6. Microtome Sectioning of Material in Paraffin

Material embedded in paraffin is almost invariably cut with a *rotary* microtome, in which the knife is stationary and the piece of tissue is moved up and down past the cutting edge. Cutting is accomplished by wedge action, like the action of a chisel or a plane. After a section has been cut, and the tissue carrier has passed the knife on the upstroke, an automatic feed mechanism advances the tissue carrier forward, and another section is cut. Successive slices remain attached to each other, forming a *ribbon* of paraffin. The successive sections cut from a piece of tissue are thus kept in *serial* order, and this order can be preserved throughout the processing of the slides. From serial slices of an organ of a plant it is possible to reconstruct the external or internal structure of the organ, of a tissue system, or even of a single cell. As an example of serial sectioning we may use the homely illustration of a loaf of bread, cut into slices and the slices laid out in order.

The piece of material to be sectioned is fastened to a mounting block, which is clamped into the microtome. Inexpensive mounting blocks can be made of hard, porous wood, such as oak or ash. The most useful sizes range from 1 by 1 by 2 cm. to 2 by 2 by 3 cm. Soak the blocks in hot canning wax. Bakelite and other plastics make excellent blocks, preferred to wood because plastic blocks do not compress when clamped into the microtome (Fig. 6.1 *A*). Wood or plastic blocks are satisfactory for most work, being inexpensive and sufficiently rigid for sections over 6 μ in thickness. For routine sampling of material, many pieces of tissue can be mounted on separate blocks, the mounting blocks numbered by means of string tags, and test sections made from each piece. The mounted blocks can be kept with the proper batches of embedded material until staining trials establish which block has the desired stage.

Metal mounting disks (Fig. 6.1 *B*) afford greater rigidity than plastic blocks and are indispensable for cutting very thin sections, or for sectioning large pieces of firm material. Disks remain cold longer after chilling, thereby keeping the paraffin block cold for sectioning. Disks are much more expensive than homemade blocks, and most laboratories have a limited supply, making them unsuitable for the routine sampling method described above. Some microtomes have a built-in tissue-mounting disk on a ball-and-socket joint. This device is satisfactory for work in which each piece of material is used up at one cutting, thus emptying the carrier for the next piece or for the use of other workers. For class use or for work requiring much sampling of diverse materials by several workers, removable disks or blocks are much more desirable.

To fasten a piece of material on a mounting block, trim the paraffin around the material so that the cutting plane is established.

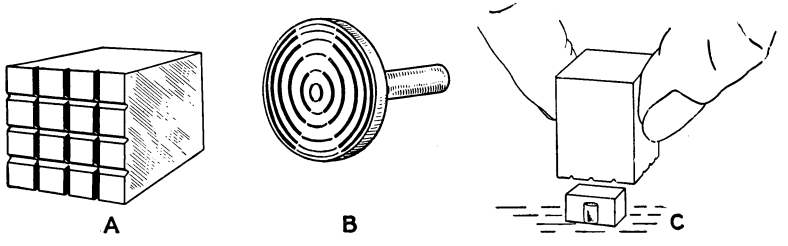

FIG. 6.1—Mounting of tissues on object blocks: *A*, wood or plastic block with scored surface; *B*, metal object disk; *C*, method of orienting paraffin block and fastening to object block.

Lay the cutting face on a clean surface. Heat the mounting block, press it firmly on the back of the specimen, and hold in contact until the wax cools (Fig. 6.1 *C*). Build up a fillet of paraffin around the specimen to afford firm bracing for the tissues (Fig. 6.2). Cool thoroughly before sectioning.

Some workers of acknowledged skill use a heavy knife for class-work, for routine preparation of teaching material, and for research. Other workers of equal ability use either a knife or a razor blade in accordance with requirements of the work in hand. For sections ranging from 8 to 15 μ in thickness, a sharp razor blade in a suitable, rigid holder will match the work of the heavy knife. The thick type of razor blade (Enders or Christy) can be stropped, used repeatedly, and discarded when stropping no longer restores the edge. In a course in microtechnique, for the routine preparation

of teaching material, and for many research tasks, razor blades are economical and entirely satisfactory.

For cutting very thin sections, uncommonly thick or large sections, or for tough materials, the heavy microtome knife is indispensable. The greater rigidity of a knife permits sectioning of material with which a flexible razor would chatter, cutting sections of uneven thickness.

The sharpening of a microtome knife is a laborious process, best learned by observing a demonstration. If a knife is badly nicked or bowed in the center it is best to send it to the manufacturer for grinding. A straight edge and a new correct bevel of the cutting edge are thus established. With a properly ground knife, occasional stropping restores the edge for a considerable time, depending on the hardness of the material being cut. A honing back is a longitudinally split metal cylinder that is slipped over the thick back of the knife for honing or stropping. The diameter of the cylinder determines the angle of the honed wedge. The metal of the cylinder is usually softer than the knife and wears away faster by honing. When the cylinder is appreciably worn, or unevenly worn, a new one should be fitted and a new wedge angle honed on the knife. It may be necessary to hone the knife on a fine gray hone using soap suds as a lubricant. When the fine hone and strop fail to restore the edge, and it is not possible to have the knife machine-ground, a new wedge and cutting edge can be established as follows. Place the cutting edge on the coarser yellow hone with the knife vertical, and make one *light* stroke, removing the cutting edge completely. Then lay the knife flat on the hone and stroke with long oblique strokes, alternating the two sides, until the two sides of the new wedge meet. Examine periodically with a microscope. Hone on the fine gray stone until the edge consists of uniform, minute serrations. Strop as usual before using.

Factors Influencing Sectioning

Successful sectioning in paraffin depends upon a number of interacting factors, the most important of which will be discussed briefly.

QUALITY OF THE PARAFFIN

The hardness of the paraffin should be appropriate for the character of the tissues, the desired thickness of the sections, and for the room temperature at which the cutting is done. The paraffin should

have a grainless or very fine-grained texture, should be free from bubbles and opaque spots, and should contain no grit or other debris.

PROPER INFILTRATION

Improperly infiltrated material breaks out of the paraffin block. Examine the cut face of the material with a hand lens or a binocular dissecting microscope. Crumbling within the tissues may indicate inadequate penetration by paraffin during infiltration or may be the result of excessive hardness or brittleness of the tissues. Breaking out of entire sections from the paraffin ribbon indicates poor adhesion between external surfaces of the piece of tissue and the paraffin. Inadequate infiltration may be due to incomplete dehydration or excessively rapid infiltration. The remedy lies in reinfiltration.

ORIENTATION OF THE MOUNTED MATERIAL

The paraffin around the piece of material should be trimmed rectangular, with the material approximately centered laterally in the paraffin. If the tissue is not vertically centered in the paraffin, the thicker layer of paraffin should be at the top, affording support against the pressure of the cutting action (Fig. 6.2 *F*). Trim the upper and lower edges of the wax so that the sections are close enough to each other on the slide for efficient use of the slide and cover glass. See Fig. 6.6 for efficient placement of sections. The edge which approaches the knife should be parallel to the knife (Fig. 6.3 *C*). For most paraffin sectioning the knife is placed at right angles to the vertical motion of the paraffin block. The other angle to be considered is the declination, or the tilt of the flat face of the knife toward the tissue (Fig. 6.3 *A, B*). This angle must be determined by trial.

RIGIDITY OF MOUNTING

The piece of tissue should be firmly attached to a mounting block or disk and supported, especially on the edge away from the knife, by a generous layer of paraffin (Fig. 6.2 *E*). The mounting block, the knife, and the knife carrier must be firmly clamped into place. Inadequate rigidity of the tissue mounting or of the knife results in alternate sections of unequal thickness. This can often be recognized in the ribbon but usually becomes evident during staining. The thicker sections will be more deeply stained than the alternating thin ones. In sections of a large stem there may be alternate deeply stained and lightly stained bands in each section.

TEMPERATURE FACTORS

Cutting is influenced by the temperature of the paraffin block, of the knife, and of the room. If the temperature of one or more of these factors is too high, compression of the sections occurs on impact with the knife. If the temperature is too low, the sections may curl, or successive sections may not adhere and thus fail to form a ribbon. Thick sections are relatively more tolerant to higher working temperatures than are very thin sections. A heavy microtome knife permits a higher temperature than does a razor blade. If the temperature is too high for the grade of paraffin being cut, cool the mounted

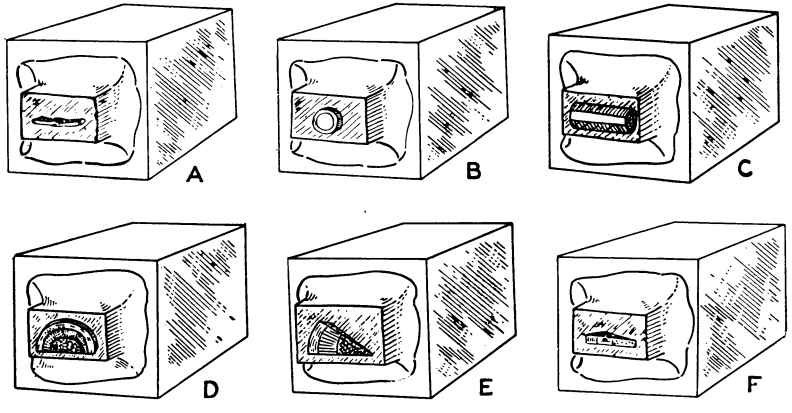

FIG. 6.2—Methods of orienting objects of various shapes: *A*, a leaf mounted for cross sections; *B*, a small stem or other cylindrical organ mounted for cross sections; *C*, for longitudinal sections; *D* and *E*, sectors mounted for cross sections; *F*, sector of large herbaceous stem mounted for radial sections.

paraffin block and the knife or razor-blade holder in a pan of ice water. Align the tissues and knife in the microtome quickly, and cut sections until the paraffin becomes too soft, when the cooling should be repeated. Knife cooling devices are described by Johansen (1940). If a refrigeration room is available, perfect control is possible by setting up the microtome in the cold room and warming a zone around the knife with a desk lamp. The author's department has a cooled microtome room with a floor space of 5 by 7 feet and a height of 8 feet. A small compressor is mounted above the room on a heavy false ceiling. Cooling coils are suspended on the ceiling. Gravity cooling provides a thermostatically controlled constant temperature of 65°F. Two microtomes and two sets of accessories are provided, and two operators have ample space. This room makes the research work-

ers, students in the technique course, and the technicians completely independent of seasonal conditions.

HARDNESS OR BRITTLENESS OF THE MATERIAL

If the above precautions are observed and satisfactory sections and ribbon are not obtainable from dry blocks of tissues, try the warm-water treatment. Plant tissues embedded in wax are not impervious to warm water. If the cutting face of a block of embedded tissues is trimmed to expose the tissues and soaked in water, the paraffin becomes translucent, water penetrates the tissues and renders many hard or brittle subjects soft enough to permit the cutting of excellent sections. Mount a specimen on a metal disk or on a block of plastic, trim as above, put into a beaker of water, and keep in a 35 to 40°C. oven for 12 hr. Objects mounted on wood blocks should be inverted in a vial of water, so that the tissues are submerged. The extent of softening should be tested after 12 hr. by cooling the material to proper cutting temperature and making trial sections. If the tissues are not soft enough, return to the oven for another 12-hr. interval, and test again. If a drop of safranin is added to the water in which the tissues are soaked, the penetration of the dye provides a good index of the depth of water penetration. Some materials, especially improperly infiltrated tissues, crumble and break out of the paraffin with this treatment. After treatment, material cannot be returned to dry storage because the wet tissues become disorganized on drying. If the hot-water method does not yield sections, the material is probably too hard to cut by the paraffin method.

The Operation of the Rotary Microtome

Having studied the foregoing discussion of some general factors that influence paraffin sectioning, we may turn to the specific operations involved. The operation of the microtome can be learned best by observing an experienced worker. Study the diagrams furnished by manufacturers, and examine your particular instrument with a view to understanding the operating principle and interaction of its parts. Some general suggestions are applicable to the operation of most types of instrument. With the tissue carrier at the upper limit of its travel, and the knife removed or at a safe distance from the path of travel of the tissue carrier, clamp the mounting block bearing the tissues into the object clamp. Manipulate the universal joint of the clamp until the forward face of the trimmed paraffin block, or the desired plane of the sections, is parallel to the knife-edge (Fig.

6.3 *C*). Move the knife carrier forward and the tissue carrier downward until the material *almost* touches the knife-edge in its downward travel. Check the setting of the thickness gauge. Make sure that the wedge-like cutting edge is tilted to have proper clearance on the return stroke (Fig. 6.3 *A, B*). This angle must be determined by trial. Inadequate clearance results in compression of the tissues by the forward flat face of the knife or by the edge of the razor-blade

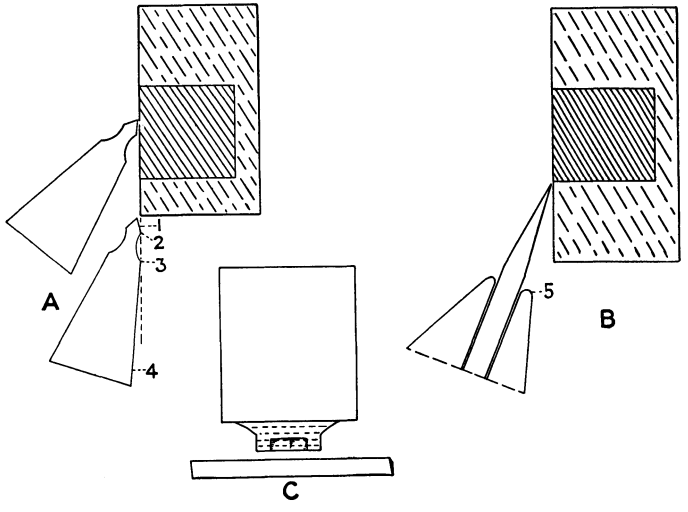

FIG. 6.3—Orientation of tissues in relation to the knife: *A,* knife with the ground cutting wedge and the hollow grind exaggerated to show necessary clearances of angles 2 and 3 and faces 1 and 4; *B,* razor-blade holder, showing declination necessary to clear edge (5) of the clamp; *C,* top view of mounting block, paraffin block, and knife-edge.

holder (Fig. 6.3 *A, B*). Too much angle results in a scraping action rather than a chisel action of the knife-edge.

Having checked the above points, turn the operating wheel slowly, and at the *top* of each upstroke, turn the hand crank of the feed mechanism one revolution, until each downstroke removes a complete slice. Clean the knife-edge by drawing the thumb and forefinger along the front and back faces of the knife, and proceed with the ribboning. Operate the wheel at such speed that there is no marked compression of each slice and successive slices adhere to form a straight ribbon. Note that an experienced worker does not turn the wheel at uniform velocity during a revolution. As the tissue approaches the knife, a snap of the wrist increases velocity considerably at the moment of contact between the tissue and the knife. This speed promotes clean

slicing and minimizes compression and wrinkling. At the moment of contact the feed mechanism is disengaged and the only wear is on sliding surfaces. High speed during the entire revolution makes violent impact between the hardened steel pawl and the much softer ratchet wheel, and the pawl may skip a tooth, or strike the top of a tooth. Excessive speed therefore produces excessive wear and is inexcusable, unless the laboratory is lavishly financed. A motor drive, in the hands of the untrained "hired help" that is sometimes used, damages the feed mechanism.

A curved ribbon may be the result of one or more of the following conditions:

1. A dull spot on the knife; shift the knife laterally in its holder or replace with a good knife.
2. The upper and lower edges of the paraffin block are not parallel; trim with a razor blade.
3. The lower edge of the paraffin block is not parallel to the knife-edge; adjust the object clamp.
4. The piece of tissue is not centered laterally in the paraffin; trim the unequal side.
5. The piece of tissue is of irregular shape and bulk. In Fig. 6.4*C* the comparatively empty right side of the paraffin will compress more than the left side, producing a curved ribbon (*B*). This may be corrected by trimming the upper face of the paraffin block (along the dotted line in Fig. 6.4*C*).

The method of straightening a slightly curved ribbon on the slide is described later. Handle the ribbon with a small brush. Do not permit a needle or scalpel to touch the knife-edge. The slightest contact will turn the fine cutting edge. For beginners a quill-shanked brush is the safest implement. Lay the segments of ribbon, in the order of removal from the knife, on clean, lintless black paper, and keep in a cool dust-free place until you are ready to attach them to slides. For handling long ribbons in serial order, cylindrical ribbon holders are manufactured. Their operation is obvious from the catalogue illustrations. The foregoing brief outline of the operation of the rotary microtome should be supplemented by observing the methods of experienced workers. Skill can be acquired only by experience with a wide range of subjects, and a thoughtful analysis of failures.

The condition of the cells and tissues in the ribbon can be judged with considerable accuracy. Examine the ribbon with a magnifier or binocular microscope. The paraffin should be firmly attached to external surfaces and should fill all wrinkles, folds, and visible cavities. Inadequate infiltration may be one cause of separation of

the tissues from the paraffin or crumbling within the tissues. If there is abundant ribbon and if seriation need not be maintained, melt a piece of ribbon on a dry, used slide, and examine quickly with a microscope. A magnification of 400 can be used. It is possible to see the chromosomes at metaphase and even at early prophase in onion root-tip cells. The position of chloroplasts in cells can be observed; the degree of granularity of cytoplasm, vacuolation, and plasmolysis can be estimated. The success of the processing can therefore be

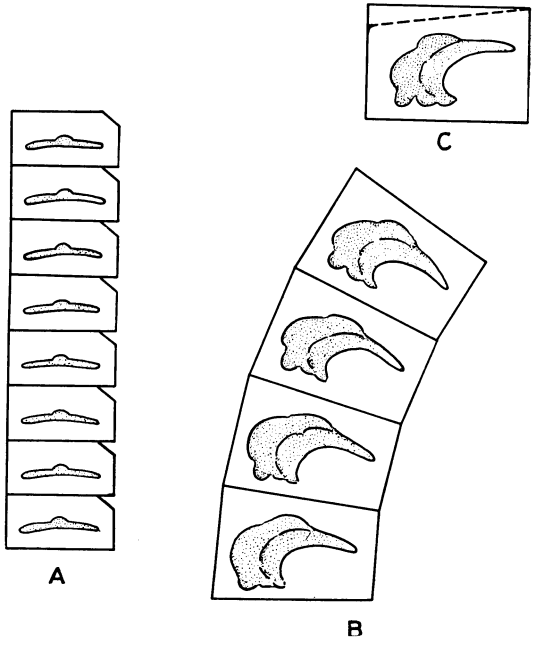

FIG. 6.4—Paraffin ribbon: *A,* straight ribbon, notched if desired by trimming one edge of the paraffin block as in Fig. 15.1*L; B,* curved ribbon; *C,* trimming of paraffin block along dotted line to correct curvature.

judged at this stage in accordance with the criteria discussed in Chap. 11.

The optimum thickness of section for any specific subject can be ascertained at this point. An experienced worker can make a good guess, subject to verification, by examining the ribbon. Study Fig. 6.5 *A,* a perspective sketch of a portion of a leaf with both layers of epidermis omitted. Assume that sections have been cut 20 μ in thickness. Note in *B* that a section of this thickness would encompass two or three layers of narrow columnar palisade cells, and consider-

able portions of interwoven spongy parenchyma. In such thick sections it is difficult to ascertain the limits of individual cells and the true location of bodies within the cells or mycelium in the tissues. A section 5 μ thick would include adequate longitudinal slices of palisade cells, but the sections cut from various portions of the irregular spongy cells would appear as separated fragments (Fig. 6.5 *C*). Sections of approximately 10 μ might be a good compromise by showing enough of the spongy cells to indicate continuity of contact.

When cutting tissues infected with a filamentous fungus, it is often necessary to make some apparently excessively thick sections in order to include sufficiently long strands of the mycelium. Lily ovules

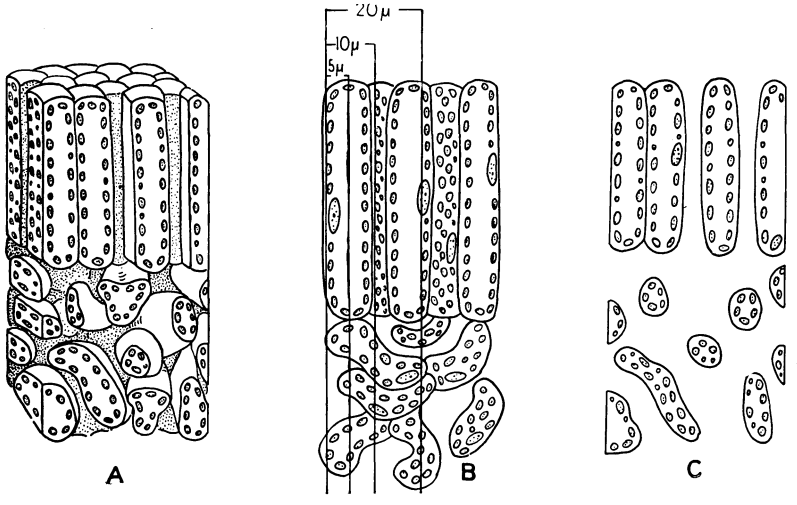

FIG. 6.5—Method of ascertaining the appropriate thickness at which sections should be cut: *A*, perspective view of leaf tissues; *B*, respective numbers of cell layers included in sections of different thickness; *C*, disjointed appearance of leaf tissues in excessively thin sections.

in the four- to eight-nucleate stage must be cut 15 to 20 μ thick to include the complete set of embryo-sac nuclei in a sufficiently high ratio of slides. Onion root-tip sections of 8 to 12 μ include enough chromosomes of the complement to show their approximate number, as well as the organization of the meristematic tissues and their derivatives, but it is desirable to have some slides of 4 to 6 μ to show certain details. Optimum thickness is a compromise between transparency and clearness of separate structures, and the preservation of the relationship and continuity of associated structures.

The very thin sections that are essential for electron microscopy can be made with the attachment supplied by the American Optical Co., Buffalo, N. Y., for their standard microtome, and with special microtomes that are advertised in current journals.

Affixing Paraffin Sections to the Slide

Paraffin sections in the form of a ribbon are fastened to a glass slide with an adhesive prior to staining. Adhesion is influenced by several factors, the most important being the following:

1. Perfectly clean slides.
2. An adhesive (fixative) suitable for the particular material.
3. Proper flattening of the sections by heat.
4. Complete hardening of the adhesive, which makes it insoluble in the reagents used in staining.

New slides should be cleaned, although they may seem to be clean. Use a soapless dish-washing detergent in 70% alcohol. The present favorite in this laboratory is a concentrated liquid detergent, two drops of which in 200 cc. of 70% alcohol makes an effective cleaner. Cleaned slides develop a film on standing, therefore it is best to clean them shortly before using. Used slides can be cleaned, with little effort and represent a considerable saving. Slides that have balsam or paraffin on them should be soaked in lead-free gasoline for several hours, wiped dry and cleaned with the detergent. Examine used slides for excessive scratches and surface corrosion. Greasiness of the slide prevents adhesion. To test for greasiness, put two drops of distilled water on the slide and spread with a scalpel. The water should spread out thin on the glass, and not roll inward like water on a hot plate.

The most extensively used adhesives are: gum arabic, obtained in granular form; commercial albumen, such as "Albusol"; fresh egg white; granular or sheet gelatin. Numerous formulas are given by Gray, and elsewhere in the literature. This chapter will present only formulas based on egg albumen and gelatin, by far the most popular and easily available adhesives.

Egg albumen: Drain the white of an egg into a graduated cylinder. Add an equal volume of water and the same volume of glycerin. Add 0.5% sodium benzoate or 1% sodium salycilate. Homogenize in a Waring blender or similar device. Filter and store most of the supply in a refrigerator. Keep a small quantity in the laboratory in a dropper bottle, using a toothpick as an applicator. Place a small

drop on the slide, smear into a thin film with the ball of a finger, flood with water, and float the ribbon.

Gelatin: Dissolve 1 g. granular or sheet gelatin in 100 cc. water at 30–35°C. Add 0.5 g. sodium benzoate or 2g. phenol. The addition of 15 cc. glycerin is optional. This stock solution can be used by several methods.

Method 1. Smear a thin film of adhesive on the slide. Flood with 4% formalin and float the ribbon. (Commercial formalin contains 40% formaldehyde gas.)

Method 2. For this one-solution formula, dilute the stock solution with water and add formalin to 4% equivalent. A few trials will determine the dilution that will hold the sections and not leave an excess of stainable gelatin under the sections and on the surrounding glass. The dilution may be as much as 1 vol. stock to 10 vol. water.

Method 3. An old formula that contains both chrome alum and formaldehyde as hardeners has been improved by Weaver by the use of a fungicide and a bactericide, which prevent spoilage of the gelatin.

Solution A		**Solution B**	
Gelatin	1 g.	Chrome alum	1 g.
Calcium propionate (Mycoban)	1 g.	Formalin (40%)	10 cc.
Benzalkonium chloride (Roccal)	1 cc.	Water	90 cc.
Water	100 cc.		

Mix 1 vol. Solution A with 9 vol. (or more) Solution B; float the ribbon and proceed as described below.

Decide on the number of pieces to be put on a slide in accordance with the size of the cover glass to be used (Fig. 6.6). Float the desired amount of ribbon on your choice of adhesive. Warm the slide over an alcohol lamp having a wire screen chimney, or on a warming plate, until the paraffin expands, undergoes a change of luster, flattens out, and *approaches* but does not reach the melting point. Keep the ribbon floating while heating to permit expansion. If the paraffin melts, cellular arrangement and cell details are distorted. An insufficiently heated ribbon does not expand or lie flat on the slide and, therefore, does not adhere well. Tough, woody, or elastic subjects are especially difficult to flatten and to attach firmly. If the heated ribbon is curved, straighten while still warm by pulling the concave ends with a pair of needles. Allow the ribbon to cool, then blot with

lintless filter paper. Wipe excess adhesive from around the edges of the ribbon to avoid leaving a ring of stainable adhesive.

The slides are now ready to be dried. Some workers prefer to dry slides at a temperature just under the melting point of the wax; however, most tissues are not damaged by drying the slides in the paraffin oven at the melting point of the wax. The adhesive becomes hardened enough in 4 hr. to hold thin sections of soft materials.

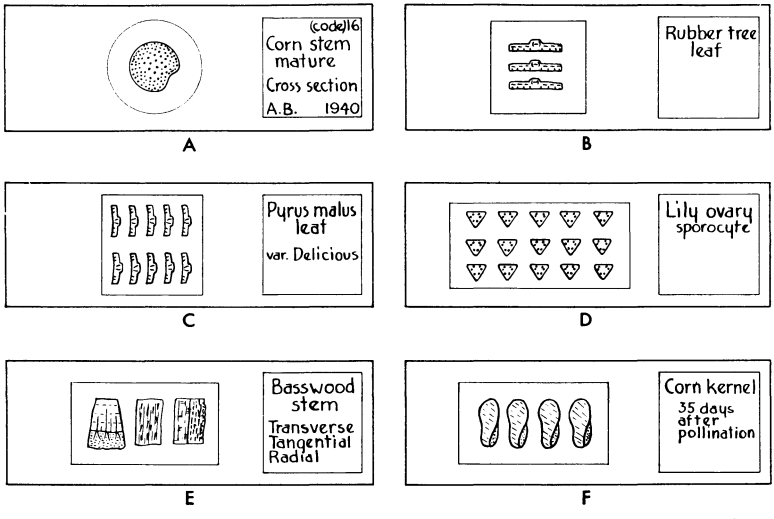

FIG. 6.6—Spacing and arrangement of sections (ribbon) in relation to size and character of subject and size of available cover glass.

Thicker sections of hard material may require 12 hr. The "Technicon" slide drier dries slides in a current of warm, filtered air, and some subjects can be dried and made ready to stain in an hour. Dried slides can be stored, possibly indefinitely, in a dust-free place until you are ready to stain them.

Numbering and Recording of Slides

It is necessary to number or otherwise mark research or demonstration slides after affixing the paraffin ribbon. The serial number or code letter of the material can be scratched on the slide with a diamond, carbide steel or carborundum pencil. Waterproof India ink diluted with an equal volume of gelatin adhesive makes an excellent slide marking ink that will adhere to clean glass through the entire staining process.

In some investigations it is necessary to make a complete series of sections from a piece of material. This may be compared to the

many sections from a complete loaf of bread, each slice having a known position in the loaf. A uniform system of placing the sections and numbering the slides should be worked out and rigidly followed. A convenient method is to place the strips of ribbon so that the sections follow the order used in writing, as follows:

1	2	3	4	5
6	7	8	9	10
11	12	13	14	15

The foregoing numbers show the successive order of the sections of a seriation as they are attached to a slide. Another convenient method of designating any given section on the above slide is as follows:

(Row	1)	1	2	3	4	5
(Row	2)	1	2	[3]	4	5
(Row	3)	1	2	3	4	5

The bracketed section is designated as *row 2, section 3*. The numbers 2–3 on the label enable the observer to relocate the section quickly.

A block of tissue may yield so much ribbon that several slides are required to mount the ribbon. In such cases seriation is maintained by mounting the ribbon on the first slide as described above. On the second slide section 16 occupies the upper left hand position as follows:

Slide 1				
1	2	3	4	5
6	7	8	9	10
11	12	13	14	15

Slide 2				
16	17	18	19	20
21	22	23	24	25
26	[27]	28	29	30

The number of sections placed on a slide depends on the space available on the slide, the size of the sections, their spacing in the ribbon, and the size of the available cover glasses. Individual sections on any slide are designated by row and section in that row. The bracketed section, which is actually twenty-seventh in the seriation, is identified by the designation *slide 2, row 3, section 2*. Each of the slides in a seriation should be engraved or marked with marking ink, giving the code number of the lot or collection of embedded material, the number of the piece taken out of that lot, and the number of the slide in the seriation. As a specific illustration, assume that from embedded batch 1 of apple leaf you have removed a piece of leaf (piece 1) and sectioned it, and obtained enough ribbon to fill three slides, with sections in serial order. The slides are numbered 1-1-1, 1-1-2, 1-1-3, meaning, in the last instance, batch (lot) 1, piece 1, slide 3. An individual section on slide 3 is then completely identifiable

as lot 1, piece 1, slide 3, row 2, section 5. This designation on a drawing or photograph makes it possible to locate the exact section used.

In some subjects the cell size or character of the cellular arrangement makes it impossible to relocate a given cell by row and section. A calibrated mechanical stage should be used to study such material. If the stage revolves, decide upon a reference point on the circular vernier, clamp the stage, and study the slide. Having found a cell which will be sought again for further study, take readings on the longitudinal and transverse verniers, and record in your notes and on drawings. It should then be possible at any future time to put the same slide on the microscope, set the verniers to the recorded readings, and locate the particular cell in the field of view.