4. Dehydration for Embedding

This operation removes water from the fixed and hardened tissues. Dehydration has some washing action, and makes the material firm and possibly hard and brittle. The process consists of treating the tissues with a series of solutions containing progressively increasing concentrations of the dehydrating agent and decreasing concentrations of water. Two contrasting methods are used to dehydrate and prepare materials for infiltration. In the first method to be described, the tissues are dehydrated in a nonsolvent of paraffin and then are transferred to a solvent. In the second method, the dehydrant is also a solvent of paraffin. The first method of dehydration also is used prior to infiltration in celloidin.

Dehydration by Nonsolvents of Paraffin

The most commonly used dehydrating agent in this category is ethyl alcohol. This is usually purchased in two grades, commercial 95% grain alcohol and absolute (anhydrous) alcohol. The solutions in the dehydrating series are made by diluting 95% alcohol with distilled water. After ascertaining the exact concentration of the alcohol purchased from a given source, it is easy to compute a table giving the respective proportions of alcohol and water for each solution in the series. However, since the series is intended to consist of a graded series of solutions rather than definite concentrations, it is quite adequate to assume the 95% commercial alcohol to be 100% and make up a series containing (approximately) 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80% alcohol by volume. Next in the series is the undiluted commercial alcohol (actual 95%), followed by anhydrous alcohol. This graded series of solutions should be kept on hand in the laboratory.

As discussed in the preceding chapter, some killing fluids require more or less prolonged washing of the tissues in water; other fluids require no washing, and dehydration is begun directly after killing or after a brief rinsing in water. Begin dehydration with a dehydrant having approximately the same percentage of water as the killing or storage fluid. For example, after *FAA,* begin dehydration in 50% alcohol. After weak chrome-acetic or the weaker Craf type formula, such as I and II, begin in 5 or 10% alcohol. The stronger formulas such as III, IV, and V, which have greater hardening action, make it possible to begin dehydration in 20 or 30% alcohol. When Craf V was in use for routine chromosome counts, root tips were transferred directly from the killing fluid into 75% alcohol. After Bouin's fluid, begin with 50% for firm subjects and 20% for delicate materials.

Solutions in the dehydrating series are changed by decanting the liquid from the tissues and promptly flooding the material with a generous volume of the solution next in the series. A piece of fine brass-wire screen or a layer of cheesecloth is used to retain materials that tend to float out of the bottle. The volatility of the solutions high in the series demands speed in making the change to avoid drying of the tissues. The material should not be permitted to become dry even for an instant at any stage in the process. Never drain the fluid from several specimen bottles, and then look on the shelf for the next reagent, only to find that the bottle is empty.

The interval in each of the solutions in the series depends on the size of the pieces, the nature of the material, and the solubility of the residual reagents left in the tissues. For root tips or small pieces of leaf use 30-min. intervals up to 70%. After a picric acid formula make each interval 1 hr. For twigs killed in *FAA* use 4- to 8-hr. intervals up to 70%. For large blocks of wood the interval should be about 12 hr. Beginning with 70%, double the previous interval for each grade. Change the cork for a thoroughly dry one when first changing to 100% alcohol. Make three changes of anhydrous alcohol. Plan the timing of the dehydration series so that the series is stopped at 70% for storage until you can resume the process.

Some workers are inclined to make an unnecessary ritual of the time element in dehydration. It is recognized that drastic changes of concentration bring about shrinkage of protoplasm and distortion of cells. Long intervals in low concentrations of dehydrating fluid, or long washing in water, tend to make tissues soft and promote disorganization. Long exposure to high concentrations or anhydrous reagents shrinks tissues and causes brittleness. With these general

precautions in mind the intervals can be regarded as sufficiently flexible to conform to the demands of other duties.

Isopropyl alcohol can be used in exactly the same manner as ethyl alcohol. Isopropyl alcohol can be purchased without restrictions, and the commercial grade can be dehydrated as described on page 29. Methyl alcohol has not been used extensively for dehydrating plant tissues. Its toxicity is objectionable, and the vigorous dehydrating action damages delicate structures.

Acetone is an excellent dehydrant. Its purchase and use present no legal, administrative, or disciplinary problems, making it a desirable substitute for ethyl alcohol. Acetone is obtainable in several grades, at prices that vary widely with the quality and source. If anhydrous acetone can be purchased in drum lots at reasonable cost, only this one grade needs to be stocked and used for all the dehydrating grades. Acetone of good quality, but not strictly water-free, can be obtained and used for the gradations, and the more expensive anhydrous grade used only for the final stages in the process. The procedure with acetone is exactly the same as with ethyl alcohol. It is permissible to change from alcohol, or a killing fluid containing alcohol, to a grade of acetone having approximately the same water concentration.

Acetone is highly volatile, and care should be taken not to permit acetone to evaporate from tissues or slides during processing.

Glycerin is used as a dehydrant, especially for algae and other delicate subjects. The high boiling point of glycerin permits the elimination of water by evaporation. The slow, progressive dehydration prevents sudden changes of concentration and minimizes plasmolysis. Material must be washed in water before using glycerin, because the evaporation process obviously does not wash residual reagents out of the tissues. Moderately firm tissues can be washed in running water, but delicate materials should be washed by diffusion. Rinse the material carefully to remove the bulk of the killing fluid, transfer to a 2-quart jar of water, and allow the jar to stand undisturbed for 2 hr. Siphon off most of the water without agitating the material, and refill the jar with water. Repeat the replacement of water at least twice, then proceed with the glycerin method.

Transfer the material to a large volume of a 5% solution of glycerin in water. Use a wide-mouthed bottle or jar and mark the level of the 5% glycerin. The volume should be so gauged that after the elimination of water the residual glycerin will more than cover

the tissues. Evaporation of water may be accomplished by several methods or combinations of methods. The most practicable are as follows:

1. In an incubator oven at 35 to 40°C.
2. In a desiccator at room temperatures or in the above oven.
3. In a vacuum desiccator or vacuum oven.

If the glycerin solution becomes colored or turbid during evaporation it may be replaced with fresh glycerin solution of the same concentration. When the volume of the solution has been reduced by evaporation to one-half of the original volume, the glycerin concentration is approximately 10%, and the liquid may be replaced with fresh 10% glycerin and the evaporation continued. Most of the water can be removed by evaporation, especially in vacuum. After a nearly anhydrous condition is attained, the tissues are firm enough to withstand transfer directly into anhydrous alcohol. Change the alcohol at least twice, and proceed with the graded transfer to the desired paraffin solvent as described below, or proceed with one of the whole-mount methods (Chap. 10).

TRANSFER TO A SOLVENT OF PARAFFIN (CLEARING)

After the use of dehydrating agents that are not solvents of paraffin, the dehydrated tissues are transferred to a solvent. The term *clearing,* applied to this transfer, is derived from the fact that some paraffin solvents render the tissues transparent. The clearing action is merely incidental to the function of the reagent, to serve as a solvent of paraffin. The most common solvents are xylene (xylol) and chloroform. Either reagent may be objectionable or even toxic to some workers. Xylene is inexpensive and is by far the most widely used solvent. Chloroform is more expensive, but it is less likely to be toxic. Benzene and toluene can be used, but their lower boiling points increase the fire hazard.

As in the case of dehydration, a graded series is used for clearing. After dehydration in ethyl alcohol, the following absolute alcohol-xylene series is used. For critical cytological work 10 gradations have been recommended. The interval in each mixture ranges from ½ hr.

Grade number	Ethyl alcohol %	Xylene %
1	75	25
2	50	50
3	25	75
4	0	100

for very small or thin pieces to 3 hr. or more for large pieces of tissue. A similar acetone-xylene series can be used.

Chloroform may be substituted for xylene in a similar series, except that more abrupt changes are permissible. A practical series is as follows:

(1) ⅓ chloroform
    ⅔ absolute alcohol
(2) ⅔ chloroform
    ⅓ absolute alcohol
(3) Pure chloroform, changed at least once

Chloroform does not make tissues as brittle as does xylene.

Trichloroethylene is a good solvent of paraffin and may be substituted for xylene in the foregoing processes. Trichloroethylene is not inflammable and is not toxic unless inhaled directly in large quantities. It dissolves Canada balsam but does not affect stained sections. This reagent deserves thorough trial with a wide range of subjects. Any reagent that decreases the hazards of fire and poisoning is worth serious consideration.

Cedar oil is an excellent clearing agent after dehydration in ethyl alcohol. The procedure is to pour a layer of cedar oil into a dry vial, then carefully pour the anhydrous alcohol containing the material over the cedar oil. The pieces gradually sink into the oil and become strikingly clear. The alcohol is removed with a pipette, and the cedar oil is rinsed out of the tissues with several changes of xylene.

Recognition of the fact that the transparency of the tissues at this stage of the process is of no value, and the widespread use of the higher alcohols for dehydration and as wax solvents, have practically eliminated the use of clearing oils.

Following dehydration in any of the butyl alcohols or dioxan, no clearing reagent is used, because these reagents are solvents of paraffin. They do not render the tissues appreciably transparent.

Dehydration in Solvents of Paraffin

THE BUTYL ALCOHOL METHOD

Normal and tertiary butyl alcohol have been introduced into microtechnique in recent years and show much promise as dehydrating and infiltrating agents. Normal butyl alcohol, also designated butanol, was the first of these higher alcohols to be used extensively. Lang's careful experiments have shown that a miscibility curve of the three components of the dehydrant may be used to ascertain the

composition of solutions for an ideal dehydrating series. For critical cytological work, follow Lang's miscibility curves (Lang, 1937) in making up a series. The following series is a simplification that has been found to give excellent results in histological and anatomical work.

Grade number	*n*-Butyl alcohol (Butanol)	Ethyl alcohol	Water
1	10	20	70
2	15	25	60
3	25	30	45
4	40	30	30
5	55	25	20
6	70	20	10
7	85	15	0
8	100	0	0

Note that each of the first six grades consists of three ingredients. The last two grades are anhydrous. Use new anhydrous butyl alcohol for Nos. 7 and 8. After being used once, No. 8 may be used to make up any of the first six grades.

After an aqueous killing fluid, wash or rinse the tissues in water, dehydrate in alcohol in the usual manner to 30%, then transfer to the above reagent 1 and follow the series. After *FAA* or other fluids having a water content of about 50%, rinse in 2 changes of 50% alcohol and begin the *n*-butly series with No. 2, in which the water content is 60%. With many histological subjects good results can be obtained by dehydrating to 50% in steps of 10%, then continuing in *n*-butyl series 3, 5, 7, and 8.

Tertiary butyl alcohol (*TBA*) is regarded by some workers as the most ideal dehydrating reagent of any thus far used (Johansen 1940). Unlike the two other butyl alcohols, its odor is agreeable. The cost is at present much too high for extensive routine work. Tertiary butyl alcohol is used in accordance with the principles of dehydration described in the preceding pages. Dehydrate in ethyl alcohol to 50%, then pass through the following series:

Grade number	95% ethyl alcohol	Absolute ethyl alcohol	*TBA*	Water
1	50		10	40
2	50		20	30
3	50		35	15
4	50		50	
5		25	75	

Make three changes of anhydrous tertiary butyl alcohol and proceed with infiltration in wax.

An unfavorable factor in the use of tertiary butyl alcohol is that it solidifies at 25.5°C., a temperature that is not uncommonly attained in laboratories and stock rooms. Provisions must be made to keep this reagent fluid for immediate use. The low boiling point of 82.8°C. presents some fire hazard.

The butyl alcohols have greatly extended the range of usefulness of the paraffin method by making it possible to cut materials that are rendered hard and brittle by ethyl or propyl alcohol or acetone.

THE DIOXAN METHOD

Dioxan, diethylene dioxide, is becoming widely accepted as a dehydrating agent and paraffin solvent in the embedding of plant materials. This reagent is miscible with water and may therefore be progressively substituted for water in the tissues. Unlike the vigorous dehydrating action of the alcohols or acetone, the substitution of water by dioxan is not associated with great plasmolyzing stresses. This fact permits dehydration by rapid substitution. Tissues do not become excessively brittle, and the histological details obtainable are equal to those obtained by other methods. The dioxan method requires much fewer separate operations than does any other method, and the operations may be at widely spaced intervals, thus reducing the burdensome routine of handling the specimens many times at frequent intervals.

Kill the material in the desired formula. After the optimum fixing interval, wash in water if required by the formula. Animal tissues are said to be transferable directly from the wash water into pure dioxan, but plant cells are plasmolyzed by such treatment.

Materials that were washed in water are transferred through the following three grades at 4- to 12-hr. intervals. Wide latitude in these intervals is permissible.

(1) 1/3 dioxan
    2/3 water
(2) 2/3 dioxan
    1/3 water
(3) Anhydrous dioxan. Replace the cork with a perfectly dry one.

Make two more changes of anhydrous dioxan after intervals of 4 to 8 hr. Proceed with progressive infiltration in paraffin as described in the next chapter.

Materials that were killed in *FAA*. or in any fluid that is followed

by rinsing in 50% alcohol, are transferred through the following series at 4- to 12-hr. intervals. For small root tips the intervals need not be over one hour.

(1) ½ dioxan
    ½ water
(2) ⅔ dioxan
    ⅓ water
(3) Two changes of anhydrous dioxan as in the previous schedule.

Infiltrate in paraffin as descibed later.

The following five-grade series is recommended for delicate or easily plasmolyzed material: 10% "commercial" dioxan in water, 25%, 50%, 75% dioxan at 1- to 4-hr. intervals. Change corks and make two or three changes of anhydrous dioxan at intervals of 1 to 12 hr., depending on the size of the pieces. The time intervals in this series are not critical. Anhydrous dioxan is a solvent of wax, but the rate of dissolving and infiltration can be increased by the addition of 5 to 10% xylene or chloroform to the last change of dioxan.

If anhydrous dioxan is difficult to obtain, a dioxan-normal butyl alcohol series may be used. The method has been tested extensively and is highly recommended. Dehydrate in the foregoing dioxan series to the commercial grade. Transfer to equal volumes of commercial dioxan and commercial butyl alcohol for 1 to 12 hr. Make two or three changes of anhydrous butyl alcohol and proceed with infiltration. A similar dioxan-tertiary butyl alcohol series also is satisfactory.

Regardless of the dehydrating agent and wax solvent that were used, it is desirable to evacuate any residual air that may remain in the tissues at this point. Place the uncorked specimen bottle into a dry jar, use a safety bottle between this jar and the aspirator, and evacuate until no more bubbles come out of the specimens (Fig. 3.1).

During the experimental period following the introduction of dioxan, unsatisfactory results were reported by many workers. Some lots of dioxan produced severe shrinkage; other purchases yielded acceptable, though variable, results. An inexpensive and satisfactory commercial grade dioxan can now be obtained.

Dehydrating agents can be re-claimed after they have been used and have absorbed some water. Commercial grades that contain a low percentage of water can be made anhydrous. It is rarely profitable to re-claim ethyl alcohol, or any other dehydrating agent that contains more than 5% water. If the water content is not over 5%, remove part of the water with anhydrous calcium chloride. Use a large jar with a layer of $CaCl_2$ at least one-third of the volume of

the container. Pour the fluid, for instance once-used anhydrous butyl alcohol, into the jar. As the alcohol accumulates, shake the jar occasionally. Allow to stand for a day and decant the liquid onto anhydrous calcium sulphate, known commercially as "Drierite." After several hours, decant and filter the fluid and it is ready for use. If the fluid has become colored from materials extracted from the tissues, distill at the boiling point of the reagent being distilled. This information can be found in a chemical handbook.

Wet calcium chloride can be regenerated by drying first in dry air, then in an oven at 110°C. Drierite can be regenerated by air drying and then heating in a furnace at 225 to 250°C. for two hours.