THE MICROTOME-CRYOSTAT FOR CUTTING WOODY TISSUES[1]

Harvey D. Erickson and *Yang-Hsien Chen*
College of Forest Resources, University of Washington, Seattle, WA 98195

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ABSTRACT

A microtome-cryostat was used on short blocks to produce high-quality frozen sections of woody tissues suitable for staining and mounting with no use of organic solvents. The method leaves the extractives intact. Temperatures about −30 C were preferred. Transverse sections were of excellent quality and were usually better than longitudinal sections. A high degree of water saturation is necessary for good ice-embedding support. Sections from 2 to 12 μm thick were cut from the wood of Douglas-fir, western hemlock, southern pine, and red alder. Phloem tissue was sectioned successfully. The cryostat method deserves more general use since it is more convenient and easier to control than the usual CO_2 cooling system.

Additional keywords: Tsuga heterophylla, Pseudotsuga menziesii, Pinus spp., *Alnus rubra, Picea alba*, wood structure, histology, cryostat, microtomy.

INTRODUCTION

The difficulties encountered in making acceptable sections for microscopy usually are based on the tendency of the cells to separate and collapse into the lumens or to be torn in one or more layers of the wall. Fragile structures such as tori in bordered pit pairs and soft tissue such as phloem and cambial tissues are subject to various degrees of damage or displacement from their normal positions. Various embedding techniques have been developed to provide a rigid matrix that will support the cells and will itself section well. Embedding agents that have found considerable use are: waxes, paraffin, celloidin, methacrylates, glycols, and epoxy resins. The use of nearly all of these agents requires slow replacement with solvents and impregnation by diffusion or carefully applied pressure, and, with resins, time to polymerize them. If shrinkage, due to phase change or polymerization takes place, an unnatural condition develops and the fine structures may be disturbed or damaged. When time is important and when natural materials in the tissue should not be dissolved out by organic solvents, most embedding processes may not be satisfactory.

Biologists have to some extent used the technique of sectioning frozen tissue to avoid these problems. Frozen tissue sections have been used in histological diagnosis during surgery for over 80 years in this country. Welch, in 1891, performed the first sectioning of frozen tissue from a lesion during surgery for immediate examination. The method became extensively used in pathological diagnosis. The frozen tissue method had some disadvantages, however: It required some special apparatus, there were difficulties in adequate freezing, adherence of the block to its mount to resist the knife force, maintaining a frozen condition during sectioning, controlling temperature, and preventing unwanted or excessive frost formation on the working surfaces.

Modifications of the method were devised by quite a number of medical researchers, some of whom are: Adamstone and Taylor (1948); Bush and Hewitt (1952); Schimizu et al. (1956); Thornburg and Mengers (1957); and White and Allen (1951). Wilcox (1964) tried the frozen section technique with decayed wood but

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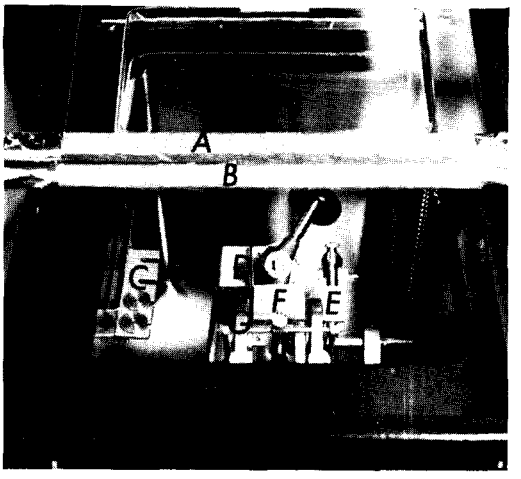

FIG. 1-A. Top and interior of microtome-cryostat. The plastic curtain is fastened to a wood strip, A, that lies on the edge of the folded-back lid and may be taped to the cabinet. The curtain has been rolled back on the wood dowel B. The curtain material is "see-through" roll film used for wrapping roasts, etc. Parts of the equipment are: C—Freezing block with specimen disc mounts; D—Specimen block on a disc set in the swivel head; E—Knife; F—Section antiroll device; G—Knife clamps.

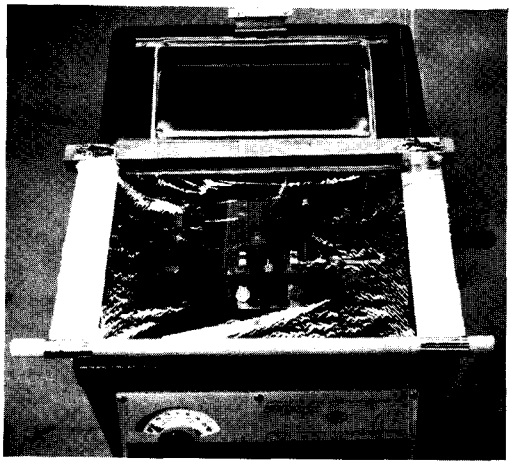

FIG. 1-B. Plastic curtain rolled out to cover the opening.

did not find it satisfactory. In all of these cases, the blocks and the microtome were cooled or frozen with CO_2 gas or dry ice. The first open-top cryostat was constructed by Chang et al. (1961). It employed a condenser cooling system for the enclosed microtome and a temperature regulator.

Most of the experience with the cryostat technique for frozen sections has been limited to homogeneous and soft tissues such as kidney, muscles, soft plant stems, and periderm (Mullick 1971). We were unable to find reports in which this technique was used on the comparatively dense and anisotropic wood tissue. Such application was explored during a study of the effects of subfreezing temperatures on wood and is the basis of this report.

MATERIALS AND PROCEDURES

The basic machine was a Microtome-Cryostat, Model CTD, with a Minot Custom rotary microtome, made by International Equipment Company. Temperature can be controlled in the cold chamber down to −30 C. The knife used was an IEC 3257. About 6 hr are required to bring the chamber temperature down to −30 C. We encountered some difficulty, when the hinged lid was kept open, in maintaining uniform optimal operating conditions because of circulating laboratory air that mixed with the air in the chamber and sometimes caused clouding, frosting on the knife, and frost buildup on the walls.

A plastic curtain was constructed and placed over the opened forward half of the top, which permitted observation into the chamber but prevented entry of ambient air (Fig. 1A and 1B). We used transparent plastic sheet that was less than 1 mil thick and the full width of the cryostat. The back edge is wrapped around and fastened to a wood or metal strip and the edge toward the operator is rolled around a wood dowel about ¾″ in diameter and fastened to it. The back strip lies either in front of the hinge of the lid or, preferably, on the front edge of the open section of the lid, and the dowel end is rolled back or forth to uncover the chamber. The back strip may be taped to the cryostat to hold it in place. This system creates more stable conditions than the opening and closing of the lid during sectioning and removal of the sections.

A room at 20 C was available in which

the relative humidity could be reduced to about 20%. Because of the lower dew point, better performance and results were obtained when this room was used. In combination with the added shield, the result was good.

The materials sectioned were green western hemlock (*Tsuga heterophylla* (Raf.) Sarg.), green Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), green red alder (*Alnus rubra*, Bong.), dried and resoaked Douglas-fir, and southern pine (probably *Pinus taeda*, L.) and cambial and phloem tissue of Douglas-fir trunk and fresh Norway spruce twig (*Picea alba*, Karst.). All figures are 1050×.

Preparation of sections

The blocks were sawn to 1 cm by 1 cm by 2 cm along the grain. From this a smaller block about 4 mm long in the fiber direction was sawn with a jeweler's saw with No. 8 blade. For cutting cross sections, the short block was generally split with a razorblade into pieces about 2.5 mm radially, or one annual ring wide, if convenient, and 8 mm tangentially. For radial and tangential sectioning, the blocks were cut to 3.5 mm on all sides.

It is very important that the specimen blocks be as nearly saturated with water as possible to obtain the highest quality sections. Fresh wood was kept wet during preparation of the blocks and then submerged in water for 1 hr before trimming. Specimen blocks prepared from dried wood were submerged in water and evacuated for ½ hr, after which they were soaked for 1 hr. When the length of the block is about equal to, or less than, the fiber length, one cycle of vacuum is often sufficient. Complete saturation to the center may not be necessary unless many serial sections are desired. The blocks were usually trimmed on the cryostat microtome and then placed in water for 12 hr at 3 C to assure saturation before freezing and cutting. If this extra freezing is undesirable, the trimming may be omitted or done under water.

The knife designed for the microtome is somewhat thinner than other brands. The knife angle setting was used mainly at 30° as suggested by the manufacturer. Minor adjustments from this angle seemed to have no noticeable effect, but we have not ex-

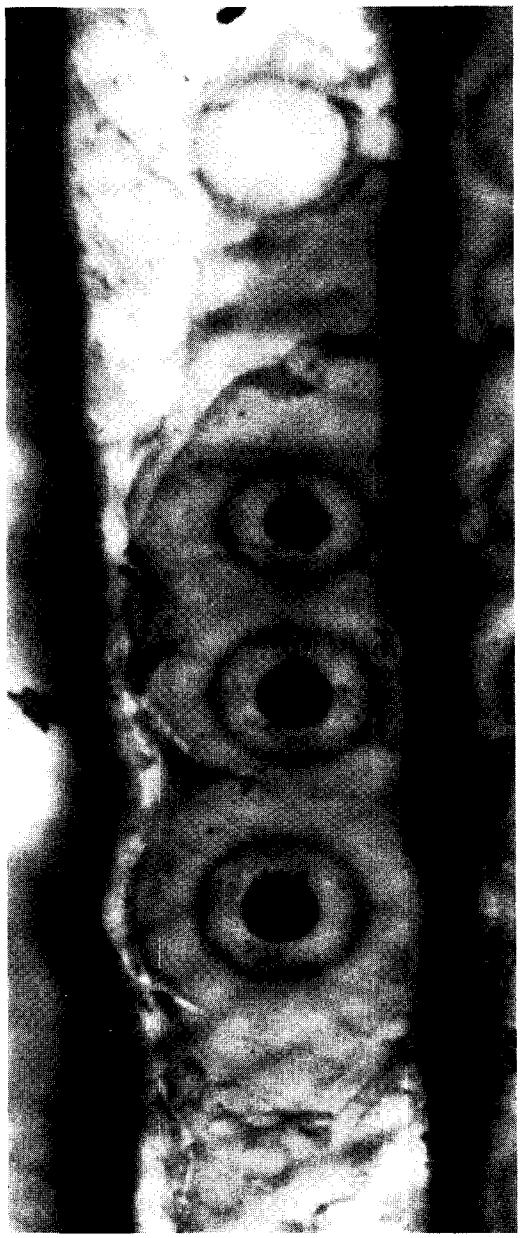

FIG. 2. Radial section of Douglas-fir showing some fracturing of layers of the tracheid wall during cutting in the direction of the fiber axis; 6 μm, Karo mount.

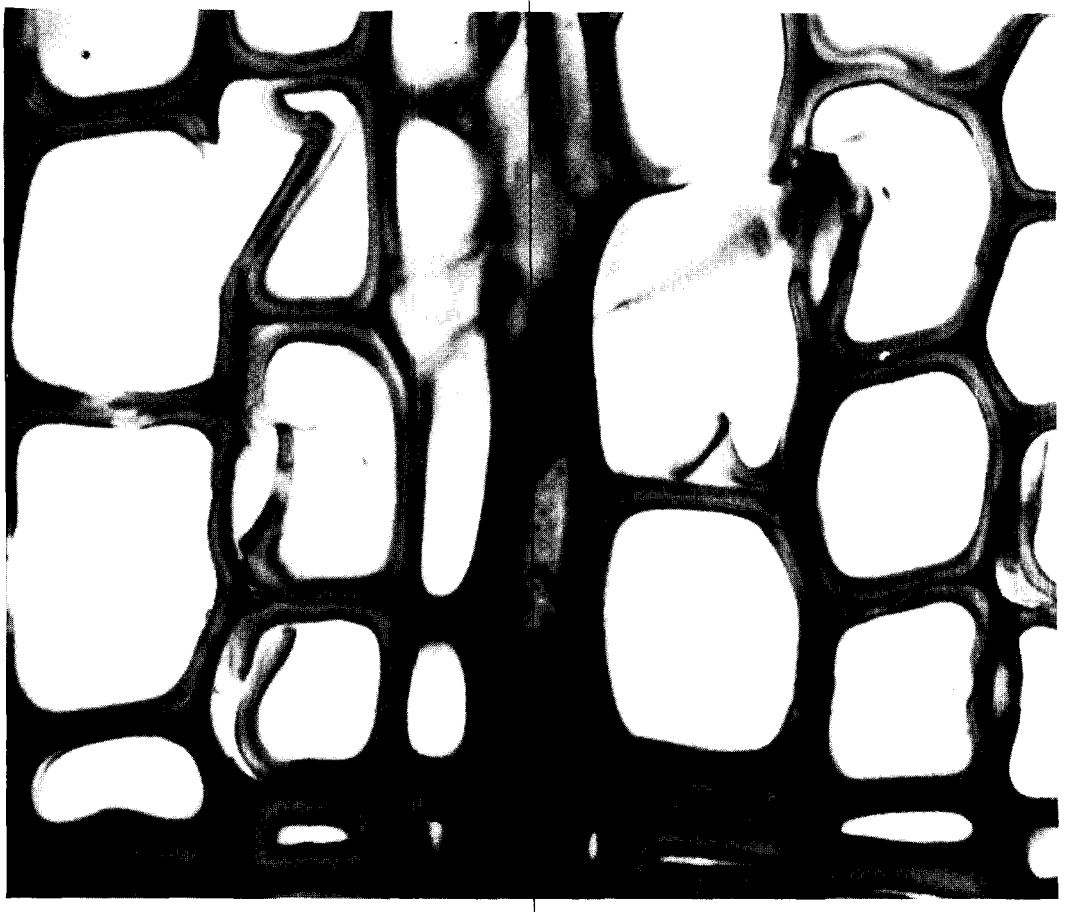

FIG. 3. Section of frozen natural western hemlock heartwood with moisture content of 50%. Breakage of tracheid walls indicates insufficient ice embeddment; 6 μm, Karo mount.

plored this topic fully. The knife edge bevel was 10.5° center line to surface. Knives were sharpened on a Shandon-Elliot automatic sharpener with the fine grinding done with ¼ μm Metadi diamond paste mixed with oil.

Sectioning procedure

The cryostat takes about 6 hr to pull down to –30 C, dial temperature. The cutting block is adhered to the metal specimen disc by placing a few drops of water on the disc, placing the block on it, and inserting the assembly into a slot provided in the quick-freeze block in the chamber. The top surface of the specimen should be wet. For wood tissue, 15 min is adequate. Protection from any drying during freezing and interim periods in sectioning can be prevented by covering it with a teflon film, a waxed cover glass, or a rubber policeman.

The anti-roll device was helpful for obtaining sections free from curling and folding. As the sections are cut and lie on the knife, they are picked off with a small, soft, compact brush and transferred to a precooled slide. Warming the bottom of the slide under the specimen by pressing it on a warm object or with the finger tip thaws the section and causes radial and tangential sections to flatten readily. Cross sections are somewhat more difficult, but with a slightly longer hold-down with the brush they will flatten adequately.

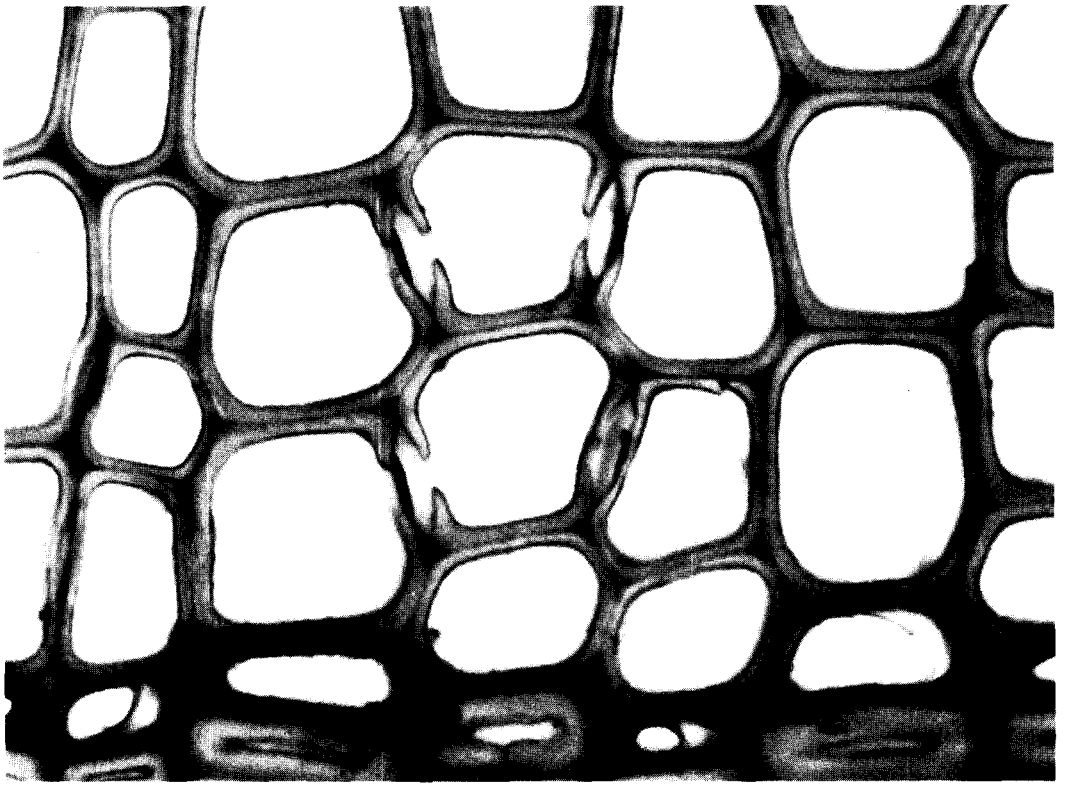

FIG. 4. Matched sample of block in Fig. 2 sectioned after thorough impregnation with water and freezing. Proper ice embeddment permits good quality sections; 6 μm, Karo mount.

Most of the sections less than 12 μm thick were stained on the slide to avoid unnecessary handling. Sequential staining with hematoxylin and safranin was used except for phloem tissue, which was dehydrated and stained with fast green. After washing, excess liquid was removed by careful blotting around the section, after which it was mounted in Karo syrup. Some sections were solvent-dehydrated and mounted with standard media.

RESULTS AND DISCUSSION

The quality of frozen-tissue sections depends upon the temperature used, sharpness of the knife, cutting speed, embedding medium, structural direction of the cut, and tissue characteristics.

Gauge temperatures from –20 to –30 C were tried in cutting western hemlock. The optimum sectioning temperature was near –30 C. With substantially warmer temperatures, the sections were less uniform in quality, and around –10 C there may be poor adhesion of the block to the specimen holder. Preventing frost formation on the knife, by excluding ambient air, minimized breaking and tearing of the sections. The walls of the chamber are the cooling surface and, as vapor condenses onto them, it causes drying of the interior air and consequent sublimation from the knife of frost formed on the moist track from contact with the wood block.

Temperature varies within the cryostat chamber with the lid closed. For example, the dial and wall temperatures were –31 C, the knife temperature was –23 C, and the air around it was –25 C. With the lid open, the knife temperature was –21 C. The relative humidity near the knife was 30%. With the lid opened for operation, the interior

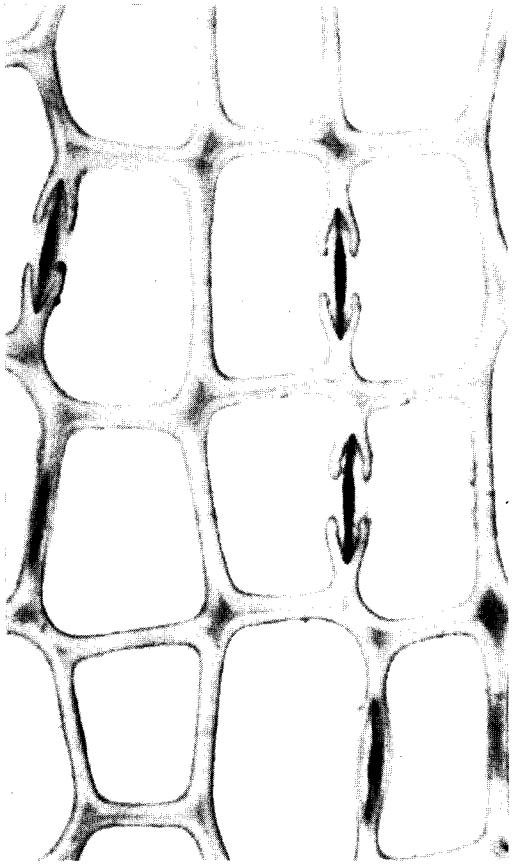

FIG. 5. Typical section cut from frozen green western hemlock sapwood thoroughly soaked before freezing and cutting; 6 μm, Karo mount.

temperature will vary according to the room air conditions and the disturbance by mixing.

Water as an embedding medium has the advantages of low viscosity, compatibility with natural and moist tissue, no disturbance of cell structure, easy and quick penetration of the cells and the cell walls, good swelling of the cell walls with accompanying softening of the walls for better cutting, and good firmness as a solid to prevent movement and separation of cell components under the severing force of the knife. There is no problem or time lost in removing the embedding medium; the only requirement is a simple phase change to liquid.

A commercial glycol liquid, cryoform, sold as an embedding medium for the usual soft tissues to be cut as frozen tissues, was tried on western hemlock. The block, 1.5 mm tangentially by 3.5 mm radially by 3 mm along the grain, was soaked in the medium for 4 days at room temperature, with a change of liquid after 2 days. Cross sections 6 μm thick were of good quality, but the sections were not of uniform thickness. Also the sections tended to curl tightly before they could be flattened on a slide.

Our early work on cutting frozen xylem was in the longitudinal plane with the knife moving parallel to the fiber axis and the knife edge oriented 90° to the fiber axis. As it turned out, this was not the best direction for good results. Some fracturing and peeling of surface layers in the knife plane were seen on the cross walls of the tracheids (Fig. 2). However, such fractures are also noticeable but to a lesser degree, perhaps, in sections cut from unsoftened wood at room temperature with a sliding microtome. Some bordered pit pairs in tangential sections showed damage to the margos and dislodged tori and, in some cases, damage to the border itself especially in thin sections 4 to 6 μm. One can not rule out some damage due to handling of the thin sections.

When the knife-edge orientation was parallel to grain and the knife moved across the fibers, either radial or tangential in the longitudinal plane, there was less damage to the bordered pits. Some fracture lines were still visible, usually near cell-wall corners, but the walls parallel to the plane of the cut were in good conditions. The preferred cutting direction for longitudinal sections was across the fiber. Cutting parallel to the fiber axis may produce a splitting action ahead of the knife causing excessive stressing of the pit borders in tangential sections and rupturing of the margo. Kennedy and Chan (1970) reported that the least damage to strength of microsections occurred when the knife edge was parallel to the grain direction and the slice angle was small. Our results are in agreement, although they used a sliding micro-

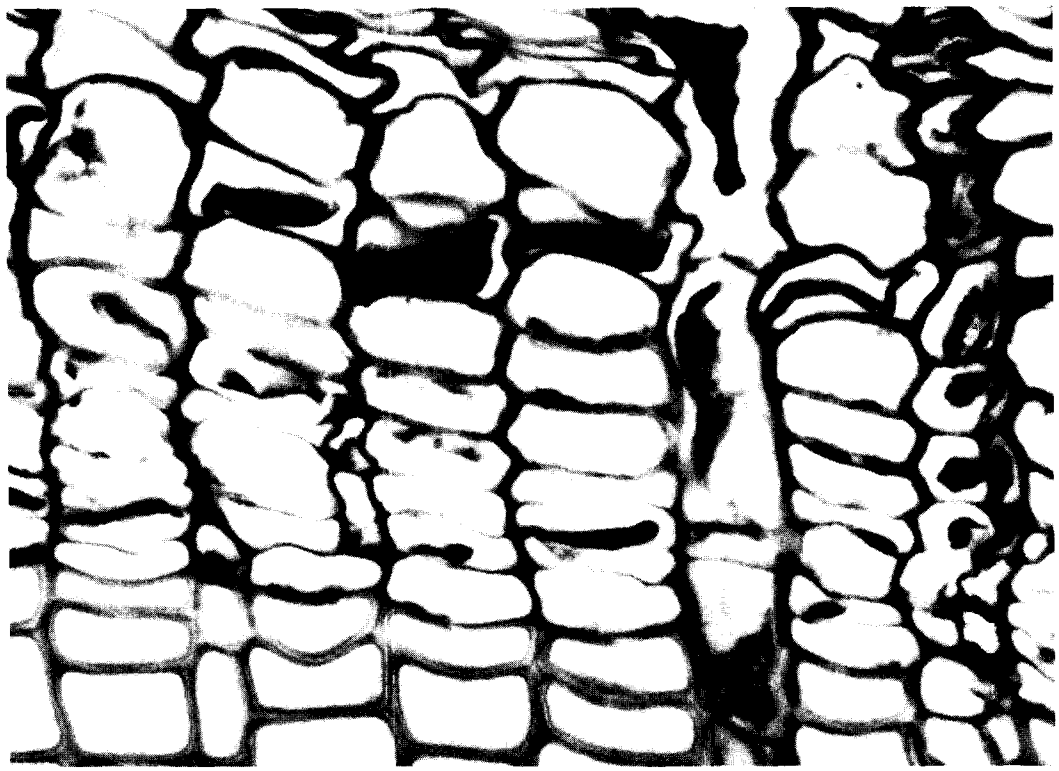

FIG. 6. Transverse section through xylem, vascular cambium, and part of the phloem of a green Norway spruce twig; 8 μm, Karo mount.

tome with a longitudinal component of friction and cutting, which was absent in our cutting with the rotary microtome.

In contrast to certain deficiencies of some longitudinal sectioning techniques, the transverse sections of frozen, water-saturated blocks were quite easy to cut and were almost free from cutting damage over large areas. Bordered pits were undamaged and tori were not torn loose. Most of our anatomical studies employed this type of section because of the ease of producing excellent stained mounts. The best cutting was in the tangential direction, parallel to the growth ring. This was almost mandatory if thick, dense latewood was present because, (1) there was less chance of breaking loose the specimen block from the base plate because of the high mechanical resistance to the knife of a wide front of hard latewood across the entire section as would occur in cutting radially, and (2) the thickness variation within the section was minimized if there was no abrupt density change in the direction of cutting. Blocks that are long and narrow, and mounted with the wide face parallel to the knife edge, will break loose more easily than short blocks (4 μm), which are either square in cross section or are widest in the cutting direction.

Satisfactory cross sections of western hemlock heartwood were not obtained when sectioned at 50% MC. Tracheid walls separated and were pushed into the lumens and tori of bordered pits were dislodged (Fig. 3). However, when matched blocks were thoroughly impregnated with water and frozen, the microtome sections were excellent, as shown in Fig. 4. High-quality sections were also obtained with southern yellow pine sapwood and Douglas-fir sap-

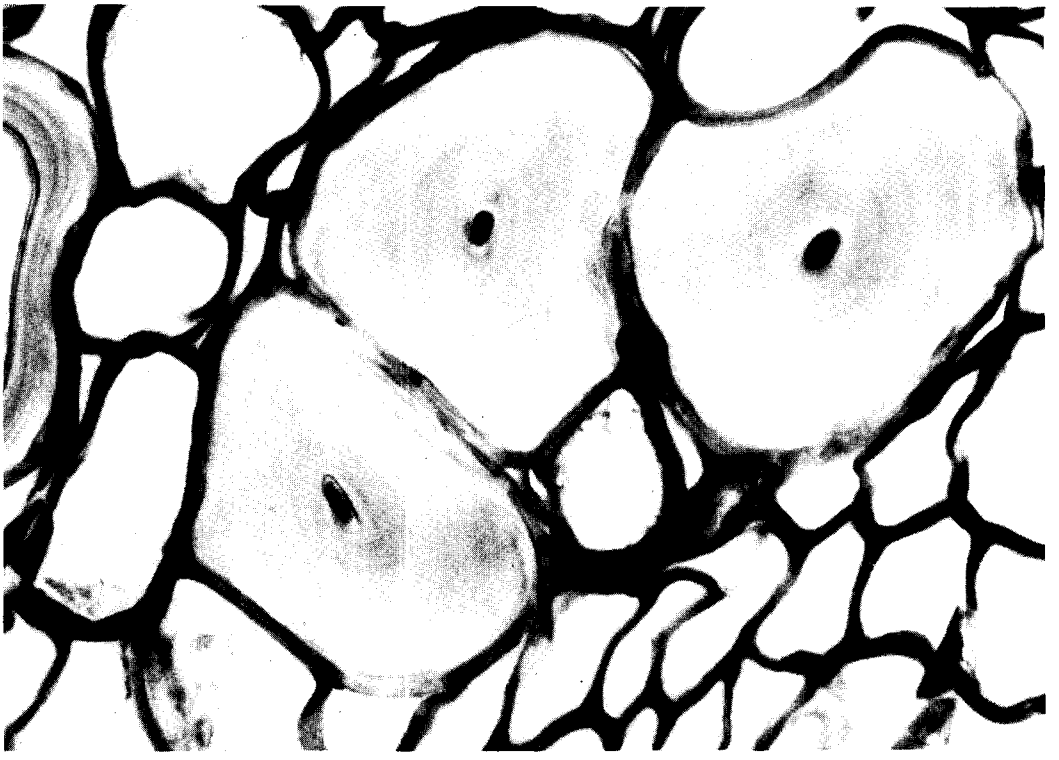

FIG. 7. Phloem tissue of green Douglas-fir stem showing sclereids with concentric layering. The section was solvent-dehydrated, stained with fast green and set in Permount; 10 μm.

wood and heartwood. The epithelial cells in southern pine were sectioned best at a thickness of about 10 μm.

Xylem sections were produced with about equal quality between 12 and 4 μm; a few were tried at 16 μm with fair results. Thin sections in the range of 4 to 6 μm were quite easily produced and were cut more easily than unembedded green xylem. Figure 5 shows a 6-μm section of western hemlock with undistorted bordered pits. Such sections are very useful for certain minute structure studies. Radial sections, 2 μm thick, of western hemlock were cut satisfactorily but similar cross sections were of erratic thickness. Radial sections 4 to 6 μm were exceptionally clear in detail of the ray cells.

Deposits in the phloem or xylem tissues are not disturbed or removed by cryostat sectioning. Deposits in the tracheids of green Douglas-fir heartwood were left intact during cutting and slide preparation. When they were present, they occurred mainly in the corners of the cells with a miniscus shape as if this were due to surface tension effects.

The effectiveness of the cryostat-microtome technique was tested on undried phloem tissue of Douglas-fir and Norway spruce. The initial trials produced variable quality; some cambial cells and sieve cells had broken or distorted walls. Additional blocks were prepared with greater precaution to prevent drying during block preparation and, after end trimming, allowing two days of soaking in the refrigerator to obtain greater water saturation. From these specimens, generally excellent sections were produced (Fig. 6) although not all areas were equal in quality. Sclereid cells, with their thick walls, cut very well (Fig. 7). This sectioning technique should be very useful for cambial and phloem tissue

studies even though these tissues are more difficult to section and process. Perhaps an agar matrix as suggested by Evenden and Schuster (1938) for immature nut tissues might help to hold the delicate sections together, although Mullick (1971) was able to cut good periderm sections using only Lab-Tek O.C.T. as a sectioning aid when cutting sections in a cryostat.

Theoretical considerations

When frozen water-embedded tissue is sectioned, there is cutting of ice in the cell lumens and other microscopic openings as well as cutting of the swollen cell walls. However, the water in the cell wall is not frozen in the conventional sense because shrinkage in wood takes place upon drying at the subfreezing temperatures used in this study (Erickson et al. 1968).

Unlike that of most solids, the melting point of ice is lowered by pressure. Thornburg and Mengers (1957) proposed that since the melting point of ice is lowered approximately 1 C for each 10^9 dynes/cm^2 of applied pressure, melting will occur in the cutting region and heat will flow into the melting zone from the adjacent areas. However, their knife temperature was much colder than the block, a fact that was not true in our study. They computed the fusion energy from a measure of the cutting resistance of the block. For a resistance of 20 gm/cm of block width, the energy dissipated per cm of knife travel will be 20 gm $\times$ 980 dynes/cm $\times$ 1 cm = 1.96×10^4 ergs. Converting to calories and dividing by the heat of fusion, this energy is sufficient to melt a layer of ice about 60 nm thick.

The thickness of the micro-melting zone will increase as both the block and knife temperatures rise. At some point it will approach the thickness of the section being cut, especially for thin sections, and the unsupported cellular material will likely lodge near the edge of the knife. Even if the whole section is not in the melt zone, a melt zone at the cutting edge of 1 μm or more probably will cause damaged surfaces on those cells that are difficult to cut.

In the sectioning of tissues, much of the heat energy developed in the cutting process is conducted into the knife and some goes into the adjacent frozen tissue. As temperature is lowered well below the freezing point, the melting zone becomes thinner because of the much greater pressure necessary to melt ice at low temperatures. A sharp knife edge will narrow the zone of cell-wall severence and allow a pressure build-up on the ice to the point where the energy absorbed will melt the ice. The pressure in kg/cm^2 that is required to melt ice increases greatly with decreasing temperature. For example, it is 1 kg at 0 C, 615 kg at –5 C, 1625 kg at –15 C and 2200 kg (31,300 lbs/in^2) at –22.1 C (Hodgman et al. 1956), which is near the actual knife temperature used in most of our cutting. Thornburg and Mengers (1957) state that at some point, as temperature is lowered, the pressure required to create a micromelt zone will exceed the mechanical strength of ice and will change the character of the cutting process. We had no evidence of such a change at the temperatures we used. We suggest, however, that the high knife-edge pressures that are required to melt ice at low temperatures may also cause mechanical failure of the ice by a combination of cleavage at or near the very edge of the knife and by tension perpendicular to the plane of the section. The crystalline structure of ice and the fracture planes would influence the cleavage patterns, but we are not aware that this information exists for water in the lumens and pit chambers of fibrous cells.

Cutting speeds that are too slow may cause refreezing of the melted water to the knife at any given point before it travels away from the bevel. We found a cutting speed of 0.3 cm/sec to be satisfactory for xylem and phloem. The exact speed is not critical but very rapid cutting produces torn and distorted cells as well as rapid dulling of the knife. This may be an expression of mechanical failure, as described above, and excessive pressure on the knife edge.

CONCLUSIONS

A cryostat with rotary microtome has been used to produce high-quality sections for study directly or after staining. No organic solvents are required; hence, there is no distortion due to solvent dehydration. Karo syrup was used for semipermanent mounts. Substances soluble in the usual microscopy solvents are not removed from the xylem or phloem leaving them in a natural conditions. It is important to saturate the cells to make maximum use of the support offered by the ice.

Temperatures in the range of –20 C to –30 C are satisfactory for most tissues. The lower temperature was generally preferred. Cuts on the cross section were easily made and were of high quality. In longitudinal sectioning, a cutting direction across the fibers gave less tendency to cleavage than did a direction parallel to the fiber axis. Cross sections of 4–6 μm thickness pose no problem in cutting. Radial sections 2 μm thick were also cut. Minimizing ambient air entry into the chamber by a plastic curtain device and working in a low-humidity room contribute to improved quality of the sections.

The method was used on several species and on phloem as well as xylem. Quick freezing of the specimen block did not appear to give adverse effects to the cell structure. The microtome-cryostat should find more extensive use in studies of xylem and phloem tissues, fibers, and glue-lines.

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