5. Infiltration and Embedding in Paraffin Wax

The paraffin matrix in which tissues are embedded serves to support the tissues against the impact of the knife and to hold the parts in proper relation to each other after the sections have been cut. These functions are best performed if all cavities within the tissues are filled with the matrix and if the matrix adheres firmly to the external and internal surfaces of the material. Infiltration consists of dissolving the paraffin in the solvent containing the tissues, gradually increasing the concentration of paraffin, and decreasing the concentration of solvent. The solvent is eliminated by decantation or evaporation, or both, depending upon the character of the solvent and the process used.

Properties and Preparation of Paraffin

The properties of the embedding paraffin are important factors in the success or failure of sectioning. Desirable properties are as follows:

1. Constant and known melting point and appropriate hardness; the waxes used for most botanical work have melting points between 50 to 55°C., with a tolerance of 2° for a given grade.
2. Smooth, even texture, with a minimum of crystalline or grainy structure.
3. Absence of particles of dirt, included water, and volatile or oily components.

Commercial paraffins from different sources differ widely in properties and suitability for embedding. Purchases made from a given source may vary from time to time — some lots giving satisfactory results, whereas other lots, treated by indentical methods, are unsatisfactory. For these reasons paraffins from the available sources should be tested as to melting point, texture, behavior under the casting methods used, and cutting properties with familiar subjects.

In most parts of the United States, the wax obtained from petroleum is known by the name paraffin, whereas in some areas the term wax is used. The two terms are used indiscriminately in this manual.

Most of the paraffins sold for domestic canning have excellent properties but are too soft for sectioning under ordinary room temperatures or for cutting very thin sections. This inexpensive paraffin is satisfactory for sectioning soft materials such as fruits, if sections over 20 μ in thickness are desired. Paraffin of excellent quality and stated melting point can be purchased from biological supply houses, but at rather high cost. Canning paraffin can be used for preliminary infiltration, and the more expensive hard paraffin used for the final embedding. Canning paraffin requires no preparation; the pieces may be put into the oven tank where melting takes place readily.

Bulk paraffin can be purchased in 10-lb. slabs at low cost from petroleum refining companies. This bulk paraffin usually contains considerable dirt but it can be purified easily. Heat a quantity in a pan until it just begins to smoke, then keep over a small flame for at least ½ hr. Avoid heating the paraffin to the ignition point. Pour the paraffin into a tall metal container, such as a tall coffee can, and permit it to cool in a warm place. This permits particles of dirt to settle. Cool until the surface begins to solidify, then decant into the oven tank. The smoking hot wax can be filtered rapidly through dry filter paper. Use a coarse filter paper and keep the sides of the metal funnel warm with a small bunsen flame.

Each purchase of paraffin should be tested by casting a test block into a mold. The paraffin test block should contain no bubbles, opaque spots, streaks or internal fractures. When the chilled block is broken, the fracture should show a grainless or finely granular surface. The paraffin should slice into thin curled shavings, not into brittle granules. Keep a test block at a temperature of 30 to 35°C. for 24 hr.; bubbles and opaque crystalline spots should not appear.

Cast blocks of good paraffin should remain free from internal defects indefinitely, especially if stored at a constant, low temperature. Occasionally, one encounters old blocks that are almost as clear as glass. Some such waxes have adequately fine grain and may cut very well. In other cases, the impact of the knife causes opaque fracturing of the wax, as when ice is struck with an axe. The wax has obviously crystallized into a coarse texture during storage. When a block of

such paraffin is melted slowly in the oven and recast into new wax, the tissues may still be in good condition.

The texture and cutting properties of paraffin can be improved by the addition of rubber and beeswax, and a hard wax, such as ceresin wax. Hance's formula is recommended. Dissolve 20 g. of crude rubber in 100 g. of smoking hot paraffin. Cool and cast into slabs like canning wax. Make up the following mixture:

Paraffin	100 g.
Rubber-paraffin mixture	4–5 g.
Beeswax	1 g.

Ceresin wax may be added to the above, 2 to 5% by weight. Heat the mixture until it just begins to smoke, filter through paper, and cool until it begins to solidify before putting into the oven tank. Tissuemat and Parlax are two commercial embedding waxes that have excellent properties.

Hard waxes and synthetic resins need continued study as hardening agents. For instance, Fisher Scientific Company's "Permount" cover glass resin contains a resin of undisclosed formulation. Dissolve 10 cc. Permount in 200 g. melted Tissuemat in a 60–80°C. oven. When the odor of the solvent, toluene, can no longer be detected, pour into a mold. Melt this very hard wax into 10 to 20 times its weight of Tissuemat. The resulting hardened wax yields thin sections, though not in the range of ultra-thin sectioning.

Apparatus

OVENS FOR INFILTRATION

A well-insulated oven with thermostat-controlled electrical heating is the most reliable type. A removable copper tank makes a suitable container for the supply of melted paraffin. The tank can be equipped with a brass petcock, but petcocks develop leakage. A more satisfactory method is to dip out the paraffin with a spoon as needed. Debris settles to the bottom of the tank, and the clear paraffin is used from the top. An incubator oven with a reliable thermostat is satisfactory for paraffin work, but the temperature in different parts of the oven is not the same and must be determined. In the latter type of oven the supply of melted paraffin may be kept in a container with removable cover and dipped out with a spoon kept hooked in the container. If it is possible to have two ovens for infiltration, use an inexpensive wooden incubator oven set for 35°C. for preliminary infiltration.

DEVICES FOR CASTING BLOCKS

Several methods are in use for casting infiltrated tissues into a mold. The most practical mold is a tray or "boat," made of heavy glazed paper, aluminum foil, or paper-backed aluminum foil. The method of making boats is shown in Fig. 5.1. Fold along the dotted lines (A) and lap the wide side to lock the narrow side as in B. Masses of loose minute objects that have been processed by centrifuging and decantation can be cast in a pyramid mold (C). Aluminum foil is folded best over a wood form. Paper-backed foil can be folded as easily as heavy paper without a form.

Soak paper boats in smoking hot canning wax until bubbles cease to come from the paper. Remove the boats from the wax, shake off surplus wax and cool the boats on a paper towel. A supply of boats of various sizes can be prepared in advance and used as needed. Boats

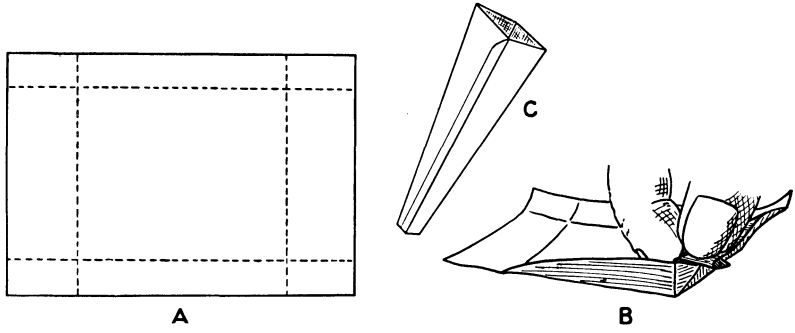

FIG. 5.1—Method of laying out (*A*) and folding (*B*) paper boats for casting paraffin blocks. *C*, pyramid mold.

of durable, flexible plastic in various sizes will probably be developed. Some brands of plastic ice cube trays have desirable properties.

Some form of hot plate is used to keep the paraffin in the boat melted while the material is being arranged. Electric plates with thermostatic control are available. A sheet-copper table is used in many laboratories. An easily controlled heating table consists of a sheet of ½-in. boiler plate, 6 to 8 in. wide and 18 to 24 in. long, mounted on legs or on a large ring stand (Fig. 5.2).

The warm-pan method must be used for materials that are very small, buoyant, or transparent. The boat is supported on a wire

triangle in a pan, which serves as an air bath, heated by a small Bunsen flame (Fig. 5.2).

Infiltration With Paraffin Wax

The following infiltration procedure may be used with any of the common solvents, if the respective specific gravities of the solvent and the wax are taken into account. Paraffin wax, either in solid or melted form, floats on chloroform and also on dioxan. Therefore, either of these solvents, used alone, provides the progressive infiltration that will be emphasized in this chapter. The addition of chloroform or xylene to dioxan, as suggested previously, merely accelerates the dissolving of the wax.

Paraffin sinks in xylene, but if the melted wax is poured into a bottle of cold xylene along the side of the bottle, a solidified layer of wax will remain on top of the solvent during preliminary infiltration at 25 to 35°C. When the solvent is warmed above the melting temperature of the wax, the nearly pure wax will sink to the bottom and envelop the tissues. The abrupt concentration gradient between

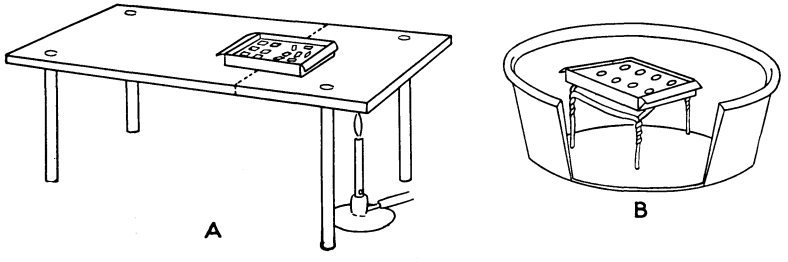

FIG. 5.2—Casting tables: *A*, boiler-plate table (the dotted line indicates the melting zone); *B*, warm-pan device.

the wax and the tissues may be destructive to fine detail. The addition of 6 to 10% chloroform by volume to xylene raises the specific gravity sufficiently to float wax chips or melted wax, and a gradual downward diffusion gradient is obtained.

Normal and tertiary butyl alcohol have a lower specific gravity than paraffin wax, and the wax sinks upon the tissues. Flotation of wax can be obtained by the addition of chloroform, 15% by volume to normal butyl alcohol, and 25% to tertiary butyl alcohol, respectively. If chloroform is not added, the addition of wax must be made by small increments, in liquid form, and each increment must be homogenized to prevent the added wax from sinking and enveloping the tissues.

If a mixture of two solvents is used, the bottle should be kept corked until the infiltration is well advanced, to prevent the differential evaporation of the two solvents in the oven.

Pour a teaspoonful of melted paraffin over the cold solvent, where the paraffin solidifies as a layer on top of the solvent. Remove the stopper and place the bottle into the 35°C. oven. The layer of paraffin does not melt, but it gradually dissolves and diffuses downward into the tissues. If all of the paraffin dissolves at this temperature, add more melted wax. If the bottle becomes filled with solution, pour some of the solution into the waste can (*not into the sink!*). Continue the addition of melted wax until a thin layer of undissolved wax remains on top of the solution. The undersurface of the layer of paraffin eventually develops a translucent, crystalline appearance. When this stage is reached, the solvent is obviously *saturated with paraffin at this temperature.* Tissues are not damaged by prolonging this infiltration at 35°C., therefore, this part of the process may be extended over 2 or 3 days.

Transfer the specimen bottle to the 53°C. oven, where the layer of solidified paraffin soon melts and continues to dissolve, and the infiltration initiated at the lower temperature is continued. If the specific gravity of the solvent has not been adjusted as described above, it is best to remove the solid supernatant wax at this point and add wax by small increments at intervals.

If the tissues are not extremely delicate or fragile, whirl the bottle *gently* until the liquid is homogeneous, as shown by the absence of refraction waves within the liquid. At intervals of 1 to 4 hr., pour off one-half of the homogenized solution into the waste can. Replace the decanted liquid with an equal volume of melted soft paraffin, and replace the bottle into the oven quickly. After four or more such partial replacements, pour off all the paraffin-solvent solution, which now consists mostly of paraffin, and replace with pure paraffin. After 1 to 4 hr. make another complete replacement and make a *button* test. Cast a button of paraffin about the size of a silver dollar by pouring some of the paraffin from the tissues into a pan of cold water. Promptly replace the specimen bottle into the oven. Allow the test disk to cool thoroughly. The cooled test button should not be greasy. Chew a piece of this paraffin. The presence of even a slight trace of xylene or other solvent is easily detected by taste. Examine for the defects and qualities described on page 31. If the test piece indicates that all the solvent has been removed, make two changes of hard paraffin or commercial casting compound at 1- to 4-hr. intervals. The material is then ready to be cast into a mold.

The use of two, or even three grades of paraffin with different melting points, used successively during infiltration, has been suggested in the literature. If a laboratory is equipped with three ovens, maintained at 40, 50 and 54°C., there may be some point to the successive use of waxes having those melting points, but if the three waxes are used at the same temperature, there seems to be little basis for the procedure. It can be demonstrated easily that high-melting-point wax that contains a trace of solvent becomes low-melting-point wax. The progressive method outlined in this chapter may be used with only one grade of wax; however, the use of inexpensive canning wax for preliminary infiltration, followed by casting in a high grade filtered wax or compounded formula, is economical and entirely satisfactory for most tasks.

A vacuum oven is an aid in the infiltration of difficult material. Cavities that resist evacuation at low temperature can be exhausted at 50°C., when the material has progressed to approximately equal volumes of wax and solvent. The low boiling point of tertiary butyl alcohol makes the use of a vacuum oven impossible with this solvent until the solvent has been almost completely replaced by wax.

Most material can be adequately evacuated by the time it is in the final solvent, and the dissolving wax will then diffuse into all spaces occupied by the solvent.

CASTING INTO A MOLD

Assume that the infiltrated material is in the final change of pure paraffin. If the oven has cooled because of frequent opening, the paraffin in the specimen bottle may have congealed. Heat the neck and upper portions of the bottle in a Bunsen flame. Never heat the *bottom* of the bottle because the tissues resting on the bottom will be overheated and ruined. Apply only enough heat to liquefy the paraffin. Slight heating repeated at 10-min. intervals is safer than melting at one heating. If you have not yet provided means of identification, write the designation of the given lot of material on a ½-cm. square of paper and put into the bottle. Select a paper or foil boat that will accommodate the pieces in the specimen bottle, without wasting space or wax. Heat one end of the casting plate with a small flame and place the empty boat at the *melting zone*. Pour the paraffin containing the tissues into the boat. Arrange the pieces with a bristle. A warmed needle may be used, especially to move pieces that have become frozen into unsatisfactory positions. Slide the boat over the edge of the melting zone toward the cold end of the plate as fast as a row of pieces is arranged. When the pieces are suitably

oriented, move the boat to the cold end of the plate. Sweep the Bunsen flame rapidly over the surface of the paraffin; this permits contraction on the upper surface while the bottom of the paraffin is cooling, thereby preventing the formation of cavities. As soon as the paraffin is hardened enough to keep the pieces of tissue from moving, float the boat in a pan of cold water and brush with the Bunsen flame again. Allow the surface to solidify, and submerge the boat in the water, holding it under with a staining jar cover or other weight. When the paraffin is thoroughly cooled, peel off and discard the paper boat.

Some purchases of paraffin wax, and occasional lots of proprietary compounded waxes, develop white areas or bubbles if kept at room temperature after casting. This can be minimized or prevented by storing the cast blocks in a refrigerator for several days. If the spots appear upon subsequent exposure to room temperature, permanent low temperature storage should be provided.

When using the warm-pan method (Fig. 5.2 *B*), place a paraffin-soaked boat on the triangle, and warm the bottom of the pan with a Bunsen flame of such size that a layer of paraffin in the boat is kept just melted. Pour the material into the boat, and arrange the pieces, occasionally flaming the top surface of the paraffin. There is little danger of overheating the material; hence the operator need not hurry in arranging the pieces. When the pieces are arranged satisfactorily, remove the burner, and pour cold water into the pan until the water level is slightly above the bottom of the boat. The pieces become hardened into place quickly. Sweep the flame over the surface of the paraffin to permit internal contraction. Complete the hardening as in the former method.

The spacing of pieces in the block depends on the size of the pieces. Root tips and small pieces of leaf can be spaced 5 mm. apart; large pieces of stem require more supporting paraffin during sectioning and should therefore be spaced at least 1 cm. apart. Very slender root tips, coniferous needles, and similar objects may be blocked in groups of three or more pieces laid parallel so that they can be microtomed simultaneously.

RECASTING

Poor paraffin of a cast block can be replaced with good paraffin, or poorly arranged material can be rearranged and recast, and excessively large pieces can be trimmed and recast. Cut the pieces out of the block, trim the pieces if desired, cut away excess paraffin if it is

of bad quality, and drop the pieces into a bottle of melted paraffin in the oven. Do not apply extra heat; the temperature should not exceed 53 to 54°C. When the old paraffin has amalgamated with the new wax, make at least one change into new casting wax and cast into blocks.

REINFILTRATION

Poorly infiltrated tissues can sometimes be salvaged by reinfiltration. This should not be attempted if there has been excessive collapse of cells, a frequent result of poor infiltration. Cut the pieces out of the paraffin block, trim away excess paraffin, and drop the pieces into anhydrous dioxan, normal butyl or tertiary butyl alcohol, xylene or chloroform. After 24 hr. at 35°C. transfer to the 53 to 54° oven and continue progressive infiltration. A vacuum oven may be used for such salvage operations.

Cast blocks should be stored under conditions that minimize damage to the tissues and to the texture of the paraffin. Trim the edges of the cast block so that both surfaces are flat. Store in a stout manila envelope or small cardboard box bearing adequate identification data. If several blocks are stored in one container, use thin cardboard separators. Box containers should be stacked so that the blocks lie flat. Stout envelopes support the blocks well enough to permit filing the envelopes in the vertical position in a filing cabinet. Storage temperatures should be low enough to prevent bending of paraffin blocks.