8. The Celloidin Method

Infiltration

The celloidin process is used for subjects that are tough, brittle, or friable (crumbly). Paraffin does not afford adequate support for sectioning such materials. One example of a subject for which the celloidin process must be used is a graft union (Fig. 13.9 *a*), in which the incompletely united members must be kept intact before and after sectioning. Another illustration is the preparation of pathological materials which may be in such disintegrated, fragile condition that the sections would fall apart without the celloidin matrix. Sectors from large trees having wood, cambium and bark tissues cannot be kept intact without embedding in celloidin (Fig. 13.9 *b, c*). Unembedded small twigs are difficult to hold and orient for longitudinal sectioning without some matrix. A twig or sector from a tree can be embedded in celloidin, blocked as shown in Fig. 8.2 *D–F,* and sectioned accurately in any desired plane. A properly selected piece of material may yield 100 sections, uniform in thickness, orientation, and staining properties. Permanent slides can thus be made by the hundreds at low cost. In some laboratories the celloidin method is neglected, or even scorned, but the above illustrations show that the process has its place in any well-equipped, versatile laboratory.

The matrix for the celloidin process is a form of nitrocellulose, known by several trade names — celloidin, collodion, Parlodion, and some less common names. This product is sold in the form of shreds or chips, packed dry or in distilled water. The latter method retards the development of a yellow color. Celloidin should be dried thoroughly before being dissolved for use. The most commonly used solvent consists of approximately equal volumes of ether and methyl alcohol. These reagents must be of the best quality and *anhydrous.* Five stock solutions of celloidin are usually used. These solutions contain, respectively, 2, 4, 6, 8, and 10 g. of dried celloidin per 100 cc. of solvent. These solutions are designated for convenience as 2% celloidin, etc.

Infiltration in celloidin consists of transferring the previously killed and dehydrated tissues into a dilute solution of celloidin, concentrating the celloidin, and finally molding the thickened celloidin into blocks containing the material. Concentrating the matrix may be accomplished by one of the following processes or by combinations of processes.

1. Transfer the tissues thorugh a graded series of celloidin solutions of increasing concentration.
2. Add chips of dry celloidin at intervals to the initial 2% solution.
3. Evaporate the solvent from a large volume of a 2% solution.

METHOD 1

Transfer the dehydrated tissues from the dehydrant into the solvent. After one to several hr., transfer to 2% celloidin, covering the material with at least five times its volume of celloidin. Fasten a dry, rolled cork into the bottle by means of wire loops (Fig. 8.1 *A, B*). Put the bottle into the 53°C. oven.

The interval in the oven varies widely. For sections of twigs having a diameter of 3 to 5 mm., 24 hr. in 2% celloidin may be enough. For larger pieces and for dense materials increase the time to 2 days or more. After the interval in 2% celloidin, *cool the bottle,* remove the stopper, and pour the celloidin into a dry pan (not into the sink!). Keep away from flames or sparks. Cover the tissues immediately with 4% celloidin. Reseal the bottle, and repeat the interval under pressure in the oven. Repeat this operation with 6, 8, and 10% celloidin. Following the last treatment, continue to thicken the celloidin by adding a chip of dry celloidin every 24 hr. When the celloidin is so thick that it just flows at room temperature, the material is ready to be hardened as described on page 81. To determine whether the consistency of the celloidin is correct for blocking, dip a thoroughly dried matchstick into the celloidin, lift out a mass of celloidin, and immerse in chloroform for 1 hr. The celloidin should become hardened into a clear, firm mass that can be sliced easily with a razor blade. A comparison of samples taken during successive stages of infiltration will show the progressive increase in firmness and improved cutting properties.

METHOD 2

The material is first given at least 48 hr. in 2% celloidin in a sealed bottle in the oven. At intervals of several days the bottle is cooled and unsealed, a chip of dried celloidin added, and the bottle

sealed and returned to the oven for the next interval. When working with delicate material, the celloidin chip should not be dropped onto the material but tied into a bag of dry cheesecloth, which is then suspended in the bottle so that the celloidin is just immersed in the solution (Fig. 8.1 *C*). The periodic addition of celloidin is continued

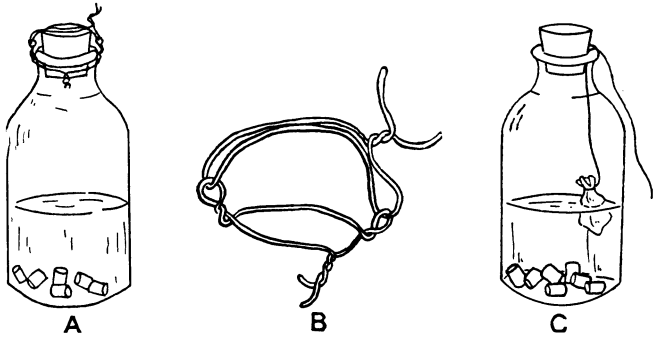

FIG. 8.1—*A*, Specimen bottle for infiltration with celloidin under pressure, with stopper fastened by wire loops; *B*, detail of wire loops for fastening cork; *C*, cheesecloth-bag method of thickening celloidin.

until the solution is thickened to the degree described in the preceding method.

METHOD 3

This method is very slow but yields superior results. The material is started in a large volume of 2% celloidin, at least four times the depth occupied by the material. Mark the initial level of the solution. Cork the bottle loosely, but wire the cork so that it cannot be pushed out. Keep the bottle in a warm place, away from flames or sparks. Slow evaporation takes place, and, when the volume is one-half the original, the solution is in approximately 4% celloidin. Add new 4% celloidin to make up the original volume. If the celloidin has become colored, replace with new 4% solution. Continue the process of slow evaporation until the material is in thick celloidin. An objectionable feature of method 3 was pointed out by Walls (1936). If the evaporation rate is too rapid, it seems that the ether of the solvent evaporates more rapidly than the methyl alcohol, and the celloidin jells before adequate thickening is obtained. This can be remedied by adding a small quantity of pure ether and continuing infiltration until the proper viscosity is attained.

A low-viscosity nitrocellulose has been recommended for rapid infiltration of firm materials (Davenport and Swank 1934; Koneff and Lyons 1937). This inexpensive celloidin forms a firm matrix, and its solutions in ether-alcohol tolerate 6% water, thus minimizing the extreme brittleness produced in woody materials by total dehydration. The above references give complete details of procedure.

Hardening and Blocking

In the celloidin process the solvent is not eliminated completely during infiltration. The thickened celloidin solution is hardened by immersion in chloroform. Remove an infiltrated piece of material and a mass of enveloping celloidin and immerse in chloroform. The celloidin loses its stickiness at once and soon becomes hardened throughout. It is best to leave the material in chloroform for 12 hr. to harden the celloidin in the innermost cells of the material. Transfer the hardened material into a mixture of approximately equal volumes of 95% ethyl alcohol and glycerin, in which the material may be stored indefinitely.

Large pieces of embedded wood may be removed from the glycerin-alcohol, clamped directly into the microtome, and sectioned. Subjects having easily separable soft tissues are often damaged by compression in the clamp. Such materials are sectioned best by mounting them on blocks of wood or plastic. The mounting block may then be clamped rigidly into the microtome clamp without damaging the tissues. A twig or other long slender object should be mounted into a plastic tube or a wood block having a hole of suitable size drilled lengthwise through the mounting block (Fig. 8.2 *A–C*). Prepare mounting blocks by drying them thoroughly in a 110°C. oven, soak in anhydrous methyl alcohol, then store in waste 4% celloidin until needed. When the material being infiltrated is put into 8% celloidin, drop a prepared mounting block into the specimen bottle and continue the infiltration.

To mount twigs for cutting transverse sections, remove the desired twig and a suitable drilled mounting block from the thickened celloidin, and push the twig into the hole, leaving 6 to 10 mm. of the twig protruding. Fill any space remaining around the twig by pushing slivers of matchstick into the hole from below. Wrap a generous mass of thick celloidin around the twig and mounting block, and harden in chloroform (Fig. 8.2 *C*). For longitudinal sections of a twig, lay the infiltrated twig on a large, undrilled block, wrap well with additional thick celloidin, and harden in chloroform.

When the surface of the celloidin is hard (2 min.), press the twig gently until it is flat on the mounting block, thus affording firmer support for sectioning. A batch of embedded material usually contains more pieces than are needed for immediate sectioning, therefore, only a few pieces need to be mounted on blocks. Most of the pieces are merely removed from the thick celloidin, hardened in chloroform, and stored in glycerin-alcohol. The pieces can be blocked at any future time by the method to be described later.

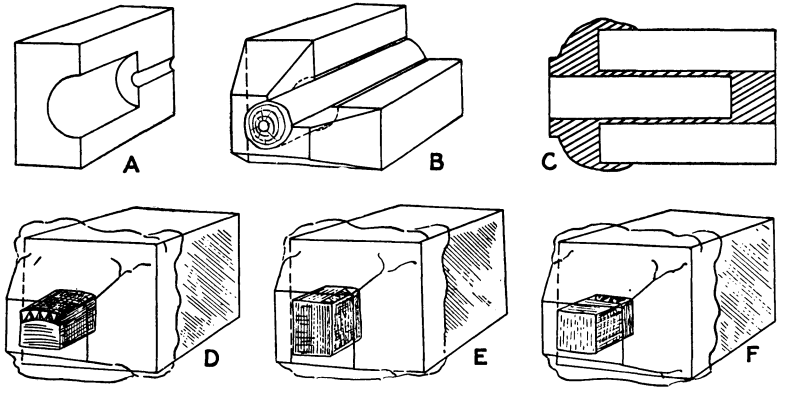

FIG. 8.2—Methods of mounting tissues on blocks for sectioning in celloidin: *A*, cutaway view of drilled block for holding long object; *B* and *C*, cutaway views of drilled block containing twig or other long object enveloped in hardened celloidin; *D–F*, infiltrated blocks of wood and bark embedded in hardened celloidin, oriented on mounting blocks to cut transverse, radial, and tangential sections, respectively.

Sectors from large limbs usually must be fastened on mounting blocks for sectioning. If the pieces have cambium and other tissues of the bark, these tissues may peel off when the piece is compressed in the microtome clamp. Mount three pieces from each subject, on separate blocks, so that transverse, radial, and tangential sections can be cut (Fig. 8.2 *D–F*). A generous wrapping of celloidin should envelop part of the mounting block as in Fig. 8.2. The rigidity of the mounting of such material can be improved by mounting the material in a recess that has been drilled about 1/16 in. deep into the end of the mounting block.

Blocking of previously hardened embedded material is a simple operation. Remove the desired pieces from the glycerin-alcohol storage fluid and soak in anhydrous *ethyl* alcohol. Change the alcohol twice at 4- to 8-hr. intervals. This removes the small amount of water

left from the storage fluid, softens the celloidin, but does not dissolve an appreciable amount. Transfer the pieces into thick celloidin of a consistency suitable for casting. Also put a supply of mounting blocks into this thick celloidin. After at least 24 hr. in thick celloidin, mount and harden as previously described. It is sometimes necessary to trim pieces of tissue prior to reblocking in order to establish the correct cutting planes. Trimming should be done when the pieces are removed from glycerin-alcohol. The glycerin prevents drying and shrinkage of the tissues during trimming.

Waste celloidin from various stages in the process can be salvaged by evaporating in a shallow pan in a place free from dust and open flames. The dried sheet is cut into shreds, dried at 53°C., and used to make solution for treating mounting blocks and to make 2 and 4% celloidin for method 1. Celloidin solution that is too discolored to be salvaged is most easily disposed of by pouring it into a pan of cold water. The celloidin hardens into a crust which can be lifted out and discarded.

Cellosolve is the trade name used for two synthetic organic compounds, ethylene-glycol-monoethyl ether and its methyl homologue. These fluids are solvents of celloidin and may ultimately replace the inflammable alcohol-ether solvent used heretofore. These solvents are not inflammable at ordinary working temperatures, therefore the entire process may be carried out in open or loosely stoppered bottles. The evaporation rate is very slow at 50 to 55°C. Methyl Cellosolve, which boils at 124.3°C., evaporates slightly faster than ethyl Cellosolve, which boils at 135.1°C. The latter solvent is preferred for fragile subjects which tend to collapse if the celloidin concentration is increased too rapidly.

An inexpensive method of using Cellosolve is to dehydrate the tissues in the appropriate grades of alcohol and to transfer to a 2% solution of celloidin in Cellosolve. Subsequent infiltration may be accomplished by successive treatment in 4, 6, 8, and 10% solutions at 50 to 55°C., or by beginning with 2% and periodically adding celloidin chips by the cheesecloth-bag method. The interval in each grade ranges from 24 hr. for small or porous pieces to a week for large blocks of wood.

Cellosolve may also be used as the dehydrating agent. Materials in which the preservation of the protoplast is not important may be transferred, after killing and washing, directly into Cellosolve. Make two or three changes of Cellosolve before beginning the infiltration. However, if the material requires thorough or gradual dehydration

to insure adhesion of the celloidin, it is better to dehydrate in ethyl alcohol or acetone and to use the much more expensive Cellosolve as the infiltration solvent.

Special Treatment of Hard Woods

The foregoing methods of infiltration yield excellent results with some soft woods such as willow, poplar, basswood, white pine, and many other woods. These can be infiltrated in celloidin without special preliminary treatment, but oak, hickory, walnut, the yellow pines, and other woods are too hard to section by the regular process. Such materials can be softened by treating with hydrofluoric acid (HF). This highly corrosive reagent is purchased in wax bottles and should be used in wax or wax-lined or plastic containers. Because of the corrosive action of the liquid and vapor on glass, metals, and the skin, HF should be used in an isolated part of the premises, away from valuable instruments. The staining and microchemical reactions of tissues are materially altered by this treatment.

Twigs having living bark tissues are first killed as usual and transferred to HF. Dry woods are prepared for treatment in HF by alternate boiling in water and exhausting in an aspirator in cold water until the pieces are saturated. The safest concentration of HF for most subjects is commercial acid diluted with approximately twice its volume of water. The duration of treatment in HF varies greatly with the hardness of the material, the size of the pieces, and other factors. As a trial, treat a hard wood such as oak for 5 days, wash in running water for at least 1 hr. to make it safe to handle the pieces, and try to cut thin slices with a sharp razor blade. An alternative method of testing is to wash the pieces in running water for 4 hr., clamp a piece into the sliding microtome, and test its cutting properties. After making a test, wash and wipe the clamp and the knife thoroughly. If the material is too hard to cut readily either freehand or with the microtome, return to the HF for another 3- to 5-day interval, and test again. When the wood seems to cut satisfactorily, wash for at least 48 hr., whether it is to be embedded in celloidin or cut without embedding. Wood or twigs that have been treated with HF and are to be put into storage without embedding should be dehydrated through 20, 40, and 60% alcohol or acetone at 4- to 8-hr. intervals, then stored in a mixture of equal volumes of alcohol, glycerin, and water.

The softening of wood can be accelerated by treating in HF under pressure. The necessary equipment is not available commercially

and must be built to specifications. A satisfactory apparatus, described by Chowdhury (1934), consists of a section of iron pipe with a threaded flange at each end. Plates are bolted to the flanges, and the upper plate is removable for introducing the specimens. The compression chamber is lead lined and is provided with a pressure gauge and a valve to which the pump is attached. Chowdhury recommends 40% HF and a pressure of 80 lb. He found that 1-in. cube blocks of *Juglans regia* were adequately softened in 3 days; blocks of *Diospyros melanoxylon,* an extremely hard wood related to our persimmon, required 7 days. The equipment necessary for this method is amply justified if a considerable amount of diagnostic work on timber woods is being carried on.

Material embedded in celloidin can be cut as soon as the celloidin has been hardened in chloroform and the volatile chloroform replaced with the glycerin-alcohol storage fluid. The cutting properties are improved by prolonged storage in glycerin-alcohol. If materials having dark-colored bark and light-colored wood are stored for several years, the storage fluid dissolves coloring matter from the bark and imparts a dark color to the wood. Stained sections from such wood do not have bright, clear colors. The stock of embedded twigs of basswood, for example, should be replaced every 3 to 5 years. Incomplete removal of killing fluids or of hydrofluoric acid results in gradual disintegration of stored material.

Sectioning

Celloidin sections are usually cut with a sliding microtome. In this type of instrument the material is stationary during the cutting stroke, while the knife carriage slides on an accurate track. An automatic or hand-operated feed mechanism moves the tissues upward between cutting strokes. The catalogues of the leading manufacturers contain instructive illustrations and descriptions of several types and price classes of sliding microtomes. Small pieces of moderately soft tissues can be cut with a razor blade in a special holder designed for the sliding microtome. The limitations of the razor blade must be determined by trial. Hard materials and large sections must be cut with a microtome knife. Various lengths and weights of knives are available. The method of sharpening a microtome knife is described in Chap. 6.

Before using the sliding microtome, wipe the track of the knife carriage with an oiled cloth and test the feed mechanism. Clamp the knife firmly into the sliding carriage. Remove a piece of blocked tissue

(Fig. 8.2), fasten into the microtome clamp, and adjust the universal joint until the desired plane of sectioning is parallel to the plane of travel of the knife (Fig. 6.3). Keep the tissues moistened with glycerin-alcohol. If the upper surface of the material is not level, trim with a razor blade, sparing the microtome knife from rough trimming work. The best cutting angle for the knife-edge, with reference to the line of travel, ranges from 30 to 40°. The vertical tilt or declination of the flat side of the knife is also subject to variation. Begin with just enough tilt to enable the back of the ground wedge to clear the tissues (Fig. 6.3 *A, B*). Bring the tissues into cutting contact with the knife, using the hand-operated feed, making each vertical feed movement *after* the knife has passed over the material on the *return stroke*. In order to avoid damaging the knife-edge, feed in steps of not over 15 μ. Make sure that there will be ample clearance between the knife carriage and the tissue carrier even after many sections have been cut.

When each stroke cuts a complete section, set the thickness gauge and the automatic feed device. A thickness of 15 to 20 μ is satisfactory for most woody subjects. Keep the material and the knife flooded with 95% alcohol while cutting sections, and transfer each section as soon as it is cut to 95% alcohol in a watch glass or other shallow container. Vary the cutting angle and declination until sections slide up onto the knife without compression, curling, or breaking. Newly embedded material is liable to be hard and brittle and to curl. Curling can be minimized by holding a finger, moistened with alcohol, in light contact with the material during the cutting stroke, until the knife has cut through the marginal celloidin and enters the material. If scratches are evident on the cut surface, the portion of the knife being used may have bad nicks. Shift the knife longitudinally in its clamp and discard the next few sections. Sections can be stained at once, or they may be stored in glycerin-alcohol indefinitely. In the case of materials that do not curl, it is possible to cut several hundred sections, store them in a bottle of glycerin-alcohol, and remove as many as needed for staining at any time.

Some materials can be cut readily enough but difficulties arise after sectioning. The sections may curl soon after removal from the knife and become increasingly tightly curled during staining and dehydration. Being made brittle by dehydration and clearing, the sections break when an attempt is made to uncurl them for mounting. The following method is usually effective for such material. Keeping the knife well flooded with alcohol, cut a section. Hold a finger under

the knife-edge and float the section onto the finger, with the concave side of the section upward. Press the section, with the concave side *down,* on a slide flooded with a thin film of glycerin-alcohol. Line up successive sections on the slide, where they lie flat; drying of the sections is prevented by the glycerin. When enough sections have been cut, press a dry slide over the sections. Transfer the slides with the sections pressed between them to a dry Petri dish, put lead weights on the top slide and fill the dish with water. As many as four pressed lots can be put into one Petri dish. The water renders the sections flexible and permits them to flatten. The sections are then floated out and stained. For prolonged storage, transfer the slides and weights to a Petri dish of glycerin-alcohol, in which the sections become hardened in a flattened condition and in which they may be kept pressed indefinitely. Sections that have been stored either floating or pressed in glycerin-alcohol are progressively transferred to water and stained.

Staining

Sections cut in celloidin on the sliding microtome do not adhere to form a ribbon. They are usually stained as loose sections floating in a watch glass or small evaporating dish. The sections are usually floated off the knife into 95% alcohol. For staining in an aqueous stain, sections are gradually transferred to water. As the first step, add about one-third as much water as there is alcohol. After 3 to 5 min. pour off half of the liquid, and add an equal volume of water. Repeat the decantation and addition of water two or three times, then drain off all the liquid, and rinse in water. From this point the sequence of operations conforms in general to Staining Chart III. Drain and cover with hemalum. After 5 min. in stain remove a section with a brush, rinse in distilled water, then in tap water, and examine with a microscope. The intensity of hemalum is correct when the cambium, phloem, cortex, pith, and xylem rays are blue, but lignified tissues are practically colorless. Nuclei should be blue-black. Drain off the stain, and rinse the sections in three to five changes of distilled water and two or three changes of tap water. Overstained sections can be destained in ½% HCl, followed by thorough washing in tap water. When the intensity of the blue color is correct, cover the sections with aqueous safranin. Some woody materials take up enough safranin in 5 to 10 min. Materials having less highly lignified cell walls may require 12 hr. After the estimated time in safranin, rinse with water

until the rinse water is colorless. Flood with 50% alcohol, in which destaining of safranin begins to take place. Do not use acetone until the anhydrous stage. After 3 to 5 min., change to 95% alcohol, in which destaining continues. At first the blue color of the hematoxylin is completely masked by the red safranin, but as destaining proceeds the blue color becomes evident. At intervals of 2 to 5 min. transfer a section to a watch glass of clean 95% alcohol, and examine with a microscope. When there is good contrast between the blue color of nonlignified tissues and the clear, brilliant red of lignified elements, drain, and rinse in five changes of anhydrous alcohol or acetone at 2- to 5-min. intervals. Drying of the sections must be avoided in making these changes. Destaining is almost entirely stopped in anhydrous acetone, and the celloidin matrix is dissolved out of the tissues. If absolute alcohol is used, make two changes of ether-alcohol solvent to dissolve the celloidin out of the tissues. Flood with fresh carbol-xylene. There should be practically no destaining action during the 5- to 10-min. interval in carbol-xylene. Rinse in five changes of xylene, and mount in balsam or synthetic resin.

If the celloidin support is dissolved out of some pathological materials, graft unions, and some fragile subjects, the sections disintegrate or lose important parts. The sections can be cleared by transferring directly from 95% alcohol to terpineol, carbol-xylene, or creosote. The clearing agent must be changed several times, and thoroughly rinsed out in xylene before mounting. The supporting celloidin is retained by this method.

To mount one section on each slide, remove a section from xylene with a small brush or section lifter and place the section on the center of a dry slide. Stained sections can be selected under a binocular microscope or a hand lens and the imperfect sections discarded. Keep the section on the slide moistened with xylene. If the section is curled, straighten with two brushes, keeping the concave side down. Place a drop of resin on the section and lower the cover glass obliquely, squeezing out air bubbles by gentle pressure or by tapping with the eraser on a pencil. Put a lead weight on the cover glass. The drop of resin should be of such size that there is no excess resin around or over the weighted cover glass. If air bubbles cannot be expelled or if too much resin was used, put the slide into a Petri dish of xylene. The cover glass and section can be slid off in a few minutes and the section remounted. It is much easier to uncover and re-cover than to clean up a messy preparation later. After 1 to 3 days of drying under pressure in a horizontal position, the weight may be removed, and the

slides labeled and boxed. Refer to Fig. 6.6 for suggestions on the selection of cover glasses of suitable size.

The foregoing method is rapid, highly productive, and entirely satisfactory with materials that do not curl during the staining process. It is often possible to stain at least 50 sections in a Petri dish or evaporating dish and to mount most of the sections before appreciable brittleness and curling develop. Sections that undergo rapid curling after removal of the matrix must be handled by other methods. Terpineol has the valuable property of clearing stained and dehydrated sections without making them brittle and without affecting the stain. The terpineol is introduced in place of carbol-xylene. Sections may be lifted singly from terpineol, rinsed in xylene, and promptly mounted. If sections curl with this method, remove the sections singly from terpineol and place them lined up in rows on a dry slide until the slide is filled. Place the sections with the concave side down, and keep them moistened with terpineol. Cover with another dry slide. Place the slides with the pressed sections into a dry Petri dish, and flood with xylene. After 4 to 8 hr. the sections will become hardened flat, and they can be floated out a few at a time, rinsed in two changes of xylene, and mounted in resin before serious curling occurs.

The foregoing staining process, using a self-mordanting hematoxylin and safranin, is but one of the many stain combinations that can be used for celloidin sections. Safranin is almost invariably one component, because of the highly lignified character of most plant subjects for which the celloidin method is used. The safranin and fast green combinations yield strikingly beautiful preparations with many subjects. A batch of blue ash stem, killed in *FAA* showed highly differential, clear and brilliant tones, whereas a batch of red elm, similarly processed, had an unattractive, hazy blue tone in tissues that should have stained green. This stain combination can be tested rapidly with a few sections from any subject and deserves a trial. Follow the sequence given in Staining Chart V, observing the precautions and modifications necessary with celloidin sections.

Iron hematoxylin is an important stain for celloidin sections, because of its sharp selectivity for the middle lamella. The sequence is the same as in Staining Chart VI, but the time in mordant and stain, respectively, need not exceed 1 hr. After the stain has been differentiated, washing in water must be very thorough because woody tissues retain the alum tenaciously, resulting in early fading of the stain in the finished preparation.

Celloidin-Paraffin Double Embedding

Double embedding consists of infiltrating and embedding tissues in celloidin and then infiltrating with paraffin. This procedure is used with materials that combine hard tissues with regions of very fragile and brittle tissues. The stems of some grasses and sedges do not become well infiltrated by celloidin, but paraffin penetrates well. The material has regions of highly lignified sclerenchyma, requiring more support than that afforded by paraffin alone.

Embed in celloidin by one of the foregoing processes and harden well in chloroform. Trim away the enveloping celloidin, exposing all cut surfaces but leaving intact the outer surface of the epidermis. Some workers use clearing oils or mixtures that clear or make the tissues transparent. This does not necessarily improve the subsequent infiltration and usually aggravates brittleness. It is adequate to change the chloroform several times to eliminate the celloidin solvent and to proceed with infiltration in paraffin. The embedded material may be cut and ribboned on a rotary microtome; very firm material must be cut on the sliding microtome.

Rapid progress is being made in the use of methacrylates, polyesthers, and various polymers for embedding, especially for ultra-thin sectioning (Newman, Borsyko, and Swerdlow, 1949; Massey, 1953; Kuhn and Lutz, 1958). The use of such materials as additives to embedding wax is being explored (see page 33). It is possible that these new matrices may replace to some extent the use of celloidin for hard tissues.