10. The Preparation of Whole Mounts and Smears

Preparations made without sectioning may be roughly classified as follows:

1. Dry preparations: herbarium sheets, Riker mounts, and bulk specimens. These methods are not within the scope of this manual.
2. Wet preparations: museum-jar preparations; bulk material for dissection.
3. Whole mounts, smears, and macerations for microscopic study.

Wet preservation may be used for a wide range of subjects in all major categories of the plant kingdom. Many subjects, such as the algae, can be preserved for critical study only by wet preservation. Entire plants or parts such as leaves, flowers, and fruits can be preserved in fluids that kill the cells, prevent decay, preserve the material in firm condition, and possibly preserve the natural colors.

The best-known preserving fluid is ethyl alcohol, usually used at a concentration of approximately 70%. This fluid preserves even the bulkiest objects. Considerable brittleness is produced, but preserved material can be made flexible for dissection by soaking it in water. Cells are plasmolyzed by alcohol, but this is not objectionable in dissections or freehand sections, in which the condition of the protoplasm is not important. Ethyl alcohol is difficult to obtain in some institutions, its use at field stations may be undesirable, and shipment of materials in alcohol involves legal technicalities. Regardless of these objections, ethyl alcohol is firmly established as a preserving fluid. Isopropyl alcohol may also be used.

Formaldehyde is an excellent preservative. This reagent is obtained as an aqueous solution containing 37 to 40% formaldehyde gas by weight. The U.S.P. (United States Pharmacopoeia) grade is adequate for preserving bulk materials. The most useful concentration for bulk preservation contains 5 parts formaldehyde solution in 95 parts of water. For massive objects the concentration must be doubled. At these concentrations the material does not become brittle, and some materials become pulpy after prolonged storage. Formaldehyde

vapor and the solution are highly irritating and poisonous, producing persistent skin and pulmonary disorders. Materials that have been stored in strong formaldehyde should be rinsed in water if used for prolonged study.

An improvement over formaldehyde for the preservation of algae is the following:

Water	93 cc.
Formaldehyde (U.S.P.)	5 cc.
Glacial acetic acid	2 cc.

Hydrodictyon and *Spirogyra* stored in this fluid for 5 years were found to be in excellent condition for whole mounts in water. A further improvement is to add glycerin.

Water	72 cc.
Formaldehyde (U.S.P.)	5 cc.
Glacial acetic acid	3 cc.
Glycerin	20 cc.

This is one of the best preservatives for unicellular, filamentous, and even the larger bulky algae, for fleshy fungi, for liverworts, and for mosses. A trace of fast green dye imparts enough color to minute or transparent subjects to make them more readily visible under the microscope. The material should be mounted on a slide in a drop of the preservative. The volatile ingredients soon evaporate, but the glycerin prevents drying of the preparation during a long period of study. Liverwort thalli can be removed from the fluid and placed in a watch glass for study. The thalli of *Marchantia,* for example, are so firm that the gametangial disks stand upright in a normal position. The glycerin keeps the material moist for hours, and specimens can be returned to the stock bottle uninjured.

The natural colors of plants can be preserved in one of several formulas. The simplest formula for green plants consists of one of the *FAA* formulas with copper sulphate added (Blaydes 1937). The following formula is usually satisfactory:

Water	35 cc.
Copper sulphate	0.2 g.

When completely dissolved, add

Glacial acetic acid	5 cc.
Formaldehyde (U.S.P.)	10 cc.
Ethyl alcohol (95%)	50 cc.

Exhausting the air from the submerged specimens with an aspirator aids penetration. If the materials can withstand brief boiling in the fluid on a water bath, penetration and fixation of the color are hastened. Materials preserved in this formula can be subsequently embedded and sectioned, provided that the pieces are small enough to insure quick penetration of the preservative and satisfactory preservation of cellular details.

Keefe's formula is one of the best and should be used if the expensive uranium salt is available.

50% alcohol	90 cc.
Formaldehyde (U.S.P.)	5 cc.
Glycerin	2.5 cc.
Glacial acetic acid	2.5 cc.
Copper chloride	10 g.
Uranium nitrate	1.5 g.

Delicate subjects may be ready to use in 48 hr., but most materials require 3 to 10 days for complete fixation of the color. Leafy plants can be treated and then mounted as herbarium specimens, in which the color will persist for many months. This formula does not preserve the colors of flowers, nor is it satisfactory for gymnosperms.

The red and yellow coloration of fruits can be preserved in the following formula (Hessler):

Water	1 l.
Zinc chloride (dissolve in boiling water and filter)	50 g.
Formaldehyde (U.S.P.)	25 cc.
Glycerin	25 cc.

Allow to settle and decant the clear liquid for use as needed.

With the introduction of dioxan into microtechnique, several workers independently developed the idea of using this reagent in killing and preserving fluids. McWhorter and Weier (1936), for example, devised the following formula:

Dioxan	50 cc.
Formalin	6 cc.
Acetic acid	5 cc.
Water	50 cc.

This solution preserves unicellular and filamentous algae, fungi, and other delicate subjects. Temporary mounts for microscopic study can be made on a slide in a drop of this fluid, or permanent slides can be made.

Temporary and Semipermanent Slides

The foregoing outline of methods of preserving bulk materials leads to a consideration of methods of preparing mounts for microscopic study. The simplest method obviously consists of mounting a small quantity of the material in a drop of water. However, water mounts dry out during prolonged study, and it is better to mount the material in 10% glycerin. As the water evaporates, introduce more glycerin solution under the cover glass at intervals, until no further evaporation takes place. Such preparations can be kept almost indefinitely if stored flat and handled with care.

The preservative and swelling action of lactic acid and phenol (carbolic acid) is utilized in an important class of formulas. More or less durable slides of algae, fungi, fern prothalli, sections, and other small objects can be made by mounting in one of these lactophenol solutions, with or without added dye. The following selected formulas are taken from Maneval's valuable compilation of these methods.

Aman's Lactophenol

Phenol (melted)	20 cc.
Lactic acid	20 cc.
Glycerin	40 cc.
Water	20 cc.

Phenol-Glycerin

Phenol (melted)	20 cc.
Glycerin	40 cc.
Water	40 cc.

If a staining effect is desired, add a 1% aqueous solution of either cotton blue, aniline blue, or acid fuchsin as follows:

Lactophenol	100 cc.
Glacial acetic acid	0 to 20 cc.
Dye solution	1 to 5 cc.

The optimum concentration of glacial acetic acid is that which produces no collapse or bursting of cells or filaments. Try the above formula and dilute with lactophenol until the best proportions are established.

Glycerin-jelly preparations are a further advance toward permanent slides and are preferred to glycerin mounts if the slides must withstand considerable use. The mounting medium is made as follows:

Gelatin	5 g.
Water	30 cc.
Glycerin	35 cc.
Phenol (dissolved in 10 drops water)	5 g.

Dissolve the gelatin in the water at 35°C.; then add the other ingredients. Filter while warm through fine silk or coarse filter paper. This mounting medium keeps well. Materials to be mounted in glycerin jelly are first stained (if necessary), then dehydrated by the glycerin evaporation process. For filamentous algae and fungi the most satisfactory stains are the self-mordanting hematoxylins and iron hematoxylin. Staining trials can be made with small quantities of the plant material until a satisfactory schedule is worked out. Then stain a large batch, and dehydrate by the glycerin method. To make a slide from the dehydrated material, place a piece of glycerin jelly about as large as a match head on a clean, dry slide, and warm until melted. Remove a quantity of the plant material from the pure glycerin, draw off excess glycerin with filter paper, and put the plant material into the warmed jelly. Lower a cover glass carefully over the material. If the material is not excessively fragile, a lead weight on the cover glass will squeeze out excess jelly and make a thinner mount. When the jelly is cool, clean off any excess around the cover glass and seal around the edge of the cover glass with a quick-drying lacquer such as Duco. Sealed preparations will keep for several years, but it is well to remember that the mounting medium is soft. Such preparations are not desirable for use by elementary students.

Permanent Slides of Whole Mounts

Permanent stained slides in a hard, durable mounting medium are much more satisfactory than soft, easily damaged, temporary or semipermanent slides. Modern methods make possible the rapid, quantity production of permanent slides almost as quickly and easily as the making of old-fashioned semipermanent slides. Filamentous algae and delicate objects may be killed in any of the formulas described in the foregoing pages. Wash out the killing or storage fluid with water and apply an appropriate stain. Any of the self-mordanting hematoxylins will give excellent results. It is best to overstain strongly, leaving the material in the stain for ½ to 1 hr. Wash in distilled water until the rinse water is no longer tinted. Destain in 1/10% HCl. Cover the material with the acid in a shallow dish and agitate gently. After 1 to 2 min. drain, rinse in tap water, and examine with a microscope. Repeat the treatment in acid until only the nuclei and pyrenoids remain blue-black, then wash thoroughly in tap water.

Iron hematoxylin will give the most critical staining of nuclear structures and pyrenoids. Mordant algae for 1 to 2 hr. Rinse quickly but thoroughly in distilled water and stain for 2 to 8 hr. Destain by

immersing in the destaining agent for 1 to 2 min., rinse in *distilled* water, examine with the microscope, and repeat the brief immersion in alum until the nuclei and pyrenoids are sharply differentiated. After the last rinsing in distilled water, wash thoroughly in tap water. Dehydration and mounting may be accomplished by one of the several methods outlined below.

THE VENETIAN TURPENTINE METHOD

The Venetian turpentine method yields excellent preparations, but this method is likely to be supplanted by modern methods using a variety of synthetic organic solvents and synthetic resins. Therefore, only a brief outline of the method will be given, and the interested reader is referred to Chamberlain for details (1932). Kill and stain the material, and dehydrate by the glycerin evaporation method. Rinse out the glycerin in 95% alcohol, then complete the dehydration in at least three changes of absolute alcohol. Transfer to 10% Venetian turpentine in absolute alcohol. Eliminate the alcohol by evaporation. When the material is in thickened turpentine, mount the desired amount of the material in a drop of that medium. Dry the slides in a horizontal position. Preparations made by this method are durable and the stains are permanent.

THE BUTYL ALCOHOL-RESIN METHOD

The term resin is used here in the broad sense to include Canada balsam as well as the increasing number of synthetic resins. Test the solubility of the currently available mounting resins in *anhydrous* normal and tertiary butyl alcohol and in dioxan, and use the solvent-resin combination that has the highest transparency and least color. The following tertiary butyl alcohol – Canada balsam procedure serves as an example of the series of operations in a whole-mount method. Stain and wash as before. Transfer a small quantity of the material into a watch glass of 50% alcohol, and observe with the microscope. If there is much distortion, try 20% alcohol on another batch. Well-hardened material can withstand 50%. When the proper starting point for dehydration is established, carry the material in steps of 20 to approximately 70% alcohol. Add to the 70% a few drops of stock solution of counterstain – eosin Y, erythrosin B, or fast green, saturated solution in absolute alcohol or in methyl Cellosolve. Leave in the counterstain until slightly overstained. This may require 4 to 12 hr. Rinse in 70% alcohol, and transfer through the following series at ½- to 1-hr. intervals:

3 parts alcohol to 1 part *TBA* (anhydrous tertiary butyl alcohol)
2 parts alcohol to 2 parts *TBA*
1 part alcohol to 3 parts *TBA*
Pure *TBA;* change twice at 15-min. intervals

Transfer to a large volume of 5% solution of balsam or synthetic resin in *TBA* in a short wide-mouthed bottle. Allow the *TBA* to evaporate slowly at a temperature of about 35°C. When the balsam is slightly more fluid than that used for covering sections, mount the material. Remove a suitable quantity of the plant material with its enveloping balsam, place on a dry, clean slide and lower a dry cover glass carefully so as not to produce bubbles or to push the plants too close to the edges. Dry the finished slides in a horizontal position. (Johansen, 1940.)

THE DIOXAN-BALSAM METHOD

This is the most promising of the newer methods of making whole mounts. The method was worked out in almost identical form independently by McWhorter and Weier (1936), Johansen (1937), and the present writer. It is probable that numerous other workers had developed similar schedules.

Stain filamentous or other delicate materials as in the preceding methods. Pass through a series of aqueous solutions of dioxan, containing the following percentages of dioxan: 20, 40, 60, 80, 90, then three or more changes of *anhydrous, chemically pure* dioxan. The interval in each should be 1 to 2 hr. Examine a few filaments under a microscope, mounted in the last fluid. If the material is in good condition, transfer to a 10% solution of balsam or resin in dioxan. Use a wide-mouthed bottle and gauge the volume of the liquid so that the material does not become exposed as the dioxan evaporates. Place the uncovered container into an oven or a dust-free place at a temperature of approximately 35°C. The dioxan evaporates in 2 to 8 hr., leaving the material in thick resin in which it is mounted. Extremely fragile materials, such as *Volvox* or *Vaucheria,* should be started in 5% resin and evaporated very slowly by keeping the container loosely covered.

This process is so rapid that it is worth the time to carry small trial lots of the material through the process at different speeds. The condition of the material can be examined at various points in the process. When the optimum or shortest safe schedule is found, the main batch of material can be carried through.

ACETOCARMINE (OR ACETO-ORCEIN) SMEARS

The acetocarmine method has become so commonplace that it may well be included in an elementary manual. This rapid method combines killing, fixing, and staining. The freshly-made preparations can be used in nonpermanent form for counting chromosomes, determining their association, and studying intimate details of structure. The slides can be made permanent if desired.

The stain is prepared by dissolving 1 g. carmine in 100 cc. of boiling 45% acetic acid (or propionic acid). Cool and decant. Add 2 drops of a saturated aqueous solution of ferric acetate, and allow to stand for 12 hr. Filter and store the main stock in a refrigerator, keeping a small quantity in a dropper bottle in the laboratory for immediate use. Some workers omit the iron salt and incorporate the correct amount of iron from the reaction between steel dissecting needles and the acetic acid in the dye. This requires considerable experience. Excessive iron prevents clear differentiation and may produce a dark granular precipitate. This can be minimized by cleaning the steel needles in 45% acetic acid periodically during dissection. If iron is added to the dye formula, use nickel or chromium plated needles or pyrex needles drawn to an abrupt, fine point.

The simplest method of using acetocarmine consists of macerating or smearing a fresh anther in a drop of the stain. Small anthers may be dropped into the dye entire, then dissected under a binocular, discarding pieces of anther wall and leaving only the masses of sporocytes. Large anthers should be sliced into the thinnest possible slices on a sheet of wet blotting paper, the fragments transferred to a drop of dye and macerated as above.

Lower a cover glass over the drop of dye containing the sporocytes, and press or tap gently. A careful sliding motion on the cover glass sometimes aids in smearing the cells into a thin film. Pass the slide quickly over an alcohol lamp flame several times. The amount of heating must be determined by trial, but do not heat to the boiling point. Drain off excess stain, and seal the edges of the cover glass with paraffin. Examine the slides with a microscope, and store satisfactory ones in a refrigerator. The color improves in a few days, reaches maximum intensity, then begins to deteriorate.

Fresh anthers are not available at all times, and it is often necessary to make extensive collections in the field for subsequent study.

Kill entire anthers, or grass spikelets, or even large portions of an inflorescence in Farmer's or Carnoy's fluid. Maize cytologists

prefer an alcohol-acid ratio of 3:1, but ratios of 1:1, 2:1 etc. may be tested until the optimum fixation is obtained for a given species or stage. Plant materials can be stored in these fluids for months at 0°C., depending on specific response. Most workers transfer the tissues after 1 or 2 days of fixation to 70% alcohol, in which tissues can be stored for many months in a refrigerator. Good preservation for as long as a year can be obtained by storing in acetic-alcohol in a freezer.

Root tip smears are being used extensively for chromosome counts and for the study of the morphology of metaphase chromosomes. The principal problem is to obtain separation and flattening of cells. Some large root tips, such as onion, may be squashed flat easily, whereas the small root tips of sweet clover resist crushing.

Fix the root tips in Farmer's or Carnoy's fluid for at least one day. Smear directly from the fixing fluid, or transfer to 70% alcohol for prolonged storage. The responses of a species to the fixing formula, the fixing interval and the permissible storage period in fixing fluid or 70% alcohol, must be determined by experimentation.

The piece of root tip that is fixed is usually at least three times as long as the most actively meristematic region. Cut off this active end into a drop of acetocarmine, and macerate into linear strips with needles. Some materials are benefited by some heating at this stage. Cover and heat carefully. Press or smear the cover until the cells are sufficiently separated and flattened.

The separation of cells can be improved by hydrolyzing the middle lamella with 5 to 10% HCl. The acid may be in water, or 70% alcohol, or in Farmer's fluid. After immersion in the acid for 5 to 30 min., return the roots to fixative, which should be changed at least once. Enzymes also have been suggested for digesting the middle lamella.

Clumping of chromosomes can be prevented, and some morphological details accentuated by pre-treatment of the living root tips. Soak the tips in a saturated aqueous solution of paradichlorobenzene for 1 to 4 hours and fix in the preferred formula. Methyl alcohol, in concentrations of 1% to 3% in water has also been used for pre-treatment.

Several readily available plants, especially *Rhoeo* and *Tradescantia,* yield excellent sporocyte smears that show the coiled chromonemata (Sax and Humphrey). Smear sporocytes on a clean slide and flood with 1% ammonium hydroxide in 30% alcohol. Make trials at intervals of 10–30 seconds. Drain and cover with aceto-

carmine. The time and temperature of heating at this stage must be determined by trial. The slide can be studied as a temporary slide, or made permanent. Variations of the acetocarmine process can be found in Smith's comprehensive review.

THE FEULGEN METHOD

The Feulgen smear or squash technique has become firmly established in cytological research. The process and allied methods are under intensive study in many laboratories; therefore, only a few citations are given here. Lillie described an error-proof method of making the Schiff reagent for the Feulgen stain (1951). Dissolve 1 g. basic fuchsin and 1.9 g. $Na_2S_2O_5$ in 100 cc. of 0.15 *N* HCl. Shake on a mechanical shaker for two hours. Add 0.5 g. fresh activated charcoal, shake for two minutes, and filter. If the reagent becomes pink, add charcoal, shake, and filter. Store the pale yellow reagent in a refrigerator.

The reagent can be used with sectioned material (Lillie, 1954). Many dyes other than fuchsin can be used to make Schiff-type reagents (Kasten, 1950).

To make squash preparations, fix tissues in Farmer's fluid. Hydrolize in *N* HCl for 8–10 min. at 60°C. The time and temperature are critical. Drain and cover with Feulgen reagent. After 1 hr. at room temperature, transfer a piece to 45% acetic acid and squash with a glass rod. Cover and seal, or make permanent.

Smear preparations can be made permanent. The process consists of dehydrating the cells and mounting in a resin. If the cells are smeared on perfectly clean glass, they will adhere during subsequent treatment. Slip the cover glass by immersing the slide in equal volumes of acetic acid and alcohol, inverted in a Petri dish, with one end of the slide held up on a glass rod. Pass the slide and its cover glass through the following fluids at 2–5 min. intervals:

(1) equal volumes of ethyl and tertiary butyl alcohol
(2) tertiary butyl alcohol, 3 changes

Place the slide with the smear upward on blotting paper, place a drop of thin balsam or resin on the smear, and lower the cover glass carefully. Place a lead weight on the cover glass. A similar acetic acid-ethyl alcohol-xylene series can be used (McClintock) but more intermediate gradations must be used to prevent collapse of the sporocytes.

A quick-freeze method of making slides permanent, using dry ice (Conger and Fairchild), paved the way for an even better method using liquid carbon dioxide (Bowen).

MACERATION

Whole mounts or sections of tissue frequently do not convey an adequate three-dimensional impression of cell structure. A valuable and much-neglected type of preparation is made by isolating complete individual cells from a mass of tissue. This is accomplished by chemical and mechanical separation of cells. Divide the material into slivers thinner than a toothpick. If the material is dry, boil in water containing a wetting agent, aspirating if necessary. Treat with one of the following macerating processes:

Schultze's Method.—Cover the material with concentrated nitric acid.
Add a few crystals of potassium chlorate.
Heat gently on a sand bath, in a closed hood, until the material is bleached white.
Wash thoroughly, and shake with glass beads until the material disintegrates.
Increase or decrease the duration of heating until entire unbroken cells can be isolated.

Jeffrey's Method.—The macerating fluid consists of equal volumes of 10% chromic acid and 10% nitric acid.
Treat for 1 to 2 days at 30 to 40°C.
Wash and shake with glass beads.

Harlow's Method.—Treat the subdivided and boiled material in chlorine water for 2 hr.
Wash in water.
Boil in 3% sodium sulphite for 15 min.
Wash and macerate.
Repeat chlorination and the sulphite bath if necessary.

Following any of these maceration treatments, wash the pulp thoroughly by decantation. A centrifuge is an aid in washing. Unstained material may be mounted in water or glycerin for study. The cells may be lightly stained in aqueous safranin, washed, and mounted. The mounting media described on pages 102–5 may be used to make semipermanent or permanent slides.