations are given for treatments (5) or (11).

(b) Action of Fixatives on Hair Cells

Petiolar hairs of *H. mantegazzianum* consist of a multicellular base of coloured epidermal cells from which protrudes a single, colourless uninucleate cell that may be 5 mm long and 0.4 mm wide. Hairs were removed by cutting through the base, maintaining the giant cell intact. Cells were mounted in water on a slide and the coverslip raised slightly above the specimen by supporting it on one or two thicknesses of coverslip fragments. Fixatives were then drawn in beneath the coverslip and changes in structure monitored by phase-contrast microscopy with Zeiss optics, photographed on Tri-X roll film developed for 800 ASA. Alternatively, hairs were mounted directly in fixative and the edges of the coverslip sealed with nail polish. This operation can be completed in 30 s and the time lost is unimportant.

The fixatives tested included 2% acetic acid, 50% ethanol, Clarke's fluid, FAA, CRAF III, 4% formaldehyde, 3 or 6% glutaraldehyde, 10% acrolein, 3% glutaraldehyde plus 10% acrolein, and 2% OsO_4 in water. The aldehydes were prepared as in Section II(a).

(c) Fixation of Hecht's Threads

Epidermal strips 5 by 5 mm were prepared from the inner epidermis of the bulb scales of white onions, and a small piece trimmed from one corner of the strip to allow later orientation. The strips were plasmolysed in 0.8M sucrose or 0.6M KNO_3 and selected cells that showed well-developed Hecht's threads and continued to stream were photographed by phase-contrast. The strips were then fixed by floating them cuticle side up on 5% glutaraldehyde prepared as in Section II(a) but containing in addition the same concentration of plasmolyticum. The same cells were relocated and rephotographed. Strips were then mounted in 2% OsO_4 in plasmolyticum for 1 hr to overnight with or without prior rinses in plasmolyticum, and rephotographed. Finally, the plasmolyticum was replaced with water in 0.1M steps, the strips dehydrated in ice-cold methyl cellosolve for 1 hr to overnight, and rephotographed.

III. OBSERVATIONS AND DISCUSSION

(a) Light and Electron Microscopy of Root Tip Cells

(i) *Potassium Dichromate* (Fig. 1).—Though the light microscope image (LM) suggests that this might be a reasonably good fixative, the electron microscope image (EM) reveals that it is in fact very destructive. Both the nucleoplasm and cytoplasm appear to be badly extracted, and the nuclear envelope, ER, and dictyosomes are unrecognizable. The vacuoles of the LM appear only as areas of extraction with undefined margins in the EM. Organelles that are probably plastids and mitochondria are present but cannot be distinguished from one another. Ribosomes appear to be destroyed, both in the cytoplasm and in the nucleolus.

(ii) *Osmium Tetroxide* (Fig. 2).—Shrinkage is evident both as undulations of the cell wall and distortion of the nucleus. Mitochondria, plastids, ER, dictyosomes, cytoplasmic and nucleolar ribosomes are all evident. Chromatin appears to be very homogeneous both in the LM and EM.

(iii) *Glutaraldehyde* (Fig. 3).—General preservation is excellent though undulation of some walls suggests there has been some shrinkage. Though membranes show no contrast, plastids, mitochondria, ER, nuclear envelope, and nucleolus are all well-defined. Vacuoles appear to be well preserved, and retain some contents.

(iv) *Glutaraldehyde-OsO₄* (Fig. 4).—Differences between this image and that shown in (iii) appear to be due chiefly to the positive membrane contrast. In the LM, the presence of the OsO₄ in the tissue enhances the reactivity of cytoplasmic organelles towards the ammoniacal silver stain, and thereby improves optical contrast.

(v) *Formaldehyde* (Fig. 5).—Cell wall undulations suggest some shrinkage, and mitochondria may be somewhat swollen, though it would require a statistical analysis of a large number of micrographs to establish this with any certainty. The general level of preservation appears to be excellent. The appearance of the vacuolar contents is unique to this fixative.

(vi) *Acrolein* (Fig. 6).—This image does not differ significantly from that produced by glutaraldehyde (cf. Figs. 3 and 4).

(vii) *FAA* (Fig. 7).—The familiar clarity and sharpness of this image in the light microscope is due largely to the destructiveness of the fixative. Most of the nucleoplasm and cytoplasm has been destroyed and the remainder coagulated into a coarse network. The clarity of the nuclear envelope in the LM is due to precipitation of material upon it from the nucleoplasm and the cytoplasm. The space around the nucleolus is clearly an artefact.

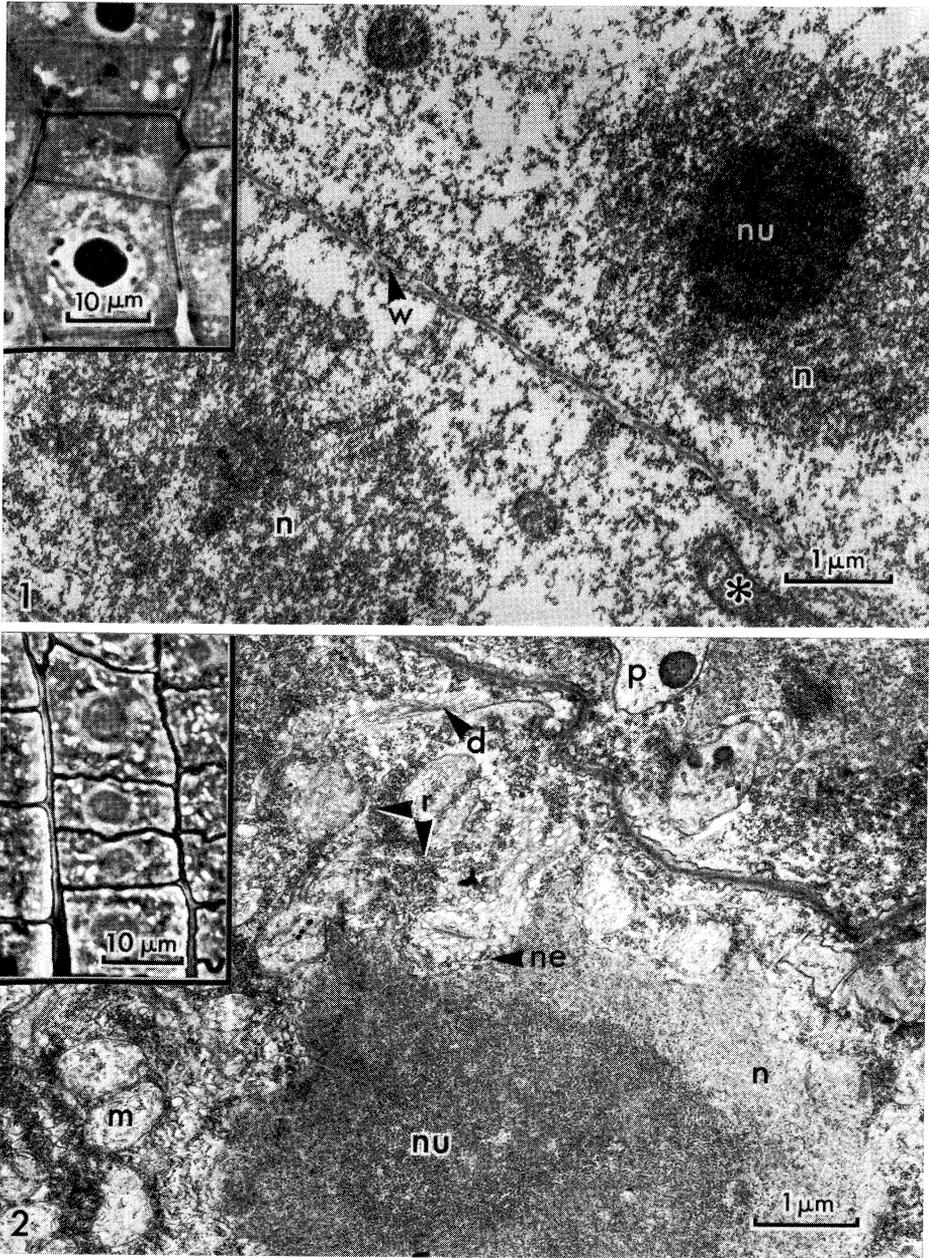
(viii) *CRAF III* (Fig. 8).—This image does not differ significantly from that produced by (vii). The coagulum is somewhat more diffuse and the wall less shrunken, and a remnant of what might have been a plastid or a mitochondrion is retained.

(ix) *Boiling Water* (Fig. 9).—This drastic treatment appears to dissolve chromatin aggregates (and chromosomes) but preserves the rest of the nucleoplasm better than FAA or CRAF III. No cytoplasmic organelles can be recognized.

(x) *Clarke's Fluid* (Fig. 10).—This fixative strongly coagulates chromatin, a fact which probably explains its popularity as a fixative for nuclei. What may be remnants of mitochondria or plastids can also be detected. Curiously, this fixative appears to do slightly less damage than FAA or CRAF III.

These observations largely speak for themselves. It is clear that Baker's (1958) term "coagulant" is ideally suited to describe the effects of the traditional botanical fixatives (FAA, CRAF), since these fixatives convert the fine structure of the cell into a stringy precipitate that is somewhat coarser than that produced by boiling. The action of the coagulants upon nuclear chromatin in the interphase nuclei illustrated here produces massive alteration in structure. Such fixatives are still the most widely used in the field of chromosome cytology and one cannot help but wonder what the relationship may be between *in vivo* chromosome structure and that which is studied by most cytogeneticists. Since nuclear and cytoplasmic fine structure is so drastically altered by these fixatives, a strong case must surely be made for attempting to phase out their use wherever possible from future studies of plant-cell structure.

Of the non-coagulants studied here, dichromate shows no promise at all. The LM image produced by this fixative offered some promise and it is clear that one may not safely deduce the quality of structural preservation by examining tissues solely by light microscopy. OsO₄ is among the most widely studied of all fixatives and the



Figs. 1–10 are electron micrographs of cells from the lateral roots of *Phaseolus vulgaris*, fixed as set out below, embedded in Spurr's resin and stained with uranyl acetate and lead citrate. The inset in each figure is a light micrograph of a section $0.25\ \mu\text{m}$ thick from the same specimen, stained with ammoniacal silver and photographed by phase-contrast. *d*, dictyosome; *er*, endoplasmic reticulum; *m*, mitochondrion; *n*, nucleus; *ne*, nuclear envelope; *nu*, nucleolus; *p*, plastid; *r*, ribosomes; *v*, vacuole; *w*, cell wall.

Fig. 1.—Potassium dichromate fixation. The asterisk shows an organelle that may be a mitochondrion.
 Fig. 2.—Osmium tetroxide fixation. There is evidence of considerable shrinkage (folded walls, distorted nucleus) and extraction of the stroma of plastids and mitochondria.

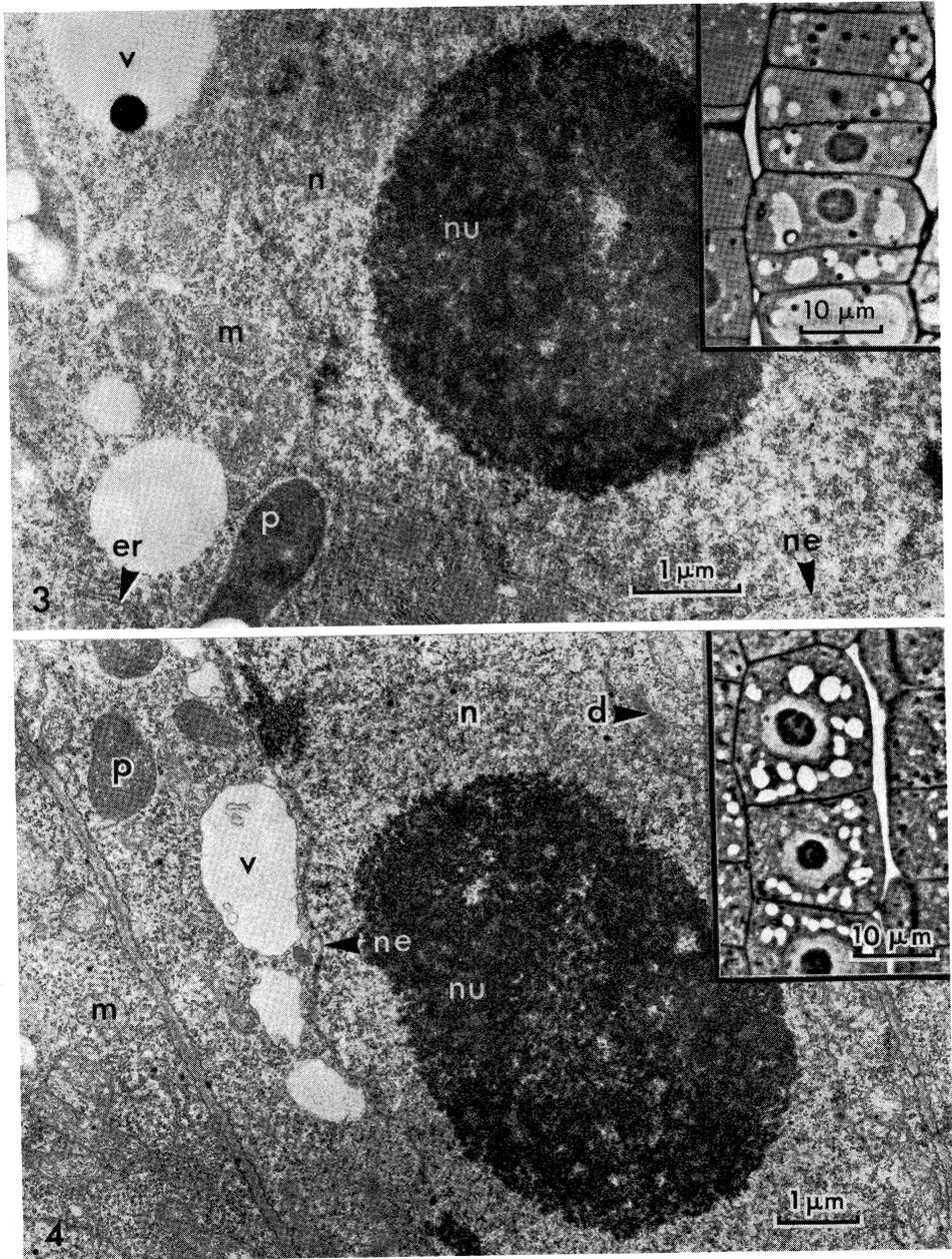


Fig. 3.—Glutaraldehyde fixation. All organelles and ground substance appear to be well preserved although all membranes lack electron contrast.

Fig. 4.—Glutaraldehyde post-fixed with osmium tetroxide. There are no detectable differences between this image and that of Figure 3 except for the positive electron contrast of all membranes.

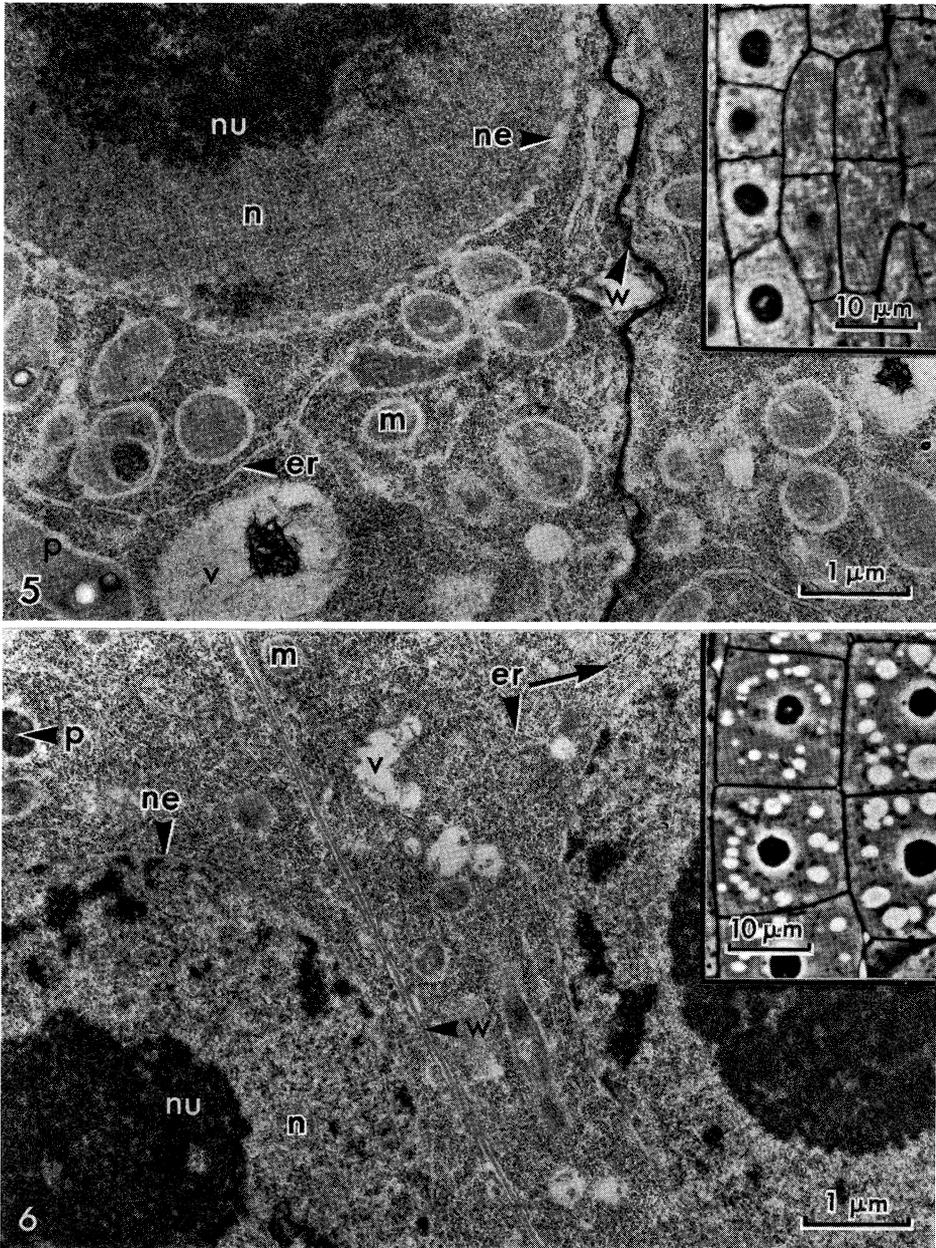


Fig. 5.—Formaldehyde fixation. Image similar to that of Figure 3 except for the fibrous precipitate present in the vacuoles and the high electron contrast of the cell walls.

Fig. 6.—Acrolein fixation. Very similar image to glutaraldehyde-fixed tissue (Fig. 3).

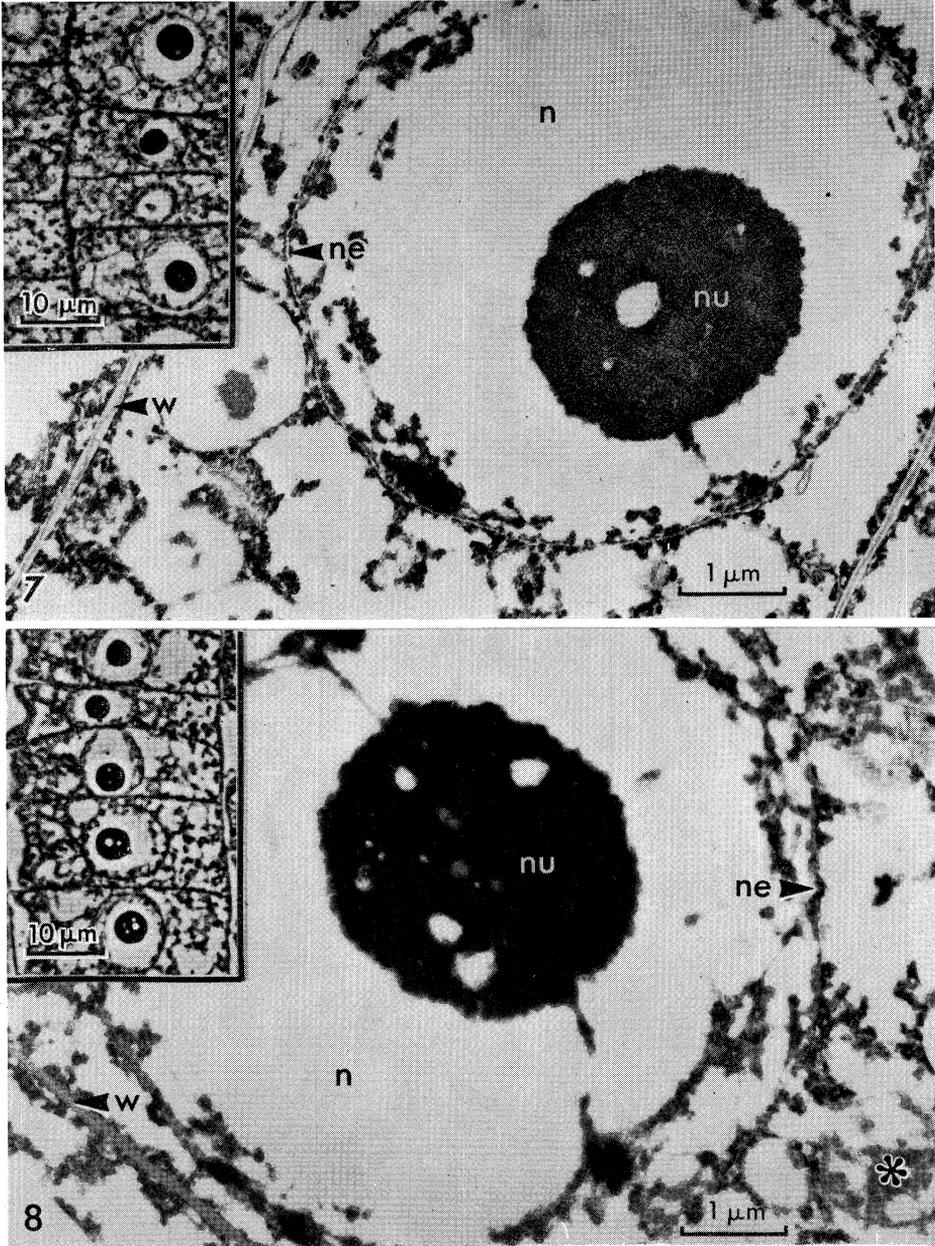


Fig. 7.—FAA. Coagulant fixation with clumped chromatin and the formation of a large perinuclear cavity. Most cytoplasmic organelles and membrane systems have been rendered unrecognizable.

Fig. 8.—CRAF III fixation. Very similar to Figure 7 though the texture of the nucleolus is somewhat different. A structure that may be a mitochondrial remnant is shown at the asterisk.

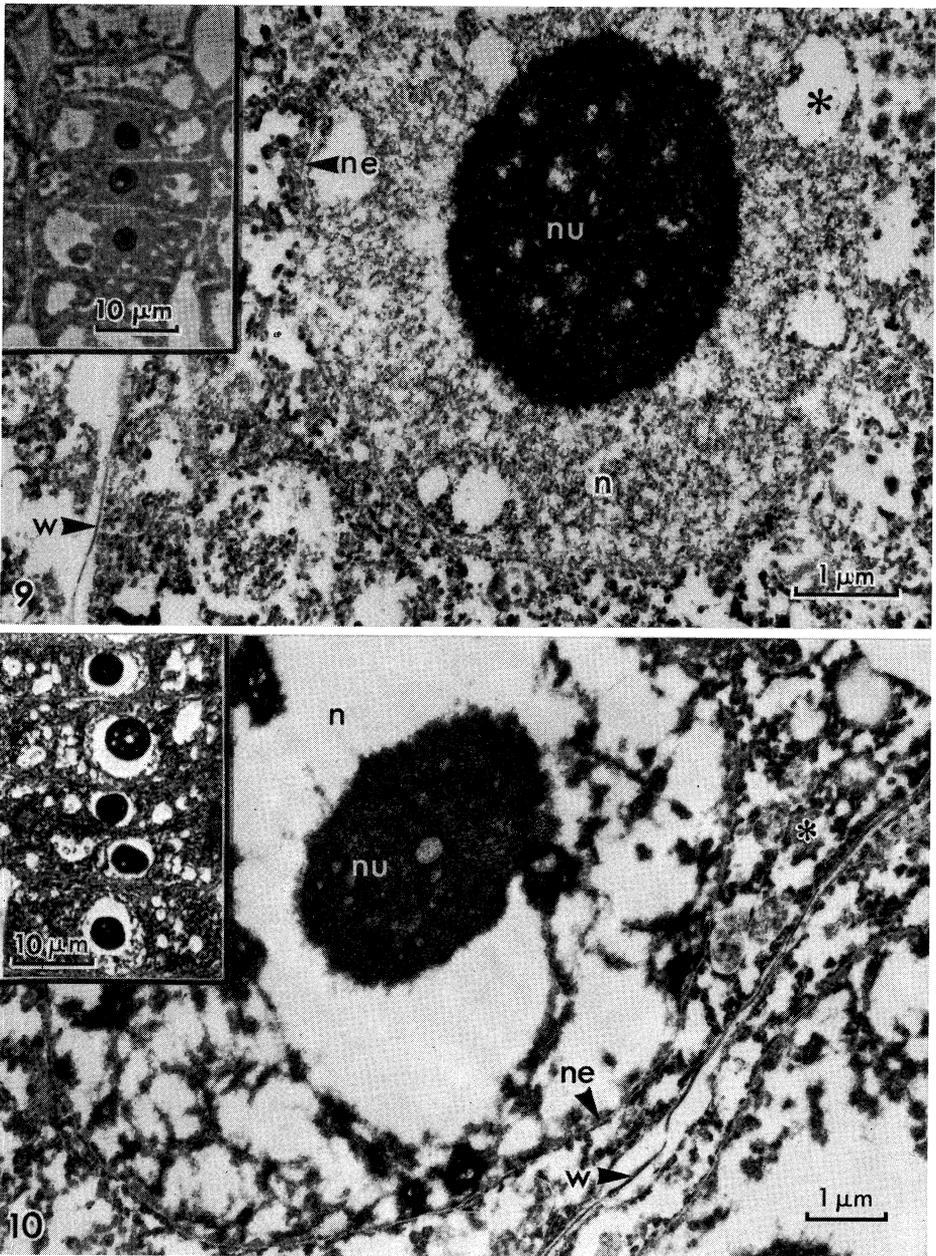


Fig. 9.—Boiled tissue. Coagulant fixation which fails to clump the chromatin. Large chromatin aggregates (asterisk) appear to have dissolved. All cytoplasmic organelles have been rendered unrecognizable. It is a moot point whether such a specimen shows more or less damage than those illustrated in Figures 7 and 8.

Fig. 10.—Clarke's fluid. Very powerful coagulant the overall effect of which is similar to that of FAA. Somewhat surprisingly, there is evidence of the retention of some cytoplasmic organelles (asterisk).

problems that surround its use on any routine basis include rather poor penetration over distances greater than 1–2 mm, great expense, and interference with many staining reactions.

These observations upon root tip cells suggest that there is little to choose between formaldehyde, acrolein, and glutaraldehyde as primary fixatives for this tissue. However, formaldehyde is very much less satisfactory than either of the other two when it is used upon more vacuolated cells and one cannot recommend widespread use of formaldehyde for general work (see p. 1241). Its ability to render insoluble certain as yet unidentified (probably phenolic) constituents of the vacuole may mean that it will come to have useful applications as a post-fixation rinse.

Feder and O'Brien (1968) recommended the use of acrolein as a general fixative for light microscopy of plant tissues, especially when the specimens were to be embedded in glycol methacrylate. The observations presented here and considerable experience since that time in the use of acrolein and glutaraldehyde, alone or in combination, lead us to believe that there is very little to choose between these substances as primary fixatives. There may be some merit in using acrolein where it is essential to attempt fixation of a large specimen for acrolein undoubtedly penetrates tissues faster than the larger molecule of glutaraldehyde. Its use as a mixture with glutaraldehyde gave marginally better results in the fixation of dense seed tissues of cereals (Swift and O'Brien 1972*a*, 1972*b*). However, it is easy to prove that albumens are not as well cross-linked by acrolein as they are by glutaraldehyde. A hen's egg fixed for 48 hr in 5% glutaraldehyde may be handled with forceps but this is not the case if 10% acrolein is used instead. This fact, combined with the extremely irritant properties of acrolein, the need to distil it freshly before use from commercial samples, the inconvenience and risk of this distillation, and the slow deterioration of pure acrolein during prolonged storage all militate against it as a general-purpose fixative. Furthermore, glutaraldehyde is now widely available in a number of different commercial grades, is relatively non-noxious to use, and its mode of action at the chemical level is under intensive study (see Hopwood 1972).

(b) Action of Fixatives on Hair Cells

If one of these cells is mounted in water and viewed with oil-immersion under phase-contrast, one may focus below the slightly patterned wall into the thin layer of parietal cytoplasm that surrounds the large central vacuole which is devoid of particulate matter. Within the cytoplasm, one can resolve numerous filamentous mitochondria, colourless amoeboid plastids with included starch granules, lipid droplets, and small disc-shaped organelles that we presume to be the dictyosomes of this cell (see also Url 1964). The mobile network of cytoplasmic vacuoles (see Figs. 11 and 24) is very evident and under certain circumstances, fine fibrils can be detected within the rapidly streaming strands of cytoplasm along which the organelles move (O'Brien and McCully 1970). The giant cell is covered by a cuticle and fixatives tend to enter most rapidly through the cut basal cells. Fixation is therefore progressive along the length of the cell from base to tip, providing an opportunity to study the sequence of fixation more than once in the same cell. In a long cell, it is not uncommon to find the basal 100 μm completely fixed while the tip 300 μm is still streaming.

The comments which follow record the major changes which take place in the appearance of these living cells when they are mounted in the fluids listed. The numbers in brackets give the time, in minutes after mounting, at which the changes were first noticed about 300 μm from the base of the cell. Figure 11 shows the appearance of the cytoplasm in the living state and Figures 12–19, 25, and 26 illustrate some of the changes induced by the treatments listed below.

(i) 2% *Acetic Acid*.—Brownian motion of lipid droplets is evident (1·5), but this motion is not obvious until streaming almost stops. The cytoplasm produces bubbles, and sheets and tubes of membrane slide around the cytoplasm–vacuole boundary. Mitochondria and dictyosomes swell (2). The cytoplasm becomes fibrous, and all organelles become hard to recognize; a few large, swollen mitochondria are still present (5). Transvacuolar strands appear stringy and devoid of a covering membrane; free membrane-bound bubbles lie in the vacuole (7). All organelles except the nucleus become impossible to recognize and a continuous network of fine fibrils forms in the cytoplasm (11). The cytoplasm begins to shrink away from the wall (17).

(ii) 50% *Ethanol*.—Brownian motion of lipid droplets is evident (6·5). Cytoplasmic vacuoles round up, mitochondria become beaded, then vesiculate (the transformation of a filamentous mitochondrion to a swollen mobile sac can take less than 2 s) (9). Dictyosomes swell up and become difficult to distinguish from swollen mitochondria. Membranous outgrowths form from the vacuolar membrane and protrude into the vacuoles (these outgrowths are very evident because they exhibit an irregular “dancing” motion) (16). A “wave” spreads over the surface of the vesiculated cytoplasm, followed instantly by the formation of a fibrous image of the cytoplasm within which organelles other than the nucleus are difficult to recognize. Crystals form in the central vacuole (19).

(iii) *Clarke's Fluid*.—The changes are too rapid to watch. Brownian motion of lipid droplets is detectable in the middle of the cell within 1 min. Fibrous, coarse cytoplasm in which only the nucleus can be recognized is present throughout the length of the cell in 3 min.

(iv) *FAA*.—Brownian motion of the lipid droplets is evident (2·5). Cytoplasmic vacuoles round up, mitochondria swell, and a fibrous network forms at the cytoplasmic surface (3·5). Membranous bubbles protrude from the cytoplasm into the vacuole, especially on transvacuolar strands (5). Longitudinally oriented fibres appear in the cytoplasm and dictyosomes and filamentous mitochondria are no longer detectable (10). Bubbles lining the vacuole disappear and the cytoplasm becomes very coarse and granular. The cytoplasm shrinks away from the wall (23).

(v) *CRAF III*.—Brownian motion of lipid droplets is evident (3). Cytoplasmic vacuoles vesiculate, mitochondria and dictyosomes swell (4). Bubbles of membrane protrude into the vacuole, and the cytoplasm becomes coarse (6). A fibrous network appears in the cytoplasm (9). The cytoplasm shrinks away from the wall and only the nucleus is recognizable (12).

(vi) *Formaldehyde* (4%).—Brownian motion of lipid droplets is evident (5). Cytoplasmic vacuoles round up, some mitochondria develop pouches, and plastids

become indistinct (6). There is a rapid outgrowth of "dancing" membranous threads from the vacuolar membrane into the vacuole (9). Brownian motion is stopped in the base of the hair, but is still occurring in the tip (28). All mitochondria show some abnormality (pouches, complete vesiculation, sharply bent shapes) and "dancing" membranous threads are still present (30).

(vii) *Osmium Tetroxide* (2%).—Brownian motion of lipid droplets is evident (2·5). Cytoplasmic vacuoles round up and swell, and mitochondria produce pouches (3). Mitochondria thicken, plastids withdraw their amoeboid processes, round up, and become invisible, their position being marked only by their starch grains (7). Cytoplasm becomes very vesiculate, but few membranous outgrowths develop. Mitochondria are stabilized in a swollen and coarse state (10). A vacuolar precipitate forms (15).

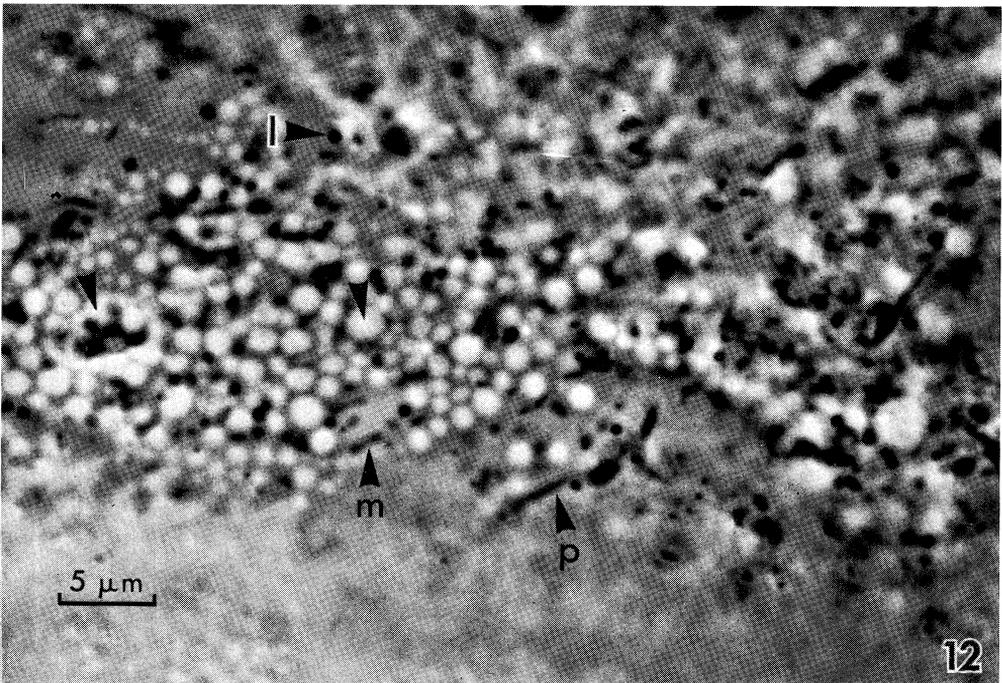
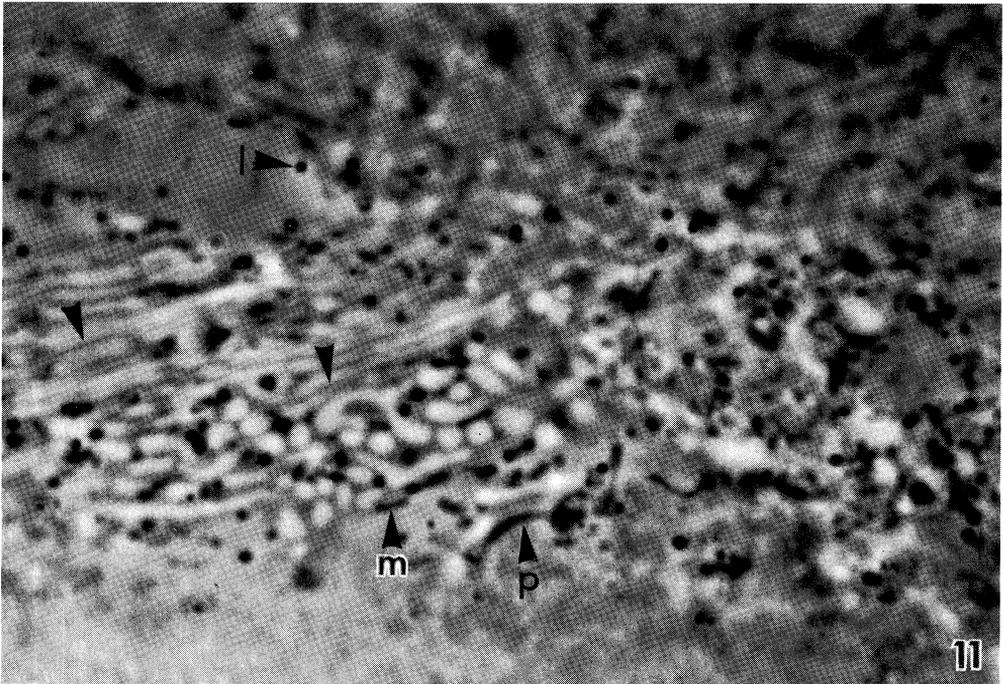
(viii) *Glutaraldehyde* (3% or 6%).—Brownian motion of lipid droplets is evident (6). Cytoplasmic vacuoles round up, and clusters of very small vacuoles form (7). Membranous outgrowths form from the vacuolar membrane and protrude into the vacuole and similar outgrowths can be detected at the cytoplasmic surface (10). Dictyosomes, mitochondria, and plastids remain apparently unchanged (15). Brownian motion stops and a faint network appears in the cytoplasm within which all organelles appear "normal" (24). The dancing motion of membranous outgrowths persists.

(ix) *Acrolein* (10%).—Brownian motion of lipid droplets is evident (1·5). Cytoplasmic vacuoles swell and round up; these changes take place at about the same time *throughout the length of the cell* (3). Small vacuoles continue to swell and the cytoplasm becomes filled with small vacuoles, but the mitochondria look normal (6). Membranous outgrowths form from the margins of the small vacuoles (7). Mitochondria remain filamentous, but develop fuzzy outlines. The cytoplasm develops a slightly coarse appearance and Brownian motion ceases (25).

The first effect of all of these fixatives is to slow down and disorganize cytoplasmic streaming. That this is a localized effect of the fixative and not part of a general response of the whole cell to injury we may infer from the fact that the cytoplasm in the tip of the cell may still be streaming even when the cytoplasm at the base of the cell has been still for 10 min. Indeed, it is an unforgettable sight to watch the progress of a "fixation front" as it moves along the length of these cells and one may readily confirm the same sequence of changes many times in the same cell. Once organized streaming has ceased in a region of cytoplasm, the mobile network of cytoplasmic vacuoles begins to fragment and round up (cf. Figs. 11 and 12; 24

Fig. 11.—Living cell, showing filamentous mitochondria, plastids, and lipid droplets and the system of cytoplasmic vacuoles, many of which are present as elongated canals in a rapidly streaming area of the cytoplasm (arrows).

Fig. 12.—The same field as that shown in Figure 11, but after 10 min fixation in 5% glutaraldehyde. All canalicular cytoplasmic vacuoles have now fragmented into vesicles, giving the cytoplasm a bubbly appearance. The same lipid droplet, plastid, and mitochondrion are labelled for reference in the two figures. No further changes were detectable by light microscopy in these cells except for the production of membranous outgrowths from the cell membrane and vacuolar membranes (see Figs. 24–27).



Figs. 11-19 are phase-contrast photomicrographs of the cytoplasm of hairs of *Heracleum mantegazzianum*. Labels as for Figures 1-10; also *l*, lipid droplet; *s*, starch grain. Figures 11 and 12 are at same magnification; the scale in Figures 13-19 is 10 μm .

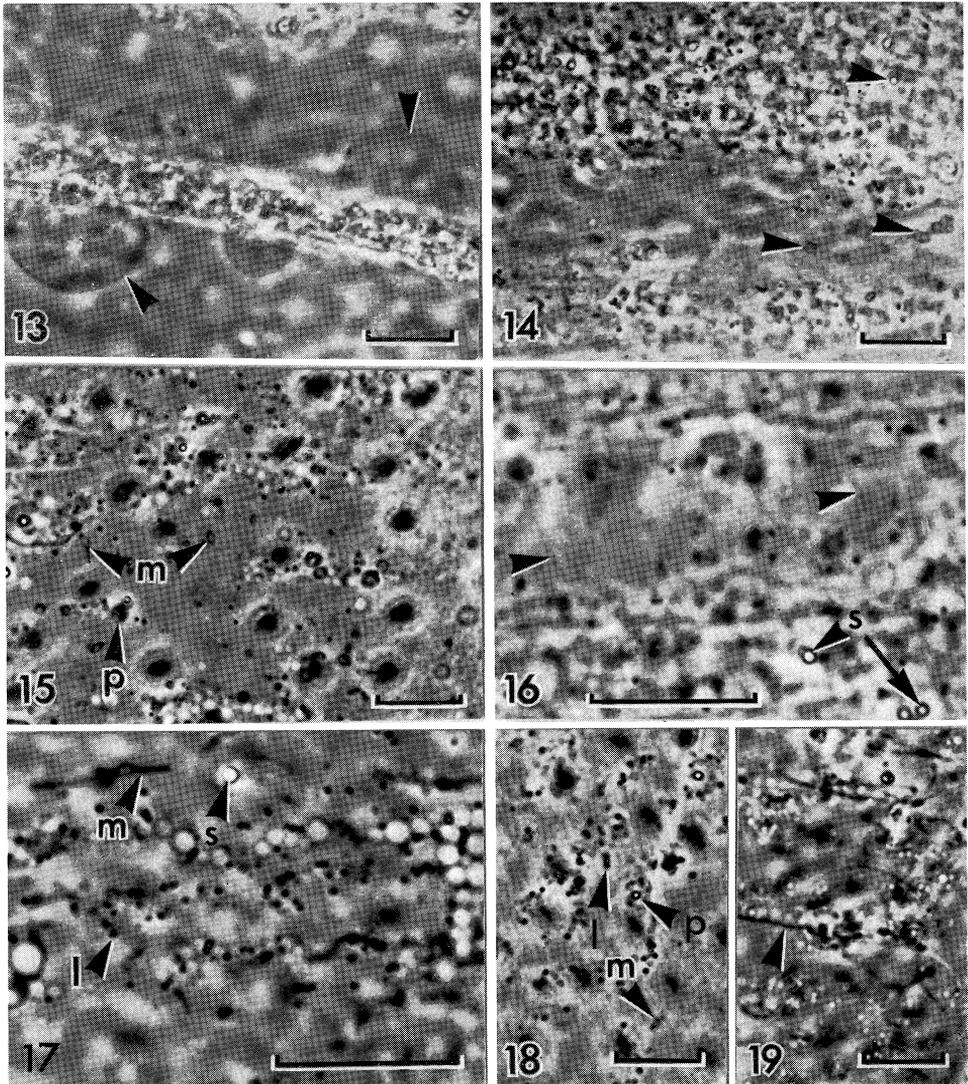


Fig. 13.—Outgrowths of membrane (arrows) from the surface of a transvacuolar strand of cytoplasm in a cell exposed to 2% acetic acid for 2 min.

Fig. 14.—Parietal cytoplasm in the same cell as that shown in Figure 13. Starch grains of plastids (top arrow) and swollen remnants of mitochondria (lower arrows) are all that remain clearly resolved.

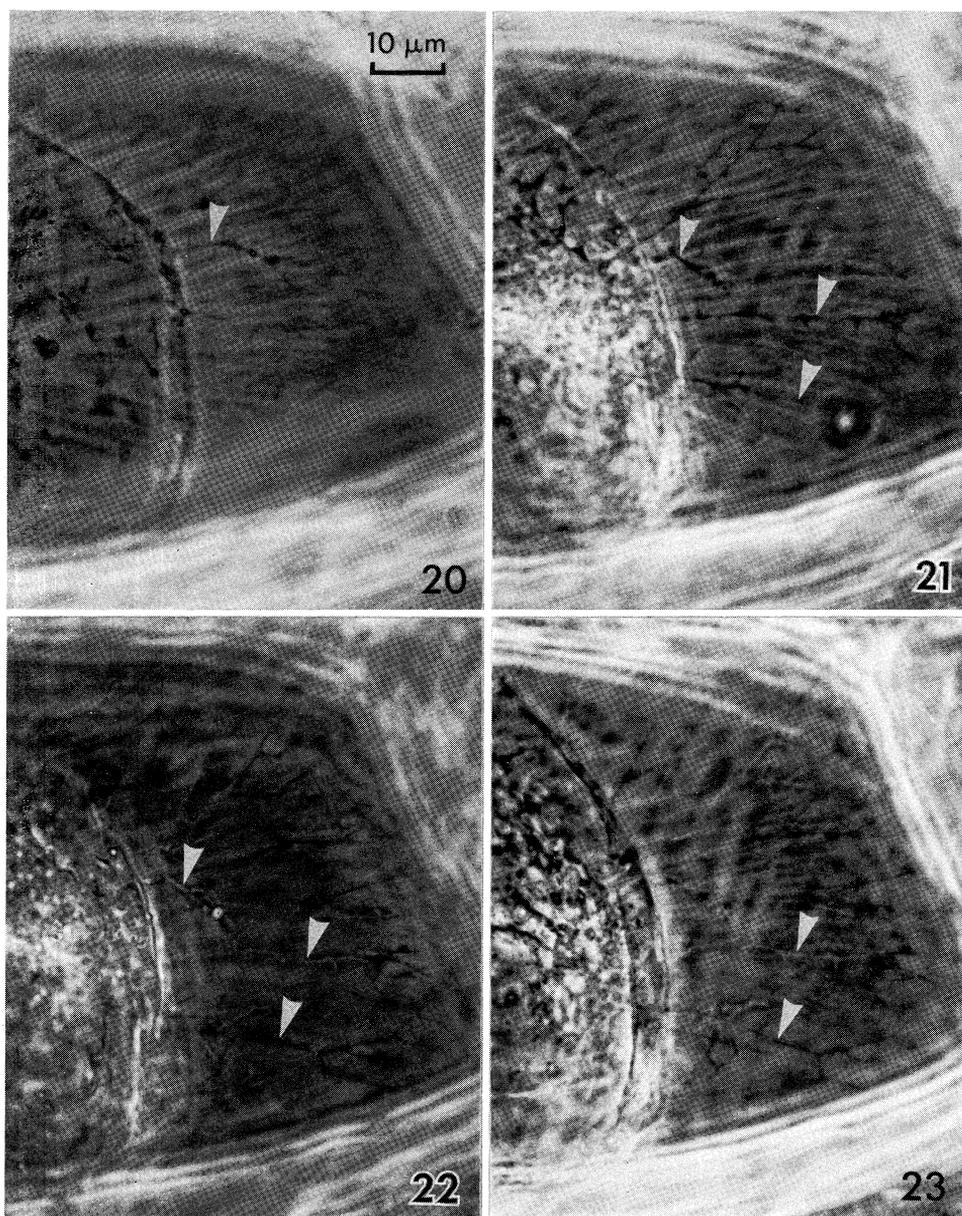
Fig. 15.—Parietal cytoplasm showing distorted mitochondria (*m*) in a cell exposed to 50% ethanol for 10 min.

Fig. 16.—Parietal cytoplasm in a cell fixed in FAA for 20 min. Most organelles are rendered unrecognizable although the starch grains of the plastids (*s*) show where these organelles were. The nature of the fine lines (arrows) is not known, though they are a response to fixation, being absent in the living cell.

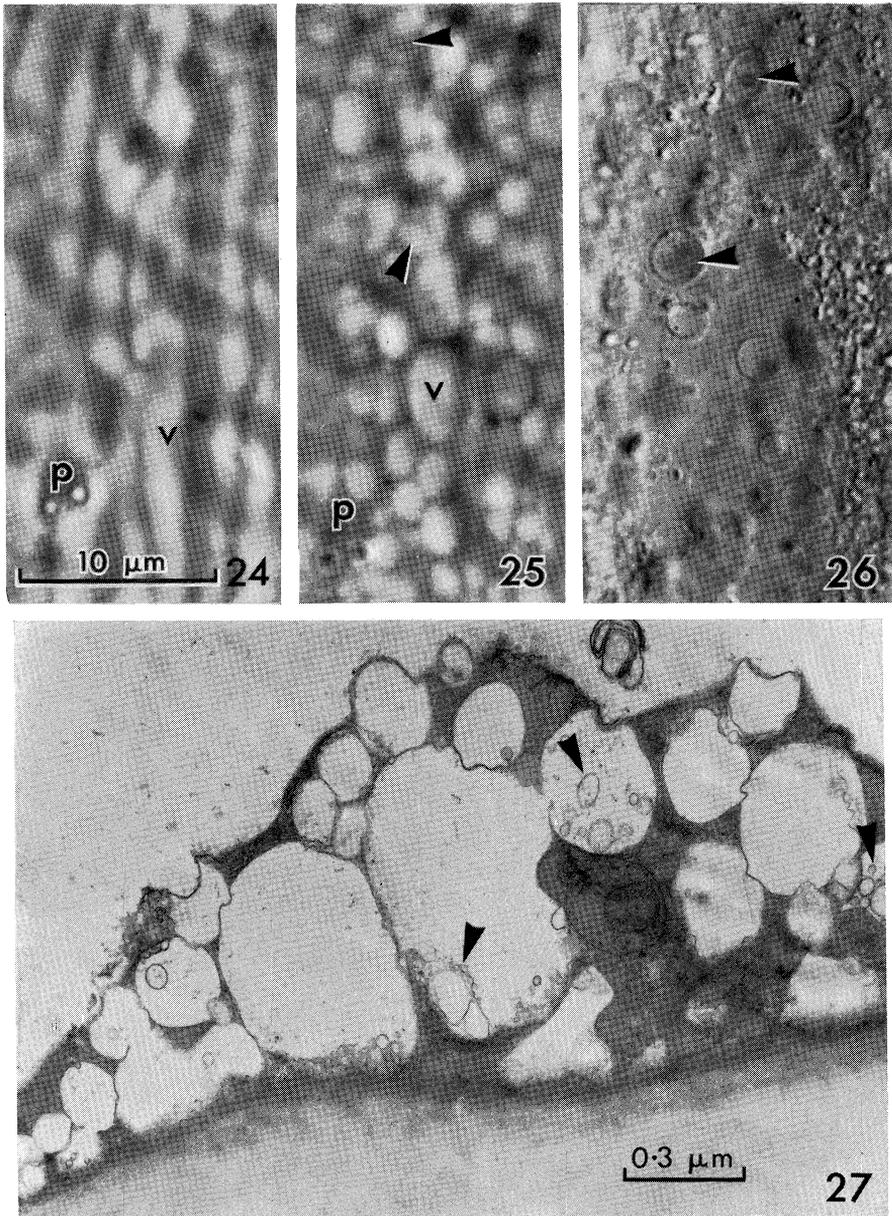
Fig. 17.—Parietal cytoplasm in a cell fixed in 2% OsO₄. Filamentous mitochondria are preserved though they appear to be somewhat fatter than in the living cell. Plastid stroma becomes unrecognizable, only the starch grains remaining visible.

Fig. 18.—Parietal cytoplasm of a cell exposed to 4% formaldehyde for 35 min. Both the mitochondria (*m*) and plastids (*p*) appear to be distorted.

Fig. 19.—The cytoplasm—central vacuole boundary in a cell exposed to 4% formaldehyde for 20 min. Long filaments (arrow) have formed from the vacuolar membrane and project into the vacuole.



Figs. 20-23.—Phase-contrast photomicrographs of plasmolysed onion epidermal cells, showing Hecht's threads (arrows) in the living cell (Fig. 20), after 10 min fixation in 5% glutaraldehyde (Fig. 21), after post-fixation in 2% OsO₄ (Fig. 22), and after dehydration in methyl cellosolve (Fig. 23). The same cell is shown in Figures 21-23, and though there are unavoidable variations in focus and optical appearance of the cell surface, it is clear that most of the threads visible in Figures 20 and 21 are still retained in Figure 23.



Figs. 24 and 25.—Phase-contrast photomicrographs of canalicular vacuoles in a *Heracleum mantegazzianum* hair in the living state (Fig. 24) and 3 min after exposure to a mixture of 5% glutaraldehyde and 10% acrolein (Fig. 25). The canals have all fragmented into vesicles, and membranous outgrowths have begun to form within them (arrows). The plastid (*p*) is the same in both pictures. Fig. 26.—Nomarski micrograph of membranous structures (arrows) forming at the surface of the cytoplasm of a *H. mantegazzianum* hair cell after 30 min fixation in glutaraldehyde. Fig. 27.—Electron micrograph of parietal cytoplasm of *H. mantegazzianum* hair after glutaraldehyde-OsO₄ fixation. The large cytoplasmic vacuoles contain a variety of membranous structures which we suspect have been produced from material similar to that illustrated in Figure 25.

and 25). It seems likely that this change results in part from the cessation of streaming (see Zirkle 1932, 1937). Once this point has been reached, the subsequent changes depend upon the nature of the fixative.

In any fixative that contains an appreciable quantity of acetic acid, the vacuolar membrane appears to separate from the cytoplasm (Fig. 13). This is accompanied by swelling and dissolution of most of the cytoplasmic organelles (Fig. 14). The other components of the acetic acid mixture appear to modify the extent to which the cytoplasm shrinks, and to determine how fibrous it becomes (Fig. 16). It is difficult to decide how much the destructive effects of these fixatives are due to the action of their components on the cytoplasm rather than to the effects of the released vacuolar contents upon an improperly stabilized cytoplasm.

Damage to the vacuolar membrane is also evident in cells fixed in formaldehyde (Fig. 19), glutaraldehyde (Figs. 12 and 25), and acrolein and appears to be least in cells fixed in osmium tetroxide (Fig. 17). However, this evident change in the structure of the vacuolar membrane is accompanied by less damage to the cytoplasmic organelles with non-coagulant fixatives. Mitochondria are deformed, but do not disappear in cells exposed to formaldehyde (Fig. 18) or osmium tetroxide (Fig. 17), while these organelles are well preserved by acrolein or glutaraldehyde (Fig. 12). Plastids appear to be damaged by osmium tetroxide or formaldehyde but well preserved by acrolein or glutaraldehyde (see also Gunning 1965). Though the cytoplasm is very vesiculate after acrolein fixation, it is never stringy or fibrous when osmium tetroxide or any of the aldehydes are used. Cells fixed in glutaraldehyde are clearly the most life-like, the only artefacts evident after 30 min fixation being the production of membranous outgrowths of the vacuolar membrane and cell membrane (Figs. 25 and 26).

Figure 27 is an electron micrograph of part of a cell after fixation in glutaraldehyde and post-fixing in osmium tetroxide. Unfortunately, we were not able to produce this figure from a region of cytoplasm previously photographed by light microscopy in the intact hair. Nonetheless, it seems likely that the membranous material that can be detected in the vacuoles of Figure 25 is the source of the vesicular structures visible inside the cytoplasmic vacuoles in Figure 27.

Finally, it is worth noting that cells fixed in acrolein tend to show the sequence of changes characteristic of this fixative more or less uniformly along their length, in contrast to glutaraldehyde fixation where the changes proceed from base to tip. This fact offers some support for the suggestion that acrolein can penetrate heavily cutinized cell walls much more readily than glutaraldehyde.

(c) *Fixation of Hecht's Threads*

This system was chosen because it was felt that it provided the ultimate challenge to any fixative. If it was possible to stabilize against dehydration the delicate cytoplasmic strands (Hecht's threads) that connect the plasmolysed cell surface to the wall, then glutaraldehyde would surely be established as a fixative that causes very little structural distortion. The results were quite clear-cut. Figures 20-23 show the same cell alive, after glutaraldehyde fixation, after osmium tetroxide fixation, and after dehydration in methyl cellosolve. Though there are slight differences in the level of focus between these micrographs, and unavoidable variation in contrast

(the refractive index of the mounting fluids is very different), it is quite clear that none of the threads present in Figure 20 have broken or changed their shape in Figure 23. Unfortunately, it was impossible to study the effects of infiltration with embedding medium upon these threads because the embedding medium and threads have too little difference in phase-contrast.

(d) *General Comments*

Though the subject has not yet been analysed by a competent scientific historian, it seems likely that coagulant fixatives have been retained in plant microtechnique because they give better results with the paraffin procedure than either osmium tetroxide or formaldehyde (see Baker 1958). The paraffin wax procedure does have some advantages over any plastic-embedding procedure so far devised since it yields ribbons of sections. However, neither acrolein nor glutaraldehyde have been widely evaluated as fixatives for paraffin wax embedding of plant tissues. In preliminary experiments with bean root tips, we found that tissues fixed in glutaraldehyde, embedded in paraffin by an automatic tissue processor, de-waxed with xylene, and returned to ethanol and then embedded in Spurr's resin had a fine structure indistinguishable from tips taken straight into Spurr's resin as described in Section II. This suggests that, perhaps unlike formaldehyde or osmium tetroxide, glutaraldehyde-fixed tissue survives the rigours of paraffin-embedding rather well. This is not to say that such tissues will survive the sectioning and conventional staining procedures of paraffin wax schedules, the steps in which we believe a great deal of structural damage occurs (see Moss 1966).

Since these results show that the coagulants are so destructive, we believe that their use should be discontinued whenever possible. This is already possible in the case of tissues which are to be embedded in epoxy resins or glycol methacrylate. For those workers who feel that they must use paraffin-embedding, surely it is worth the effort to discover if glutaraldehyde can be substituted for the destructive coagulants? Familiarity with the coagulant image should not blind us to the undeniable fact that that image is a faithless representation of the *in vivo* structure.

The observation that all fixatives slow down and disorganize streaming raises an important caveat in the interpretation of electron micrographs. A great deal of descriptive electron microscopy is concerned with attempting to relate temporal processes to the image of the fixed state. If organelle A is close to B in the micrograph, it is often asserted that this makes it likely that there may be exchange between A and B. To those who regularly watch actively streaming cells, this kind of argument has always been a dubious one but it must be admitted that it would be possible for spatial associations to be maintained between cellular organelles even in the presence of streaming. However, these present observations show that a period of 5–20 min elapses between the disorganization of streaming and gelation of the cytoplasm. During that time organelles diffuse by Brownian motion. Organelles such as dictyosomes end up fixed many micrometres removed from the site where they stopped streaming and the displacement during this period of the submicroscopic vesicles (5–10 nm) that one does not usually see by light microscopy must surely be even greater. Just how much may one infer with any confidence from spatial association

between organelles after 5–20 min of Brownian motion prior to their “fixation” in space?

Finally, there is the question of the changes that occur during fixation at the vacuolar membrane, where they are easy to see, and at the cell membrane where visualization is much more difficult. These changes produce pleiomorphic structures that are not usually visible in healthy cells but which can often be seen in cells that have been examined under anoxic conditions for 30 min–1 hr, or which have been held plasmolysed for some time, especially if salts are used as the plasmolyticum. These phenomena were noticed many years ago and the production of extreme forms of these structures is recorded by Strugger (1949) as a class experiment, to demonstrate the effect of salts upon the structure of the cytoplasm. The nature of these structures has not been adequately explored by modern electron microscopy but one thing is clear. They form in cells treated with any of the aldehydes, and none of these fixatives will stop their formation once they have been initiated. We believe that it is likely that many of the structures currently identified as “lomasomes”, or “paramural bodies” (Marchant and Robards 1968) may have originated in this manner. Some will object that paramural bodies cannot be artefacts since they are seen in freeze-etched preparations. However, it is rare for freeze-etched preparations to be made without first protecting the tissue from ice damage by treating it with 10–30% glycerol, a 1–3M solution! Few cells will fail to initiate plasmolysis in 1–3M glycerol. There is pressing need for a decisive study of paramural bodies.

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