FIXATION OF JUVENILE CAMBIUM FROM TWO CONIFEROUS SPECIES FOR ULTRASTRUCTURAL STUDY

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ABSTRACT

Samples of dormant and of actively growing cambial-zone tissue collected in June and March, respectively, from plantations of young white spruce (*Picea glauca* [Moench] Voss) and red pine (*Pinus resinosa* Ait.) near Ottawa, Canada, were fixed in five different solutions at three temperatures. Fixation quality was evaluated by electron microscopy.

Not all cellular organelles were preserved equally well by the same fixative in the active and in the dormant conditions, or in both spruce and pine. In general, our best results were obtained with Karnovsky's fixative solution. Somewhat less satisfactory results were obtained with a glutaraldehyde-acrolein mixture followed by straight glutaraldehyde. Poorer results were obtained with a trialdehyde solution while a glutaraldehyde-osmium tetroxide cocktail undoubtedly provided the worst fixation.

Different fixative temperatures had a marked effect on fixation quality only when phosphate buffer was used. There was little gained by prolonging the period of fixation beyond a few hours.

Keywords: *Picea glauca, Pinus resinosa,* cambium, cytology, fixation, organelles, membranes, chemical fixatives, buffers, temperature effects, ultrastructure, electron microscopy.

INTRODUCTION

Studies of cellular ultrastructure in the cambial region of woody plants have been frustrated over the years by the problem of fixing this delicate tissue in its natural state for microscopic study (Srivastava and O'Brien 1966; Kidwai and Robards 1969b; Timell 1973). A variety of chemical formulations and techniques have been employed, but none appear to be fully successful in preserving both the cambial initials and their immediate vascular derivatives free of alteration (Robards and Kidwai 1969).

Good fixation of the cambial zone is more readily obtained during dormancy than during active growth (Robards and Kidwai 1969; Timell 1973). The problem seems to be most pronounced with the long fragile fusiform initials of conifers.

This investigation was undertaken to examine and evaluate a limited number of fixative solutions and techniques on both active and dormant cambia of two typical conifers. We hoped that improved fixation, particularly of the active cambium, might be obtained.

MATERIALS AND METHODS

Collection and subdivision of test material

Material for study was cut from the upper stems of 6- to 8-year-old white spruce (*Picea glauca* [Moench] Voss) and red pine (*Pinus resinosa* Ait.) growing in the Greenbelt Forest Reserve south of Ottawa, Ontario. One tree was sampled for each fixative trial.

Samples of actively dividing cambium were collected about the end of June. The fixing solutions designated "ambient" were equilibrated to the shade temperature of about 27 C prior to use, while others were kept on ice. Samples of dormant cambium collected near the beginning of the following March were all fixed on ice since the ambient temperature was about 0 C.

Just after mid-March, dormant cambium collected at about 0 C was fixed in solutions held in a water bath at an above-ambient temperature of about 27 C. Therefore, the elevated temperature fixations G_{27}, K_{27}, and GA_{27} relate only to dormant cambium.

Discs 1.3–2.5 cm in diameter and 1.0–1.5

TABLE 1. *Summary of fixation methods*

Fixative	Fixative Buffer pH 7.2	Symbol	Temp.	Time h	Postfix[a] Buffer pH 7.2	Washer Buffer pH 7.2
		GLUTARALDEHYDE (G)				
5% glutaraldehyde + 7% salts[b]	0.1M phosphate	Gi	ice	2	Veronal acetate + 7% salts[b]	0.1M phosphate
		Gai	amb. + ice	2 + 22		
		G_{27}	27°C	2		
		GLUTARALDEHYDE-OSMIUM COCKTAIL (GO)				
2% glutaraldehyde 1% osmium tetroxide	0.1M sodium cacodylate	GO	ice	2	0.1M sodium cacodylate	0.1M sodium cacodylate
		KARNOVSKY (K)				
2% glutaraldehyde 1% formaldehyde + 0.025M $CaCl_2$	0.1M sodium cacodylate	Ka	amb.	2	0.1M sodium cacodylate	0.1M sodium cacodylate
		K_{27}	27°C	2		
		GLUTARALDEHYDE-ACROLEIN (GA)				
3% glutaraldehyde 3% acrolein	0.1M sodium cacodylate	GAa	amb.	3	0.1M sodium cacodylate	0.1M sodium cacodylate
		GA_{27}	27°C	2		
		TRIALDEHYDE (TA)				
3% glutaraldehyde 2% formaldehyde 1% acrolein + 2.5% DMSO[c] and 0.001M $CaCl_2$	0.1M sodium cacodylate	TA	ice	3-4	0.1M sodium cacodylate + 0.2M sucrose	0.1M sodium cacodylate + 0.2M sucrose

[a]Postfixation was in 1% OsO_4 for 2 h at 8°C.
[b]Salt solution consisted of 8% NaCl, 0.4% KCl and 0.2% $CaCl_2$.
[c]Dimethylsulfoxide.

cm in length were cut immediately from excised stem sections using a fine-toothed fretsaw. Fixative solution was introduced into the kerfs while sawing, and the severed discs were dropped immediately into more fixative solution. The discs were then cleaved axially along radii into wedges 1–2 mm wide at the cambium. Bark and wood were trimmed away to within 0.5–1.0 mm of the cambial zone, and the specimens were transferred to fresh fixative solution.

Following buffer washes and storage overnight at about 8 C, the specimens were further subdivided by trimming a few mm from their ends and bisecting them both radially and transversely. After more washes, the material was postfixed in osmium tetroxide (OsO_4), washed and stored overnight in buffer at 8 C.

Fixatives

As summarized in Table 1, the study essentially covered five different fixative solutions together with three variations in fixation temperature. The fixative solutions examined were (1) glutaraldehyde (G), (2) glutaraldehyde-osmium tetroxide cocktail (GO), (3) Karnovsky (K), (4) glutaraldehyde-acrolein (GA), and (5) trialdehyde (TA).

The straight glutaraldehyde fixative (G) followed by the modification of Palade's postfixative attributed to Zetterqvist (cited in Pease 1964) had been the standard fixative in this laboratory and was included as a basis for comparison. It was hoped that the GO cocktail might improve preservation of microtubules, cytoplasmic fibers, ribosome complexes, polysaccharide- and lipid-containing structures, and avoid the possible extraction of incompletely fixed materials by the buffer washes between the prefix and the postfix.

Because of the large block size necessary to avoid mechanical damage to the fragile cambial zone cells, cells near the

interior of the block could suffer ultrastructural alterations resulting from isolation and probable anoxia before they could be killed by the slowly penetrating glutaraldehyde; it was therefore desirable to employ the more rapidly penetrating aldehydes, formaldehyde and acrolein, which could be expected to kill large volumes of tissue swiftly (Feder and O'Brien 1968). It was hoped that their combination with glutaraldehyde (K, GA) might overcome the slow, weak crosslinking of formaldehyde and the possible disruption of microtubules by acrolein (Sandborn 1966; Schultz and Case 1968). Dimethylsulfoxide (DMSO) was expected to aid the penetration of all components (TA).

In attempting to standardize the fixatives, postfixatives, and buffers and adapt them to cambial tissue, some modifications were made to the fixative recipes and procedures taken from the literature. For instance, the postfix time following the GO cocktail was reduced to one-third that used by Franke et al. (1969) because it was suspected that a long OsO_4 treatment following GO fixation would be unnecessary and could be detrimental. The K fixative (Karnovsky 1965) was diluted somewhat, and cacodylate buffer was used for the postfix. The GA fixative (Hess 1965) was used in 0.1 M rather than 0.2 M sodium cacodylate buffer. It was felt that 16 h of TA treatment followed by up to 24 h in 2% OsO_4 postfix as utilized for amphibian embryos (Kalt and Tandler 1971) would be needless for cambium as reasonably adequate fixation was obtainable from much shorter fixation durations using the G fixative. Also, it was hoped that a shorter treatment might avoid the detrimental effects of DMSO indicated by our previous limited experience and that of others (Jones 1969; Kidwai and Robards 1969a).

Sodium cacodylate buffer was used because of its apparent widespread successful use in ultrastructure fixation and its ease and simplicity of preparation. The 0.1 M concentration was used because cambium fixed in more concentrated solutions had shown unacceptable plasmolysis. The PIPES (piperazine-NN′-bis-2-ethanesulphonic acid) buffer was not employed because several previous attempts to obtain fixation of cambium with the system so successfully utilized by others (Salema and Brandão 1973) had yielded very poor results, i.e. uneven fixation, cytoplasmic precipitation, rupture of cellular and organelle membrane systems, and often organelles so indistinct as to be unrecognizable.

No attempt was made to determine the osmolalities of the fixatives, mainly because it seems unclear whether or not the solute concentration as it is commonly measured by freezing point depression, really has any significance in relation to the osmotic events hypothesized to occur before, during, and after cell death and fixation.

The elevated temperature fixatives were made up from 70% ampouled glutaraldehyde, and the stock OsO_4 was made by dissolving vial-sealed anhydrous OsO_4 in distilled water.[1] Ampouled 8% glutaraldehyde and ampouled 4% OsO_4 were used for all other fixatives.[2] Formaldehyde was made up as an 8% stock solution from paraformaldehyde immediately before use.

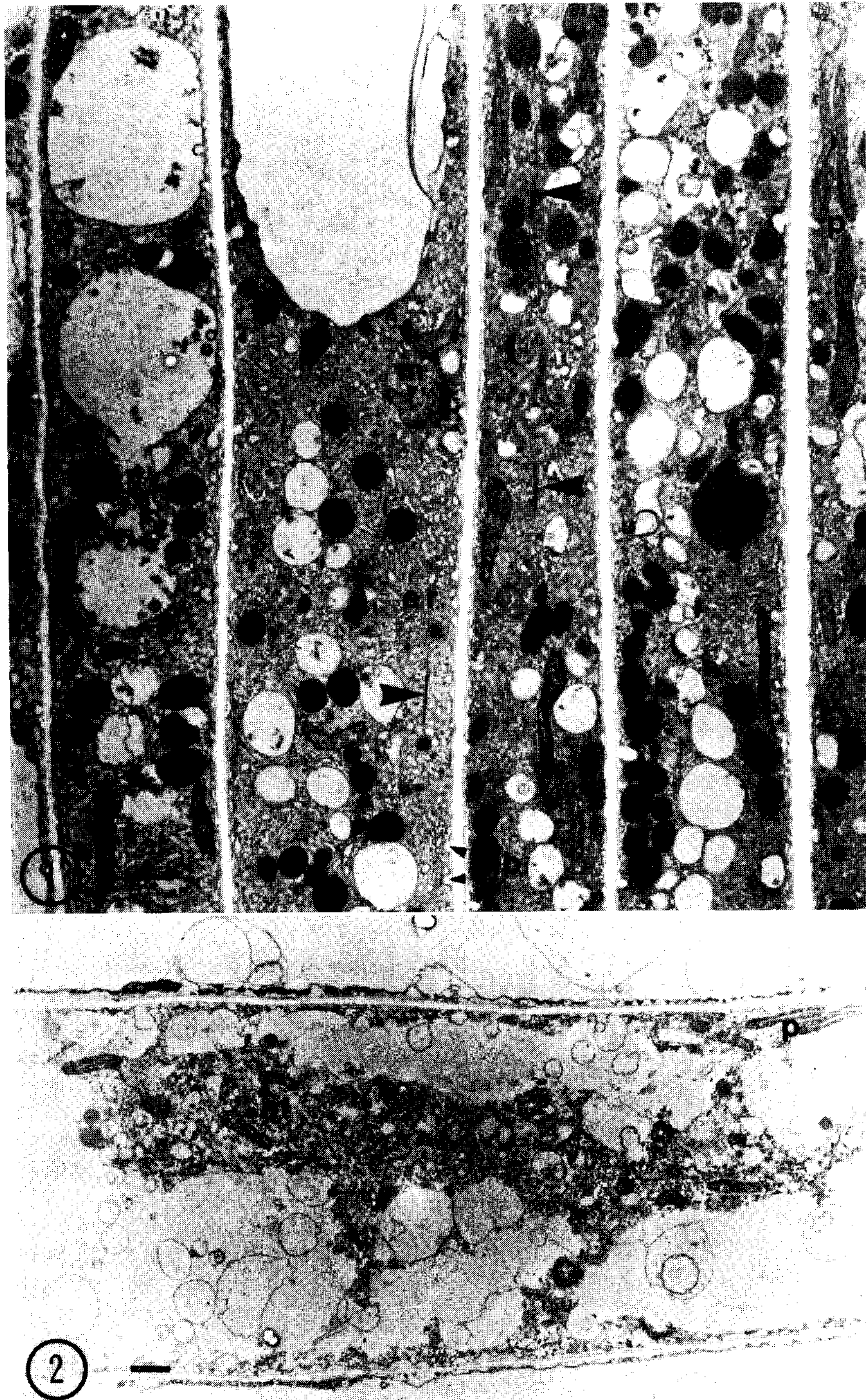
Preparation of material for microscopy

After fixation, the specimens were dehydrated through a graded (10%) series of cold spectrographic grade acetone to Spurr's resin. Infiltration took place over 4–5 days at room temperature. Polymerization in flat molds (70 C for two days) was preceded by a short period of evacuation to remove air bubbles from the resin and blocks as soon as they had warmed to 70 C.

Thin sections were cut with a diamond knife using either a Reichert OmU2 or an LKB Ultratome III. Sectioning was oriented in the radial plane and usually included an area of grazed radial cambial

[1] Both supplied by Ladd Research Industries, Inc., through Otto C. Watzka & Co., Ltd., P.O. Box 4010, Montreal, PQ Canada H3Z 1A6.

[2] Both supplied by Polysciences, Inc., Paul Valley Industrial Park, Warrington, PA 18976.



KEY TO ABBREVIATIONS IN FIGURES

cp Cell plate; *d* dictyosome; *er* endoplasmic reticulum; *lw* wall bordering latewood; *m* mitochondrion; *n* nucleus; *p* plastid; *plb* plasmalemma bleb; *plB* plasmalemma balloon; *sv* spiny-coated vesicle; *v* vacuole; *vs* vacuolate spherosome. All bars represent one micrometer.

walls and, in active cambium, at least one phragmoplast. Wherever possible, sections were taken from about 0.5 mm depth or less. Sections were stained with uranyl acetate and lead citrate (Reynolds 1963) and examined with a Philips EM-100 electron microscope.

Nomarski interference contrast light microscopy observations were made on freehand sections of active state cambium, and on entire African violet (*Saintpaulia* sp.) leaf epidermal hairs collected by severing the basal cell. The killing and fixing rates of 3% glutaraldehyde in sodium cacodylate or PIPES buffer were studied by observing the cells as the fixative solutions were drawn under the cover slip and contacted the sections or hairs.

The influence of tissue and fixative temperature on cytoplasmic streaming and fixation artifact formation was investigated because it was felt that cooling should generally lower the activity and increase the viscosity of the cytoplasm so that the cytoplasmic turbidity and membrane rupture accompanying fixative contact would be minimized. Since a cold stage was unavailable, slides and fixatives were chilled with ice prior to observation, and the temperature of the preparation could neither be monitored nor maintained uniformly.

RESULTS

The methods of collection and dissection of cambial tissue are perhaps equally as important as the fixative and fixation procedures utilized. Mechanical stress must be minimal. Best results are obtained when blocks are long enough to prevent excessive turgor loss by severing of the long fusiform cells. Cuts oriented parallel to the cell axis cause the least cambial compression.

Ultrastructural observations were made on the cambial initials and their least differentiated derivatives. The dormant cambial zone (Fig. 1) consisted of 4–6 layers of principally undifferentiated cells. The cells in the central 2–3 layers were often radially narrowest and structurally indistinguishable. The actively growing cambial zone (Fig. 2) was much wider. Nevertheless, 2–4 layers of radially narrow, thin-walled meristematic cells could be distinguished from the enlarging mother cells and their derivatives.

Electron microscope observations

A critical appraisal of the occurrence, abundance, ultrastructural morphology, and clarity of selected cell components provided the basis for comparing the different fixatives and methods. Criteria for judging the quality of the fixation image were obtained by perusing the literature, with particular emphasis given to published micrographs of cambium.

For convenience of discussion, our observations on the quality of preservation of selected cellular components are presented in relation to the five fixative solutions examined.

Glutaraldehyde fixative (G)

Ice and ambient temperatures

Fixation obtained with glutaraldehyde used at ice temperatures for two hours (Gi) or at ambient temperatures for two hours followed by extended cold fixation (Gai) was judged to be of average quality (Figs. 3, 4).

Microtubules:

—fairly dark, well defined, generally of common occurrence.

—present in mitotic spindle, phragmoplast, and cell cortex.

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FIG. 1. K_a-fixed cambial zone of dormant red pine. The cell on the extreme right side of the micrograph abuts the latewood. Portions of two cytoplasmic fibers are visible (large arrowheads). ×5,200.

FIG. 2. A phragmoplast in GA_a-fixed red pine cambium. Microtubules are absent, there are many small vacuoles, and the plastids exhibit wide central intralamellar spaces. ×4,200.

Cytoplasmic fibers:
All spruce and Gi-fixed active pine cambia: fairly abundant dark fibers exhibiting straight microfilaments.

All Gai-fixed pine: fibers few, pale, and felty.

Gi-fixed dormant pine: fibers similar, but rare.

Mitochondria:
—some membrane pouchings in all samples.

Active cambia: mitochondrial profiles typically spherical or rodlike with moderately dense matrices and prominent DNA loci.

Dormant cambia: more sacklike and less dense.

Plastids:
—smooth outline, elongate, with plastoglobuli and intralamellar inclusions.

Active cambia: matrices dense with clear DNA loci.

Dormant cambia: matrices lighter with diffuse loci.

Dictyosomes:
—cisternae generally straight, evenly spaced and stacked.

Active cambia: some cupping and skewing of cisternae.

Vesicles:
—clear, grey, and spiny-coated generally common.
—cored vesicles present in Gi-fixed active spruce and Gai-fixed active spruce and pine cambia.

Endoplasmic reticulum:
—no pronounced swelling or breakage.
—rough endoplasmic reticulum present but ribosomes were mostly detached, particularly with Gi fixation.

Ribosomes:
—pale and diffuse in outline, polysomes absent after Gi fixation.

Granular microbodies:
—present in all samples.

Multivesiculate bodies:
—present in all samples, characterized by small vesicles in a large lumen.

Tonoplast:
—variable. Large vacuoles often ruptured and reformed into smaller vacuoles.
—most disruption seen in active cambia fixed by Gai.

Inclusions: common. Tubules occurred in Gi-fixed spruce vacuoles.

Plasmalemma:
Active cambia: variably plasmolyzed with large invaginations.

Dormant cambia: mostly non-plasmolyzed.
Pine—fine undulations.
Spruce—many small blebs into cytoplasm, which were more abundant after Gi fixation.
Paramural bodies: vesicles and tubules relatively common in invaginations after Gai fixation. Membrane fold inclusions occurred only after Gai.

Spherosomes:
—some contained an electron-lucid area.
—typically smaller and fewer in active cambial cells than in dormant ones, in which they often appeared to be coalescing.

At 27 C
Different results were obtained by using G at 27 C on cold dormant cambia (Fig. 5).

Microtubules:
Spruce—same as above.
Pine—rare and diffuse.

Cytoplasmic fibers:
—pale, felty, rare.

Mitochondria:
—usually swollen, extracted, and often pouched.

Plastids:
—extracted, sometimes swollen but most often shrunken-appearing with wavy membranes.
—dense intralamellar inclusions occurred as with other G fixations, but pale plastoglobuli were only found in pine plastids.

Dictyosomes:
—cisternae often widely skewed and erratically spaced with wrinkled membranes and wide uneven lacunae.

Vesicles:
—profiles irregular, sometimes ruptured.
—mostly clear, some grey, spiny-coated and cored vesicles absent.

Endoplasmic reticulum:
—often extensively swollen, membranes wavy.
—rough endoplasmic reticulum present but ribosomes mostly detached.

Ribosomes:
—equivalent to Gi fixation.

Granular microbodies:
—some found in pine, but none in spruce.

Multivesiculate bodies:
—absent from all samples.

Tonoplast:
—frequently ruptured.
—many small vacuoles which often contained vesicles.

Plasmalemma:
—poorly fixed.
Paramural bodies: vesicle- and tubule-containing blebs particularly numerous in spruce cambium.

Spherosomes:
—appeared essentially the same as with Gi and Gai fixation.

Glutaraldehyde-osmium tetroxide cocktail (GO)

An example of the preservation obtained with this fixative is shown in Fig. 6.

Microtubules, Cytoplasmic fibers:
—absent.

Mitochondria:
Active cambia: badly swollen and extracted.

Dormant cambia: moderately swollen and extracted.

Plastids:
—saccous, highly extracted.
—intralamellar inclusions lacking, plastoglobuli pale and rare.

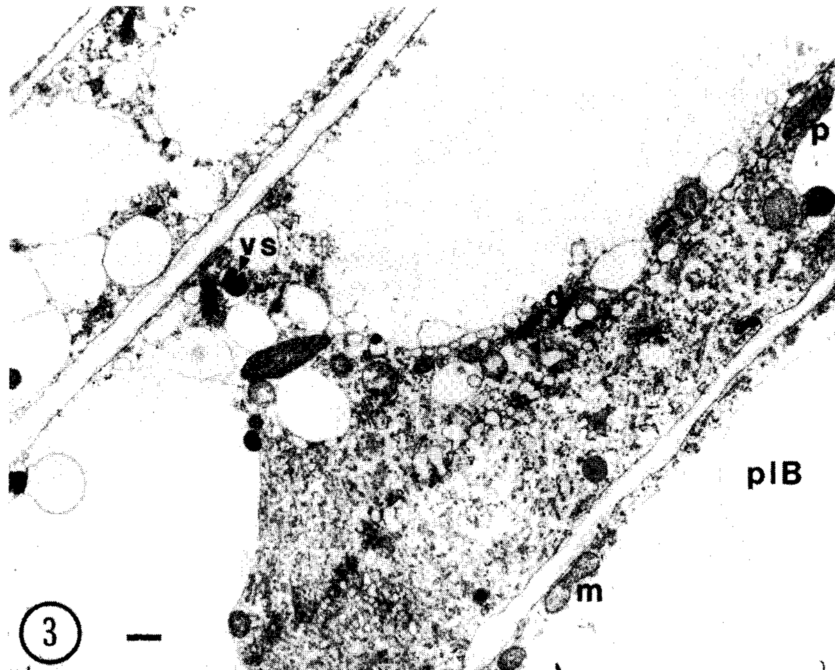
Dictyosomes:
—cisternae wrinkled and erratically and widely spaced.

Vesicles:
—often irregularly shaped.
—mostly clear except for a few grey and cored ones in active cells. Spiny-coated vesicles absent.

Endoplasmic reticulum:
—usually a highly swollen jumble of irregular vesicles and erratic crinkly lamellae.
—ribosomes mostly detached.

Ribosomes:
—large, dark and prominent.
—occurred singly, in clumps, and in large spiral polysomes in the cytoplasm.

Granular microbodies:
—found only in dormant cells.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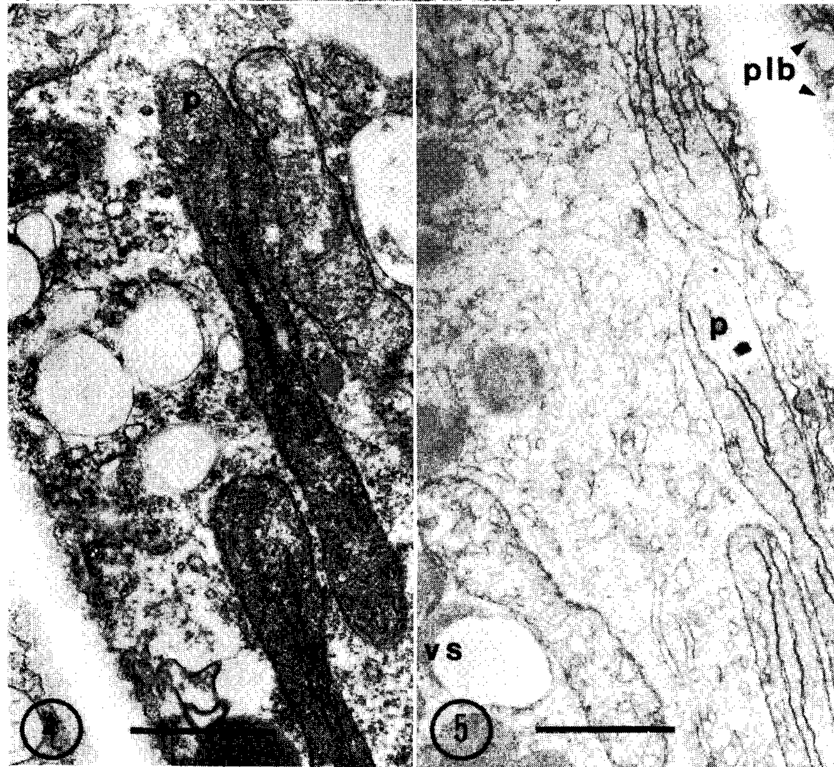
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Multivesiculate bodies:
—present, contained small dense vesicles.

Tonoplast:
Active cambia: variable. Sometimes most large vacuoles were intact, but at other times most appeared ruptured, especially in spruce. The membrane was darker and smoother than it usually was with other fixatives.

Dormant cambia: vacuolar system less well preserved. Vacuoles were often angular and inclusions relatively common.

Plasmalemma:
—appeared to be the best fixed of all the membranes, being dense with a distinct trilaminate structure.

Active cambia: moderately plasmolyzed with large smooth waves.

Dormant cambia: negligibly plasmolyzed with only small undulations.

Paramural bodies: limited to one or two large vesicles.

Spherosomes:
Pine—often extensively peripherally vesiculated.
Spruce—only a few with peripheral vesiculation.

Karnovsky's fixative (K)

Examples of preservation by this fixative are shown in Figs. 1 and 7.

Microtubules:
—appeared exceptionally well preserved.
—abundant in division figure, phragmoplast, and the cell cortex.

Cytoplasmic fibers:
Active cambia:
Spruce—fibers numerous and dark, often showing individual microfilaments.
Pine—fibers rare, faint, without resolvable microfilaments.

Dormant cambia: fibers dark and generally common, sometimes felty.

Mitochondria:
Active cambia: mostly rod-shaped or oval.
Pine—some doughnut and dumbbell configurations.

Dormant cambia: generally less dense and more nearly spherical in profile, although doughnut forms did occur.

Plastids:
—similar to G-fixed plastids but with somewhat lighter matrices and some distention of the internal lamellar spaces, especially in dormant cambia.
—dense plastoglobuli present, but intralamellar inclusions rare in dormant cell plastids and absent from active ones.

Dictyosomes:
—similar to G-fixation image.
—some slight cupping in active pine.

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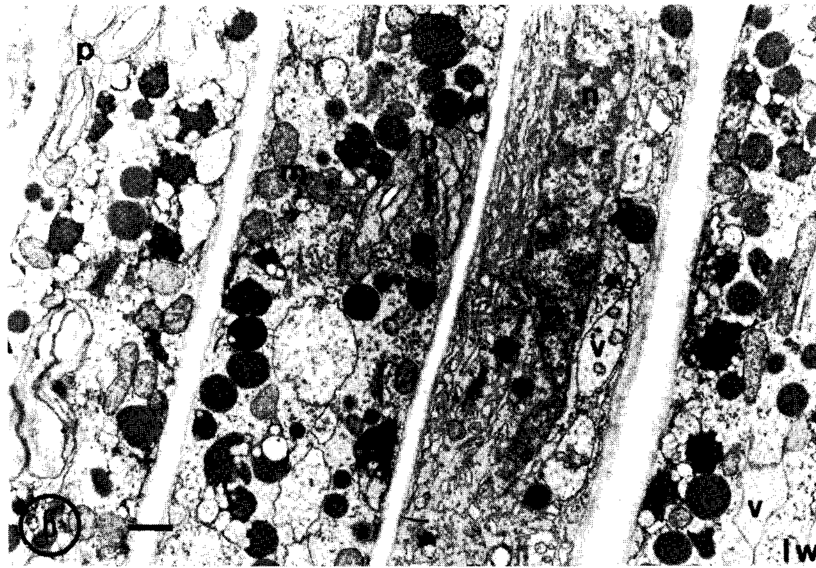
FIG. 3. Gi-fixed red pine cambial zone, showing a phragmoplast containing abundant microtubules. One spherosome contains an electron-lucid area. Part of a large plasmalemma balloon, most of which is excluded from this micrograph, protrudes into the large central vacuole of an adjacent cell. ×4,900.

FIG. 4. Part of a dormant cambial cell of Gai-fixed white spruce exhibiting narrow-cristate, light-matrix mitochondria and electron-dense plastids, one of which has an osmiophilic intralamellar inclusion. The plane of sectioning is perhaps responsible for the obscurity of lamellar membranes. The finely undulate plasmalemma is devoid of blebs in this region. ×19,000.

FIG. 5. Part of a dormant cambial cell of white spruce following G_{27} fixation. Plastids and cytoplasm are extracted, and the plasmalemma is highly folded with many small blebs. ×18,000.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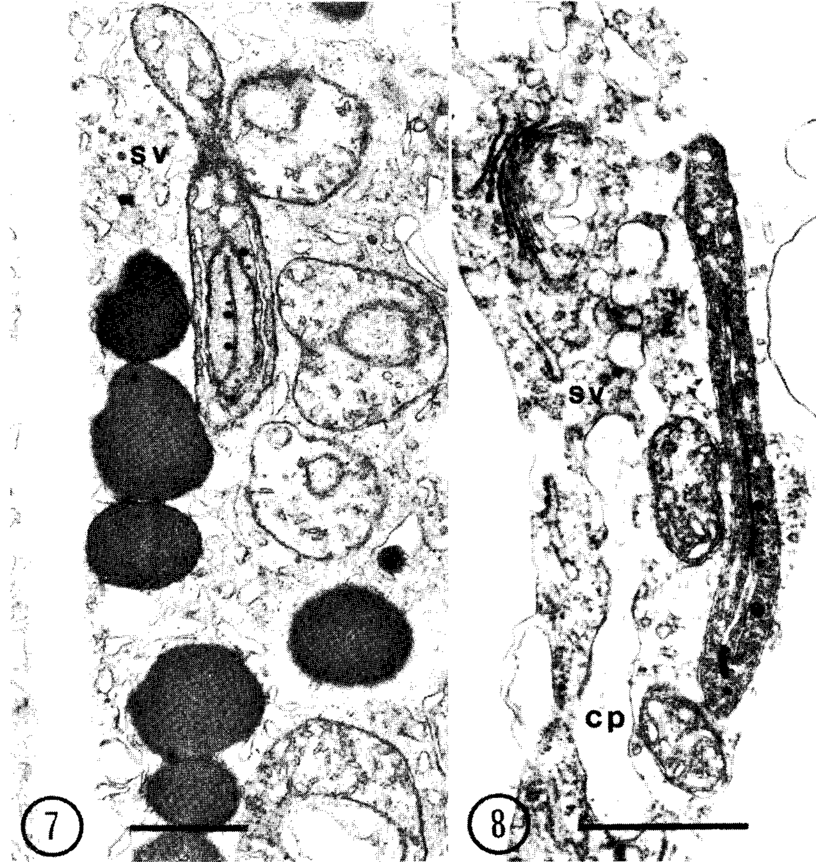
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Vesicles:
Active cambia: all types abundant and well fixed.

Dormant cambia: spiny-coated vesicles rare and cored ones lacking.

Endoplasmic reticulum:
—both smooth and rough endoplasmic reticulum appeared slightly distended, especially in dormant cells.
—ribosomes sometimes detached.

Ribosomes:
—large and dense, occurred in large polysomes and cytoplasmic clusters.

Granular microbodies:
—present.
Pine—common.

Multivesiculate bodies:
Pine—common.
Spruce—rare in dormant cambia and absent from active cambia.

Tonoplast:
—often ruptured in active cells but mainly intact in dormant ones.

Plasmalemma:
—negligibly plasmolyzed and mostly intact.

Active cambia: smooth with small undulations and very few blebs.

Dormant cambia: commonly convoluted into many blebs, more so in spruce than in pine.

Paramural bodies: rare, usually contained vesicles.

Spherosomes:
—occasionally contained an electron-lucid area.
—often appeared to be coalescing in dormant cells.

Except for the following observations, fixation of dormant cambia at 27 C produced results indistinguishable from the above.

Cytoplasmic fibers:
—the most affected structures, pale and without definite microfilaments.

Mitochondria:
—considerable variation, but profiles generally tended to be spherical.

Plastids:
—generally paler than above, and intralamellar spaces seemed swollen.
All cellular membranes tended to be somewhat more wavy.

Glutaraldehyde-acrolein fixative (GA)

Preservation representative of this fixative is shown in Figs. 2 and 8.

Microtubules:
—cortical microtubules present but not abundant in active cambia and scarce in dormant cambia.
—spindle and phragmoplast microtubules absent.

Cytoplasmic fibers:
—exceptionally large, dark and abundant, with resolvable microfilaments.

Mitochondria:
Active cambia: tended to have a swol-

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FIG. 6. Cambial zone of GO-fixed dormant red pine. Plastids are swollen and extracted, and many spherosomes are peripherally vesiculate. Mitochondrial fixation in this specimen is better than average for this fixative. ×6,000.

FIG. 7. K_a-fixed cambial cells of dormant red pine showing doughnut mitochondria and undulate plasmalemmae with some blebs. ×15,700.

FIG. 8. A dividing red pine cambial cell fixed with GA_a, showing cell plate segments, a plastid with osmiophilic intralamellar inclusions, and swollen-cristate, dense-matrix mitochondria. ×22,500.

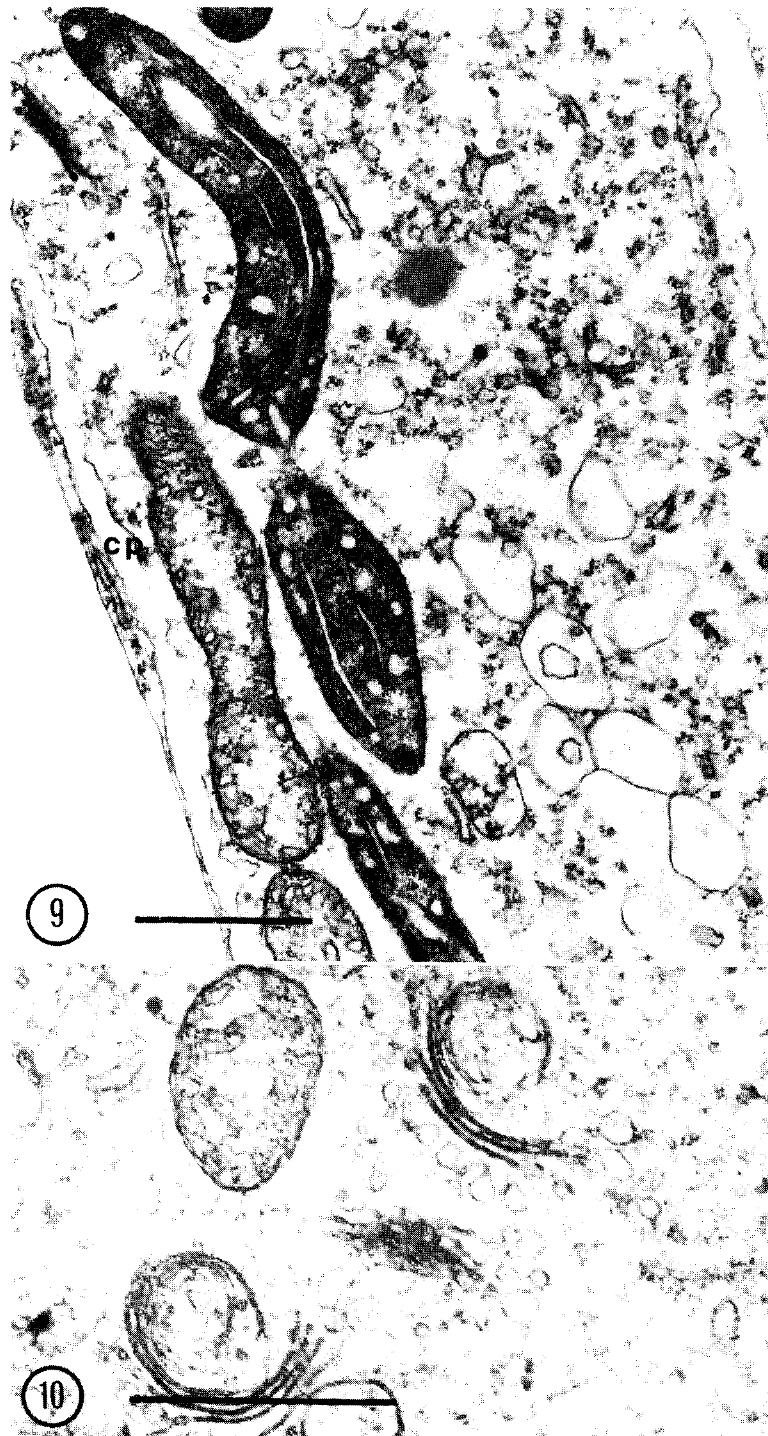

FIG. 9. A dividing cambial cell of TA-fixed white spruce. Narrow-cristate mitochondria and dense plastids border the cell plate. ×23,500.

FIG. 10. Curled dictyosomes in a TA-fixed red pine cambial cell. × 35,000.

len-cristate, dense-matrix form, especially in pine.

Dormant cambia: had the more commonly observed narrow-cristate, light-matrix form.

Plastids:
Active cambia: very dense matrix, wide intralamellar spaces.

Dormant cambia: lighter matrix, membranes sometimes wavy, contained more plastoglobuli and intralamellar deposits than those in active cambia.

Dictyosomes:
—well fixed, with dark, often straight, evenly spaced membranes and cisternae.

Vesicles:
—abundant.
Spruce—all types present.
Pine—cored vesicles not found in dormant state.

Endoplasmic reticulum:
—not swollen.
—rough lamellae common in both active and dormant material.

Ribosomes:
—dense, sharp profiles, and commonly in large polysomes.

Granular microbodies, Multivesiculate bodies:
—common in active cambia only.

Tonoplast:
—seemed mainly intact but was disrupted in some areas.

Inclusions: precipitates and various-sized vesicles and tubules often present in smaller vacuoles. Tubular-appearing structures sometimes crossed the main cell vacuole in near-parallel array.

Plasmalemma:
Active cambia: commonly plasmolyzed and frequently broken, particularly in pine.

Dormant cambia:
Spruce—convoluted into many blebs.
Pine—tended to be wavy with fewer blebs.

Paramural bodies: vesicles, fine tubules and microfibrillar or amorphous material fairly common.

Spherosomes:
—exceptionally small and dense, non-coalescing, and lacking lucid areas.

There were no noticeable effects other than those noted below when dormant cambia were fixed in GA at 27 C.

Microtubules:
—well fixed and fairly abundant in both species.

Mitochondria:
—some in spruce closely resembled the swollen-cristate, dense-matrix active cambial form.

Vesicles:
—spiny-coated vesicles seemed more distinct than in cambia fixed by any of the other methods.

Granular microbodies:
—none found in spruce.

Trialdehyde fixative (TA)

Examples of the results obtained with this fixative are presented in Figs. 9 and 10.

Microtubules:
Active cambia: almost totally lost.

Dormant cambia: only a few, but distinct and seemed well fixed.

Cytoplasmic fibers:
—generally not abundant, dark, with usually unresolvable microfilaments.

Mitochondria:
—typical narrow-cristate G morphology with dumbbell and doughnut forms occurring in pine.

Plastids:
Active cambia: very dark matrices, with even membrane spacings. Plastoglobuli and intralamellar inclusions common in spruce but less common in pine.

Dormant cambia: matrices lighter, membranes unevenly spaced. Some plastoglobuli, but intralamellar inclusions almost entirely lacking.

Dictyosomes:
Active cambia: membranes dense, often unevenly spaced, cisternae skewed.
Pine—cisternae commonly crescent- or ring-shaped.

Dormant cambia: more evenly structured, often appeared to be interconnected by a network of tubules.

Vesicles:
—clear, grey, and distinct spiny-coated vesicles common, cored vesicles absent.
—vesicle profiles sometimes irregular in active cambia.

Endoplasmic reticulum:
—smooth and rough lamellae were common, as were vesicular and branched forms.
—lamellae somewhat erratically swollen in dormant cambia.

Ribosomes:
—abundant, distinct, frequently in polysomes.

Granular microbodies:
—dense, finely granular, common in all samples.

Multivesiculate bodies:
—bodies containing large vesicles were common in all samples.

Tonoplast:
Active cambia: tonoplast reasonably intact, but more often ruptured in pine than in spruce.

Dormant cambia: most vacuoles intact, sometimes contained vesicles.

Plasmalemma:
Active cambia: only slightly plasmolyzed, smoothly undulate with some large invaginations.

Dormant cambia: not plasmolyzed.
Pine—only small undulations.
Spruce—ruffled into many small blebs.
Paramural bodies: some vesiculate and tubulate bodies, especially in active spruce.

Spherosomes:
—always small.
—occasionally contained a lucid area in dormant cells.

Light microscope observations

Our observations by Nomarski interference contrast microscopy of intact, actively streaming cells in freehand sections of cambium revealed no significant differences in the apparent killing and fixing rates of 3% glutaraldehyde, whether the vehicle was cacodylate or PIPES buffer. Penetration was not problematical, and killing occurred more or less simultaneously throughout cells and sections. Total cessation of cytoplasmic streaming and apparent cytoplasmic gelling occurred within 15–20 sec of fixative contact, but Brownian motion of vacuolar particles could persist for a much longer time.

Visible artifact formation was largely restricted to the conversion of the canalicular vacuolar system maintained by cytoplasmic streaming into arrays of small spherical vacuoles. There was no obvious difference in either the type of artifact or the degree of artifact formation between the two buffers. None of the observations could explain or have predicted the poor ultrastructural preservation we had obtained with PIPES buffer.

The African violet (*Saintpaulia* sp.) leaf epidermis hairs examined by Nomarski interference contrast microscopy averaged 3 mm in length and consisted of a single file of at least 10–12 macroscopically pigmentless cells. The cylindrical cells decreased in size markedly from the large basal cell

to the tiny conical tip cell. Observation of fixation was far easier than with cambium sections because of the generally much larger cell size and the one-cell-thick subject. The fixative was unable to penetrate the cuticle and external walls and therefore could only enter via the severed basal cell and progress acropetally through the hair, a situation perhaps simulating that of a column of cells extending from the surface to the centre of a tissue block. Thus, observations of hair fixation could yield insight into the effects of block size and fixative composition on fixation.

Killing and fixing rates comparable to those in cambial sections were observed in the most basal hair cells. The rate of fixative action decreased dramatically with increasing distance from the base of the hair. More than 30 min could elapse before fixative action was observable in an approximately median cell (ca. 1.5 mm from base) and the fixation front could take a further 5–10 min to acropetally span the cell. Pronounced artifact formation similar to that described by O'Brien et al. (1973) was evident only in the slowly killed and fixed cells. As before, no features attributable to the buffer type were discerned.

Cooling the cambial cells slowed cyclosis, but it was not observed to be halted even after chilling for ½ h. This probably relates to the inadequately controlled conditions. However, cytoplasmic streaming in these cold-hardy conifers seems resistant to cold. Streaming was observed to resume after a few minutes of microscopic examination in dormant cambia that had been collected and maintained at below freezing temperature until sectioned.

Streaming in African violet hair cells was more readily slowed but was rarely observed to be halted because cell death seemed to occur very quickly once streaming ceased. Better temperature control was definitely needed.

Cytoplasmic strands and canaliform vacuoles were still present in all genera when streaming was slowed or stopped. Fixative contact created turbulence and vesiculation equivalent to that seen when rapidly streaming cytoplasm was fixed at room temperature.

DISCUSSION

The slightly beneficial effect of the longer (Gai) as compared to the shorter (Gi) duration glutaraldehyde fixation on spruce and the more noticeable effect on pine (particularly dormant pine) may indicate a generic difference, as may the poorer fixation of spruce by GO as compared to pine. Both genera responded similarly to the other fixatives, although pine seemed more damaged by G_{27} than did spruce. It must be recalled that as different trees were sampled for each fixative treatment, the variation between individuals was uncontrolled.

The overall retention of polyribosomal aggregates by Gai fixation and their loss with Gi and G_{27} are unlikely to be related to the initial temperatures of the fixatives, as these temperatures were identical for dormant cambia fixed with Gi and Gai. It seems more probable that insufficient stabilization occurred during the shorter fixation period, and that this may have been dependent upon the intracellular glutaraldehyde concentration attained. Glutaraldehyde is known to penetrate slowly (Feder and O'Brien 1968), and our observations on epidermal hairs show that gelling protoplasm rapidly becomes a profound barrier to glutaraldehyde diffusion, probably resulting in a steeply decreasing intracellular glutaraldehyde concentration gradient. The accompanying slowing of killing and stabilization is paralleled by an increase in the diversity and degree of visible artifacts formed. Polysomes may require more extensive crosslinking and hence a relatively greater glutaraldehyde concentration than other organelles. Alternatively, polysome stabilization reactions with glutaraldehyde may simply proceed more slowly than those of other organelles.

Polysome retention by all other fixatives may indicate that the nature of the buffer has a bearing on polysome retention or disruption. Phosphate buffer is known to cause tissue extraction (Salema and Brandão 1973), but whether glutaraldehyde

penetration into cambium is slower in phosphate than in cacodylate buffer is unknown. The G fixative contained a larger variety of salts (Table 1) than did the other fixative solutions, but the effect on glutaraldehyde penetration is unclear. The presence of buffer salts and of electrolytes has been reported to both increase and decrease the rate of osmium penetration (Hayat 1970). These salts were also present in the phosphate buffered postfixative. As the buffer solution used with OsO_4 has considerable influence on tissue ultrastructure, more so than has the buffer used with the aldehydes (Trump and Ericsson 1965; Hayat 1970), it seems possible that the postfixative could also have been involved in polysome disruption.

The relative uniformity of fixation quality regardless of fixation temperatures with cacodylate buffered fixatives and the poor fixation of dormant cambium by G_{27} imply that fixation temperature per se is less important for good preservation than the particular buffer and fixing agent employed at a certain temperature. Since buffer ions are thought by some (Hopwood 1969) to penetrate tissues before certain fixatives, it may be possible that the phosphate buffer ions influenced cellular ultrastructure even before killing occurred. Phosphate buffer has been suspected of causing, or shown to cause considerable extraction, particularly of tissue protein (Trump and Bulger 1966; Hopwood 1969; Simkins 1972; Salema and Brandão 1973).

The ultrastructural damage characterizing G_{27} fixation (Fig. 5) most probably occurred during the interval between cell death and accumulation of an intracellular glutaraldehyde concentration sufficient to stabilize the ultrastructure. Observations of the progress of a "fixation front" along a hair cell and of the killed cytoplasm behind it indicate that a greater concentration of glutaraldehyde is required for stabilization than is required for killing, and that slow fixation generates excessive artifact formation.

The damage may have resulted from the (perhaps synergistically) combined effects of autolysis and phosphate buffer disruption, occurring at rates preferentially enhanced relatively to the rates of glutaraldehyde penetration and action. The nature of the dormant tissue probably was an important factor in this enhancement, as good results were achieved when active cambium was fixed with Gai at the same initial temperature (27 C) as G_{27}. The high cytoplasmic density and probably much higher osmotic pressure of the dormant cambium undoubtedly retarded glutaraldehyde penetration (perhaps more so than that of the buffer), resulting in a prolonged period between cell death and stabilization when autolysis and buffer-induced changes could occur.

It seems unlikely that the brand of glutaraldehyde used influenced the fixation quality greatly, as the cacodylate buffered fixatives K_{27} and GA_{27} contained the same stock glutaraldehyde as G_{27}, but the fixation results were relatively unaffected by fixation temperature. The mixtures of aldehydes probably penetrated and therefore fixed faster than did glutaraldehyde alone. Some interaction of glutaraldehyde with formaldehyde and acrolein may have been involved in this, but the buffer may also have been a factor significant to both penetration speed and the degree of tissue extraction. Although both acrolein and formaldehyde have been shown to penetrate faster than glutaraldehyde, stabilization (as judged by cessation of Brownian movement) apparently occurs after approximately the same fixation interval with all three aldehydes (O'Brien et al. 1973). It seems probable then that the combination of slow glutaraldehyde penetration and the extractive nature of phosphate buffer resulted in the poor G_{27} fixation of dormant cambium.

Our light microscope observations tend to negate temperature as a critical factor in determining the quality of aldehyde fixation of actively streaming, highly vacuolate cells, at least with respect to cytoplasmic features visible with the light microscope. Fixed cytoplasm appeared about the same regardless of the temperature variations. However, fixations that appear adequate

and identical with the light microscope may not appear so with the electron microscope.

More recently, Mersey and McCully (1976) reported on a similar though well-controlled and more extensive series of experiments utilizing phase contrast optics, a microscope cold stage, and tomato petiolar hairs. They also found that the fixation image of chilled hairs fixed with cold fixative was equivalent to that of room temperature hairs fixed with room temperature fixative. Vesiculation of the canaliform vacuolar system occurred whether the fixative was 3% glutaraldehyde in phosphate buffer, Karnovsky's fixative, or acrolein.

It seems then, that the main effect of cold temperature could be the minimizing of cytoplasmic disruption and extraction during the interval between killing and definite stabilization, the interval when vacuolar contents may be released into the cytoplasm and the cell may be permeable to detrimental elements in the extracellular environment. It also appears that temperature may only be of great importance when certain buffers, fixing agents, and tissue types are combined.

The fixation image produced by GO (Fig. 6) lacks definite features of glutaraldehyde fixation such as microtubule and microfilament retention, and appears to be primarily that of OsO_4 fixation. It is known that the degree of tissue extraction caused by OsO_4 depends on the buffer (Trump and Ericsson 1965; Trump and Bulger 1966; Hopwood 1969), but good results have been obtained with both GO and OsO_4 fixatives buffered with cacodylate (Trump and Ericsson 1965; Franke et al. 1969; Hirsch and Fedorko 1968).

It seems more likely that insufficient reactive glutaraldehyde was available in our 2:1 ratio fixative to counterbalance the undesirable effects of OsO_4. The 2:1 ratio is not necessarily faulty, since good fixation of a wide variety of tissue types has been achieved using either of a 2:1 or 1:1 glutaraldehyde:OsO_4 ratio fixative (Hirsch and Fedorko 1968; Franke et al. 1969). Hirsch and Fedorko (1968) obtained similar fixations with mixtures of from 0.5 to 2.5% of each of the components. However, glutaraldehyde from different sources can behave differently (Weakley 1974), and it may be that our stock glutaraldehyde contained relatively little reactive aldehyde, or was highly susceptible to oxidation by OsO_4 to forms incapable of securely crosslinking proteins. Alternatively, our stock OsO_4 was more highly reactive than the OsO_4 used by others. Either way, the resultant preponderance of reactive Os compounds in the fixative, combined with the probably unnecessary OsO_4 postfixation, may have destabilized most glutaraldehyde crosslinks and resulted in the loss and swelling of cellular components.

Perhaps the 6:1 glutaraldehyde:OsO_4 ratio used by Trump and Bulger (1966) would have yielded better results than our 2:1 ratio. The higher glutaraldehyde concentration should overwhelm the oxidation reaction so that fixation by glutaraldehyde could proceed. Reduction of OsO_4 occurred in the 6:1 solution, but as long as the components were chilled prior to mixing and the mixture was kept cold, the reduction of OsO_4 occurred too slowly to interfere with fixation (Trump and Bulger 1966). Discoloration occurring during fixation with our 2:1 solution indicated a slight degree of OsO_4 reduction, which would not be expected to influence the fixation image.

A particular fixative usually failed to preserve all organelles within the same cell equally well. The performance of individual fixatives also varied with the seasonal activity of the cambia and between spruce and pine. Besides generic differences, a causative factor could have been differences in response to the osmolality of the fixative. It is surprising that dormant cambial cells were fixed so well by the same fixatives used for active cambial cells, unless there is actually little seasonal variation in the osmotic pressure of the cytoplasm, and the multitudinous large spherosomes which usually pack the dormant cells are not osmotically important during fixation.

It may also be possible that dormant cells do not respond to osmotic stress in the

same manner as do active cells. The manner in which fixation affects the osmotic properties of cells is not yet well understood. It has been reported that glutaraldehyde, acrolein, and OsO_4 destroy the cell's ability to respond to osmotic stress (Carstensen et al. 1971; Penttila et al. 1974), but other reports indicate that cells retain osmotic properties after aldehyde fixation and only become totally permeable after OsO_4 fixation. Penttila et al. (1974) have suggested that a small amount of protein per unit area may preclude sufficiently rigid crosslinking to provide osmotic insensitivity. If so, the highly vacuolate active cambial cells should remain osmotically sensitive following aldehyde fixation, but the much less vacuolate, more densely cytoplasmic dormant cambial cells should lose their osmotic sensitivity. The degree to which membrane selective permeability is altered (Salema and Brandão 1973) should also influence the osmotic sensitivity of the fixed cells.

Perhaps our fixation could have been improved by using more concentrated fixative solutions such as the "high osmolality" Karnovsky fixative with which Timell (1973) obtained excellent fixation of both active and dormant cambial-zone tissues. There is some evidence that a high tonicity solution may yield better penetration (Hopwood 1969), but other studies (Bone and Denton 1971; Weakley 1974) indicate that aldehyde fixation can reduce the osmotic pressure of tissues by about 40%. Thus an isotonic fixative solution could become sufficiently hypertonic in relation to the fixed tissue to cause osmotic stress. As noted by Rasmussen (1974), the total osmolality of the solution should not be so important as the concentration of the component (usually felt to be the buffer (Yamamoto 1963; Weakley 1974)) which is unable to cross membranes freely.

Arborgh et al. (1976) have shown that osmotic damage is dependent on the effective osmotic pressure of the fixative solution as determined by the vehicle concentration, and is not related to the total osmotic pressure. The glutaraldehyde concentration is immaterial, except that a higher concentration seems to provide better stabilization. Since only the aldehyde concentration of Timell's (1973) "high osmolality" Karnovsky fixative differed from that of our Karnovsky fixative, the effective osmotic pressure should have been about the same and the consideration of factors causing differences in fixation quality can be limited to such items as the total aldehyde concentration, duration of fixation, and specimen characteristics. Fixative properties other than osmotic pressure were certainly of greater importance for the preservation of many cellular components.

Microtubule preservation was undoubtedly related to the fixing agent used. Both Os and acrolein are known to disrupt microtubules (Hepler and Palevitz 1974), and in this study microtubules were disrupted to varying degrees by GO (totally or nearly so), TA, and GA. Summer microtubules were nearly completely destroyed by TA fixation, but winter microtubules were fixed nearly as well by TA as by K. This could indicate that winter microtubules differ from their summer counterparts or that the destructive components of the fixative (acrolein and DMSO) were perhaps unable to act in the same manner in both dormant and active tissue, or to penetrate simultaneously with the stabilizing components.

Cortical microtubules were apparently well fixed by GA, but spindle and phragmoplast microtubules were totally lost, implying that they are more labile than cortical microtubules. There may be as many as four physiologically distinct types of microtubules in the same cell (Behnke and Forer 1967).

Spindle and phragmoplast microtubules were only retained by G and K fixatives, and K fixation was superior to G fixation. Schultz and Case (1968) also found a synergistic interaction between the microtubule-retaining abilities of glutaraldehyde and formaldehyde. The aldehydes obviously countered the microtubule depolymerizing effects of the Ca ions (Weisenberg 1972) present in K fixative. Luftig et

al. (1976) have found that both cacodylate and phosphate buffers caused extensive microtubule depolymerization and proposed for optimal microtubule retention a fixation medium which contains PIPES buffer and known microtubule polymerization agents.

The fixation quality of cytoplasmic fibers was more difficult to judge than that of microtubules because a fiber could vary considerably in appearance along its slightly undulate length. Even the apparently best-fixed fibers had disarrayed and felty portions, which suggested different functional states within the fiber. Recently, Nagai et al. (1976) have shown that microfilament arrangements in contractile plasmodial strands of *Physarum polycephalum* change from a paracrystalline arrangement of straight parallel cross-linked microfilaments when the strand is maximally-elongated to a feltlike network of entangled microfilaments when the strand is maximally-contracted. Similar activity could explain some of the heterogeneity of cambial fibers, although fixation was also a factor.

Straight microfilaments were commonly seen after G fixation, with waviness and felty masses being more common after Ka and GA fixations. Perhaps the cytoplasmic fibers were stabilized by G after they had become static and elongated, whereas Ka and GA may have retained them in more active, or more fully contracted, configurations. However, the more poorly fixed fibers appeared to have degenerated by a loss of microfilament arrangement and electron density. Spooner et al. (1971) have suggested that disarrayed microfilaments are more labile than ordered ones.

Microfilaments have been reported to be less well preserved by acrolein or glutaraldehyde/acrolein than by glutaraldehyde or glutaraldehyde/formaldehyde; and to be more often retained if an initial ambient temperature fixation is continued in the cold (O'Brien and Thimann 1966; Parthasarthy and Mühlethaler 1972). In our material, cytoplasmic fibers were disarrayed by G_{27} and K_{27} fixation, but seemed otherwise unaffected by fixation temperature and duration. Contrary to the above reports, cytoplasmic fibers were usually abundant and thick following fixation in acrolein-containing fixatives, and were characterized by exceptional electron density. Acrolein probably allowed the good results with GA_{27}. The scarcity of fibers after TA fixation and their total absence after GO fixation probably indicate DMSO- and Os-lability of the fibers.

The generic difference was quite pronounced; of the best fixatives (Gi, Gai, GA, Ka), only GA preserved pine microfilaments as well as it did spruce microfilaments. This suggests that there may be several types of microfilaments, as seems to be the case with microtubules (Behnke and Forer 1967).

Mitochondrial configurations also varied with the fixative. Narrow-cristate, light-matrix rod- or oval-shaped profiles were typical of G fixation (Figs. 3, 4), except G_{27} and GO which produced swollen and extracted circular profiles. After K (Fig. 7) and TA fixations, the typical G configurations were augmented by doughnut and dumbbell forms, both of which occurred more commonly in pine and were nearly exclusive to the active state. Such configurations may represent different views of curved discs (Öpik 1968), and seem related to formaldehyde fixation. If they represent dynamic mitochondrial configurations, their absence would mean that mitochondria had changed shape prior to stabilization. However, O'Brien et al. (1973) have reported the universal occurrence of abnormalities in mitochondria fixed by formaldehyde.

Mitochondrial fixation quality appeared to be the most uniform after GA fixation, although there was a definite seasonal variation in mitochondrial configuration. Active state mitochondria (Fig. 8) were characterized by a swollen-cristate, dense-matrix form, particularly in pine; dormant state mitochondria assumed the narrow-cristate, light-matrix form. The condensed form could be an artifact, or a functional configuration retained by acrolein. The

seasonal variation could reflect the different cellular activity levels, as mitochondria apparently become condensed when carrying out oxidative phosphorylation and transform to the narrow-cristate, light-matrix form when performing succinate-induced electron transport (Öpik 1968; Hackenbrock 1972).

The small bulges and pouches observed in mitochondria after G and K fixations could represent normal pleomorphic structures. However, they seemed to be more common with poorer fixations (G_{27} and GO), and there is light microscopic evidence that mitochondrial pouching is a fixation artifact (O'Brien et al. 1973).

Dictyosomes were relatively insensitive to variations in fixation conditions (except for G_{27} and GO), but there were some notable features and generic differences. The cupping and skewing of active cambial dictyosomes following G and K fixations were only occasionally observed in spruce but were quite common and more pronounced in pine. Dictyosomes in TA-fixed active pine showed an extreme form of this in which one or more cisternae were complete rings around which the other cisternae curled and skewed (Fig. 10). Dictyosomal cupping and skewing were rarely found after GA fixation (Fig. 8), which could mean that their formation is promoted by glutaraldehyde and formaldehyde and prevented by acrolein in some instances. However, such forms may not be artifacts because dictyosomal activity and morphology can change considerably with the physiological state of the cell. Curled, ringlike, loosely associated dictyosomes have been reported as typical of "resting, replicating, or regenerating dictyosomes" in either quiescent or meristematic higher plant cells, or to be induced by CO_2 or KCN treatment (Morré et al. 1971; Mollenhauer and Morré 1966a).

Also exceptionally common after TA fixation (particularly in pine and active cambia) were complex networks of tubules which frequently appeared to be linking dictyosomes, and dictyosomes with fenestrated or tubular cisternal extensions.

If the curled and interconnected dictyosomes and tubular networks are the vital forms, then the separate flattened discoid dictyosomes typical of glutaraldehyde fixation are probably artifacts. It seems possible that the random cytoplasmic movements that can occur during the interval between initial contact and the final stabilization by glutaraldehyde (O'Brien et al. 1973) could both dissociate and redistribute fixative-altered dictyosomes. Tubular networks and cisternal extensions could fragment into groups of vesicles as large cell vacuoles do; and cisternae could flatten, similarly to the rounding-up of mitochondria (O'Brien et al. 1973). The combination of aldehydes and DMSO may penetrate and fix so rapidly that this could not occur; or TA may not be as membrane-disruptive as other fixatives.

Evidence that acrolein-containing fixatives may provide the best stabilization of membranous structures is provided by the most labile cellular membrane, the tonoplast, which showed least apparent rupturing when fixed by TA and GA. The most complex tonoplast elaborations occurred after GA fixation. Thick, unbranched, straight tubules which appeared to be tonoplast invaginations were often seen in scalariform arrangements radially crossing the large vacuoles of active cambial cells. The smaller vacuoles in dormant cells were often crammed with smaller bent and coiled tubules. These inclusions were usually most frequently observed in cells having the least apparent tonoplast breakage, suggesting that they may occur in vivo.

However, the least-ruptured plasmalemmae (Ka- and TA-fixed) were associated with the fewest numbers of vesiculate and tubulate paramural bodies. These were most abundant when somewhat more plasmalemma breakage was apparent (GA and Gai fixation), which could indicate that they are artifacts arising in conjunction with membrane breakage. The real or artifactual nature of paramural bodies and similar membrane elaborations has been an unresolved controversy since the first of these structures was observed.

It does at least seem possible to conclude that the poorer the fixation, the less complex and detailed is the cellular ultrastructure. Compare, for example, the results we obtained from GO and K fixations. Esau et al. (1966) observed that fixation with OsO_4 left the plasmalemma smooth and undulate, but after sequential glutaraldehyde-OsO_4 fixation, it was irregular and associated with paramural bodies. The contrast between the almost diagrammatic image produced by $KMnO_4$ fixation and that of standard double fixation is well known. Nevertheless, this does not mean that the tonoplast and plasmalemma elaborations and complex dictyosome configurations cannot be artifacts.

Of the remaining cellular components, spherosomes showed the most definite fixation-dependent variations. The apparent fusing of dormant cell spherosomes seems to be enhanced by glutaraldehyde and discouraged by acrolein and formaldehyde, as fusion configurations were relatively common after G fixation, rare after K, and absent after GA or TA fixation. There was a direct relationship between spherosome size and apparent confluency. Bounding membranes were always so difficult to resolve that it was impossible to determine whether fusion had actually occurred, and it is quite possible that the large G-fixed spherosomes were merely deformed by crowding and not confluent. Electron density varied inversely with the spherosome size, indicating that the spherosomes had either swollen or shrunk.

Electron-lucid areas (particularly common in pine) were not seen in GA-fixed spherosomes, were rare after TA fixation, and were most common after G (Figs. 3 and 5) and K fixations. Extensive peripheral vesiculation of spherosomes occurred almost exclusively in GO-fixed pine (Fig. 6). Kidwai and Robards (1969b) felt that spherosome vacuolation represented cytoplasmic vacuole formation by the digestion of the stroma at the onset of cambial activity, but this interpretation fails to account for the above fixative-dependent variations. Neither does the conversion of spherosomes into fat or oil droplets through intermediate forms having clear central cores (Frey-Wyssling et al. 1965) agree with our observations.

It seems more probable that since the stroma is heterogeneous (Sorokin and Sorokin 1966; Frey-Wyssling et al. 1965; Gahan 1968), differential stabilization of the various stroma components and alterations in the permeability of the limiting membrane could permit the leaching out of various components and the swelling or shrinking of the spherosomes. The presence of lucid areas representing the loss of lipidic stroma materials might be expected following G and K fixations because, although both glutaraldehyde and formaldehyde react with lipids, the reactions are sufficiently slow and weak to allow lipid extraction (Jones 1969; Sabatini et al. 1963). Glutaraldehyde may even extract some lipids (Trump and Bulger 1966), whereas acrolein is thought to prevent the loss of lipids by rapidly crosslinking them to proteins (Jones 1969; Sabatini et al. 1963). The lucid areas might also represent bubbles of fluid imbibed by the spherosomes after the stromata had expanded maximally.

OsO_4 is known to be a poor protein fixative and to react primarily with lipids (Hayat 1970), but it also may destroy some fatty acids and lipidic substances (Hopwood 1969). Peripheral spherosomal vesiculation probably indicates the loss of some stroma proteins and lipids different from those potentially lost with G and K fixation.

The predominance of lucid areas and peripheral vesiculation in pine spherosomes seems to represent a generic difference in stroma composition.

SUMMARY AND CONCLUSIONS

None of the fixative solutions tested provided artifact-free fixation, but considering overall fixation quality, our best results were obtained with Karnovsky's fixative (K), followed by the glutaraldehyde-acrolein combination (GA), and the glutaraldehyde fixative (G).

Slightly better results (mainly polyribosome retention) were provided by long-

duration G fixation (Gai), but generally differences between the short and long duration fixations were so slight that prolonged fixation may be considered unnecessary except with certain material (e.g. dormant pine cambium).

Fixation temperature was only critical for above-ambient temperature fixation of dormant cambia in a phosphate-buffered fixative.

Rapidity of fixative action was probably more important for fixation quality than were other factors; and since the speed of glutaraldehyde penetration decelerates rapidly with the depth of penetration, blocks should be cut as small as possible while avoiding mechanical damage to the tissue.

For any one fixative, the fixation image and quality varied between active and dormant cambia and between genera. Variations were more pronounced with some fixatives than with others.

Generic differences in response to fixation were more pronounced with some organelles than with others, and the particular organelle(s) most affected varied with the fixative.

Some organelles were apparently more sensitive to fixation conditions than others and as a result all organelles were not preserved equally well by the same fixative. In choosing a fixative then, it is necessary to decide which cytological features are most important to fix well.

The ultrastructural appearance of some organelles such as mitochondria and cytoplasmic fibers was altered by individual fixing agents such as formaldehyde and acrolein. Therefore, any ultrastructural study of a cell component should utilize various fixatives. For example, cytoplasmic fibers were exceedingly dense after fixation by acrolein-containing fixatives, but the microfilaments tended to be wavy and unevenly spaced. Following glutaraldehyde fixation, the microfilaments tended to be straight and evenly spaced, as though cross-linked by uniformly sized bonds. The fibers, however, were so pale that they blended into the ground plasm, particularly in dormant cells.

Light microscope observations should also be included in cytological studies, as they yield information on the original cell structure and how it is affected by treatments such as temperature variations and fixation.

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ERRATUM

I am indebted to Prof. Robert J. Hoyle, Jr. for drawing my attention to a slip in my paper "Sampling Strategies for Destructive Testing." [Wood and Fiber 7 (1975): 178–186.] The "greater than" on the top line of the second column of page 182 should read "less than." The Monte Carlo study was, however, carried out correctly.

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