3. Killing, Fixing, and Storing Plant Tissues

One of the most critical operations in the processing of tissues is the killing of the protoplasm. The stopping of life processes within the cells should be accomplished with the minimum structural disturbance within the cells and minimum distortion of the arrangement of cells in the tissues. In addition to killing the protoplasm, the killing fluid or the subsequent processing must retain or fix the undistorted structure and render the mass of material firm enough to withstand the necessary handling.

No single substance has been found to meet the requirements of successful preservation. The formulas used for this purpose consist of ingredients in such proportions that there is a balance between the respective shrinking and swelling actions of the ingredients. The numerous formulas found in the literature are variations of a comparatively few fundamental formulas, and the chemical substances in the formulas are few in number. Any formula should be regarded as a starting point for experiments to determine the proper balance of ingredients for specific subjects. The formulas recommended in this chapter have been found to be satisfactory for a diversity of subjects.

Preparation of Stock Solutions and Killing Formulas

The following reagents and stock solutions are used in a wide range of killing (fixing) formulas:

Glacial acetic acid.
1% acetic acid (approximately), made by adding 10 cc. of glacial acetic acid to 990 cc. of water.
10% acetic acid, made on the same basis as the above.
Propionic acid may be substituted for acetic acid in the above.
1% chromic acid. (10 g. chromic anhydride crystals per liter.)
Formalin, the trade name used for an aqueous solution of formaldehyde, containing 37 to 40% formaldehyde gas by weight.

Picric acid, saturated aqueous solution.
2% osmic acid. 2 g. crystals in 100 cc. of 1% chromic acid, or in 100 cc. of water.
Ethyl alcohol; commercial 95% grade and anhydrous grade.

The use of stock solutions of 1% and 10% acetic or propionic acid is advocated because the error involved in measuring a small volume, say 1 cc., of glacial acetic acid is much greater than in measuring 10 cc. of 10% acid.

Apparatus

Use specimen bottles that hold a generous quantity of killing fluid, especially with bulky or succulent materials that may dilute the formula. After washing and partial dehydration, materials may be transferred to smaller bottles or vials for the remainder of the process.

When the pieces of plant material are dropped into the killing fluid, the hairs, stomates, folds, and other cavities of plant organs retain air bubbles which retard penetration by reagents. If the pieces do not sink at once, attach the bottle to an aspirator, and apply suction for repeated short intervals until the pieces sink, if not to the bottom of the liquid, at least under the surface. Use a safety bottle (Fig. 3.1 *A*) to keep water from backing into the specimen

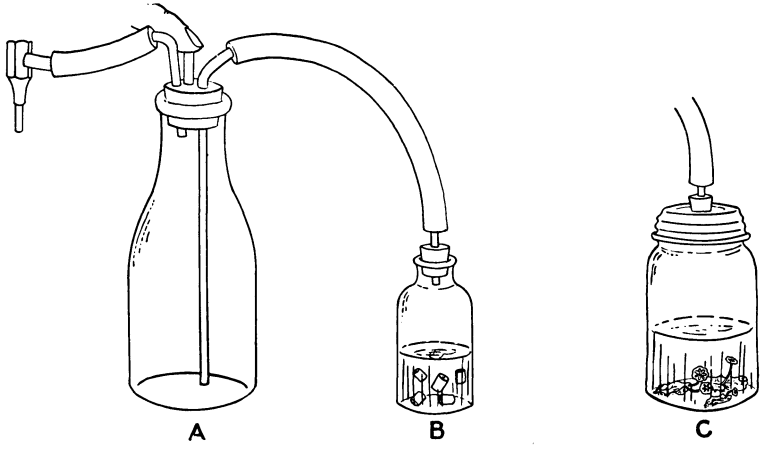

FIG. 3.1—Aspirator setup for pumping specimens in killing fluid: *A*, safety bottle with finger valve or glass stop clock; *B*, specimen bottle or large empty bottle into which specimen bottle is placed; *C*, pint jar used as container for large specimens.

bottle. Tapping the specimen bottle gently against the sink aids in the loosening of air bubbles within or on the specimen. Highly buoyant materials should be placed into a tall vial of the killing fluid and held below the surface by means of a plug of cheesecloth. A screw-topped wide-mouthed bottle is necessary for evacuating large objects (Fig. 3.1 *C*). When most of the pieces remain submerged after the suction is released, push any floating pieces under the surface with a matchstick, and most of them will then sink. Remove and discard all pieces that do not sink after pumping and submersion.

Materials from which it is difficult to evacuate air do not become infiltrated readily and should be pumped again when nearing the end of the dehydrating series, and again when in the final change of paraffin solvent, before any paraffin has been added. Connect a second safety bottle between the regular safety bottle and the specimen. The possible entry of water vapor into the specimen bottle when the pump is shut off is prevented by having a deep layer of calcium chloride and a layer of cotton in the second safety bottle. The ingenious and precisely controllable vacuum apparatus of Wittlake (1942) may be used for the killing, as well as the subsequent operations of embedding.

Killing and Fixing of Tissues

Killing solutions may be grouped into types on the basis of the ingredients used. Some formulas are stable and may be kept on hand ready for immediate use. Other formulas must be made up immediately before use. The formulas given on the following pages have been computed so that they can be made up from the above stock solutions by volumetric measurements. The system of letters and numbers used in this manual to designate killing fluids is explained later in this chapter.

The length of time necessary to bring about killing and hardening of material varies greatly and is determined by the character of the fluid used, the bulk of the individual pieces, and the resistance of materials to penetration by reagents. Fluids of the anhydrous type, such as Carnoy's absolute alcohol-glacial acetic acid formula, penetrate small objects almost instantaneously, and killing and hardening are a matter of minutes. The chrome-acetic fluids penetrate slowly into the interior of a piece of tissue, and have poor hardening action. Recommendations concerning the duration in killing fluids are given in the description of the various formulas. Washing of tissues, which

is necessary after some killing fluids, is discussed in connection with specific formulas.

One of the most useful types of killing and preserving fluid, known as *FAA,* is represented by the following formula:

Ethyl alcohol (95%)	50 cc.
Glacial acetic acid	5 cc.
Formaldehyde (37–40%)	10 cc.
Water	35 cc.

Propionic acid may also be used, the formula is then designated *FPA.*

Several modifications may be found in the literature. This fluid is stable, has good hardening action, and material may be stored in it for years. These properties make this formula suitable for large or impervious objects such as woody twigs, tough herbaceous stems, and old roots. The high concentration of alcohol is likely to produce shrinkage of succulent materials, although it is possible to develop a formula for some apparently tender subjects and even for filamentous algae. A balanced formula can be worked out by varying the acetic acid, which has a swelling action on protoplasm, from 2 to 6% by volume. The formaldehyde and alcohol, which have a shrinking action, should be held at the indicated concentrations. When making trials of variations from the fundamental formulas, kill a trial lot or batch of material in the formula to be tested, and a check lot in a standard formula, and carry the batches through identical processing simultaneously, so that differences in cellular detail will be the result of variations of formula.

Pieces of thin leaf are killed and hardened in 12 hr. The actual killing of the protoplasm probably occurs in much less time. Thick leaves or pieces of small stem require at least 24 hr. Woody twigs should be kept in *FAA* at least a week before continuing the processing for embedding. Materials do not need to be washed after *FAA.* The ingredients of this fluid are soluble in the dehydrating agents and are thus removed before infiltration is begun.

An extensively used formula consists of *FAA* containing bichloride of mercury ($HgCl_2$) to saturation. This fluid penetrates and hardens tissues rapidly. It preserves bacterial zoogloea in plant tissues, thus being useful in pathological studies. The alcohol may be increased to 70%. Prolonged storage in fluids containing bichloride is undesirable. The tissues should be transferred after 48 hr., or at most a week, to a fresh solution of the original formula which does not

TABLE 3.1

KILLING FLUIDS OF THE CHROME-ACETIC AND FLEMMING TYPE *

(The numbers in the columns represent cubic centimeters of the designated reagents)

	Chrome-acetic					Flemming type			
Stock solution	Weak I	Weak II	Medium I	Medium II	Strong	Weak	Medium	Strong	Chamberlain
1% chromic acid	30	50	50	70	97	25	50	75	96
1% acetic acid	70	50	.....	.....	.....	10	.....	.....	.....
10% acetic acid	.....	.....	10	20	.....	.....	10	.....	.....
Glacial acetic acid	.....	.....	.....	.....	3	.....	.....	5	3
2% osmic acid	.....	.....	.....	.....	.....	10	10	20	1
Water	.....	.....	40	10	.....	55	30	.....	.....

* The formulas in Tables 3.1 and 3.2 have been arranged and arbitrarily numbered, beginning with the weaker solutions.

contain the bichloride of mercury. After four or five changes of the latter solution, tissues may be stored indefinitely in the last change.

Chromic acid and acetic acid are the ingredients of an important class of fluids, the *chrome-acetic* formulas. These fluids are not used as extensively as some years ago. Table 3.1 gives the proportions of five formulas. Because of widespread use of the terms weak, medium, and strong for fluids of the type given in Table 3.2, these terms are retained for the series of modifications in the table. The weaker solutions are suitable for succulent or delicate subjects, the strong solution for firm subjects. If this type of fluid is to be used for a critical study, a balanced formula should be worked out by balancing the shrinking action of chromic acid and the swelling action of acetic acid.

The above formulas are not very satisfactory for bulky or woody subjects because of poor penetrating ability. Use these mixtures for filamentous and thalloid plants, root tips, floral organs, and small sections of leaves or stems. The length of time required to kill materials varies greatly. Filamentous algae are probably killed in a few minutes. Small pieces of leaf or root tips require about 12 hr. Larger pieces of tissue should have at least 24 hr. The progressive destruction of chlorophyll from the cut edges inward is a good gauge of the rate of action. Prolonged storage in chrome-acetic produces brittleness of the tissues and muddy staining effects. Therefore, these fluids are not suitable for storage and the tissues must be processed after the optimum interval necessary for killing.

Materials killed in the fluids given in Table 3.1 should be washed in running water. Various devices may be used for accomplishing this prolonged washing. The simplest method is to tie a strip of cheesecloth over the wide mouth of the bottle containing the tissues and to allow a slow stream of water to flow into the bottle. More vigorous washing action can be obtained by inserting the water inlet tube to the bottom of the specimen bottle. These fluids do not have good hardening action, so it is best to avoid violent motion of the pieces. Firm materials can be washed in a vertical length of 1-in. glass tube with a stopper at the lower end, admitting a stream of water through a small tube, the waste water leaving through cheesecloth tied over the upper end of the large tube.

Osmic acid is used in a class of formulas known as the Flemming fluids. These fluids are indispensable for cytological studies but are seldom justifiable for histological work. Osmic acid is expensive, its vapors are highly irritating, and it blackens tissues, making it necessary to bleach sections before staining. Osmic acid preserves chromosome details with great fidelity, but has no special virtues for the preparation of slides of such subjects as corn stem or apple leaf for anatomical or histological study. Osmic acid has poor penetrating ability and is therefore not satisfactory for bulky objects. The formulas given in Table 3.1 will serve for preliminary tests, subject to experimental variation of proportions. Because of the blackening action and poor hardening properties of the Flemming fluids, material should be washed in water and processed immediately after killing. The intervals for killing are approximately those given for chrome-acetic.

Table 3.2 gives several formulas based on the Nawaschin formula, containing chromic acid, acetic acid, and formaldehyde. Numerous modifications may be found in the literature. The name Craf has been coined for this widely used type of fluid. For critical work on specific subjects, experiment with variations of the formulas in the table. The acetic acid should be varied from 0.7 to 5% glacial acetic acid equivalent by volume. The optimum chromic acid and formaldehyde concentrations for many subjects are the proportions given in formula V. The other formulas in the table, including Nawaschin's original formula, also give good results with specific subjects. The formaldehyde should be added immediately before using. If one of these formulas is to be used for making extensive collections in the field, it will be found convenient to make up the desired mixture of the chromic and acetic acids, adding the measured volume of for-

TABLE 3.2

KILLING FLUIDS BASED ON THE NAWASCHIN AND BOUIN FORMULAS

(The numbers in the columns represent cubic centimeters of the designated reagents)

	Nawaschin type (Craf)						Bouin	Allen-Bouin type		
Stock solution	Nawaschin	I	II	III	IV	V		I	II	III
1% chromic acid	75	20	20	30	40	50	.....	50	50	25
1% acetic acid	.....	75	....	....	....	....	.....	....	....	....
10% acetic acid	.....	....	10	20	30	35	.....	20	....	40
Glacial acetic acid	5	....	....	....	....	....	5	....	5	....
Formaldehyde 37–40% aqueous	20	5	5	10	10	15	25	10	10	10
Picric acid saturated aqueous	.....	....	....	....	....	....	75	20	35	25
Water	.....	....	65	40	20	....	.....	....	....	....

maldehyde before using. A few hours after the formaldehyde is added a perceptible change of color takes place in the liquid, and after several days the chromic acid becomes changed to an olive or green compound. Long before this condition is reached, killing action has been completed, and the altered fluid then serves as an excellent hardening and preserving agent. Material may be left in these fluids for as long as 5 years and yield excellent histological preparations. The effect of prolonged storage on critical cytological details deserves further study. The minimum time for small masses of soft tissue is 12 hr., but it is obvious from the foregoing remarks that several days at least can be allowed to insure hardening without danger of distortion or darkening of the material. A further advantage of the Nawaschin type fluid is that materials need not be subsequently washed in water, thus avoiding the possible softening and pulping of material.

Bouin's fluid, given in Table 3.2, has long and deservedly been a favorite. It is excellent for root tips, especially for telophase figures, and has been used successfully for embryo sac studies. The complete mixture is stable and may be kept on hand in the laboratory or carried to the field ready for use. A minimum interval of 12 hr. is suggested for finely divided material. Larger pieces such as thick root tips or mature tissues should have at least 48 hr. Prolonged storage is regarded as undesirable. After the optimum interval in the killing fluid, the material is not washed in water, but is rinsed several times in 20% alcohol or acetone. Dehydration is then continued as described later.

The addition of chromic acid and urea to Bouin's fluid makes what is known as the Allen-Bouin formula. For cytological work use the original formula, as given in the reference manuals, or one of the formulas (lacking urea) given in Table 3.2. For further trials vary the glacial acetic acid equivalent from 1 to 4% by volume. The formaldehyde should be added immediately before using. Tests have shown that tissues may be left in these solutions for several months. It is probable that hardening of the material reaches a maximum in less than a week. Dehydration and subsequent processing are carried out as with Craf.

Farmer's fluid and Carnoy's fluid have limited uses in histology. These fluids kill protoplasm by rapid and probably violent dehydration. Because of their ability to penetrate very rapidly, these fluids have some value for processing extremely downy, resinous, or impermeable structures that must be preserved entire. The fluid may be used alone, followed in 1 hr. or less by the subsequent operations of the paraffin process. An alternative method consists of first immersing the materials in a Carnoy or Farmer formula (the time ranging from an instantaneous dip to 10 min.) and then treating in one of the more critical fluids. Two widely used formulas are as follows:

1. Farmer's fluid
   Anhydrous ethyl alcohol ..... 75 cc.
   Glacial acetic acid ..... 25 cc.
2. Carnoy's fluid
   Anhydrous ethyl alcohol ..... 60 cc.
   Glacial acetic acid ..... 10 cc.
   Chloroform ..... 30 cc.

The fluids given thus far in this chapter produce an *acid fixation image,* preserving particularly well the chromosomes, nucleoli, and the spindle mechanism. Nucleoplasm and mitochondria are dissolved; cytoplasm is rendered in fibrillar or alveolar form. This type of image is preferred for most studies of plant structure.

In certain cytological studies it is desirable to preserve mitochondria and allied cytoplasmic structures. In such cases a fixing fluid that produces a *basic fixation image* is used. Such fluids preserve mitochondria, nucleoplasm, and in some instances nucleoli and vacuoles. Chromatin and the spindle mechanism are dissolved. For serious studies in this field of cytology each worker must work out specific techniques based on an extensive literature. However, it is possible

to produce slides showing mitochondria adequately for teaching purposes, using Zirkle's modification of Erliki's fluid.

Water	400 cc.
Potassium bichromate	2.5 g.
Ammonium bichromate	2.5 g.
Cupric sulphate	2.0 g.

Fix for 24 to 48 hr., wash in water, dehydrate and embed in paraffin.

The desirability of wetting agents and penetrants in microtechnique has been apparent to experienced workers for many years. The rapid development of numerous wetting agents in recent years has led to considerable experimentation, with the expected diverse results. The most prominent unfavorable effect of wetting agents are the peeling of cuticle and epidermis, and varying degrees of cell distortion. Further experimentation with the increasing number of available substances is certainly desirable.

The wetting action of a substance can be tested easily. Make a series of solutions of the substance to be tested by diluting a 1:1000 stock solution. Cut uniform pieces of a highly pubescent leaf and drop alternating pieces into distilled water and into the dilutions of the wetting agent. Note the relative time required for the leaf pieces to sink, and use the most dilute wetting agent that will sink the tissues after brief aspirating. Determine whether the wetting agent forms a precipitate or cloudiness with the killing fluid. If a reaction occurs, do not add the wetting agent to the fixing fluid, but sink the tissues in the wetting agent, rinse with water and cover with the killing fluid. The final criterion is the condition of the tissues in the finished slide, compared with tissues processed without the wetting agent.

A workable terminology for designating killing fluid formulas is a great convenience for giving oral or written instructions, or making routine records. The name of the investigator who first devised a type of formula is not always a satisfactory designation because the proportions of the ingredients are necessarily varied for different subjects. An arbitrary number is not sufficiently descriptive, except among a group of closely associated workers. The terminology proposed here is a compromise, the type of formula is indicated by a name or abbreviation, and the proportion of ingredients by a percentage figure. The proportion of a solid like chromic acid is given as precentage by weight; liquids like melted glacial acetic acid are given as percentage by volume. For instance, the time-honored chrome-acetic has numerous variants, one of which is C-A 0.5-0.5, meaning 0.5% chromic acid

by weight, and 0.5% acetic acid by volume. Table 3.1 gives the proportions of stock solutions used to make 100 cc. of mixtures in this category.

A variant of the Nawaschin formula, Craf 0.20-1.0-10.0, contains 0.2% chromic acid, 1.0% acetic acid, and 10.0% commercial formaldehyde solution. A variant of the Allen-Bouin formula is designated A-B 0.20-4.0-10.0-25.0, containing in addition to the ingredients of Craf, a saturated aqueous solution of picric acid, 25.0% by volume.

The foregoing system of terminology is accurate, descriptive, and convenient and has been used successfully by beginners and advanced workers.