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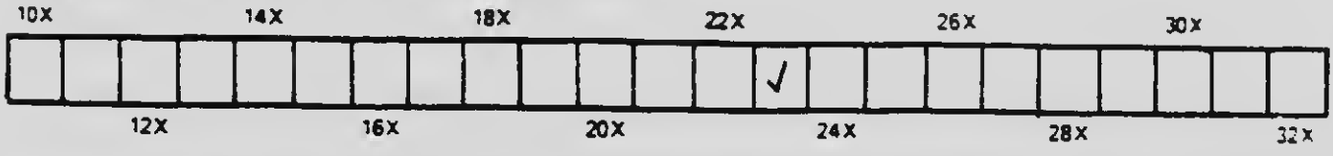

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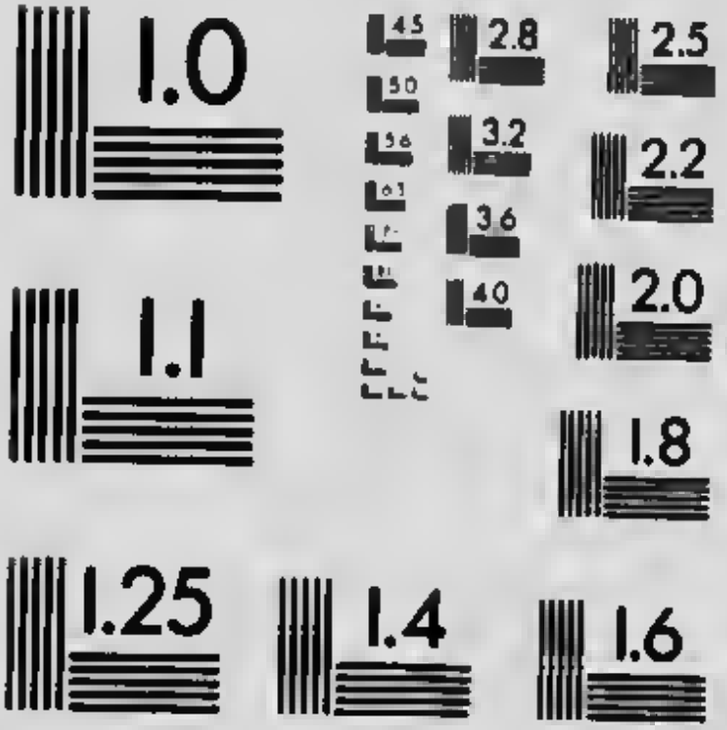

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BOTANICAL MICROTECHNIQUE

BY—

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KINGSTON

The Jackson Press

1911

PREFACE.

The object of this little book is to supply students of botany with detailed and explicit directions in preparing permanent mounts of all plant structures. The writer has tried to make all the instructions so full and exact that a student will be able to avoid the numerous mistakes that it is so easy to make in this work.

While the principal methods are those which are in common use in practical plant histology, many of the details are entirely new and are the results of the writer's experiments during the past few years. Microtechnique is becoming ever more and more important in botany, the old days of the hand-lens as the botanist's sole optical instrument are gone forever, and the use of the compound microscope has become general. Now that ecology has taken its proper place as one of the main divisions of botany, a new field in histological research is opened up, that of the influence of environment upon plant tissue, and in this field a knowledge of microtechnique is essential.

Besides being a necessary factor in botanical research, microtechnique is of great educational value as a training in attention to details. Without the most scrupulous attention to each detail of the work success is impossible, and accuracy, neatness and perseverance are the price of a perfect mount.

No directions are given here on the use of the microscope, as no student will start microtechnique until he has had at least a year's work with this instrument.

Acknowledgments are due to the Spencer Lens Co. of Buffalo, N.Y., for kindly supplying cuts for Figs. 1, 2 and 3.

A. B. K.

Queen's University,
Kingston, Canada,
August, 1911.

CONTENTS.

EQUIPMENT.

Microtome—The Bausch and Lomb Students' Microtome is suited to all classes of sectioning, the knife is easy to sharpen (as it fits the automatic strop) and I have found it heavy enough for the hardest tissues.

Hone—A soft blue-green hone should be used and should be kept flooded with water while in use.

Strop—The Keenoh Automatic Strop takes the small blade of the Students' Microtome, and sharpens it perfectly from within 2 cm. of the heel to the tip.

Paraffin Bath—The Naples Paraffin Bath is perfectly satisfactory. If electricity is used for heating a board bearing a 32, a 16, an 8, a 5 and a 2 candle power bulb should be placed under the bath and an asbestos jacket and lid over the bath. If gas is used, the bath should be provided with a thermo-regulator and a micro-burner.

Balance—A balance which will weigh accurately to at least centigrams is a necessity.

Turntable—This is necessary for ringing glycerine-gelatine mounts.

Burners—A Bunsen burner with rubber tubing will be needed.

Alcohol Lamps—One lamp of about 150 cc. capacity should be provided for each two workers.

Stender Dishes—Five Stender Dishes with accurately fitting covers should be allowed for each worker, and twenty more should be available for keeping stains in during the session.

Minots—Three should be provided for each worker.

Crystallization Dishes—In the methods here outlined these are the most generally used pieces of glassware, and eight should be allowed for each worker. The most useful sizes are 45 mm. in diameter by 25 mm. in height, and 65 mm. in diameter by 40 mm. in height.

Petrie Dishes—Both dish and cover make excellent shallow dishes, one of 50 mm. diameter and one of 100 mm. diameter should be allowed for each worker.

Balsam Bottles—One should be provided for each worker.

Graduates—One of 20 cc., two of 100 cc. and one of 250 or 500 cc. should be available.

Glass Funnels—Several of different sizes should be in the laboratory.

Specimen Jars—At least 4 jars with tightly fitting ground covers 75 mm. in height and 40 mm. in diameter, and 3 jars 100 mm. in height and 75 mm. in diameter will be required for celloidin work.

Porcelain Dishes—One of 100 cc. capacity, and one of 400 cc. capacity, should be available.

Iron Stand—An iron stand with ring and wire gauze should be provided.

Bottles—Ordinary bottles will in time accumulate in the laboratory as they are emptied of the reagents which they contained. Many of them of various sizes will be necessary. Glass-stoppered bottles will be needed for acids, etc., and glass-stoppered ones of dark glass for some reagents.

Vials—Tube vials with corks for the preservation of material should be ordered by the gross. The most useful sizes are 15 mm. in diameter by 60 mm. in height, 20 mm. in diameter by 75 mm. in height, and 25 mm. in diameter by 100 mm. in height. The intermediate size will be needed in greater quantities than the other two sizes. It should be specified in ordering that the corks must be good resilient ones, and not the hard objects full of large holes, and about as effective as wooden plugs, which are often supplied.

Beakers—Beakers of about 150 cc., 250 cc. and 350 cc. capacity should be provided.

Slides—The quantity of slides required will of course depend upon the number of preparations to be put up during the session. Only first-class slides should be used. A good slide should be of clear white glass, free from flaws, perfectly flat, well ground on the edges and well polished. Slides should be 75 x 25 mm. The B. & L. No. 1292 is an excellent slide.

Cover-glasses—Only No. 1 cover-glasses should be used and the bulk of the supply should be circles of 12, 15, 18 and 25 mm. diameter. The 15 and 18 mm. are the most useful sizes. More cover-glasses than slides should be ordered as some will be broken in cleaning and handling.

Slide Boxes—The most satisfactory slide box for general use is either the Spencer Lens Co.'s No. 1215 or Bausch and Lomb's No. 1934, both of which hold 25 slides.

Labels—Gummed labels will be required for use on vials, reagent bottles, stender dishes and slides. For the three former uses labels about 35 x 20 mm. are good, but for labelling slides they should be exactly 24 mm. square.

Pipettes—Wherever a pipette is mentioned in these pages, one with a rubber bulb is meant. One should be allowed for each worker and several kept on hand for use in staining material on the slide.

Glass Rods—A dozen or more glass rods of 3 mm. diameter and 15 cm. long should be made and their cut ends rounded by heating.

Camels-hair Brushes—These will be needed for various purposes.

Filter Paper—This will be required for filtering, for blotting the ends of slides and for absorbing reagents from slides. It is best to order the large sheets and cut in pieces of the required sizes.

Trays—One or two large tin trays should be provided to catch drippings while imbedding in paraffin.

Large Dishes—Two large enamel dishes about 8 cm. deep will be required for the immersion in water of paper baskets containing material imbedded in paraffin.

Brass Wire Gauze—A strip of brass wire gauze, 100 wires to the inch, 7 cm. wide, should be obtained from which to make the baskets described in the chapter in "The Basket Method." The length of the strip will of course depend upon the number of baskets to be made. Each basket requires a piece 7 cm. x 8 cm. One basket should be allowed to each worker and a reserve of two or three kept on hand. A strip of brass wire gauze 50 wires to the inch, about 2 cm. wide by 20 cm. long, will be required in making the washing apparatus described in "The Paraffin Method."

Pine Blocks—These should be sawed from a piece of clear pine about 5 x 10 cm. in cross-section. Some should be about 15 mm. wide, others about 30 cm. wide. From these blocks for both the paraffin and celloidin methods may be conveniently split.

Tacks—A package of 1½ oz. steel tacks will be required for pinning the paper round the blocks in the celloidin method.

Instruments—Most of these will be included in the student's personal set, but the following, being used only occasionally, may well form part of the laboratory equipment. A thin-bladed paring knife for trimming paraffin blocks, a pair of tongs for handling hot dishes, an old scalpel for using hot to cement paraffin blocks to the wooden blocks, a couple of pairs of old forceps at least

12 cm. long for heating while embedding, and a large pair of scissors.

The student's set should include the following: A pair of cover-glass forceps with thin, flat, straight points (Fig. 1), for

Fig. 1.

handling large sections and cover-glasses, a pair of small forceps with very fine straight smooth points for drawing small sections up on the slide and for handling Algae, etc; a large scalpel with a long cutting edge (Fig. 2) for cutting material, a small fine

Fig. 2.

thin-pointed scalpel (Fig. 3) for fine work and handling paraffin ribbons, and a couple of needles mounted in handles.

Fig. 3.

REAGENTS.

It is obviously impossible to give the quantities of reagents that will be required as it will depend upon the amount of work done, but in the following list those of which comparatively large quantities will be needed are placed first.

95% Alcohol.
Absolute Alcohol.
Xylol.
Turpentine.
Chloroform.
Sulphuric Ether.
Distilled Water.
Glycerine.
Paraffin (melting pt. 45°C).
Schering's Celloidin.
Canada Balsam (pure or dissolved in chloroform).
Gelatine.
Agar-agar (the dried Alga).
Glacial Acetic Acid.
Hydrochloric Acid.
Nitric Acid.
Sulphuric Acid.
Chromic Acid (crystals).
Picric Acid (crystals).
Thymol (crystals).
Ferric Alum.
Borax.
Copper Sulphate.
Potassium Hydroxide.
Iodine.
Chloriodide of Zinc.
Phenol.
Phloroglucin.
Potassium Bichromate.
Mercury.
Berry's Oil Varnish.

The following Grubler's Stains—(dry):
Fuchsin.
Acid Fuchsin.
Methyl Blue.
Methyl Violet 5 B.
Methyl Green.
Methylene Blue.
Malachite Green.
Nachtblau.
Eosin.
Safranin.
Aurantia.
Orange G.
Congo Red.
Vesuvin.
Carmine.
Haematoxylin.

COLLECTING AND PRESERVING MATERIAL.

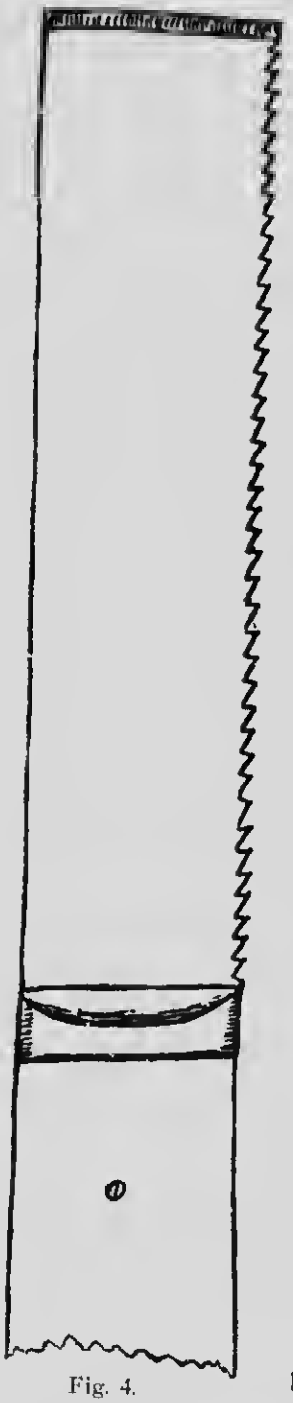

Fig. 4.

Vials of different sizes containing the various fixing solutions should be taken into the field. Material should always be cut, not pulled up or broken off, as this ruptures the tissues. The most useful collecting tool is a knife with a blade about 12 cm. long and 3 cm. wide, with the back filed into a saw and the square point ground to a chisel edge. Such a tool may be made from a butcher knife by any good mechanic and serves both as a trow calnd saw. It should be carried in a leather case. A pair of blunt-pointed scissors should also be carried for cutting leaves into pieces of suitable size and one blade of a pocket knife should be kept very sharp for cutting stems. Stems should be cut squarely across with a rolling motion so that the blade sinks gradually towards the centre until the stem is completely severed.

Material should be cut into small pieces, the exact size depending upon the permeability of the material. Stems should be cut into pieces about 15 mm. long, leaves in pieces about 6 mm. wide by 15 mm. long, and all ovaries, etc., should have a portion of either the top or bottom or sides cut away. All woody material should be placed in a solution consisting of one-half 95% alcohol and one-half glycerine. This solution softens lignified tissue and the longer the material remains in it the better it will cut.

Herbaceous stems, leaves, etc., should be placed in the following solution:—

Chromic acid (crystals) ..... 1 grm.
Glacial acetic acid ..... 1 cc.
Water ..... 98 cc.

This solution fixes thoroughly and is perfectly satisfactory for all soft material except such delicate objects as fern prothallia, antheridia of Marchantia, etc., in which it may cause plasmolysis. For such material the following weaker chromo-acetic solution should be used:

Chromic acid (crystals) ..... ·3 grm.
Glacial acetic acid ..... ·7 cc.
Water ..... 99 cc.

About ten times the bulk of these solutions should be used as there is material to be fixed. Material may be left in these chromo-acetic solutions until required for use. I have made slides from material which has remained in them for three years and found it to be in perfect condition.

Algae should be collected in vials of water, examined microscopically the same day, and the collections which contain forms to be preserved transferred to the following solution:

Chromic acid (crystals) ..... ·2 grm.
Glacial acetic acid ..... 1 cc.
Water ..... 100 cc.

where they may be left until needed.

Vials containing material should be labelled at once with the name of the structure (stem, root, etc.), the name of the species, and the date. In the case of Algae or material for ecological slides, the locality and habitat should also be recorded on the label.

MICROCHEMICAL TESTS.

Microchemical tests are important in revealing the character of cell-walls and of cell contents. The material to be tested should be fresh. It should be sectioned, a section placed on a slide, the reagent applied, a cover-glass placed over it and examined under the microscope. While making microchemical tests cover the stage of the microscope with a sheet of glass. Tests for the most important constituents of plants are given below.

Cellulose—Chloroiodide of zinc turns cellulose violet. This reagent may be prepared by dissolving chloroiodide of zinc in 10% less than its own weight of water and adding sufficient metallic iodine to give the solution a dark brown color.

Lignified Tissue—Apply a saturated aqueous solution of phloroglucin followed by a drop of concentrated hydrochloric acid. After a few minutes the lignified tissue becomes pink and finally rose-purple.

Starch—Add a dilute aqueous solution of iodine which turns starch blue.

Sugars—Test by applying Haine's solution and heating gently, when any of the sugars give a reddish-brown coloration. Haine's solution is made up as follows:

Copper sulphate	2 grms.
Potassium hydroxide	6 grms.
Glycerine	15 cc.
Distilled water	160 cc.

Proteids—Apply Millon's reagent and warm gently, when proteids turn brick-red. This reagent is prepared by dissolving 1 cc. of mercury in 9 cc. of concentrated nitric acid and adding 10 cc. of water.

Fats and Oils—Leave material in alcannin solution for six hours, when fats and oils will be red. This solution may be prepared by making a saturated solution of alcannin in absolute alcohol and adding an equal volume of water. As alcannin also colors tannins and resins red, the tannins, if their presence be suspected, should be extracted by boiling in water, and the test for resins should be made if there is any likelihood of their occurrence.

Suberized Tissue—Treat with a concentrated aqueous solution of potassium hydroxide, when suberized tissue turns yellow.

Resins—Leave sections for a week in a concentrated aqueous solution of copper acetate, when the resins will be emerald-green.

Gums and Mucilages—Apply the following solution: ¼ alcohol (95%), ¼ glycerine, ¼ water, with enough methylene blue to give a dark blue color. Allow this solution to act for about 10 mins., then wash in water and then thoroughly in 95% alcohol. The gums or mucilages in the cells will be dark blue.

STAINING.

The object of staining is to differentiate one tissue from another, as lignified tissue from cellulose, or one element of the cell from another, as the nucleus from the cytoplasm. Or sometimes a single stain is used to render cell-walls more clearly visible.

In order to stain effectively one must not only know what stains color certain tissues or certain elements, but must also know what feature of the preparation is to be brought out most clearly. Thus in most stems it is desired to differentiate lignified tissue from parenchyma, in seeds to emphasize the aleurone grains, and so on. When in the case of some structures consisting of but one kind of tissue only a single tissue is necessary, congo red, eosin or methyl green will be found to be satisfactory.

The great majority of structures require double-staining, when the second stain is termed the counter-stain, and it is important to remember that the following stain lignified tissue:—fuchsin, methyl violet, safranin, aurantia, malachite green and nachtblau, while those that stain cellulose are: methyl blue, congo red, eosin, vesuvin, and haematoxylin.

The following color the nucleus:—acid fuchsin, haematoxylin and aurantia.

In selecting two stains for double-staining it is of course important to choose stains which contrast well. In differentiating lignified tissue and cellulose, the following are particularly good combinations:—

Fuchsin and methyl blue, stains lignified tissue red and cellulose blue.

Methyl violet and congo red—lignified tissue violet and cellulose orange-red.

Methyl violet and eosin—lignified tissue violet and cellulose red.

Aurantia and methyl blue—lignified tissue orange and cellulose blue.

Safranin and haematoxylin—lignified tissue red and cellulose purple.

Methyl violet and vesuvin—lignified tissue violet and cellulose brown.

Malachite green and eosin—lignified tissue green and cellulose red.

These are only a few of the combinations which may be made, and the very best staining for a particular structure can only be ascertained by trying one combination after another.

All the stains mentioned above, with the exception of haematoxylin, should be made up by dissolving 1 gram of the stain in 100 cc. of 70% alcohol.

There are many formulae for making up haematoxylin, of which perhaps Erlich's is preferable for cellulose. It is made as follows:

Distilled water	50 cc.
Absolute alcohol	50 cc.
Glycerine	50 cc.
Glacial acetic acid	5 cc.
Haematoxylin	1 grm.
Ferric alum in excess.	

Keep in a dark place until the color becomes a deep red. In staining nuclei and filamentous Algae with haematoxylin, two solutions should be used—a 3% aqueous solution of ferric alum and a ½% aqueous solution of haematoxylin. For the method of using this stain see under "The Glycerine-gelatine Method."

For aleurone grains the best stain is borax-carmine, made up as follows: Make a 4% solution of borax in water, add 3% carmine, and then add to this solution an equal volume of 70% alcohol. Allow to stand 48 hours and filter. Dilute one part of the solution with 200 parts of water and allow sections to stain for 6 to 12 hours. The prolonged action of the stain thus diluted gives a far better differentiation than a shorter staining in the concentrated solution.

As to the time required for staining, no exact rules can be given, as it varies with the different stains and with the tissue to be stained. But the following data for the most useful stains may at least furnish a basis upon which to work: Fuchsin, 5 mins.; methyl violet, methyl blue, congo red, acid fuchsin, aurantia and eosin, 10 mins.; safranin and malachite green, 15 mins.; and haematoxylin, from 30 mins. to several hours.

In double-staining the sections should first be placed in the stain which colors the lignified tissue, washed in water until no more stain comes out and then in 95% alcohol until the stain is very faint in, or entirely removed from, the cellulose. If now the stain is too light in the lignified portions the sections must be restained and washed as before. Next place the sections in the counter-stain and examine a section after washing in water, from time to time, in order

to see when staining has proceeded far enough. The stain should be a little deeper than the color desired in the finished mount. When this point is reached, wash the sections in water until no more stain comes out and then in 95% alcohol until the stain no longer comes off in clouds.

The above procedure applies to all stains except fuchsin, which requires the use of a saturated aqueous solution of picric acid to extract the stain from the cellulose. The sections should be washed in water after removal from the fuchsin, then in 95% alcohol then in the picric acid until the lignified tissue is brownish, then washed well in water and then in 95% alcohol.

The details of staining sections of various structures are given under the different methods

CLEANING OF SLIDES AND COVER-GLASSES, ETC.

Before sectioning any material, have on hand a supply of clean slides and cover-glasses. Slides may be cleaned by placing them in 95% alcohol in a stender dish and wiping dry with a towel free from it. Cover-glasses should be placed in a minot full of 95% alcohol and dried with a piece of cheese-cloth. The above method will clean most new slides and cover-glasses, but if they still look smeary they should be placed for 12 hours or more in the following solution:

Potassium bichromate	10 grms.
Sulphuric acid	50 cc.
Water	50 cc.

They should then be washed well in water, then in 95% alcohol, and dried.

When washing, reject all chipped or scratched slides and any that are not perfectly flat.

Old slides with mounts on them may be cleaned by soaking in water to remove the labels, allowing them to dry and soaking in turpentine for a day or two; then removing the cover-glasses, placing the latter in 95% alcohol, wiping the slides with a towel, placing them in 95% alcohol and wiping them dry.

Preparation of Balsam. The pure Canada balsam should be dissolved in chloroform until it is of such consistency as to drop freely from the rod, but not thin enough to run over the slide when placed upon it. If too thick it is hard to work with and dries slowly; if too thin air-bubbles will appear under the cover-glass as it dries. The balsam bottle should always be kept perfectly clean, particular attention being paid to the neck and the inside of the cover, as otherwise the cover will be cemented on. The cleaning should be done with a cloth wet with turpentine.

THE BASKET METHOD.

This method is for use on woody material which when sectioned will have no loose parts. It may also be used on many of the firmer herbaceous stems and roots but does not usually give as good results with these as the celloidin or paraffin methods.

The first requisite for this method is a basket made of brass wire gauze, 100 wires to the inch. The gauze should be cut into pieces 7 cm. by 8 cm. A cardboard model should be made exactly the size shown in Fig. 5. The model should be laid on the piece of gauze and the outline drawn in pencil. The other lines should then be drawn with a ruler.

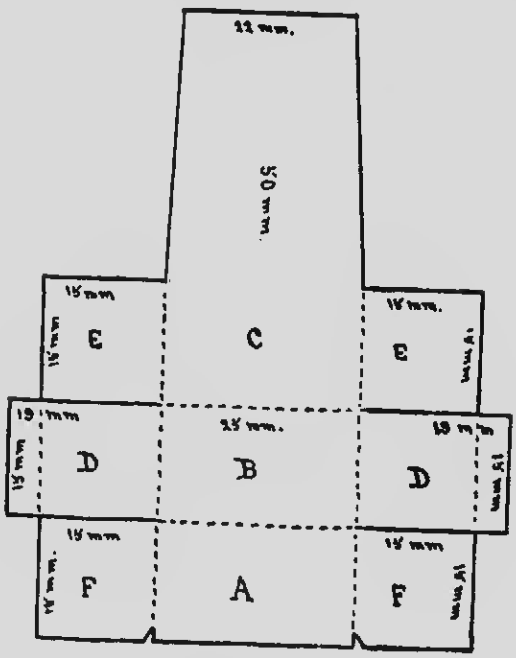

Fig. 5.

The gauze should be cut along the heavy lines, the notches at the bottom of the model not being cut out as they are merely to give points to draw the vertical lines to. The flap *A*, together with the flaps *F* should then be folded up along the dotted line to form a right angle with the flap *B*, and the flap *C*, together with the flaps *E* folded up at right angles with *B*. Next the flaps lettered *D* should be folded

up along the dotted line, then the flaps *E* folded in, and next the flap *F* folded in. Then the three flaps *D*, *E*, and *F*, forming the sides of the basket, should be held together by bending the outer portions of the flaps *D* along the dotted line over the edges of the other two flaps and clinching them. Finally, the top 5 mm. of *C* should be folded over to form a hook for hanging the basket on the rim of the stender dish.

The next requisite is to have the microtome knife very sharp, that is, to hone and strop it until it will cut a hair held between the thumb and finger and cut it cleaa without any bending over or dragging. It is also important to notice that the character of the edge required will vary with the hardness of the material to be cut. If it is only moderately hard, as in the case of most material, then the usual tapering razor edge is needed, but if it is very hard, as wood of *Carpinus* or *Quercus*, then the edge should be a knife edge and not a razor edge. Such an edge may be obtained by honing the knife with the back very slightly raised instead of honing with the knife perfectly flat.

Now set the knife so that it makes an angle of about 30° with the bed of the microtome. Wash the material in water, place some water on the blade of the knife with a camels-hair brush, and fix the material firmly in the microtome clamp so that about 1 cm. projects above the clamp. Run the wheel of the microtome down so that the top of the object is just below the level of the knife, bring the knife forward until it just touches the knife, then feed it up about 15 microns (3 of the small markings on the wheel), and draw the knife across the object with a rapid steady stroke. Continue to feed the material up by turning the wheel with the forefinger of the left hand and cut sections as thin as possible without tearing. If material does not cut well, try the knife at various angles. Be careful that the feeding up is done while the knife is at the back of the bed and not at the front, and also that it is done before the stroke is made and not while the knife is cutting. When several sections have accumulated on the knife, remove them with a camels-hair brush and transfer them to one of the larger size crystallizing dishes filled with water.

When the whole piece of material has been sectioned down to within a millimeter or so of the jaws of the clamp, the thinnest and most perfect sections should be selected and transferred with the forceps to the basket which has been previously placed in a small dish of 95% alcohol. Several more sections should be selected than will

be required for mounting so that the best may again be selected after staining. The basket will accommodate and stain perfectly 100 sections 8 mm. in diameter and more of smaller size.

Hang the basket on the rim of a slender dish containing the first stain and see that the stain reaches nearly to the top of the basket but not quite flush with the top. During staining shake the basket gently at frequent intervals so as to shift sections which may be lying in too close contact. When staining is completed wash the basket and contents in water until no more stain comes out, then in 95% alcohol in a small dish until the stain is very light in, or entirely removed from, the tissues to be stained by the counter-stain. If it washes out evenly from all tissues, replace in the stain and the wash as before. Now transfer to the counter-stain and shake at frequent intervals. While counter-staining pick out a section from time to time, wash it in water, then in 95% alcohol and examine it under the microscope to see when staining has proceeded far enough. When the sections are satisfactorily stained wash in water until no more stain comes out, and then in 95% alcohol until the stain no longer comes off in clouds.

The above method of staining applies to all stains except fuchsin, which differs in requiring the use of a saturated aqueous solution of picric acid after the washing in 95% alcohol to extract the fuchsin from the cellulose. Sections should remain in picric acid until the lignified tissue is brownish. Then wash well in water and then in 95% alcohol. If sections are too deeply stained, leave them for some time in 95% alcohol, which will extract the stain.

When the sections are perfectly stained, blot the bottom of the basket on a pad of filter-paper and transfer to a small dish half-full of absolute alcohol. Leave the basket in the absolute alcohol for a few minutes to dehydrate, then pick the sections out of the basket one at a time with the forceps and place them in a small dish one-third full of xylol. If the xylol turns "milky" it shows that the sections were not perfectly dehydrated and they should be placed in fresh absolute alcohol. If, however, the xylol only becomes slightly cloudy, the sections should be left in it as it will clear up in a few minutes.

As soon as the sections are perfectly clear, pick one out with the forceps, place it in the centre of a clean slide, and carefully place from one to three drops of balsam upon it. The amount of balsam required varies with the size of the cover-glass, with the consistency of the balsam and with the thickness of the section. Next take up a clean cover-glass with the forceps, place the side of the cover-glass furthest from the forceps in contact with the slide and gently lower

it upon the section. Enough balsam should be used to run freely to the edge of the cover-glass but not beyond. If the balsam has hardened a little and does not run quite to the edge of the cover-glass, heat the slide gently over an alcohol flame. If the cover-glass is lowered as described above no air-bubbles should be included, but should any be present heat the slide just under the bubbles until they expand, and press very gently on the cover-glass until they are expelled at the edge. If sections are large but one should be placed on a slide, if small three or more should be mounted on each slide. When mounting small sections be careful to keep them from running to the edge of the cover-glass, which they usually have a tendency to do. To avoid this let the balsam harden until the surface begins to look a little wrinkled, then warm the cover-glass in the alcohol flame by passing it rapidly through the flame three or four times and lower it upon the sections. Proceed as described above until a few more sections than required are mounted so that after the mounts are dry the best may be selected. Then label one of the slides with a temporary label bearing the name of the structure and the name of the species, and place the slides in a slide-box standing on end so that the slides will remain horizontal, and leave them thus for 48 hours or longer. When the slides are dry, that is, when the cover-glass does not move on a slight lateral pressure, label them on the left hand side with neatly written labels bearing the data on the vial from which the material was taken and your initials. Always be careful that everything about a slide is true and neat, that the section and cover-glass are in the centre of the slide, that the balsam does not extend beyond the cover-glass, that the label is neatly written, the words on it evenly spaced, and that it is placed squarely on the end. Attention to these details will not turn a poor preparation into a good one but they will enhance the value of a good one.

THE PARAFFIN METHOD.

The paraffin method should be used on all soft material which will have no loose parts when sectioned, as leaves or herbaceous stems, and on all soft material of which very thin sections (less than 10 microns) are desired. It should also be used when serial sections (that is, when many sections are to be mounted on a slide in the order in which they were cut) are required. This method should never be used on woody material as it renders it as hard as bone.

As this method is a long one, several pieces of different material should be put through at the same time and it is consequently most important to keep each piece of material identified all through the process. Therefore make a list of the material and if all the material is taken from the vial, make a sketch of a typical piece of each structure. If all the material is not used, keep the vial at hand for reference. If pieces of different material resemble one another very greatly (as for instance two pieces cut from leaves of two different species of *Gramineae*) they should be kept separate throughout the process. Three or more pieces of each structure should be taken. The material should be washed in running water for 12 hours or more in a washing apparatus made in the following way: -Fit several wide-mouthed bottles about 8 cm. in height with double-bored rubber corks. Bend glass tubing, which fits the holes in the corks tightly, in such a way as to make a gentle curve through a right angle. Cut one piece of tubing for each bottle about 10 cm. from the elbow on one side and 5 cm. on the other. Cut another piece of tubing about 5 cm. from the elbow on each side. Run one end of the shorter piece through the cork, allowing it to project about 15 mm. below the cork, and with fine copper wire bind a piece of brass wire gauze, 50 wires to the inch, over the end of the tube. Insert the long end of the other tube through the second hole in the cork and push it down until the end is within 5 mm. of the bottom of the bottle. Now join up the bottles in series with rubber tubing and attach to a tap with rubber tubing in such a way that the water will flow in at the long glass tube and out at the tube covered with wire gauze. Attach a rubber tube to the last bottle to run the waste water into the sink and turn on a small stream of water.

Now make up about 300 cc each of the following grades of alcohol:—15%, 35%, 50%, 70%, and 85%. [illegible] may be made from

95% alcohol and in this case 95% alcohol may be treated as 100% and the percentages made up accordingly.

After washing, the material should be placed in a covered dish and treated successively with 15%, 35%, 50%, 70%, 85% and 95% alcohol. Each grade should be allowed to act for six hours or for a longer period if it is more convenient. The alcohols after use should be poured back into their bottles. In changing the alcohols it is best to strain the alcohol off through a piece of brass wire gauze 50 wires to the inch, so that the material may not be handled more than is necessary. Then the material should be placed in absolute alcohol for 12 hours or longer. The absolute alcohol should not be put back in the bottle but should be kept for slide-washing, etc.

Next treat the material for six hours or longer with the following solutions:—

75% absolute alcohol, 25% xylol,
50% absolute alcohol, 50% xylol,
25% absolute alcohol, 75% xylol.

Then place the material in pure xylol for six hours or longer. By this time the material should be translucent.

The object of treating with the graded alcohols is to thoroughly dehydrate the material, and the material is transferred to xylol because this reagent is on the one hand miscible with alcohol and on the other hand is a solvent of paraffin.

Place the dish on the paraffin bath and add a large piece of paraffin (melting point 45°C.) to the xylol. The piece of paraffin should be about twice the bulk of the xylol present. The object of adding the paraffin in a lump is that it may dissolve slowly and so bring the material gradually into a more and more concentrated solution of paraffin. When the paraffin has dissolved take the dish off the bath and see if at room temperature the mixture begins to solidify. If it does not, add another lump of paraffin and replace on the water bath. If it does tend to solidify replace it on the bath, and transfer the material with warmed forceps to the vials on the bath which should be half full of melted paraffin (m.p. 45°C.). At the end of an hour change the paraffin in the vials so as to get rid of any xylol which may have been carried over. Now the material should be left on the bath for about 5 hours. This part of the process is termed infiltration and the time required will vary with the ease with which the material may be penetarted. Most material will, however, be perfectly infiltrated in 6 hours. During infiltration the temperature of the paraffin bath must be kept not more than four

degrees above the melting point of the paraffin or the material will be rendered extremely brittle and ruined.

The temperature of the bath is far more easily kept constant if a jacket and tight-fitting lid of heavy sheet asbestos are placed on the bath in such a way as to leave a dead-air space between the bath and the asbestos. With this covering on the bath electricity may be used for heating, by having a board bearing a 32, a 16, an 8, a 5 and a 2 candle-power bulb under the bath. This does away with the danger of fire, which is ever present when gas is used, no matter how carefully it is guarded against. When the number of lights to turn on for a certain temperature has been ascertained, the temperature may be kept constant within two degrees. The bath should first be heated up to the required temperature with a Bunsen burner.

If electricity is not available a micro-burner, attached with flexible metal gas-tubing, and a thermo-regulator should be used. While the material is infiltrating paper baskets should be prepared in which to imbed it. First three soft-wood blocks of the following sizes should be made:

30 mm. x 24 mm. x 12 mm.
20 mm. x 14 mm. x 14 mm.
20 mm. x 13 mm. x 9 mm.

Take a strip of paper (with a hard surface) 78 mm. wide and 150 mm. long, place the largest block with its 30 mm. edge on the 78 mm. edge of the paper, and in the centre of this edge of the paper. Wrap up the block tightly in the paper, creasing it firmly at each edge, until exactly two laps are round the block. Cut off the piece of paper projecting. Now wrap up the ends of the block as you would wrap up a paper parcel, being careful that the last fold down on the sides is made in such a way that these flaps will serve as handles as shown in Fig. 6. Now cut out the 30 x 12 face which is between the handles with a sharp scalpel, extract the block, and the basket is complete.

Fig. 6.

To make the second-sized basket, take a strip of paper 54 mm wide and 150 mm. long, place a 14 mm. edge of the second block in

the centre of the 54 mm. edge of the paper and proceed as for the first basket. This will give a deep basket as shown in Fig. 7.

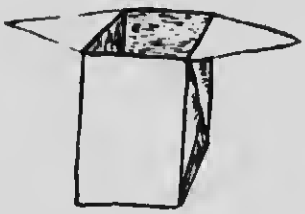

Fig. 7.

To make the smallest basket, take a strip of paper 53 mm. wide and 150 mm. long. Place the 13 mm. edge of the third block in the centre of the 55 mm. edge of the paper and proceed as above. This will give a small basket of the same shape as the first basket.

See what shapes will be best adapted to each piece of material and make up a basket for each piece.

Now fill each basket full of melted paraffin, pour it out again and let the baskets cool. This will give the baskets a lining of paraffin which will render them stiffer and easier to handle. Write on the side of each basket in pencil the name of the structure to be imbedded in it

When the material is infiltrated, heat a porcelain dish of paraffin to just above the melting point. Pour a layer of melted paraffin about 3 mm. deep into a basket, let it become viscid (not solid) by cooling, pick a piece of material out of a vial with an old pair of forceps warmed in a low Bunsen flame, place the material in the basket in the proper position and fill up the basket with melted paraffin. Blow on the surface of the paraffin until a scum is formed, and plunge the basket into a pan of cold water.

Particular attention should be paid to the position in which the material is imbedded, and the long axis of the object should always coincide with either the long axis on the vertical axis of the basket, as if it does not it will lead to complications in trimming the paraffin blocks. Leave the baskets in the water for six hours, or over night, then take them out and allow them to dry.

The next step is to tear off the paper and trim the paraffin so as to form a rectangular block with the material in the centre, and cement it to a block of wood of about the same size with hot paraffin. The material must be placed on the wooden block in such a way that either a true cross-section or a true longitudinal section can be cut.

Allow the paraffin used for cementing to become perfectly hard and clamp the wooden block in the microtome.

Now we have reached a point where the further procedure will depend upon the material to be cut. If it will not contain loose parts when sectioned the knife should be set at an angle of about 30° with the bed of the microtome, the sections cut as thin as possible without tearing, and allowed to fall on a sheet of paper placed over the pan of the microtome. Whether they roll up or not is immaterial. If, however, the sections will contain loose parts, as in the case of sporangia, etc., the knife must be set at right angles to the microtome bed, and the sections must come off in the form of a ribbon.

We will now follow through the process for sections containing no loose parts. They should be brushed with a camels-hair brush into a wire-gauze basket, the basket placed in hot turpentine to dissolve off the paraffin, the bottom of the basket blotted on a pad of filter paper, and then transferred to a dish of xylol to complete the removal of the paraffin. Blot the bottom of the basket again and transfer it to a dish of absolute alcohol. Now the sections may be stained and mounted exactly as given in "The Basket Method." There are, however, one or two difficulties in mounting which arise from the delicacy or the small size of the sections which are often cut by this method. If the sections are very delicate, and difficult to handle with the forceps, the xylol containing the sections should be poured into a petrie dish, the slide should be placed on the edge of the dish so that half of it is under the xylol and the section should be carefully drawn up to to the slide with the fine smooth-pointed forceps. If the sections are extremely small they will be hard to handle with the forceps and will also tend to run to the edge of the cover-glass when it is placed on [illegible], and should consequently be treated as follows:—Draw the sections, together with as little xylol as possible, up into a pipette. Hold the pipette vertically and allow the sections to settle to the tip. Place the drop containing the sections on a slide. Take up all the xylol possible with filter paper. Cover the sections with balsam, gather them to the centre of the slide with a needle wet with xylol. Allow the balsam to harden until it becomes wrinkled at the edges and apply a cover-glass. If the balsam does not flow to the edge of the cover-glass, heat the slide gently over an alcohol flame.

If the sections contain loose parts, they should come off in ribbons. If the paraffin will not ribbon it is because the temperature is too low, and if it is not feasible to raise the temperature of the room, then the knife should be warmed. If the ribbons are not straight it is

because the front and back top edges of the block are not parallel and they should be trimmed until true. The ribbons should be as thin as it is possible to cut them without tearing or wrinkling them too much. The ribbons should be cut off the edge of the microtome knife with the fine-pointed scalpel, being extremely careful not to injure the edge of the knife, and laid on paper.

A solution of 1% agar-agar should be prepared by dissolving 1 dgm. of agar-agar in 100 cc. of boiling water and filtering while hot. The hot-water funnel needed for this operation and also for making glycerine-gelatine may be readily made by cutting a round hole in the bottom near the edge of a deep pressed tin pan, inserting a bored rubber cork in the hole, passing the stem of a funnel through the cork and cutting off the stem about a centimetre below the cork. The pan should be filled with water to within a centimeter of the top of the funnel, set on a ring-stand and a Bunsen burner placed beneath the pan at the opposite side to the cork. The water should be heated just to the boiling point, a large filter paper placed in the funnel and the hot solution poured into it. After filtering, add a crystal of thymol to the solution as a preservative.

Prepare a thin celloidin solution by adding to 5 cc. of equal parts absolute alcohol and ether all the celloidin that will dissolve in it, and then adding to this solution 45 cc. of equal parts absolute alcohol and ether. Place in a bottle with a camels-hair brush inserted in the cork.

With a glass rod place a small drop of agar-agar solution on a slide and rub it over the centre of the slide with the finger. Cut a section out of the ribbon with the fine-pointed scalpel, place the section in the centre of the slide, and stand the slide inclined at an angle of about 30 degrees with the horizontal until perfectly dry. Apply a thin film of the celloidin solution carefully over the surface of the section and allow it to dry thoroughly. Mount ten or a dozen sections in the same manner. Now place 8 stender dishes in a row, put 120 cc. of xylol in the first, 120 cc. of absolute alcohol in the second, 120 cc. of stain in the third, water in the fourth, 95% alcohol in the fifth, 120 cc. of the counter-stain in the sixth, water in the seventh, and 95% alcohol in the eighth. Good combinations of stains to use are aurantia and methyl blue, methyl violet and congo red, methyl violet and eosin or safranin and methyl blue. Haematoxylin should not be used as it stains the agar-agar, and fuchsin is not advisable because of the treatment with picric acid which it entails. If nuclei are to be particularly brought out, acid fuchsin and methyl blue is a good combination. Now place a sheet of glass and a pad of filter-

paper in front of the stender dishes, and place three dishes, one containing absolute alcohol, the second half absolute alcohol and half ether, and the third xylol, together with there pipettes near the sheet of glass. Also cut some narrow strips of filter paper.

Now take a slide with a section mounted upon it, heat it in the flame of an alcohol lamp until the paraffin just melts, and place the slide in the stender dish of xylol. Treat four slides in the same manner and leave them in the xylol for about 5 minutes, then blot the end of the slides on the pad of filter paper, transfer them to the absolute alcohol, and leave them there for about three minutes. Transfer to the stain and leave them until stained deeply enough, wash in the stender-dish of water, then in the dish of 95% alcohol until the stain ceases to come off in clouds. If now the section is too pale, restrain it, and put through the water and 95% alcohol again. It is a good plan to carry the four slides together as far as the first stender-dish of water and then to finish each separately. As soon as the first four slides have been removed from the xylol replace them by four others and in this way keep a constant supply of slides on the move.

After the washing in 95% alcohol transfer to the counter-stain and when sufficiently stained, wash in water and then in 95% alcohol. When the counter-stain no longer comes off in clouds, remove the slide from the alcohol, place it on the glass plate and with a pipette apply very carefully several drops of alcohol-ether. Absorb the alcohol-ether from round the section with a strip of filter paper. Apply absolute alcohol in the same way and absorb it until the section is merely moist. Now apply xylol with a pipette, absorb excess with filter-paper, apply a second lot of xylol and absorb until the section is merely moist. Apply balsam, allow it to harden slightly and place a cover-glass in position. If the balsam has hardened too much and will not flow out to the edge of the cover-glass, heat the slide gently over an alcohol flame.

By the time the second application of xylol is made the section should be perfectly clear and free from all celloidin and paraffin. If it is not, repeat the treatment with alcohol-ether, absolute alcohol and xylol.

THE CELLOIDIN METHOD.

The celloidin method should be used for all hard materials which need imbedding as seeds, stems with loose cortex which peels off readily, etc., and is also an excellent method for handling any material of which sections thinner than 10 microns are not required. The advantages of this method are that no heat is required, consequently woody tissues are not hardened, that on account of the transparency of the celloidin the material can be observed at all stages of the process, that the celloidin matrix holds all loose parts together and the sections can be stained without being previously fixed to a slide, and finally that it is quicker than the paraffin method. The material should be washed for 12 hours or longer in running water, then left for six hours or longer if convenient, in each of the following grades of alcohol: 15%, 35%, 50%, 70%, 85%, 95%, and absolute. It should then be transferred to a mixture of one-half absolute alcohol and one-half sulphuric ether and left for 24 hours. This last mixture should be in a specimen jar about 75 mm. in height and 40 mm. in diameter and may be used for three or four lots of material. While the material is being dehydrated in the series of alcohols, a thick solution of celloidin should be made up by adding to about 200 cc. of alcohol-ether mixture all the Schering's celloidin that will dissolve.

Now take three specimen jars of the size mentioned above; in one put about 30 cc. of the thick celloidin and label it Celloidin 3. In a second jar put a mixture of two-thirds Celloidin 3 and one-third alcohol-ether and label it Celloidin 2. In the third jar put a mixture of two-thirds Celloidin 2 and one third alcohol-ether and label it Celloidin 1. Now after the treatment with alcohol-ether transfer the material successively to Celloidin 1, Celloidin 2 and Celloidin 3, leaving it for 24 hours in each solution.

While the material is passing through the celloidin solutions boxes for imbedding in should be prepared. To make these, cut a soft wood block of suitable size for each piece of material, wrap a strip of bond paper twice round it, so that from 5 to 15 mm. (depending upon the depth of the object to be imbedded) projects above the top of the block, and fasten the paper with a small tack. Thus the paper forms the sides, and the top of the block the bottom, of the box. The paper must be wrapped very tightly or the box will leak.

When the material is ready to imbed, have at hand a bottle of Celloidin 3, a small wide-mouthed bottle of alcohol ether, a specimen jar about 100 mm. high and 75 mm. in diameter one-third full of chloroform, a pipette, and the boxes. Pour enough Celloidin 3 into a box to nearly fill it, dip the flat pointed forceps in alcohol ether, pick a piece of material out of the Celloidin 3, place it in its proper position in the thick celloidin in the box, quickly drop a few drops of chloroform from a pipette on top of the celloidin containing the material, and drop the box into the jar of chloroform. The object of the chloroform is to harden the celloidin, which it does to a very marked degree. Great care should be exercised to see that the material is in its proper position in the box, that is, that it is either truly vertical if cross sections of the material are required, or truly horizontal if longitudinal sections are desired.

The boxes should now be left in the chloroform for 24 hours, then taken out, the tack pulled out and the paper stripped off, the name of the structure and the species written on the block in pencil and the block with the material on placed in a jar of 82% alcohol. The material may be left there until needed. When sections are required, trim away superfluous celloidin from round the material, clamp the block in the microtome, and set the knife so as to make an angle of about 30 degrees with the bed of the microtome, being careful, however, to see that the knife will cut clear across the celloidin matrix. Wet the celloidin block and the knife with 70% alcohol with a camels-hair brush, cut sections as thin as possible without tearing, and transfer them with the brush to a dish of 70% alcohol. The cut surface of the block must be kept constantly flooded with 70% alcohol, as if it becomes dry the material will be ruined, and the knife must also be kept wet with 70% alcohol so that the sections will slide readily up onto it. If the whole block is not cut up, replace it at once in the 82% alcohol.

In cutting material containing spores it will be found that some of the spores will tend to drop out, but this may be prevented entirely by applying a coating of Celloidin 1 with a brush, dropping a drop of chloroform upon this and allowing it to harden for a few moments between the cutting of every two or three sections.

After the sections are cut the procedure depends upon the character of the sections. If they contain no loose parts the celloidin matrix may be dissolved in alcohol-ether, the sections transferred to absolute alcohol, and stained, etc., in the wire-gauze basket as outlined in "The Basket Method."

If the sections contain loose parts, the best sections should be picked out with the flat pointed forceps and placed in a wire gauze basket which has been previously placed in a dish of 70% alcohol. The number of sections to be handled at a time will depend upon their size,—if large only about 8 should be taken, if small, from 12 to 20 may be handled.

As the majority of stains color the collodion matrix permanently, there are but few which can be used when the matrix is to be mounted with the section and these are: aurantia, eosin, acid fuchsin, orange G and borax carmine diluted 1 to 200. The first four stains may all be washed out of the matrix in 70% alcohol and the dilute borax carmine does not stain it at all if not allowed to act for more than 12 hours. The best combination to use for double staining is aurantia eosin. For nuclei use acid fuchsin and for staining protoplasm use borax carmine.

In double staining place the basket in aurantia for about ten minutes, wash in water until no more stain is given off, then in 70% alcohol until the matrix is free from color. Place in eosin for ten minutes, wash in water until no more stain comes off and then in 70% alcohol until the matrix is perfectly clear by transmitted light. When the matrix is colorless by transmitted light there may still be a yellowish tint visible on looking at the surface, but this will disappear in the subsequent processes. While staining and washing, keep shaking the basket so as to keep the sections from remaining in too close contact. Now remove the sections from the basket, which should be left in the last washing dish of 70% alcohol, one at a time. Place the section in a dish of 95% alcohol for about 15 seconds, then in a dish of absolute alcohol for about 15 seconds, and then transfer to a dish of chloroform. Carry all the sections through to the chloroform in the same manner. It is probable that the matrix will turn cloudy in the chloroform as the sections cannot usually be 'clyedrated' sufficiently in the absolute alcohol without danger of dissolving the matrix. To remove the cloudiness replace the sections in the absolute alcohol, leave them there until perfectly clear, and then transfer to the chloroform. Leave them in the chloroform for a few minutes, pick out a section, place it on a slide, put two or three drops of balsam on it and place on a cover-glass.

THE GLYCERINE GELATINE METHOD.

This method should be used for all filamentous Algæ and Phycomycetes, and for any other soft structures which will shrink on treatment with the alcohols or xylol and chloroform.

The glycerine gelatine should be prepared as follows: Dissolve 1 part of best quality gelatine in 6 parts of hot water. Add 7 parts of glycerine and 1% of phenol. Filter through filter-paper in a hot water funnel, into a wide mouthed bottle.

Many things may be mounted without any staining, but most material is improved by careful staining, which may be done as follows:- The material should be washed in running water for an hour to take out the fixing solution. Then place a little of the material in a minot in a 3% solution of ferric alum, leave it for a few minutes and examine it under the microscope to see if any shrinking occurs. If any shrinkage takes place, weaken the solution until a concentration is found which causes no shrinking. Then place all the material in this solution and leave it for two hours.

Next wash in running water for half an hour, then stain in 1% aqueous solution of hæmatoxylin in a minot for from 2 to 24 hours. Material should be examined from time to time while staining in order to see when the staining is deep enough. It should be stained a little deeper than the color desired in the finished mount. Now wash in running water for half an hour and place it again in the ferric alum solution until the stain is of the required depth. To determine this, examine a little of the material under the microscope every few minutes. Now wash in running water for 6 hours or more, then put material in 10% glycerine (1 part glycerine to 9 of water) in a minot and allow it to remain uncovered until it has concentrated to the consistency of pure glycerine. Place the bottle of glycerine-gelatine in a dish of water and heat the water until the glycerine-gelatine melts. Place a little of the material on a warm slide with the fine pointed forceps, separate out the filaments (if it is an Alga or Phycomycete) carefully, add two or more drops of glycerine-gelatine with a glass rod, warm an 18 mm. cover-glass and place it in position.

Set the slide aside for several days and then spin a ring of Berry's Hard-oil Varnish round the edge of the cover-glass. This is done by spinning the slide on a turntable and holding a camels-hair brush charged with varnish lightly at the edge of the cover-glass. Let the varnish dry for 24 hours or longer and label the slides.

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