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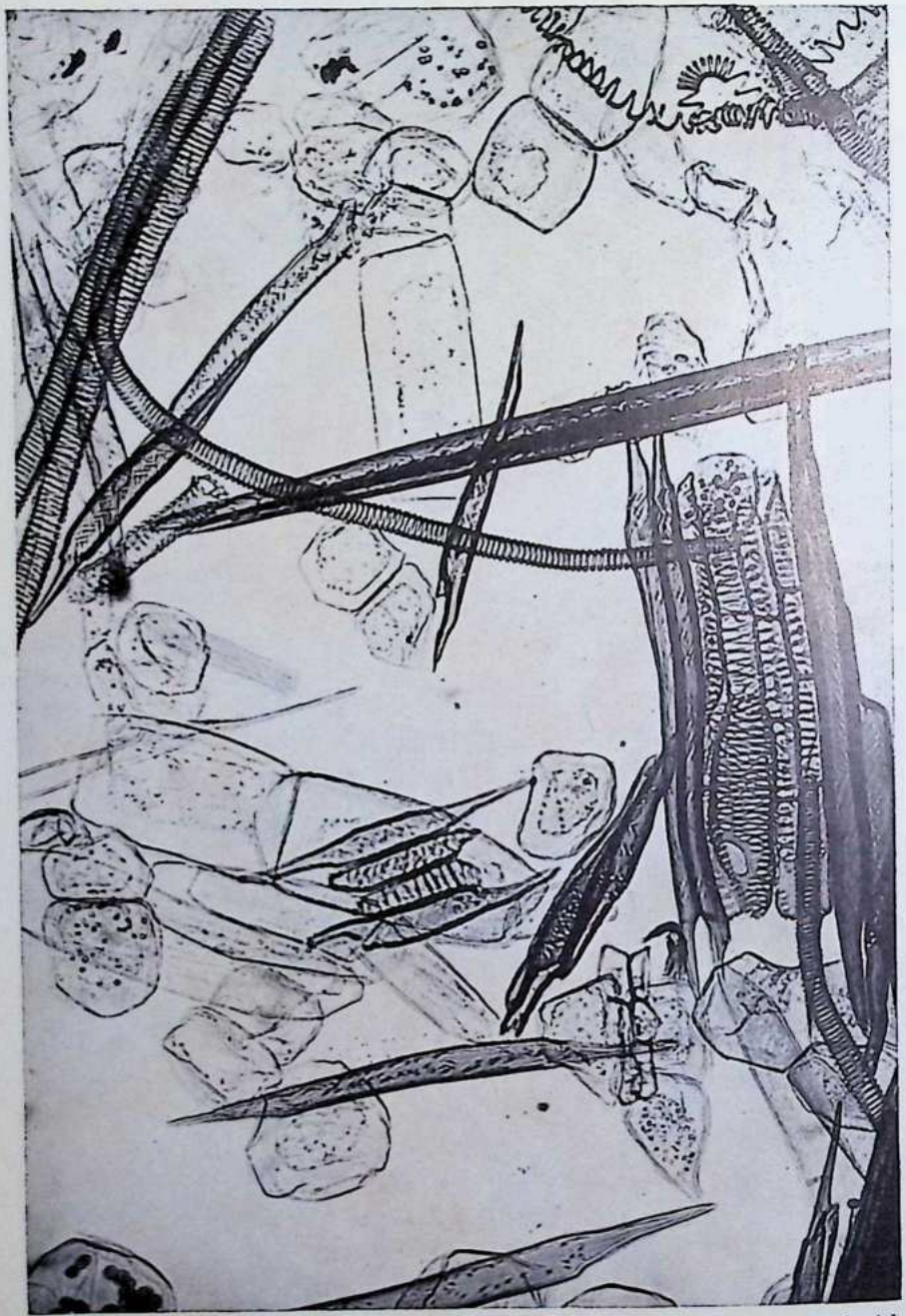

Isolated cells from macerated herbaceous stem of *Pelargonium zonale*. Stained with safranin, dehydrated with hygrobutol, and infiltrated with balsam.

PLANT MICROTECHNIQUE

BY

DONALD ALEXANDER JOHANSEN

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PREFACE

Four considerations prompted the preparation of the present text: (1) the acute necessity for a sifting and synthesis of the hundreds of methods and procedures that have been proposed during the past ten years of rapid development in microtechnique; (2) the need for a manual of modern botanical technique methods by botanists lacking special training in that field but who must prepare slides as part of their work in other fields; (3) to bring together the accumulated and mostly unpublished results of some sixteen years of extensive personal experience in collecting materials and preparing slides of plants from over the entire range of the plant kingdom; and (4) to provide an answer to the increasingly numerous enquiries received by the author concerning the methods he employs in routine and research work.

The main purpose of the book is to acquaint the user with the principles and procedures of all phases of botanical microtechnique. The specific aim is to enable elementary and advanced students, instructors and research investigators to prepare their own microscope slides of plant materials.

The text is in no sense an encyclopedia of botanical microtechnique methods. Many proposed fixing fluids, staining and dehydration methods, etc., have been omitted because it was concluded after thorough trial that they had not demonstrated their superiority over accepted procedures. With but few exceptions, every procedure cited has been carefully tested by the author or by students under his immediate supervision. Methods which are questionable under certain circumstances are so indicated. Such minor changes as might be required to adapt older schedules to modern conditions have been incorporated. In general, if a procedure or formula has not been credited to a specified person, the author may be held responsible.

The text has been divided, for reasons of convenience and experience, into two sections.

The first section describes the apparatus, reagents, dyes, etc., and the general methods universally employed by botanical technicians. Partiality in the selection of methods for presentation has been avoided, but it must be confessed that experience has engendered a preference for the paraffin over the celloidin method. Each method, however, has been described in sufficient detail to permit mastery in all its various phases. Inessentials have been eliminated and procedures made as concise and explicit as possible. The chapters on Whole-mount Methods, Smear Methods, and Cytological Methods constitute features that have never before been included as such in any text.

The second section takes up all plant phyla in phylogenetic order and gives as detailed directions or suggestions as are available for the treatment of specific groups in each phylum. The plants described are mainly those occurring naturally in the United States and Canada. The chapters on the algal phyla are particularly comprehensive. At the beginning of each chapter are given both general and specific suggestions regarding the collection, preservation, cultivation and manipulation of each phylum as an entirety, following which the orders and families are taken up in succession and more detailed directions are cited for genera and species. Innumerable procedures are described for the first time. Whenever references to other sources are not mentioned, the technical treatment recommended for a particular plant or group of plants is the one which has been found preferable from the author's personal experience.

References to the literature are intended primarily for the guidance of those who may wish to pursue a topic further, and secondarily to indicate the sources of statements or for more detailed descriptions of procedures. Only articles and books cited in the text, therefore, are included in the bibliography at the end of the second section.

A chapter on photomicrography has not been included because of the large number of excellent texts on the subject now available. Paleobotanical methods are omitted since suitable material is not available to most botanists and because the author has had insufficient personal experience in this field.

The figures have in general been selected to illustrate the results of a given technical treatment on the material concerned. Except as otherwise noted, all slides from which the photomicrographs have been made were prepared by the author. The photographs are practically all the work of a former student, Mr. John D. Poindexter.

For assistance in solving difficult technique problems and for a critical reading of portions of the first section, the author is deeply indebted to Miss Enid A. Larson. Portions of the second section have been read, and numerous suggestions made, by Dr. G. M. Smith. The author must also acknowledge the advice and assistance, particularly in connection with the commercial aspects of microtechnique, rendered by Dr. George H. Conant over a period of many years.

Botanical microtechnique is a science in which such rapid progress is being made at present that it is difficult for a person, without access to a large botanical library which receives all current journals, to keep abreast with proposed new procedures and changes in older methods. The author, therefore, would appreciate it if users of the text would favor him with reprints of their articles, or would otherwise keep him informed concerning new or improved methods.

Stanford University, Calif.,
May, 1939.

Donald A. Johansen.

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SECTION I

GENERAL METHODS

CHAPTER I

INTRODUCTION

Immense strides have been made during the past decade in all aspects of microscopical technique, and even greater progress has been made in revealing the life histories of plants and their phylogenetic relationships. There has thus been rendered imperative an attempt to correlate the information provided by these two sources and to digest it in such a manner that it may be of the optimum service to workers in all the various botanical sciences. The present text constitutes an attempt at such a correlation.

Modern synthetic chemistry has made available innumerable reagents with which both botanical and zoological technicians have recently begun to experiment. Many of the attempts turned out to have been founded upon overoptimism: there seems to be currently prevalent a delusion that there exists such a thing as a single foolproof cure-all for every one of the difficulties with which technicians are continually confronted. Dioxan may be cited as an example: expectations at first were very high, but this reagent soon turned out to have only a limited, though valuable, application. Experiments with other reagents are continuing, and every encouragement should be extended to such efforts. However, statements with regard to the applicability of a new reagent should always be made with due caution: it would be well continually to bear in mind that the innately complex structural differences among plants will always prevent any one reagent or method from being of universal application.

Although the older methods and ideas in vogue among botanical technicians often verged on superstition, there are nevertheless many sound conceptions which have been evolved and have survived all attempts at obliteration. The more prominent of the older superstitions were that absolute ethyl alcohol must always be used to ensure complete dehydration and that a clearing agent must be used to render the tissues perfectly transparent before they were in a fit condition to be infiltrated with paraffin. On the other hand, methods immediately became more refined when botanical technicians got rid of a procedure, apparently borrowed from clinical laboratories, in which time is the essence of any method, and began to use a graduated series of reagents for both dehydration and infiltration. None of the newer reagents provided by modern

chemistry has demonstrated its ability to work successfully at a given single unitary concentration, and it is extremely doubtful if any ever will, for the nature of plants cannot be changed any more than can that of human beings.

Each species of plants must be treated as individualistically as are specimens of *Homo sapiens*. The days when both plants and people were treated en masse have passed. To deal successfully with a given species, the technician must know something of the life history, the manner through which each stage is passed, the physical structure and chemical properties of the various tissues composing the organism and each of its parts, and the probable reactions of the latter to the reagents which it is proposed to use upon them. To this must be combined an essential knowledge of the physical and chemical characteristics of each reagent and of dyes and stains, their interactions with one another, and their general effects upon plant tissues.

The amazing manual skill of the older technicians has been supplanted by procedures which are partly physical but mainly chemical. In place of learning how to wield a razor and how to keep it sharp, the present-day technician must learn how to handle chemicals. Probably no other profession so abounds with "tricks" peculiar to it, as does microtechnique. A technician becomes successful only to the extent that he masters these strategems, in addition to inventing a few more of his own. It is not possible to describe all the artifices. After a thorough mastery of methods, one instinctively learns about them and recognizes the moment when it becomes necessary to apply one of them. Some small or relatively insignificant matter very frequently determines the success or failure of a procedure. Accidents and near accidents occur all the time: the mark of the technician is his ability to avoid them and to plan detours around obstacles placed in the path by the plants themselves. Experience showed that there was more than one way of killing a cat; likewise, there are different methods of dealing with obdurate plant tissues. The only trouble is that the other and correct method is not always immediately plain; more or less experimentation may be required, but this occasionally gives one the chance to devise a new method which would be of great service to other technicians. As a matter of fact, very many methods have resulted because some one could not get the customary procedure to work as it supposedly should.

The beginning technician and those who are proceeding for the first time should always follow directions exactly as given. It is highly presumptuous to imagine that one knows more about the procedure, even before attempting to use it, than did the originator. One is not at liberty to proceed differently until and unless one is convinced that the nature of the material demands a digression. The formulae of most

reagents have been determined by carefully controlled experiments; the schedules for all the principal staining methods generally represent the combined experience of many competent workers over long periods and with all sorts of plant tissues under a variety of conditions. The iron-acetocarmin method, for example, as devised by Belling, has been standardized in its basic principles, and one should first learn to obtain exactly the same results as do other workers before one starts in to make modifications. Others are prevented from understanding and interpreting one's results if undesirable or unnecessary modifications are made in familiar procedures.

A word of encouragement is in order. Many occasions will arise when one experiences a feeling of helplessness when confronted with the task of dealing with an unfamiliar plant. Even experienced technicians often find themselves in such situations. Their procedure is to employ a killing and fixing fluid with whose reactions they are familiar, then to use dehydration, infiltration and staining methods with which they have had successful previous experience, and finally to observe the results in the completed preparation. A little study will quickly reveal where improvements should be made when the rest of the material is to be worked up. This is only a sort of trial-and-error method, of course, but if each step is carried out after due consideration, the result is generally fairly satisfactory, and only minor changes need to be made afterward. The beginner should follow the same procedure. If the plant concerned is not specifically described in the text, then the methods recommended for the most closely related forms should be used for guidance.

Persons who happen to be afflicted with color blindness are under a handicap in microtechnique since the ability to judge variations in stain reactions is one of the prerequisites for successful work.

CHAPTER II

LABORATORY RULES

These rules are the self-imposed regulations of all competent technicians; by following them, the beginning technician will avoid considerable trouble.

1. First and foremost: Keep everything clean.

2. Know what you are doing. If in doubt, stop at once, and orient yourself before proceeding further. Do not try to rush things: there is no more certain way of courting grief. There will, of course, be frequent occasions for becoming exasperated with the proverbial innate perversity of inanimate things, but one should never permit such irritations to exhaust his patience.

3. Keep your desk or table in order. Have a definite place for every object. Label all chemicals, reagents, and solutions; do not trust your memory or senses to recognize them.

4. Use only clean glass vessels in preparing reagents, except in such rare cases as when special containers might be indicated. Always clean the glassware while still damp, or place it temporarily in a dishpan with running water.

5. Keep your hands clean and dry, especially when mounting paraffin ribbons on slides. Be careful not to leave traces of poisonous substances (*e.g.*, mercuric chloride and phenol) on your hands or clothes. It would be wise to wear a rubber apron in order to protect the clothing.

6. Use acids with great caution. The fumes of most acids are extremely irritating. Always pour acids into water, never water into acids. If heat is evolved, add small quantities at a time and cool between additions.

7. Keep containers holding anhydrous solutions tightly corked or stoppered. Do not use the dregs, as of absolute alcohol, for completing dehydration, for such remnants are no longer anhydrous. Use vaseline or petrolatum on the covers and edges of all stenders and coplins containing volatile or hygroscopic fluids, if they are to be left standing for more than a day or two.

8. Keep card or other simplified records, as accurately as possible, of all materials. Record on each card all data concerning the manipulation of the material. Do not leave anything of consequence to memory.

9. In weighing solid chemicals, take care to avoid contamination. Protect the pans of scales or balances with paper.

10. Do not throw solids or celloidin solutions into the sink. Flush the sink with water when pouring acids or stains into it.

11. Do not use pipettes indiscriminately. Have one for each type of alcohol, one for xylol, one for stains, another for acids, etc.

12. Take extreme care to avoid contamination of osmic acid solutions: they are very expensive. Never breathe the fumes of osmic acid, nor permit them to come near the eyes.

13. Keep balsam containers out of the light. The balsam might become acid and is then ruinous to stains.

14. In collecting material, remember that changes in the cells occur rapidly after removal from the plant, from water, etc. Shorten the time elapsing between removal and placing into the killing fluid as much as possible. It should not exceed more than a few seconds. Avoid crushing. Remove all superfluous tissue and cut into as small pieces as practicable.

15. Before starting to kill and fix tissues, be sure you have selected what you consider to be the proper solution. Better still, use several different fluids at the first trial, and later select for future collections the one giving the best fixation. Make up the solutions and have ready for use before starting work on the plants.

16. Study staining schedules carefully before starting to use one with which you are unfamiliar. Make certain that all the reagents called for are at hand.

17. Budget your time both a day and several days ahead. Plan future operations far enough ahead to ensure that the most may be made of the available time. Experienced technicians frequently have as many as a dozen operations proceeding simultaneously.

18. Remember not to leave tissues too long in killing solutions, in the dehydrating fluids, and in the paraffin oven.

19. Examine all your preparations critically and also, whenever possible, obtain the opinions of competent specialists. Never be satisfied with mediocre results. Judge on the basis of killing and fixing, infiltration, microtoming, staining, and mounting. Be honest in your judgment, even if you feel that poor results were the fault of the material or the schedule followed. You will be a better technician if you blame yourself first and your materials and schedules afterward.

20. Finally, under no circumstances become discouraged if your first efforts culminate miserably, but *try again*. Seek for the cause or causes of the failure. As Bolles-Lee, one of the greatest of zoological technicians, has truly said, even the most experienced technicians often turn out perfectly atrocious preparations on their preliminary trials.

CHAPTER III

APPARATUS

It is presumed that a person commencing the study of plant microtechnique intends to familiarize himself, to some extent at least, with the majority of the different methods. On this basis, each student should provide himself with the supplies noted in the following summarized list. Some of the items will be discussed at greater length.

One or more boxes of slides of the standard size, 25 × 75 mm. (1 × 3 inches).
Coverslips: A medium thickness, generally known as No. 1, is preferable. As a start, a half ounce each of the following sizes will serve: 22 mm. squares, 18 or 22 mm. circles, and 22 × 40 mm. rectangles.
100-cc. graduate (also 50-cc. and 500-cc. sizes if desired).
A dozen ordinary pipettes.
Giant pipette.
Large all-steel scalpel, or sharp pocketknife.
Scalpel with ebony handle and long, thin, straight blade; intended primarily for trimming paraffin blocks for microtoming.
Several needles, in holders.
2 or more camel's-hair brushes, assorted sizes.
2 pair of scissors, small and large.
Forceps: a strong one for handling slides, and a narrow-pointed one for use with coverslips.
Waterproof India ink and fine-pointed pen.
12 or more solid (Syracuse) watch glasses.
10 or more Coplin jars.
8 or more Stender dishes.
Several flat staining dishes.
8 or more bottles of 100-cc. capacity.
Several bottles of 500-cc. capacity.
Alcohol lamp: (Use only clean ethyl alcohol; traces of xylol, etc., will cause coverslips to become smudged.)
Balsam bottle, with glass-rod dropper.
6 or more square Petri dishes (for smears).
Suction pump for water faucet.
Grease or china-marking pencil (red).

Microscopes.—A microscope is, of course, necessary, but all that is required in microtechnique is an inexpensive one with a low-power objective and a single ocular. A microscope intended for research purposes emphatically should not be employed, as the chances are that it will soon be somewhat damaged by chemicals and minor accidents. The stage of the microscope should be protected by a glass plate; a large

lantern-slide cover serves the purpose admirably. It should not be fastened to the stage, as it is frequently easier to move the glass than a wet slide placed upon the glass. Also one might wish to examine a completed preparation without getting it wet, for which purpose it is merely necessary to withdraw the glass plate.

If the student has not had previous experience in the manipulation of the microscope, he should first inform himself fully upon the subject. The optical companies usually provide small handbooks to accompany their own instruments. One can pursue with great profit Simon H. Gage's "The Microscope" (Comstock Publishing Company, Ithaca, N. Y., 15th ed., 1932).

Microtomes.—Although not required at the beginning, a microtome soon becomes an absolute necessity. The modern microtomes are, on the whole, very efficient precision machines. They are of two general types, the sliding and the rotary. Technicians are not in agreement as to which type is the best; the choice of either type apparently depends upon the possession of "mechanical ability." Those who have a feeling for mechanical skill will do their best work with the sliding microtome; beginners, and even those with little or no aptitude in the use of machines, should place their main reliance upon the rotary microtome.

The rotary microtome should be used only for sectioning material embedded in paraffin; in all other methods requiring the cutting of sections, a sliding microtome is indicated.

Many students find it desirable to begin with a simple form of the sliding microtome, and to progress to a larger and somewhat more complicated as well as more accurate machine as skill increases.

There are two types of rotary microtomes. In the older type, as exemplified by the Minot microtome manufactured by the Bausch and Lomb Optical Company, the forward movement is directly related to the up-and-down movement. For optimum results, the two movements should be separated, but satisfactory sections can be cut on the Minot models if due care is exercised. In the new Spencer No. 820 microtome, the horizontal and vertical movements are wholly independent, giving greater stability and precision. The universal joint clamp for holding the object in this microtome, though of simple and rigid construction, is a little difficult for beginners to manage.

Knives.—Microtome knives are easily available, not too expensive, and those intended for rotary machines are interchangeable. Each student will find it more convenient to purchase his own knife, rather than to depend upon a laboratory knife which is liable to have been handled by careless students. Some sliding microtome knives have special two-pronged handles for clamping into the sliding block. The personal possession of such a knife is at the student's option; if he pre-

fers the sliding to the rotary microtome for cutting paraffin sections, it would be better to secure a knife intended for use in the former. Knives intended for rotary machines may also be used in some types of sliding microtomes.

The student should learn how to sharpen his own microtome knives, as a sharp knife is absolutely essential if perfect sections are to be cut. Microtome knives should not be trusted to the "key maker and knife grinder" in a dingy basement shop, nor to those who sharpen knives for surgeons. Most such persons will only ruin the knife. If the edge has a few bad nicks, it is better to send the knife to the manufacturer for resharpening. Two hones should be available: a yellow Belgian hone for the preliminary stages and a fine carborundum hone for the finishing, but many people use only the Belgian hone.

Place the back on the knife and screw in the handle. Flood the Belgian hone with clean water and place on the table so that one end is toward you. Place the knife at the far end of the hone, as close to the handle as possible and with the edge of the knife pointed in your direction, then draw the knife diagonally toward you in such a manner that the end opposite the handle reaches the near end of the hone. Do not exert any pressure on the knife; its own weight is all that should be allowed. Next turn the knife over so that it points toward the far end of the hone and with the handle end again close to the hone. Move diagonally toward the far end of the hone, then turn over and repeat the entire process for about 10 minutes. To detect nicks in the knife, draw the nail of the thumb cautiously over the edge (turning the finger over far enough to avoid cutting the ball), holding the knife pointed away from you and moving the nail in the same direction; the nail will be momentarily stopped by even tiny nicks. If any nicks are encountered, continue honing until they have disappeared.

There are a number of automatic sharpeners on the market. The necessary skill required for manipulating these sharpeners is much less than that required for stone honing, and is quickly acquired if the manufacturer's directions are followed.

In stropping, use a stiff leather strop, or one mounted on a wooden block, not one which is so soft as to bend the edge of the knife. The honing back should also be placed on the knife when stropping the final edge. Knives are stropped in the reverse of the directions employed during honing (otherwise, of course, the strop will be cut), and movements must also be diagonal. Never strike the knife against the strop with the grand flourish that barbers affect.

Any particular portion of the knife-edge should be used only once, and not too large a number of sections should be cut at this point. As soon as the edge shows signs of becoming dull, which can readily be told

when the sections begin to become rough and torn, move to a new portion, or remove, clean, and resharpen or strop. Always keep the back (the paraffin or object side) of the knife clean. Learn to do this with the ball of the forefinger or little finger, quickly, automatically and frequently.

Safety Razor Blade Holders.—The optical companies advertise clamps designed to hold a blade of the Gillette safety razor type, but none of these holders has any great merit. The new Craig-Wilson holder is superior to all others. This holder is so constructed that ice water may be run through it when very thin sections are required; or if sections 25μ thick or thicker are wanted, lukewarm water can be run through and long ribbons obtained. When using this clamp, it must be kept in mind that the blade is not straight, as is the microtome knife, but is bent into a curve. The holder should not be inclined toward the paraffin block as far as a knife is inclined, but should be as nearly vertical as possible without permitting the paraffin block to scrape against any part of the holder.

Safety Razor Blades.—There is much difference of opinion as to which brand of safety-razor blades is best for use in microtoming. As all who use safety blades for shaving know only too well, there is a very great lack of uniformity in all brands on the market. Manufacturers' statements are so unreliable as to be meaningless. The only thing one can do is to get any brand of the Gillette type, but to avoid those which are so thin as to be flimsy, and try one brand after another until a suitable one is encountered. The writer prefers Dublekeen blades. It is advisable to purchase a good quality Twinplex stropper and strop the blades occasionally, first being sure to remove any adhering paraffin by wiping the blade with xylol.

Paraffin Embedding Ovens.—It is now relatively easy to procure a satisfactory paraffin bath or oven. The various forms range all the way from an electric globe (carbon filament) suspended over a tumbler, in which the paraffin is only partially melted, to the large Lillie water bath with as many as 16 separate compartments. In localities where the daily and yearly ranges in temperature are slight, a simple form of oven is preferable, but in other places an oven with double, insulated walls will be necessary. The writer has used for many years a simple Thelco Electric Oven (manufactured by the Thermo Electric Instrument Company, Newark, N. J.). In large laboratories, where the number of students is considerable, one or more ovens of greater capacity are necessary. Excellent vacuum ovens have recently appeared on the market.

Staining Dishes.—Two types of staining dishes are available for staining sections mounted on slides: the Stender dish and the Coplin jar (familiarly known as "stenders" and "coplins"). The latter is difficult to clean because of its construction but has the advantage over the

Stender dish in that the slides are always in position. Some technicians prefer one type, some the other. As a general rule, coplins are used to hold stains and for the series of alcohols used in staining processes while stenders are used for washes, mordants, etc. In the more precise work, only a few slides are handled at a time and this is most conveniently done in coplins. If a large number of slides are to be stained by a standardized procedure and if the slides do not need to be handled individually until the process has been completed, small battery jars, each with a capacity of about 1 liter of solution, may be used. With such a container, some form of slide holder is necessary. One or two have recently appeared on the market, but it is just as easy to devise a suitable holder. One of the author's students removed the wire coil from inside the roller of an old-style window shade and cut it into convenient lengths. Or stainless steel wire of the proper gage (about 0.06 inch in diameter) can be wound around a broom handle; the coil should be about $\frac{7}{8}$ inch in diameter. The slides are inserted between the coils. A metallic rack holding 50 slides has been used for years by the writer; it should not be used in any solutions other than plain water, alcohol, or xylol—never in staining solutions and mordants.

Slides.—Many brands of slides now on the market are worthless. Slides of American manufacture are usually more dependable than those imported from other countries. It is well known that slides of European, and particularly of German, manufacture sold in the United States are generally those remaining over after the perfect ones have been picked out for home consumption. Dealers who furnish high-quality slides are listed in the chapter on Sources of Materials.

The standard thickness of slides is exactly 1 mm.; this is a rather thin slide more suitable for critical cytological work than for general purposes. Some brands appear slightly greenish when viewed edgewise, but there is no particular virtue residing in the claim that slides should appear perfectly white. Greenish slides are less prone to corrosion.

Depression Slides.—Depression slides (usually called "culture slides" in most catalogues), in which to mount bulky objects permanently, are expensive and for some purposes are unsatisfactory because the cavities are of a standard depth and width. The use of the dental engine and suitable abrasives mounted on mandrels has been suggested for making cavities of the exact dimensions required. The dental engine has its drawbacks, but there are now on the market a number of small electric grinders or hand pieces which are more easily manipulated and which can be utilized to grind cavities of any dimensions, provided mounted abrasive wheels of the proper shapes are used. The Handee grinder (manufactured by the Chicago Wheel and Manufacturing Company, 1101 West Monroe Street, Chicago, Ill.) has been used by the writer with very satisfactory results. Use the green mounted wheels, which contain

silicon or carbide abrasives, and always lubricate the site with a saturated solution of gum camphor in oil of turpentine. There is always considerable wheel wear when grinding glass; consequently the wheels should not be pressed too hard into the glass. Fairly thick slides should be selected. The ground portion has a very rough appearance when the grinding is completed, but after the cavity is filled with balsam the rough edges almost completely disappear and do not interfere with observations under the microscope.

Coverslips.—The usual run of coverslips (or cover-glasses) are no better than most slides. It is an anomaly that some of the most extensively advertised brands are, in the opinion of numerous competent critics, the poorest of all in quality. Coverslips of Japanese manufacture have, in the past, been of fairly uniform quality, relatively cheap, and satisfactory for general purposes. Coverslips that are of strictly American manufacture are somewhat high in price but are usually to be preferred. Certain manufacturers who claim that their coverslips are of American manufacture are nevertheless practicing a deception: in order to take advantage of lower import duties, the glass is imported in sheets and cut up in this country. Coverslips cut from this kind of glass are usually brittle and are difficult to clean, not to mention their tendency to corrode.

Coverslips come in four widely variable groups of thicknesses, designated by the Nos. 0, 1, 2, and 3. Number 1 coverslips, which should be about 0.17 mm. in thickness, are the standard. Squares and circles come in the following standard sizes: 15, 18, 22, and 25 mm.; rectangles are either 22 or 24 mm. wide and 30, 40, 50, 60, or 70 mm. in length. Coverslips of other dimensions may be purchased on special order. Unless the coverslips are obtained from a thoroughly reliable firm, the designated thickness should never be taken for granted but it should be checked by measuring with a cover-glass gauge. If an immersion lens is to be used on the completed preparation, the coverslips should be as large as possible and No. 0 is the preferable thickness. Round coverslips are indicated only with materials such as are run up by whole-mount methods, freehand sections, when the mounts are to be sealed on a turntable, and in similar cases. The large coverslips and slides used by zoologists and pathologists for very large sections are so rarely employed in botanical technique that they need not be discussed here.

Cleaning Slides and Glassware.—One should never believe statements of the manufacturers that slides and coverslips are ready for use but should always clean them, preferably in an acid cleaning fluid. Slides on which paraffin sections are to be mounted or on which smears are to be made should always be chemically clean, otherwise the sections are certain to be washed off. The fluid which is most generally used for cleaning glassware consists of:

Potassium dichromate	20 g.
Water	100 cc.
Concentrated sulphuric acid	100 cc.

Dissolve the dichromate in the water, add the acid cautiously in small amounts, and cool the mixture between each addition of acid. The mixture must be stored in glass containers and may be used repeatedly until it becomes too dark. Immerse the glassware for a few hours, and wash thoroughly in running water, finally rinsing with distilled water. Another mixture consists of 1 part of concentrated nitric acid and 4 parts of concentrated hydrochloric acid. It does not keep so long, and the fumes from the acids may be annoying. Immerse glassware for several hours, then wash in running water.

If it is intended that photomicrographs are later to be made, the slides and coverslips should never have been used before. If the slides have previously had sections attached to them with adhesive, the images of the earlier sections are rather certain to appear on the negative, particularly when the exposure is over a long period.

Slide Containers.—Containers for finished preparations are available in several different types. Cheap, flimsy cardboard boxes should be avoided. Wooden rack boxes with slots for 25 slides are more commonly used than any other type. They are convenient, permanent, and easily handled. There are two types: one with the cover fitting over shoulders on the base portion, and the other with the cover snapping into a depression cut in the inner side of the base. The latter type is used by commercial concerns since the slides are more easily held in position for shipping, but the former type is otherwise preferable. Similar boxes holding 12 slides each are also available, as are hinged wooden or combination wood-cardboard boxes with a capacity of 100 slides each. All such boxes should be stood on end so that the slides lie flat.

Slides should always be stored flat, particularly if they are whole-mount preparations. If whole mounts are stored on end or on one side, the material tends to flow toward the lower end, or even from under the coverslip. If the balsam is not thoroughly solidified under the coverslip, air pockets may also appear. Consequently, certain types of slide holders, which carry the slides upright, cannot be recommended. These holders, which may be large affairs constructed of metal, also generally lack easy portability and are difficult to keep in order. There is no really good container on the market for large numbers of slides.

Giant Pipette.—This useful tool is made by purchasing a rubber bulb about 8.5 cm. in length at a drugstore and attaching to it a piece of glass tubing of suitable diameter and about 25 cm. in length.

A giant pipette may be used for removing unicellular and colonial algae from pools, making changes of dehydrating fluids on materials to be mounted entire, and for countless other purposes.

CHAPTER IV

REAGENTS

GENERAL REAGENTS

The alcohols have long been the most important of the general reagents used in microtechnique. No other reagents have such diversified uses. There are a large number of different kinds of alcohols, but only ethyl alcohol can be classified as a general reagent. Formalin comes next as the most widely used reagent.

Ethyl Alcohol.—Whenever the term "alcohol" is used, whether in the present text or elsewhere, ethyl alcohol is invariably meant: if any other kind of alcohol is to be used, the exact kind is, and should always be, mentioned.

Pure ethyl alcohol is commonly called "100%" or "absolute alcohol," and in the trade it is known as "200-proof" (abbreviated, rather confusingly, to 200%). It is sometimes rather expensive and not always easily obtainable. It may occasionally be necessary for one to undertake the tedious process of making his own, but in such instances one must be careful not to violate government regulations. The most satisfactory method is distillation over calcium chloride. Shake up the lower alcohol with the chloride and allow to stand for some time. Put some of the mixture, together with some fresh chloride, in a glass still. Heat with an electric hot plate and distill over the alcohol slowly. The water will remain in combination with the chloride. A second method involves the use of cupric sulphate. Heat some of the crystals until only a white powder remains. Add this anhydrous sulphate to a quantity of ordinary 95% ethyl alcohol. The water is immediately absorbed by the sulphate, which returns to the original blue color. Continue adding calcined sulphate until it no longer turns blue. Finally filter quickly into a dry, tightly stoppered bottle. Some workers keep a little bag of the anhydrous sulphate in the bottle to insure its freedom from water.

A very sensitive test for the detection of traces of water in absolute alcohol is to add a few drops of the suspected fluid to a solution of liquid paraffin in anhydrous chloroform: if any moisture is present, it causes an immediate turbidity.

Ethyl alcohol is more commonly used at 95% or 96% strength, which is sufficiently strong for all except a few special purposes. Most botanical technicians have overemphasized the use of absolute alcohol. Some claim that tissues and slides must be passed through several changes of absolute alcohol to ensure a complete dehydration. The writer's experi-

ence, as well as that of many others, proves that this expensive process is unnecessary. In the tertiary butyl alcohol dehydration method, absolute alcohol is necessary at only one brief stage; a great saving of time and money is thus secured in comparison to the expensive and tedious older xylol method. In staining slides, if a clove oil counterstain is to be used, 95% alcohol dehydrates sufficiently, but 100% alcohol must be added to the first clove oil wash to remove traces of moisture that might be carried over.

The stock dilutions of alcohol are 15, 35, 50, 70, and 85%, although, of course, the series may be as extended as desired. These dilutions are made from 95% ethyl alcohol (never 100%) and distilled water. The quickest method of obtaining a given percentage of alcohol is to fill a 100-cc. graduate to the percentage required (as expressed in cubic centimeters) with the alcohol to be diluted, and then fill up to the percentage of the latter with distilled water. For example, if 35% alcohol is to be made from 85%, fill the graduate to the 35-cc. mark with 85% alcohol, then the rest of the way up to the 85-cc. mark with water. In the average laboratory it would be better to multiply the amounts just given by five, and to use 500-cc. bottles as containers.

Methyl Alcohol.—This poisonous fluid is also known as "wood alcohol." It is cheaper than ethyl alcohol but is of about 90% strength. It is scarcely ever specified in botanical microtechnique but may be used with caution if ethyl alcohol is not available. The "methylated spirits" frequently mentioned in English papers is ethyl alcohol containing about 10% methyl alcohol.

Formalin.—Formalin is also known as formol and formolose and in commerce is a 36% to 40% aqueous solution of formaldehyde. When, for instance, a 5% formalin solution is specified, what is meant is 5 cc. of commercial formalin to 95 cc. of water, alcohol, or other fluid. The commercial formalin generally used is certain to contain some formic acid, but it is ordinarily unnecessary to remove the acid. In fact the presence of the acid is sometimes a distinct advantage, but if neutral formalin is specifically indicated, one must resort to distillation. Add sodium bicarbonate to a flask of formalin, and distill only enough for immediate needs since the formic acid will soon reappear.

If the formalin has partially or wholly decomposed, which is indicated by the presence of a white precipitate, it is useless for critical work. It has been claimed that the addition of a little glycerin retards decomposition.

DEHYDRATING REAGENTS

Reagents which possess hygroscopic properties are those most commonly used for dehydrating tissues. The perfect dehydrating fluid is

one which mixes equally well with water, ethyl alcohol, balsam, and paraffin and which does not produce desiccation of the tissues (Johansen 1935). At the present time only two such fluids are known, *viz.*, dioxan and tertiary butyl alcohol, although other fluids are commonly used for dehydrating. The latter fluids are not miscible with paraffin and balsam, consequently another fluid which does mix with the two latter substances must follow.

Acetone.—Acetone is a satisfactory and safe dehydrating fluid but is not a paraffin solvent. Secondary or tertiary butyl alcohol, chloroform, or benzene may be used after acetone and preceding infiltration. Acetone dissolves fats, resins, waxes, and oils, and it precipitates albumins.

Dioxan (1,4-dioxan) is diethylene oxide or dehydrated diethylene glycol (Graupner and Weissburger 1931). With the exception of certain Rhodophyta it normally does not harden or shrink plant tissues. The principal disadvantage inherent in using dioxan is that it is considerably heavier than melted paraffin. It is therefore necessary to take all precautions to remove every trace of dioxan before embedding can be done. This difficulty can in fact be circumvented by using a fluid lighter than melted paraffin (such as chloroform, benzol, or secondary butyl alcohol) to wash out most of the dioxan before proceeding to the infiltration with paraffin. The use of calcium chloride to ensure perfect dehydration is advisable, especially in damp climates, but there is considerable danger of the chloride penetrating the tissues.

The Eastman Kodak Company product (#2144) is the only one which at present can be unreservedly recommended. Other brands contain considerable water and other impurities which will cause trouble.

Ethyl Alcohol.—Formerly ethyl alcohol was the only dehydrating fluid in general use, but as it has come to be suspected of shrinking and hardening tissues, it is now replaced by other fluids. However, one may safely go as far as 50% alcohol and then transfer to some other fluid which is miscible neither with water nor with grades of alcohol lower than 75% or 80% but is miscible with paraffin or balsam.

Hygrobutol.—This is a special type of tertiary butyl alcohol especially prepared for dehydration of materials intended for mounting entire in balsam (Johansen 1937–1938). Although somewhat more expensive, it may be substituted for the plain tertiary butyl alcohol in paraffin infiltration schedules.

Iso-propyl Alcohol.—This alcohol may be substituted for ethyl alcohol, except in killing fluids, with equally satisfactory results. Materials are said not to be hardened so much as by ethyl alcohol.

Methylal.—Methylal is so like dioxan in all its characteristics that there is no apparent difference between the two for microtechnique purposes. Methylal is several times as expensive as dioxan.

Normal Butyl Alcohol.—This type of butyl alcohol has a different molecular structure from tertiary butyl alcohol, lacks the soapy feeling of the latter, has a slight shrinking effect on many tissues, and frequently tends to harden others. Commercial normal butyl alcohol usually contains around 10% water; it may be made absolute by standing over calcium chloride until 24 hours after gas evolution has ceased. A pure, anhydrous product is on the market. It is soluble in water to the extent of only 8% by volume.

Normal Propyl Alcohol.—The possibilities of normal propyl alcohol in plant microtechnique have not been sufficiently investigated. Animal tissues have been run directly from water through three changes of normal propyl alcohol into paraffin. The fluid is claimed not to harden or shrink animal tissues.

Tertiary Butyl Alcohol.—Probably the safest dehydrating fluid for the beginner in technique is tertiary butyl alcohol; it is also the least expensive of all. It is superior to dioxan in that it is lighter than melted paraffin, otherwise the two fluids are somewhat alike in their microtechnical properties. The product derived from petroleum is more satisfactory than other types and more dependable for the best results. Like dioxan, tertiary butyl alcohol will mix with all reagents in common laboratory use.

CLEARING REAGENTS

The majority of clearing reagents are not miscible with water; tissues must therefore be completely dehydrated before a clearing agent can be employed.

Beechwood Creosote.—In order to avoid excessive hardening by the higher alcohols when dehydrating certain types of plant materials (such as fern prothallia), which are unusually fragile and liable to become damaged by pressure, the material may be carried as far as 80% alcohol and then transferred to beechwood creosote to complete the dehydration. Two changes of the creosote usually suffice. It may then be removed by any balsam solvent (dioxan, hygrobutol, benzol, or toluol), the materials transferred to balsam diluted at least five times with the solvent, and the latter evaporated gradually.

Some counterstains are soluble in beechwood creosote. Fern prothallia, for example, may first be stained with Harris' hematoxylin, run up to 80% alcohol, transferred to clear creosote, placed in creosote containing about 0.5% fast green dye, cleared with another change of creosote, and finally infiltrated with balsam.

The only brand of beechwood creosote that will give satisfactory results is Hartmann and Hauer's. Some brands are synthetic and wholly useless.

Benzol, Toluol.—These fluids work almost as well as xylol. Great care must be taken when using these fluids because of their explosive character.

Bergamot Oil.—This was a favorite with the older botanists. One may begin with the material in 95% alcohol and, by adding a drop at a time, gradually replace the alcohol with the oil. Some oil will remain in the material after embedding, but this is sometimes more advantageous than otherwise. Bergamot oil does not affect coal-tar dyes if used in the clearing of stained preparations.

Cedar Oil.—Good cedar oil is not easy to obtain. There is one type for immersion lenses and another for clearing. Most of the cedar oil on the market has been adulterated with xylol or other solvents. If there is much danger of the material becoming too brittle when other fluids are used, resort to cedar oil. Use like xylol, but a less close series of percentages may be used. Clearing is rather slow. Tissues may be left in cedar oil indefinitely without appreciable damage.

Chloroform.—Chloroform is the indicated clearing reagent for some kinds of material and occasionally had to be used in place of xylol before the butyl alcohols came into use. In the final stages of infiltration it is more easily removed than xylol but not so readily as the butyl alcohols. Equal parts of chloroform and carbon bisulphide sometimes constitute an excellent clearing agent. Chloroform hardens celloidin. It has been accused of spoiling delicate stain combinations, consequently one should avoid using it on stained slides.

Clove Oil.—The reagent most commonly used for clearing sections on the slide before mounting in balsam is clove oil. Before the mounting is done, all traces of the oil should be removed by washing the slide in xylol, otherwise the stains are apt to fade. Clove oil renders tissues brittle if they remain in it for any length of time. Clove oil contains about 82% eugenol. While no data are on record, eugenol may conceivably be substituted for clove oil if necessary, but it is far more expensive.

Terpineol.—Terpineol is a constituent of many essential oils and is of general value, although more appreciated by zoologists than botanists for clearing materials embedded in celloidin. It may be used as a substitute for absolute ethyl alcohol whenever this fluid is called for (Wetzel 1931) and in bringing slides from water to xylol without using alcohol (Volkmann 1933). Terpineol is harmless to most stains. In the writer's experience, the use of this fluid is fraught with danger because it appears to shrink tissues excessively.

Trichloroethylene.—This fluid is an excellent substitute for xylol. When used as a clearing agent, extraction of stains has not been observed. Paraffin is completely soluble in it, as is balsam. It has been claimed that slides cleared in trichloroethylene and mounted in balsam dissolved

in this solvent dry far more quickly than those mounted with the balsam dissolved in xylol (Oltman 1935).

Xylol.—Most technicians use the term "xylol" in place of the chemically more accurate designation "xylene." Tissues must be completely dehydrated before being gradually brought into pure xylol. This is a time-consuming and expensive procedure as xylol is generally considered to be miscible with absolute ethyl alcohol only (this is not strictly true, as traces of 95% alcohol will mix with xylol). A very close series of xylol and absolute alcohol is required. Several hours in each mixture are necessary, and the pure xylol needs to be changed several times. The transfer from xylol to paraffin must also be gradual and is just as tedious a process. Every trace of xylol must be removed from the tissues before the latter are embedded, otherwise the paraffin will crystallize. Tissues tend to become excessively hardened if left too long in xylol.

Xylol is still the prime reagent for clearing sections previous to mounting in balsam or other resinous medium.

Xylol should always be free from water and acids. To test a sample for freedom from water, add some of it to paraffin oil. If a cloudiness appears, the presence of water is indicated.

ADHESIVES

In botanical microtechnique, adhesives are fluids or substances used for affixing paraffin sections to slides.

Haupt's Adhesive.—This is by far the best fluid yet devised for the affixing of sections to the slide and may also be used for affixing unicellular and many colonial algae. To make it, dissolve 1 g. plain Knox gelatin (use the type that comes four envelopes to the package) or other pure, finely divided gelatin in 100 cc. distilled water at a temperature of 30°C. When completely dissolved, add 2 g. phenol crystals and 15 cc. c.p. glycerin. Stir well, then filter. A 3 to 4% aqueous solution of formalin is used for floating sections. Place a drop of the adhesive on the clean slide, smear evenly so as to leave a barely perceptible layer, then immediately add the formalin solution by means of a pipette. Place the sections on the formalin solution, and put the slide on a warming table (at a temperature of between 40 and 43°C. for paraffin with a melting point around 58 to 60°C.) until the sections have straightened out. Remove the slide from the warming table, and set aside until the water becomes cool; then drain off excess water, arrange the sections as desired, and set the slide aside to dry.

By putting mounted but still wet slides in a drying oven together with a watch glass or two of full strength formalin, the fumes of the formalin will further assist in coagulating the gelatin of the adhesive.

Mayer's Adhesive.—This is the older standard adhesive. To the white of a fresh egg add about an equal quantity of c.p. glycerin and 1 g. of sodium salicylate or a crushed crystal of thymol. Shake well, and filter through sterile cotton or two or three thicknesses of sterile cheesecloth. Use clean tap water for floating sections. While it will keep for as long as 6 months, solutions more than a month old lose their adhesive quality. If eggs are cheap and easily available, one may use filtered white of egg alone. Mayer's adhesive possesses less holding quality than Haupt's and also has the annoying property of absorbing coal-tar dyes.

Celloidin.—Thick woody sections and serial sections of certain marine algae which are retained on the slide with the utmost difficulty may be coated with a 1 to 2% solution of celloidin (in equal parts of absolute alcohol and ether) after first having been affixed with Haupt's adhesive and thoroughly dried. Remove the paraffin with carbol-xylol (10% phenol in xylol) and the latter with 95% ethyl alcohol. Under ordinary circumstances the interpolation of a 1% solution of celloidin in equal parts of absolute alcohol and ether in the staining schedule between the absolute alcohol-xylol and 95% alcohol stages will make the majority of sections adhere firmly to the slides.

Ullrich's Adhesive.—To 100 cc. of distilled water add 1 cc. of standard water-glass solution and 1 cc. of concentrated ammonia. Dry thoroughly in the air after stretching the sections with the aid of heat. Dissolve the paraffin with xylol as usual and bring down the series of alcohols to 70%. To this percentage add a trace of hydrochloric acid.

BLEACHING REAGENTS

Bleaching methods which are of general application are cited below, although other methods are being discussed elsewhere. In using bleachers, always watch the progress of the action, and stop it as soon as complete, otherwise the continued action will hydrolyze or macerate the tissues.

Hydrogen Peroxide.—The peroxide as it usually comes is of 2 to 3% strength. It may be used full strength or diluted to as high as 50% with either water or 50 or 70% ethyl alcohol. Merck's Superoxol is a peroxide of 30% strength and may be recommended where a powerful oxidizing bleacher is required.

Peroxide-Ammonia.—Ammonia water will accelerate the bleaching action of hydrogen peroxide.

Hydrogen peroxide (10% strength)	10 cc.
Water	200 cc.
Ammonia water	1 cc.

Transfer the material or mounted slides from water, and wash thoroughly in water after bleaching has been accomplished.

EMBEDDING MEDIA

Paraffin.—The problem of obtaining a satisfactory quality of paraffin for embedding purposes has long been a vexing one. Most paraffins on the market are worthless. There are many so-called "grades" offered, designated by the temperature at which they are supposed to melt and which, illogically, governs the selling price. One should never trust the statements which appear on the wrappers. The majority commonly crystallize readily after being cooled, despite all precautions. The consistency of a paraffin is a matter of greater practical importance than its melting point. It is recommended that one either prepare his own embedding paraffin or procure a guaranteed product from one of the reputable supply concerns.

For the infiltrating, ordinary Parowax serves satisfactorily. The Standard Oil Company of Indiana brand is superior to others.

Rubber can be mixed with paraffin to improve the consistency of the latter and thus make microtoming easier. A stock solution of crude rubber in Parowax should first be prepared. Obtain some crude Ceylon rubber in the form of thin rolled sheets (but do not use dental rubber). A rubber which melts at the melting point of the Parowax is worthless; what is needed is one that melts very slowly just below the smoking point of the Parowax. Cut up the rubber into as small pieces as possible with a stout pair of scissors. Heat the Parowax in a suitable vessel until it is very hot but not actually smoking, then adjust the heat so that this temperature is retained, and add the rubber in small quantities at a time, stirring the mixture occasionally. Depending somewhat on the size of the pieces of rubber, it should require many hours to melt the rubber thoroughly and completely. Finally pour the mixture into a clean tin can, cool, and later cut away the tin and remove the mass. Approximately 20 g. of rubber can be dissolved in 100 g. of Parowax. The embedding mixture consists of: crude mixture, 3 to 5 g.; Parowax, 100 g., ceresin wax, 3 to 5 g. These proportions may be varied slightly to meet special needs.

The same embedding mixture is on the market under the trade name Parlax.

Celloidin, Colloidin, Parlodion.—These are forms of nitrocellulose, highly inflammable but nonexplosive. They come either in the form of tablets or as shreds. The shredded form is stored in water, and should be prepared according to the directions on the label. Various brands are on the market and most of them are unduly expensive. No particular brand appears to be generally preferred over others.

Glycol Stearate.—This is a water-soluble wax melting at 48.5°C. Small portions of tissue may be placed directly into the melted wax (in a paraffin oven) from water. Leave the material in the wax for about 48

hours and give six successive changes of wax during this period. Solidify at room temperature. Sections may be cut very thin with a sliding microtome. Dissolve out the wax with chloroform, pass to absolute alcohol, and thence to the staining process.

MOUNTING MEDIA

Dammar Balsam.—Gum dammar, as this balsam is sometimes also called, is considered to be superior to Canada balsam by many microscopists. It is not readily obtainable in a condition suitable for immediate use; the crude gum may have to be purchased and prepared for use. It comes in the form of lumps of various sizes mixed with more or less powdered material and debris. Pick out the lumps, melt over a hot flame in a suitable container and pour the melted balsam into the desired solvent, which should preferably be benzol as it dries more quickly than xylol. Next filter through coarse filter paper placed in a ridged funnel, and filter again through finer filter paper if the first filtrate shows some cloudiness due to dust or finer specks of debris. Preparations mounted in dammar balsam do not fade around the periphery as frequently do those mounted in Canada balsam.

Canada Balsam.—Formerly one had to make up his own balsam from the solid, dry substance, but this has now been obviated and neutral, filtered balsam in the liquid form or dissolved in xylol or benzol may be readily purchased. For freehand sections, the solution as it comes is of about the right consistency, but it will sometimes need to be diluted slightly for paraffin sections. Xylol, dioxan, or trichloroethylene may be used as diluent. Balsam should never, under any circumstances, be heated to melt it. If the balsam comes from the jobber as a very dilute, darkly colored liquid, it has been spoilt by the application of heat and the use of too much solvent. It is highly essential that the balsam be kept from becoming acid. Keep the bottle in the dark when not in use, or paint it with black Duco. It would be well, as an added precaution, to keep a piece of clean marble in the bottle, replacing it occasionally with a fresh one. Always mount sections in Canada balsam from xylol, benzol, or trichloroethylene.

Euparal.—This mixture, popular with English technicians, is a combination of camsal, sandarac, eucalyptol, and paraldehyde, with a much higher index of refraction than Canada balsam. It has the property of intensifying hematoxylin stains, and it is often the practice to use this medium on preparations stained with hematoxylin. Mount from 95% alcohol.

Euparal has a slight solvent action on celloidin, but this may be taken advantage of to make curled or too stiff sections unroll and flatten out

before adding the cover-glass. If the euparal gets cloudy before mounting can be completed, warm the slide gently until the cloudiness disappears.

Diaphane.—The American equivalent of euparal is diaphane, but it has a slightly lower refractive index. Mounting may be from either 95% or absolute alcohol, but one should work quickly to avoid clouding. Diaphane is said to be nonoxidant and to preserve delicate stains well.

Styrax.—Styrax is a synthetic mounting resin with a very high refractive index, commonly employed for the mounting of diatoms to bring out the sculpturing of their "shells." It is best diluted, if necessary, with benzol. Mount from xylol or benzol.

Hyrax.—This medium is similar to styrax and is used for the same purpose. Fine details in smears stained with brazilin are well brought out. Mount from xylol.

Cedar Oil.—The immersion type of cedar oil hardens only along the periphery of coverslips, remaining liquid inside. Mount preferably from xylol. Stains seem to be well preserved.

Venetian Turpentine.—The resin of *Larix europaea* yields Venetian Turpentine, known also as Venice Turpentine and Turpentine Venetian. It is used only in the so-called Venetian Turpentine (whole mount) method. It usually has the fault of crystallizing in time and thus spoiling the preparations.

Lactophenol.—As a mounting medium, lactophenol consists of equal parts of phenol crystals, lactic acid, glycerin, and distilled water. Sometimes 2 parts of glycerin are added to 1 part each of the other reagents. For special purposes other ingredients are sometimes added, such as stains to color otherwise colorless tissues and copper acetate to preserve green pigments.

Karo.—Karo is an excellent substitute for glycerin jelly when employed for the same purposes. The blue-label type is preferable. Karo is a mixture of dextrose, dextrin, and maltose, but the sugars do not crystallize on drying. Materials may be mounted directly in Karo from water or the lower alcohols, but for more delicate forms, the Karo should be diluted with water or weak alcohol and concentrated by evaporation to a mounting consistency. When hard, Karo is as firm as balsam and ringing of the coverslips is not necessary, except in very moist climates.

Synthetic Resins.—During the last few years a large number of synthetic resins have appeared upon the market, and several have already been shown to have excellent possibilities as substitutes for Canada balsam. The one which seems to be the most satisfactory is Clarite (formerly known as Nevillite V); it is colorless, strictly neutral, inert, homogeneous and dries quickly. An 80% solution in xylol is to be preferred for mounting plant specimens, but a 60% solution in toluol has been recommended for thin sections of animal tissues. The resin is

intolerant of water; consequently it cannot be substituted for balsam in the hygrobutol method. There are other synthetic resins which are soluble in water but insoluble in hydrocarbons (such as Abopon and Stacol) and which form quick-drying, colorless, and nonhygroscopic mounting media. The pH of these resins varies from 6.65 to 8.0, therefore further investigation of their effect upon stain preservation is required.

SOLUBILITY OF VARIOUS REAGENTS IN WATER, ABSOLUTE ETHYL ALCOHOL, PARAFFIN, AND CELLOIDIN

	Water	Absolute alcohol, or lower percentage	Paraffin	Celloidin	B.P., degrees Centigrade
Acetone	x	x	—	x	56
Alcohol, benzyl	—	90	x	—	204.7
Alcohol, absolute ethyl	x	x	—	x	78
Alcohol, iso-propyl	x	x	—	—	82–83
Alcohol, normal butyl	8.3 parts	x	x	—	117
Alcohol, propyl	x	x	x	—	97.4
Alcohol, secondary butyl	29 parts	70	x	—	99.8
Alcohol, tertiary butyl	x	x	x	*	82.9
Anilin oil	—	90	x	—	245
Beechwood creosote	Very slight	80	—	—	200–250
Benzol (benzene)	—	x	x	—	80
Bergamot oil	—	80	x	—	183
Carbon bisulphide	—	x	x	—	46
Carbon tetrachloride	—	x	x	—	76.5
Cedar oil	—	95	x	—	237
Chloroform	—	x	x	—	61
Clove oil	—	90	—	x	250
Dioxan	x	x	At 56°	Slightly	102
Ether	12:1	x	x	x	34
Methyl benzoate	—	90	—	x	199
Methylal	Very slight	x	Slightly	—	45.5
Oil of origanum	—	90	Slightly	—	(?)
Petroleum ether	—	x	x	—	30–50
Terpineol	—	90	—	—	218
Toluol (toluene)	—	x	x	—	110
Trichloroethylene	—	x	x	—	87
Xylol (Xylene)	—	x	x	—	140

x = mixes or dissolves.
— = does not mix or dissolve.
*Mixes with celloidin dissolved in absolute alcohol-ether.

Glycerin Jelly.—Dissolve 1 part by weight of a high-quality gelatin in 6 parts by weight of distilled water for 2 hours or longer. Next add 7 parts glycerin, and to each 100 g. add 1 g. phenol crystals. Warm for

15 minutes, stirring continually until the flakes produced by the addition of the phenol disappear. While still warm, filter through two or three thicknesses of cheesecloth into a convenient bottle. The mixture solidifies on cooling. The whole may be remelted by heating gently, but it is preferable to cut out small portions and melt these since the mixture deteriorates on continual remelting.

CHAPTER V

KILLING AND FIXATION

The killing and fixing of the material to be made into permanent preparations are important processes, and the student should constantly bear this in mind. The day when a piece of tissue was dropped into any fluid which happened to be at hand has passed. Today the most competent technicians are exceedingly critical of the methods employed both by themselves and by others, and very rightly so. The student who expects recognition of his published work by others must devote the most careful and critical experimentation to secure the proper fixation of the material with which he may be working. These preliminary processes, in fact, are the most important of all, since everything else depends upon them.

NATURE OF KILLING AND FIXATION

Although the action of the substances used in killing and fixing fluids has been studied rather exhaustively, habit and prejudice rather than reasoned choice often determine what reagent will be used. It should be remembered that mixing together diverse reagents will not combine the excellent properties of each, but will often result in difficulties. Certain reagents which afford fine fixation when used alone or in certain combinations are mutually antagonistic when used in other combinations.

Killing and fixing are two distinct processes, but both are usually obtained by means of a single fluid which in turn is commonly a mixture of various chemical reagents.

The term *killing* means the sudden and permanent termination of the life processes. It does not apply solely to an entire organism, but to the individual cells of which the organism or tissues are composed. As long as a single cell capable of reproducing itself remains alive and receives nourishment, the organism is not yet dead. Any reagent used for purposes of killing must, therefore, reach every cell in the organism or piece of detached tissue if it is to do its work completely and effectively.

Killing invariably precedes fixation since the reagents which do the killing penetrate tissues faster than those upon which responsibility for fixation rests. Alcohol, for example, is primarily a killing reagent.

Fixation is a process very difficult to describe in terms that are easily understood. It is commonly said to be the preservation of all cellular and structural elements in as nearly the natural living condition as possi-

ble. Or, to put it a little differently, a good fixative is one that changes the cell chemistry the least and preserves the cell structure the best (Schiller 1930). What one actually sees after fixation is always a picture entirely different from the picture during the living condition, as it is utterly impossible to preserve anything in the exact condition in which it existed during life. Fixation is thus an entirely empirical process. The success or failure of fixation is judged by the quality of its usefulness when the completed preparation is examined. That is, it is good if the technician finds what he desires to be revealed. Many technicians do not need exact fixation; they are satisfied with a remote approximation. For example, cytologists who spend most of their time counting chromosomes ask only that the chromosomes be spread out to facilitate counting, that the chromosomes be darkly stained, and that the cytoplasm be so fixed (or partially dissolved) that it stains lightly or not at all. Accurate preservation of internal chromosomal structure and of the cytoplasm is not necessary for them.

Fixation, consequently, is something that can be judged in the subjective sense only, since there is no objective standard for the judgment of the quality of fixation. Judgment is based solely on the personal attitude of the observer. Opinions, obviously, are about as numerous as the individuals expressing them. If one of these individuals were to be asked to explain the reasons for his opinion and why it clashes with the opinions of other technicians, he is generally at a loss for a satisfactory and unbiased explanation. This is not surprising since many technicians labor under the mistaken assumption that "perfect fixation" actually exists.

Fixation is required in order that structures, which are obscured or entirely invisible when cells are observed in the living condition, may be seen more distinctly, and in order that soft structures may be hardened sufficiently for further treatment. This explanation is the one most commonly given. The point at issue is the fact that the evidence for the existence of many so-called "structures" not visible in living cells is almost wholly oblique. To understand fixation more fully, it becomes necessary to know exactly what technicians mean by structures. There is a sharp difference in the final picture of the effects of fixatives on fixed structures and on the nonfixed structural elements of protoplasm.

There are three concomitant criteria for the definition of a structure: (1) A structure is something that can easily be seen and recognized at all times in the living condition. Lignified plant cells, the nucleus, and chromosomes are examples. (2) A structure is always accompanied by an investing membrane, or may itself be made up of membranes. Structures coming under this classification are not always easily visible in the living condition and are more characteristic of plant cells than of

animal cells. Vacuoles are an example. (3) One test of the reality of the structures seen in fixed material is their appearance under a diversity of conditions and after the use of a wide variety of fixing fluids. Spindle fibers are considered to be an example of such structures.

Originally the term fixation was applied to the grosser structures which can be preserved without any relation to the change of the colloidal state which is the fundamental structure of protoplasm. These structures are not ordinarily affected by fixatives; at least, not in the violent manner that cytoplasm is acted upon. The earlier plant morphologists were interested more in definite cell regions or elements than in cellular contents. They were therefore not disturbed by the difficult artifact question, since what they saw in their preparations could also be seen in the living tissues. They fixed materials so that they could stain things seen in living cells and thereby be enabled to make more precise observations. To them fixation meant merely the preservation of structure, and from this point of view it is possible to describe fixation as being good or bad. This idea has been carried over into cytology, often with unfortunate results.

If the existence of a structure cannot be demonstrated by vital observation, it has been contended that such a structure cannot be other than some colloidal artifact since it becomes visible only after the living colloidal structure has undergone an irreversible change of condition as a result of fixation. The appearance of this structure in a fixed and stained preparation is said to be merely some one phase of the images occurring during necrobiosis, the result of the toxic action of the fixing reagents (Yamaha 1927).

Most technicians now grant that the fundamental structure of protoplasm is colloidal. To state the case more fully, each protoplasmic structure, such as nucleus, plastids, and cytoplasm, is an intricate colloidal system, and the phase boundary where each colloidal system meets, as at nuclear membranes and the tonoplast, is still another colloidal system. These colloidal systems are all believed to belong to the hydrophilous colloid group. This, it should be emphasized, is the case with protoplasm in the living condition only. When the reagents in a fixative come into contact with protoplasm, the colloidal system undergoes an irreversible change of state. As a consequence, the state of dispersion, the grade of hydration, and every other physicochemical property which accompanies these properties, are subject to marked and sometimes violent changes. What actually happens is that, when protoplasm is acted upon by a fixing fluid, it goes through various colloidal changes before it finally attains the irreversible state. This has been demonstrated time and again by the fact that the instant when the action of the fixing fluid was terminated has a direct bearing on the appearance of the

protoplasmic structure in the completed preparation. If the time of action was brief, the final picture is very different from the one presented when the time of action was too prolonged. This means that all the structural changes preceding the final irreversible coagulation of protoplasm can easily be followed out. We can, for example, observe the formation of new dispersed phases in the hyaloplasm, the dissolution or disappearance, transformation, and fusion of existing dispersed phases such as plastids and mitochondria.

The ultimate aim in fixing protoplasm is to make invisible or barely visible structures easily observable. The increase of optical contrast between structures is generally considered to be more important than the preservation of the substances forming these structures. A marked optical contrast results from the great difference in the physicochemical characteristics among the various colloidal systems making up protoplasm, and another results from the distinct phase boundaries of these colloidal systems. Now, when the final irreversible change in the colloidal system occurs as a result of fixation, what has taken place is actually *destruction*. The changes in optical differentiation just mentioned are the results of the destruction of the original colloidal systems. In the original condition (*i.e.*, in the living condition), there is little or no optical differentiation; in the final condition there is usually great optical contrast, provided a suitable fixing fluid has been chosen. The final condition depends both on the nature and on the concentration of the chemical reagents composing the fixing fluid, and on the particular nature of the protoplasm. The latter expression is to be interpreted as meaning the difference in the nature of the protoplasm between, say, a red alga and an angiosperm root tip.

According to most authorities, the hydrogen-ion concentration (pH) of the fixative influences the destructive action rather than the preservative action. For example, the higher the concentration of hydrogen ions, the less perfect is the preservation of purely cytoplasmic structures. Protoplasm, regarded as similar to a protein solution, coagulates most easily at the isoelectric point and absorbs the minimum quantity of the various ions. Karyoplasm, with respect to the isoelectric point, is known to be more acidic than cytoplasm. Since ordinary fixatives are strongly acid, the lower the pH of a fixative, the less is its coagulating power and the more is its dissolving power; this explains why strongly acidic fluids give such poor fixation of vacuoles, for example. The dissolving action is considerable in those fluids, such as Gilson's or Petrunkevitsch's modification thereof, whose pH is less than 1.

To put it briefly, fixation is a contradictory process, consisting as it does of both the preservation of some structures and the destruction of

others. The latter factor is to the microscopist actually the more important of the two since it is by this means that optical contrast is obtained. Since staining of tissues is but another method of enhancing optical differences, it in turn is dependent upon the nature of the fixed protoplasm. Therefore, if the staining is not clear and sharp, the more or less illogical conclusion is that the protoplasm was imperfectly fixed. It is really possible to get perfect preservation of structures but to have practically no optical or stain contrast, as all experienced technicians are fully aware.

The failure to get sharp staining following "perfect" fixation is to a considerable extent also bound up with the chemical properties of the various dyes and the circumstances under which they combine with the different types of fixed substances. This obviously is strictly a chemical problem, and extremely little at present is known about it. The presence of excessive amounts of phlobaphene (tannin) compounds can cause complications during both fixation and staining, and, as another example, the practice in some quarters of mixing potassium bichromate and chromic acid (which really gives an excessive amount of chromic acid) gives poor fixation and difficult staining. In any event, it is agreed that whatever the mechanism of fixation and staining, all other conditions being equal, the type of fixation employed greatly influences the ultimate staining of the tissues (Naylor 1926; Sheinin and Davenport 1931; Tolstoouhov 1927, 1928).

One effect of fixation is the hardening of the tissues, but hardening is ordinarily brought about by the dehydrating fluids. The latter differ considerably in the degree of their hardening effects, but as a rule any tissue that becomes hardened by one reagent is liable to become more or less hardened by all other reagents. The tissue hardening that occurs after fixation is presumed to be caused by precipitation, but swelling or shrinkage may also influence it (Sheinin and Davenport 1931). There is apparently no correlation between tissue hardening and tissue preservation. While good tissue hardening does not necessarily result in good tissue preservation, the latter must be preceded by the former.

Certain powerful fixatives, such as Carnoy-LeBrun's fluid, bring about their effects by desiccating the tissues. Some plasmolysis, therefore, must be expected.

It has recently been demonstrated that much of the criticism leveled against certain killing and fixing fluids was not merited since the trouble was actually caused by inadequate and, to a certain extent, improper dehydration methods. It should be borne in mind that killing and fixing are only two phases of the entire process of transforming living materials into permanent preparations and that each phase must be examined both

independently and in conjunction with each other phase. Violent dehydration methods can easily give a false fixation picture, and it is illogical to fasten the blame on the fixing fluid.

PRINCIPLES INVOLVED IN COMBINING REAGENTS IN KILLING AND FIXING FLUIDS

Each reagent used for purposes of killing and fixing has both its advantages and its disadvantages.

Some reagents used for killing purposes penetrate rapidly, and others penetrate slowly. Both classifications in turn include reagents which simply do what is expected of them and have no further effect upon the tissues, whereas other reagents will damage the tissues if their destructive effects are not deterred by the addition of other reagents, or by washing them out. Osmium tetroxide may be taken as an illustration: it is the fumes that actually do the killing, which occurs within a few minutes; if the substance is allowed to react for longer than ½ hour or is used in too large concentrations, the tissues become first brown, then blacker and blacker, and will eventually become reduced to a charred mass.

The reagents used for fixation may be classified similarly, but the definition is far sharper among this class of reagents than with those used primarily for killing.

The characteristics of the more important reagents used for killing and fixing are noted below. The general principle involved in the combining of these and other substances to form a killing and fixing fluid is to secure a balance between all the properties of the reagents involved. A substance which tends to shrink cytoplasm, for instance, should be combined with one which tends to swell cytoplasm. In other words, a disadvantage inherent in one reagent can be counterbalanced by that peculiar to some other reagent if the two disadvantages are of a directly opposite nature. Two reagents which have the identical disadvantages should never be combined by themselves.

Moreover, substances which are easily oxidized should not, as a rule, be combined with reagents that are powerful reducers.

Practically all suggested formulae are flexible insofar as the quantities or proportions of each reagent are concerned. In fact, most proposed formulae are merely variations of some one basic formula. No one fluid serves equally well for all organisms because of the immense diversity in structure, chemical and physical organization, etc., among plants. Some elasticity is necessary, and the proportions of each reagent in any given formula require adjustment according to the species, with relation to its structure and composition. If the standard proportions do not give satisfactory fixation, it is ordinarily possible to determine which reagent needs to be increased or decreased, as the case may be, or some other

combination might be experimented with. A formula intended primarily for cytological purposes does not always give good fixation for anatomical purposes, and vice versa.

It is therefore apparent from the foregoing discussion that the choice of the killing and fixing fluid is an important matter. The nature of the material being worked upon should first be determined—whether it is soft and easily damaged or tough, resistant, and not readily penetrated by reagents in solution. Next the purpose for which the material is being prepared should be considered. A fluid which might give good preservation of root tips does not necessarily render the chromosomes of the mitotic figures in the tips in a fit condition for critical study, consequently there will be numerous occasions when it becomes necessary to sacrifice some desirable qualities for the sake of others.

HANDLING OF MATERIAL

The greatest care should be exercised in handling tissues or organisms preparatory to placing them in the killing fluid. Very small organisms, such as filamentous, colonial, and unicellular algae and filamentous fungi, *Euglena*, small Bryophyta, may be placed entire in the fluid. With larger organisms, more or less dissection or reduction to smaller portions is required. Avoid pressure of any sort, and work quickly so that a minimum time will elapse between removal from the plant, from the aquatic habitat, etc., and placing in the killing fluid.

The smaller the piece of tissue and the larger the surface exposed to the direct action of the killing fluid, the better the killing and fixation. The weaker fixatives penetrate with difficulty. Occasionally it will be found that the outer layers of cells are overfixed, while the innermost portions are badly underfixed. This is caused by poor penetration of the fixative portion of the fluid. The remedy, of course, is to use one of stronger penetrating power. Acetic-alcohol and picro-aceto-formalin combinations penetrate rapidly and easily, while mixtures containing chromic and osmic acids penetrate poorly. Buds which are covered with dense hairs should be treated first with a strong alcoholic solution for a few minutes and then placed in another fluid, preferably an aqueous one. For example, buds of nearly all the Asteraceae are beautifully fixed if they are placed in Carnoy's fluid at the time of collecting and after not more than 10 minutes transferred to Navashin's fluid. All superfluous tissue should be carefully removed. In general, organs or pieces of tissue, for the optimum results, should never exceed 5 mm. in any one direction.

FIXATION IMAGES

Fixation images are of two types, and the characteristics of each are so pronounced that the technician should learn to recognize them promptly.

1. *Acid Fixation Image.*—Chromatin, nucleoli, and spindle fibers preserved; cytoplasm fixed as stringy spongioplasm; nucleoplasm and mitochondria dissolved.

2. *Basic Fixation Image.*—Resting chromatin and spindle fibers dissolved; nucleoli, nucleoplasm, and mitochondria preserved; cytoplasm fixed as hyaloplasm, and vacuoles more or less preserved.

Nearly all the fixing fluids in common use give the acid fixation image. Very few fluids giving the basic image have been compounded, and the most useful ones have been developed only during the past few years (Zirkle 1927 *et seq.*). However, there are variations in each image according to the material; for instance, mitochondria are occasionally found after distinctly acid fluids were used. Investigations on the effect of fixing fluids have generally failed to take into account the changes produced by the dehydrating reagents. The correct procedure for describing the final effect should include both the killing and fixing fluid, as well as the dehydrating reagent and the infiltrating medium. The picture given by Bouin's fluid with absolute ethyl alcohol and xylol as the dehydrating fluids is quite different when tertiary butyl alcohol is used for dehydrating.

With certain reagents there is some overlapping of the two images. Copper bichromate, for example, if used in a solution at pH 4.8, preserves both chromatin and mitochondria (Zirkle 1928).

If the fixing fluid contains two or more substances, the resulting fixation image is determined primarily by the component which penetrates more rapidly (Zirkle 1933*b*). The various reagents, it will be recalled, penetrate at different rates, and also according to their respective concentrations.

For most purposes a fluid giving an acid image will be the most satisfactory, particularly since the majority of the preparations that the technician has become accustomed to observing have been from such fluids. The basic fluids are employed mainly for studies on cytoplasmic structure and for cytoplasmic inclusions such as mitochondria.

KILLING AND FIXING FLUID REAGENTS

Under this heading will be described the substances most commonly used in killing and fixing fluids, and the formulae for the various fluids will be given in a succeeding section. The student should familiarize himself with these substances and be able to recognize all of them instantly by means of appearance, odor, and other characteristics.

Absolute Ethyl Alcohol.—This is a fair killing and fixing fluid if immediate results are desired. The time should be very short; in any event, not over 1 hour should elapse before the fluid is poured off, fresh

absolute ethyl alcohol is added to ensure complete dehydration and the infiltration then begun.

95% Ethyl Alcohol.—This is a fair general preservative but has nothing whatever to recommend it for even passable work. Alcohol is a reducing agent and is easily oxidized to acetaldehyde and the latter in turn to acetic acid. Chromic acid, potassium bichromate, and osmium tetroxide should therefore not be mixed with this alcohol. It is much more useful and efficient when in combination with formalin and glacial acetic or propionic acid. Unless some glycerin be added, tissues left in 95% alcohol become too brittle for further use. Material which has been killed and fixed in most of the standard fluids may, after the original fluid has been thoroughly washed out, be kept indefinitely in 70% ethyl alcohol. Proteins are precipitated by alcohol; the precipitates are insoluble. Nucleic acid is precipitated, but not insolubly. Fats and phospholipides are dissolved.

Alcoholic fluids should not be used at low temperatures.

Chloroform.—Chloroform is never used alone. It is a constituent of Carnoy's fluids.

Formalin.—It is best to use only the chemically pure solution, which costs but a trifle more than the ordinary brands. Used alone, fair fixation sometimes occurs, but the results are generally unpredictable because some types of material are shrunken and overfixed, while others may become swollen and vacuolized. Formalin as a rule penetrates slowly (Underhill 1932), and when used alone gives the basic fixation image. Formalin is one of the best hardening agents. It does not precipitate proteins or render them insoluble in water but merely prevents ethyl alcohol from hardening them excessively, by exerting a different hardening effect of its own. Formalin neither preserves nor destroys fats but more or less preserves phospholipides. In using formalin in combination with other reagents, such as chromic acid, it should be kept in mind that formaldehyde is a powerful reducing agent; it becomes oxidized to formic acid. The fumes of formalin are extremely irritating to mucous membranes.

Formalin may be neutralized by adding about 5% pyridine.

Most marine algae are successfully fixed in sea water to which has been added from 6 to 10% formalin.

Glacial Acetic Acid.—This acid is very commonly employed as a constituent of fixing fluids, on the assumption that its presumed tendency to swell cytoplasm counteracts the shrinking effects of certain other reagents, such as chromic acid and formalin. Actually, however, acetic acid shrinks tissues (Zirkle 1928*b*). Its usefulness lies in the fact that it is a fat-soluble acid which penetrates rapidly and produces the acid fixation image in the tissues. The acid otherwise is more of a preservative than a

fixative, as may be recalled from its use in the pickling industry; it preserves both ordinary proteins and fats, but they can subsequently be dissolved in water. It is primarily a chromatin preservative.

Fixation images identical with that of acetic acid are produced by ferric, cupric, chromic, and mercuric acetates. Propionic, butyric, and valeric acids may, under most circumstances, afford better fixation than acetic acid.

Nitric Acid.—Little use is made of this acid in botanical microtechnique, but it has been included in certain fixatives.

Picric Acid.—This acid, which comes in the form of a yellow crystalline solid, sometimes slightly moistened as a precaution against explosions, is invariably used in the form of a saturated solution. It shrinks strongly and penetrates tissues readily. Proteins, nucleoproteins, and nucleic acid are all precipitated. In whatever solution or fluid it is employed, it should always be washed out with 70% ethyl alcohol, never with water, unless the fluid contains some other reagent which indissolubly precipitates chromatin. The washing may be facilitated by warming the wash alcohols to as high as 40°C. It is not always necessary to get rid of all the yellow color imparted to the tissues; if this is found necessary, lithium carbonate may be added to the 70% wash alcohol until the color has disappeared from the tissues.

Anthraquinone is superior to picric acid as a killing reagent and may be substituted; it is used in exactly the same way.

Chromic Acid.—Microtechnicians, and particularly those engaged in cytological work, can scarcely get along without chromic acid. It is the aqueous solution of chromic anhydride, which comes in the form of reddish-brown, extremely deliquescent crystals. Chromic anhydride should not be dissolved in alcohol as it will quickly become reduced to chromous oxide or sesquioxide, neither of which is of any value as a fixative. Chromic acid is the basis of a long series of killing and fixing fluids, which commonly also contain acetic acid.

Chromic acid tends to shrink tissues; to counteract this tendency, other substances are usually mixed with it. It does not penetrate very well into many types of tissues. Some technicians contend that at low concentrations the acid exerts a considerable solvent action upon tissues, but this apparently is more true of animal than of plant tissues. The one great defect of chromic acid resides in the fact that, because it is a powerful hardening agent, it tends to cause tissues to become brittle. The acid is one of the most powerful precipitants of proteins, nucleoproteins, and nucleic acid, none of which can later be dissolved. Fats are not affected nor are lipoids.

Chromic acid stock solutions are best kept in strengths of 2 and 10%. Practically all formulae in general use are based upon dilutions of these

stock solutions. Large volumes of any killing fluid containing chromic acid should be used. Such fluids should always be washed out thoroughly with either running water or frequent changes of large volumes of water.

Fixation with chromic acid and other chromic compounds is essentially a tanning process, and tissues consequently become more or less brownish in color. The tanning is most pronounced after the use of those fluids which contain both chromic acid and potassium bichromate (*e.g.*, La Cour's fluids). If the sections appear to have too much chromate fixation, which is invariably the case after such fluids, bleaching may be effected by dipping the slides into 1% aqueous potassium permanganate for about 1 minute, rinsing very briefly in water, then dipping in 5% aqueous oxalic acid for 1 minute, and finally washing thoroughly with water. Or differential acidification (page 169) may be attempted.

As a rule, a cytological fixative weak in chromic acid will cause chromosomes to become attenuated and to reveal constrictions that a fixative strong in the acid will not show up.

Osmic Acid.—Osmic acid is the name commonly given to osmium tetroxide, although this compound is not strictly an acid. It is an extremely expensive substance and usually comes in the solid form in 0.5- or 1-g. sealed tubes. The weight cited is not always accurate but because of the extreme difficulty of checking up on this point, any probable discrepancies may for most practical purposes be ignored. Solutions of osmic acid are very readily reduced by the presence of the least bit of organic matter. Exposure of plain aqueous solutions to the light does not necessarily bring about reduction, as is commonly believed, provided that the water is absolutely pure and quite free from dust. Such a solution should be kept in a thoroughly cleaned dropping bottle with grooved stopper. Before breaking the tube in which the acid comes, clean it carefully, removing every evidence of the label, and finally rinse in several changes of pure water. It would be a far more satisfactory procedure to purchase a 1% solution in 1% chromic acid direct from the manufacturer.

Reduction of solutions in distilled water may as a rule be prevented by the addition of (1) a small amount of sodium iodate, (2) about 10 drops of a 5% aqueous solution of mercuric chloride to each 100 cc. of 1% osmic acid solution, or (3) enough potassium permanganate to give a barely perceptible rosy tinge to the solution, adding more of the permanganate whenever the solution becomes colorless.

Osmium tetroxide penetrates plant tissues poorly. The fumes or vapor kill just as efficiently as does the solution, especially spores, zoospores, small unicellular algae, and similar organisms. The solution, however, should never be used in a strength exceeding 2%. Many workers consider 0.1 to 0.5% to be strong enough. Unacidified osmium tetroxide

precipitates proteins instantly and coarsely; acid solutions do so slowly and more evenly. On the other hand, each of the two solutions works in the opposite way on nucleoproteins. Osmium is the only reagent which preserves both fats and conjugated lipides. Objects should always be as small as possible, whether for killing by the fumes or the solution. Fixation is complete as soon as the material has become brown throughout. Killing fluids containing osmium tetroxide must always be thoroughly washed out before dehydration is begun. If the material has been left in the killing fluid too long, it will become blackened, with the result that it is necessary to bleach the sections before a proper stain reaction can be secured.

Under certain circumstances the use of osmic acid is contraindicated. For example, it should not be used on the Rhodophyta because it aggravates the natural tendency of this group of plants to break to pieces during almost any stage of the subsequent processes; nor, except for special purposes, should it be used on tissues rich in oily substances.

Mercuric Chloride.—Corrosive sublimate is a synonym for this extremely poisonous substance. Mercuric chloride is never used alone. It is a rapid fixer and a powerful precipitant of proteins and nucleic acid, but it tends to shrink tissues. Transparent tissues are rendered opaque as soon as fixed. Aqueous solutions should always be based upon distilled water. The sublimate must be very carefully washed out after fixation. Aqueous solutions may be washed out with water, although it would be preferable to use an alcohol of a strength below 70%. Alcoholic solutions should be washed with alcohol of the same percentage as that in the killing fluid. In the case of material which is to be embedded, the deposits left by the mercuric salt are more easily removed from the sections. Transfer the slides from 70% alcohol to 50% alcohol to which are added about 1 g. of iodine and 2 g. of potassium iodide per 100 cc. After a few minutes, transfer to a 0.2% solution of sodium thiosulphate (ordinary photographers' hypo is satisfactory) to remove the iodine, then proceed to the staining after a thorough washing in water. The hypo solution must be kept up to, but not beyond, its original strength by the addition of a tiny crystal from time to time.

Material killed in solutions containing mercuric chloride should be embedded as quickly as possible.

The use of mercuric chloride as the main or a minor component of a killing fluid to be used on material intended for cytological study is not to be recommended.

Iodine.—A weak aqueous solution of iodine and potassium iodide is excellent for fixing microscopic plant forms; glacial acetic acid and formalin may advantageously be added. Penetration is rapid. Wash out thoroughly with water.

Potassium Bichromate.—This substance is not often mentioned in the literature on botanical microtechnique, but it is a decidedly useful substance. It is the principal ingredient of many fluids devised for the study of mitochondria. It is a superb hardening agent but has the serious fault of penetrating so slowly and poorly that some plasmolysis must be expected in large pieces of tissue. It has no effect on fats, but its effect on mitochondria will be noted in the following discussion.

The bichromate reacts in two entirely different ways, depending upon whether it is used alone or in combination with an acid. If the solution containing the bichromate is acidified to make it more acid than pH 4.6, the fixation image is that of chromic acid. The chromosomes are well fixed, cytoplasm and chromatin are precipitated as networks, and mitochondria are dissolved. When the solution is less acid or more alkaline (*i.e.*, with a pH of over 5.2), the chromosomes are dissolved, a chromatin network is not apparent, the cytoplasm is homogeneously preserved, and the mitochondria are well fixed.

It appears clear from the preceding discussion that mixtures including potassium bichromate with chromic acid, and sometimes also acetic acid, are illogical since the only effect is that of an excessive proportion of chromic acid.

Other Reagents.—Dozens, if not hundreds, of other substances have been proposed as ingredients of all sorts of mixtures. No good reason exists for the use of some of them, but others, which might after all be found to have valuable properties, were employed at a time when too little was known concerning the effects both of these substances and of the other reagents with which they were mixed, and they were consequently discarded as of no value.

KILLING AND FIXING FLUIDS

Killing and fixing fluids, in the present text at least, are classified according to whether they produce a basic or an acidic fixation image. Those which give a basic image are too few in number to require subclassification, whereas there is practically no logical method of differentiating the numerous concoctions that give acid images. All classifications which have previously been proposed are wholly arbitrary and need not be considered further.

Mixtures Giving Acid Fixation Images

In the following discussion the various mixtures are grouped according to whether the principal ingredient is formalin, glacial acetic acid, chromic acid, or various combinations of these three substances, either alone or with the addition of other reagents.

The optimum time required for fixation to be effected and the method of washing out the fluid are given for each formula.

Acetic Acid-Alcohol Mixtures

Carnoy's Fluids.—Two different fluids bear Carnoy's name. In the literature there has been hardly any mention as to which was used, which may provide an explanation for the inequalities in published reports. The second fluid is the one more commonly employed. The formula for Farmer's fluid is identical with the first one noted below.

(1) 100% ethyl alcohol	15 cc.
Glacial acetic acid	5 cc.
(2) 100% ethyl alcohol	30 cc.
Glacial acetic acid	5 cc.
Chloroform	15 cc.

Action of both fluids is very rapid, because of the great penetrating power of each. For root tips, 15 minutes is long enough; for anthers, 1 hour should suffice. The practice of certain botanists of leaving material in such violent fluids for over 48 hours is beyond all reason and merely leads to overfixation and the production of artifacts such as cytomixis. These fluids alone are not to be recommended for studies on plant cytology, but for such difficult subjects as the ova of *Ascaris* and most insects the second formula is about the best yet devised. Wash in two changes of 95% ethyl alcohol and proceed to paraffin as quickly as possible.

Carnoy-LeBrun's Fluid.—This mixture is probably the most rapid in action of all. It is not especially recommended for routine fixation but might be tried where rapid action and strong penetration are required. Wash in 95% ethyl alcohol.

100% ethyl alcohol	10 cc.
Glacial acetic acid	10 cc.
Chloroform	10 cc.
Mercuric chloride	To saturation.

Gilson's Fluid.—This is a rather unusual combination that has been recommended by some workers for the fleshy fungi, particularly for the softer, gelatinous forms such as *Tremella*. The mixture does not keep for more than a day. Allow to react for 18 to 20 hours. Wash out thoroughly with 50% ethyl alcohol, and remove the mercuric deposits from the sections.

60% ethyl alcohol	50.0 cc.
Distilled water	440.0 cc.
Glacial acetic acid	2.0 cc.
Nitric acid, 46° strength, about an 80% solution	7.5 cc.
Mercuric chloride	10.0 g.

Petrunkevitsch's Fluid.—This is a variation of Gilson's fluid, which may be employed when large masses of tissue must be penetrated in order to reach deeply embedded portions. It has given good preservation of megagametophytes in angiosperms. Care must be taken not to overfix. Most ovaries require 12 to 20 hours. Wash out as directed for Gilson's fluid, and embed as quickly as possible.

40% ethyl alcohol	125.0 cc.
Glacial acetic acid	27.5 cc.
Concentrated nitric acid	2.5 cc.
Mercuric chloride	To saturation.

Formalin-Acetic Acid-Alcohol Mixtures

Formalin-Aceto-Alcohol.—This fluid, more familiarly known as FAA, might almost be called the "standard preservative" of botanical microtechnique, since it is used more extensively than any other. The variations that have been proposed are almost endless, and some have even been given the names of the technicians who first mentioned them (Langdon, Rawlins, Lavdowsky, among others). The standard proportions are:

50% (or 70%) ethyl alcohol	90 cc.
Glacial acetic acid	5 cc.
Formalin	5 cc.

Some technicians habitually use 50% alcohol, others use 70%; the lower percentage should be employed with the more delicate materials, especially the thalloid Bryophyta. The proportions of both the acetic acid and the formalin may—indeed, they sometimes must be—varied according to the nature of the material, as determined by experience. For hard woody materials, for instance, it would be advisable to decrease the amount of acetic acid and to increase the formalin, since the latter penetrates more slowly than the former.

This reagent may be used with almost any plant material intended for anatomical or morphological study. It is unsuitable for chromosome studies. Material may be left in it almost indefinitely without appreciable damage; this property of nearly perfect preservation makes formalin-aceto-alcohol the ideal fluid to take on long collecting trips. The minimum time of fixation is 18 hours. If the tertiary butyl alcohol dehydration method is employed, it is unnecessary to wash out the killing fluid; one may go directly to the 50% stage of that method. If other methods are used, washing in two changes of 50% ethyl alcohol is all that is necessary. Woody materials should be washed for two days in running water and softened for three to six weeks in a 50% aqueous solution of hydrofluoric acid, if the use of the desilicifying acid appears necessary.

Formalin-Propiono-Alcohol.—Since propionic acid appears to give more adequate fixation than acetic acid, it may be substituted for the latter, in the same proportions, in the formula given above. The time of fixation and manner of washing are identical.

Chromic Acid and Acetic Acid Mixtures

Chromic acid is rarely used alone, but combinations of chromic and acetic acids in various proportions, to which formalin is sometimes added (see the following section), are extensively employed. Such combinations are almost endless, consequently the formulae for only a few will be cited here. By studying those given, the student can easily adjust the proportions of the two acids to fit any particular circumstance. A large volume of fluid containing these two acids should be used, and it cannot be used more than once. The time required is rarely less than 24 hours; a few days' immersion will usually do no appreciable harm. After fixation, all fluids which contain chromic acid as an ingredient should be washed out thoroughly with water, otherwise staining of the sections will be obscured and differentiation rather poor.

The chromic acid should be made up as a stock solution of 1%, 2%, or even 10% in distilled water. The tendency of the crystals of chromic anhydride, from which chromic acid is prepared, to deliquesce so readily makes it a disagreeable task to weigh out small quantities at a time. The aqueous solution is perfectly stable. Practically all other formulae are based upon one of these percentage solutions.

Stock Chrom-Acetic Fluid.—A stock solution used by some workers as a general purpose fluid consists of 1 g. chromic acid, 1 cc. glacial acetic acid and 100 cc. water and is commonly known as "1% chrom-acetic." It is, however, not a very precise fixative.

Weak Chrom-Acetic.—This is a more precise formula and may be used for filamentous algae and fungi, Bryophyta, prothallia of the Pteridophyta, moss capsules, and similar subjects that are easily penetrated.

10% aqueous chromic acid	2.5 cc.
10% aqueous acetic acid	5.0 cc.
Distilled water	To 100.0 cc.

Medium Chrom-Acetic.—This is an excellent mixture for root tips and small ovaries or isolated ovules with megagametophytes. About 2% of maltose or urea or 0.3 to 0.5% saponin should be added to facilitate penetration.

10% aqueous chromic acid	7 cc.
10% aqueous acetic acid	10 cc.
Distilled water	To 100 cc.

Strong Chrom-Acetic.—For woody materials, tough leaves, and similar objects. As indicated for the preceding mixture, the addition of maltose, urea, or saponin will be found beneficial.

10% aqueous chromic acid	1 cc.
10% aqueous acetic acid	10 cc.
Distilled water	To 100 cc.

Chromic, Acetic, and Osmic Acid Mixtures

These fluids constitute the so-called Flemming fluids, named after their originator. They represent the mixtures which will, for the various types of material for which each is best adapted, afford by far the most truthful representations obtainable by present methods. The following solutions should be made up only just before being used.

Weak Chrom-Osmo-Acetic.—For more delicate tissues.

10% aqueous chromic acid	1.5 cc.
10% aqueous acetic acid	1.0 cc.
2% osmic acid in 2% aqueous chromic acid	5.0 cc.
Distilled water	96.5 cc.

Strong Chrom-Osmo-Acetic.—For more resistant tissues.

10% aqueous chromic acid	3.1 cc.
10% aqueous acetic acid	30.0 cc.
2% osmic acid in 2% aqueous chromic acid	12.0 cc.
Distilled water	11.9 cc.

Taylor's Chrom-Osmo-Acetic for Smears.—This is a very satisfactory killing fluid for smear preparations and gives fairly faithful preservation of chromosomal structures. It is offered as a substitute for Navashin's fluid, commonly used for similar purposes. The mixture should be made up with a 10-cc. pipette graduated to hundredths. The amount of maltose to be added should be determined by experiment on each species. The purpose of the maltose is to preserve the identity of trabants (satellites) and to avoid the obliteration of constrictions.

10% aqueous chromic acid	0.20 cc.
10% aqueous acetic acid	2.00 cc.
2% osmic acid in 2% aqueous chromic acid	1.50 cc.
Distilled water	8.30 cc.
Maltose (approximately)	0.15 g.

Taylor's Modified Benda's Fluid.—Benda's was one of the earliest fluids used in investigations on chromatin and is still preferred by some workers. It is valuable in the study of prophase stages of meiosis in microsporocytes.

10% aqueous chromic acid	3.1 cc.
Glacial acetic acid	8 drops
2% osmic acid in 2% aqueous chromic acid	12.0 cc.
Distilled water	41.9 cc.

Chamberlain's Chrom-Osmo-Acetic.—This combination is suitable only for fresh-water algae, filamentous fungi, and similar organisms and should not be used for root tips, stem tips, or other morphological material.

Chromic acid	1 g.
Glacial acetic acid	3 cc.
1% aqueous osmic acid	1 cc.
Distilled water	100 cc.

Chromic Acid, Acetic Acid, and Formalin Mixtures

Mixtures of these three reagents constitute the so-called Navashin fluids, of which a considerable number of modifications have been proposed, some bearing the names of the originators. All are designed more for cytological than for morphological fixation purposes. They are excellent for smears of microsporocytes, for anthers, buds and root tips. Many cytologists fix buds first in Carnoy's fluid for from 5 to 10 minutes, then pour off the fluid and replace with a Navashin mixture. This procedure is more satisfactory on the Asteraceae than on species from other families and is better for buds covered with dense hairs or which are difficult for the aqueous solution to penetrate. Microtoming may be difficult with some materials, and the sections are liable to become loosened from the slides.

If only a small quantity is needed for immediate use, the following modification may be used. Belling's variation, which is always made up in two parts and mixed just before being used, is to be recommended under other circumstances. The great majority of other proposed combinations differ merely in the proportion of formalin to be added. This does not constitute sufficient reason for giving them new names.

Taylor's Modified Karpechenko Fluid.

10% aqueous chromic acid	1.5 cc.
10% aqueous acetic acid	10.0 cc.
Formalin	0.83 cc.
Distilled water	23.67 cc.

Belling's Modified Navashin Fluid.—Make up the two solutions separately, mixing equal volumes of each just before using. For metaphase smear preparations, Solution B may be composed of 100 cc. formalin and 275 cc. distilled water. Three hours appears to be long enough for most smears, but immersion for as long as 12 hours does no injury. After fixation, transfer smear preparations to a stender of

0.5% aqueous chromic acid for not longer than 10 minutes, to remove the formalin, then proceed with the staining.

Buds, root tips, and similar materials may be left in the fluid almost indefinitely. Shortly after being mixed, the solution will turn a brownish-green to greenish color, which indicates the reduction of the chromic acid. Wash thoroughly in several changes of water when ready to commence the dehydration.

Solution A:	Chromic acid crystals	5 g.
	Glacial acetic acid	50 cc.
	Distilled water	320 cc.
Solution B:	Formalin	200 cc.
	Distilled water	175 cc.
	Saponin	3 g.

Randolph's Modified Navashin Fluid.—The following fluid, to which the abbreviation Craf has been applied, is claimed to be superior to other Navashin-type mixtures (Randolph 1935). Fix for 12 to 24 hours. Transfer the material, without washing, directly to 70% ethyl alcohol, changing the alcohol three or four times at 15-minute intervals.

Solution A:	Chromic acid	1 g.
	Glacial acetic acid	7 cc.
	Distilled water	92 cc.
Solution B:	Neutral formalin	30 cc.
	Distilled water	70 cc.

Mix equal portions of A and B just before using.

Potassium Bichromate Mixtures

As has been noted in the general discussion above, the mixing of potassium bichromate with chromic acid is illogical and gives unsatisfactory results; therefore fluids of this type are scarcely to be recommended. LaCour's fluids, which have come into favor in some English institutions but have not met with the same reception in the United States, are in this category. Tissues become so heavily tanned that they are hard to bleach sufficiently and staining has always been poor. In fact the use of these fluids has been claimed by some observers to be the direct cause of the erroneous interpretations of certain cytological phenomena by one school of thought in that field.

The first fluid mentioned below has considerable to recommend it for certain special purposes, but the others are included merely as a matter of record.

Tellyesnicky's Fluid.—In this mixture potassium bichromate is substituted for chromic acid. It has been used on the Bryophyta and also on leaves with excellent results. It might be tried if a similar

fluid containing chromic acid fails to work. Fix 24 to 48 hours, preferably changing once, and wash out in running water for 12 hours.

Potassium bichromate	3 g.
Glacial acetic acid	5 cc.
Distilled water	100 cc.

LaCour's 2BE Fluid.

1% aqueous chromic acid	90 cc.
Potassium bichromate	1 g.
Saponin	0.05 g.
5% aqueous acetic acid	10 cc.
2% aqueous osmic acid	15 cc.
Distilled water	45 cc.

LaCour's 2BD Fluid.

1% aqueous chromic acid	100 cc.
1% aqueous potassium bichromate	100 cc.
Saponin	0.1 g.
2% aqueous osmic acid	30 cc.
5% aqueous acetic acid	30 cc.

Picric Acid Mixtures

The first such mixture to be proposed was the familiar Bouin's fluid, so widely used by zoological technicians. The original mixture is hardly suitable for plant material since the tissues are apt to become brittle and difficulty is often experienced in microtoming the material. The original formula is as follows:

Saturated aqueous picric acid	75 cc.
Formalin	25 cc.
Glacial acetic acid	5 cc.

Anthraquinone may be substituted for the picric acid and should give more satisfactory results. Fix tissues for 24 hours, rinse quickly with water, then wash thoroughly with 50% alcohol.

Allen's Modified Bouin's Fluid (Allen's B-15).—This fluid has proved to be very satisfactory with the buds of many species which give poor results with alcoholic and chrom-acetic fixatives. Immediately before using, heat 100 cc. Bouin's fluid to 37°C. and add 1.5 g. chromic acid. Stir thoroughly, then add 2 g. urea. Keep the fluid at between 37 and 39°C. while the material is being placed in it; when finished, allow it to cool gradually. The fluid will become greenish within ½ hour and rapidly loses its efficiency. Four hours' time should give thorough fixation, but the material may be allowed to remain in the fluid overnight. Wash with frequent changes of 70% ethyl alcohol over two days or until no more yellow color is extracted.

The morphology of metaphase and anaphase chromosomes is well brought out by this mixture, but it is not suitable for other stages in either mitosis or meiosis. Cleland adds 1 g. of chromic acid to freshly prepared Bouin's fluid and substitutes 1 g. of maltose or lactose for the urea. With these modifications he has secured satisfactory results with the very difficult Onagraceae. Staining is usually brilliant with iron hematoxylin.

Sass' Modified Bouin's Fluid.—Beautiful results are obtained with buds and anthers of the Liliaceae by means of this mixture. Dehydrate, without washing out the mixture, in grades of acetone (5, 10, 15%, etc.), and clear in five grades of acetone-xylol or acetone-tertiary butyl alcohol.

1% aqueous chromic acid	50 cc.
Saturated aqueous picric acid	35 cc.
Formalin	10 cc.
Glacial acetic acid	5 cc.

Mercuric Chloride Mixtures

Schaudinn's Fluid.—This mixture is widely employed on the Protozoa and related organisms. It may be used to fix on the slide plant spermatozoids and zoospores and certain of the flagellated unicellular algae. For this purpose it is used at a temperature of 70°C. It was originally made by adding 10 cc. of absolute ethyl alcohol to 20 cc. of a saturated aqueous solution of mercuric chloride. A later version is to add 10 cc. of absolute ethyl alcohol to 40 cc. (or twice the original quantity) of the chloride. Most protozoologists add from 1 to 5 parts of glacial acetic acid just before using, but it seems better to dilute the fluid one-half with water and to add 2% glacial acetic acid immediately before using. Fix for several hours, and wash out with a medium strength alcohol, to which is added a little iodine solution to remove any mercury deposits.

Worcester's Fluid.—Worcester's fluid has been found to give excellent results with plant tissues whose cells are congested with various substances. The enzyme-secreting cells of seedlings are well fixed. Allow to react about 20 hours, and wash thoroughly with 70% alcohol to which is added about 1% potassium iodide.

Saturated aqueous mercuric chloride	96 cc.
Formalin	4 cc.
10% aqueous glacial acetic acid	10 cc.

Mixtures Giving Basic Fixation Images

Zirkle-Erliki Fluid.—For mitochondria. The image is completely basic, and all chromatin is dissolved (Zirkle 1934). Fix for 48 hours; wash with water.

Potassium bichromate	1.25 g.
Ammonium bichromate	1.25 g.
Cupric sulphate	1.00 g.
Distilled water	200.0 cc.

Zirkle's Reduced Chromic Fluid.—For mitochondria and vacuoles (Zirkle 1932). Formalin when added to solutions containing unreduced chromium compounds immediately reduces the latter, consequently such mixtures are unsatisfactory. This disadvantage is obviated by using a reduced chromium salt, such as chromic sulphate.

Chromium sulphate	5 g.
Cupric oxide	Slight excess
Formalin	10–50 cc.
Distilled water	90–50 cc.

(Total amount of fluids should be 100 cc.)

Fix 48 hours; wash with water. The purpose of the copper is to bring the pH to 4.6. The concentration of the formalin depends upon the material being fixed and must be determined by experiment. If used in too great a concentration, there will be some plasmolysis and swelling of the vacuoles; if in too low a concentration, the mixture may be so diluted by the liquid contents of the tissue that an erratic fixation image results. Even when this fixative seems to be at its best, perfect preservation of the vacuoles and their tonoplasts does not always result.

CHAPTER VI

STAINS

The subject of dyes or stains and methods of using them has become one of vast proportions. The number of dyes available is enormous, and ways of employing stains are almost as numerous as the workers using a particular dye or combination of stains. Standardization in the manufacture and certification of dyes of American manufacture has been attained, but the majority of technicians are still individualistic in the utilization of these stains. These facts, however, need deter no one because the most convincing and satisfactory results have been, and still are being, obtained with a few well-known dyes. For example, plant morphologists use safranin and fast green more extensively than any other combination of stains; cytologists consider that crystal violet has no rival as a stain for dividing chromatin; the bacteriologist would be almost helpless without methylene blue; and Delafield's or Harris' hematoxylin with a counterstain of eosin is in universal use by zoological and clinical technicians. The preparation and use of stains has all but become a science in itself, and there exists a most valuable journal, *Stain Technology*, devoted to these and related subjects. Most of the abstracting journals, American and foreign, devote special sections to stains and staining methods. One should make it a habit to read these abstracts in order to keep abreast of current trends. Experimenting with new dyes and new staining schedules is a most fascinating occupation, and there is a very great deal yet to be learned about stains and their utilization.

It is not proposed to burden the present text with accounts of the history of stains, their preparation, chemical constitution, and similar purely technical details. For such information, the student cannot do better than consult the very valuable manual by Dr. H. J. Conn, "Biological Stains" (Biological Stain Commission, Geneva, N. Y., 3d ed., 1936). The greater part of the information contained in the remainder of this chapter has been derived from the publications of the Stain Commission and other reliable sources.

The older botanical technicians placed much of their reliance upon natural or textile dyes. Very few of the natural, and practically none of the technical, dyes are still in use. The latter were always of doubtful purity since manufacturing methods could not always be so standardized

as to give an identical product each time a batch was prepared, and they were too often insoluble in the technician's customary solvents. Nearly all the dyes now used in biological technique are synthetic chemical compounds made from the substances found in coal tar. The two types of dyes, natural and coal tar, are being discussed separately.

THE NATURAL DYES

Only three natural dyes are still used by botanical technicians. These dyes have not yet been manufactured synthetically. All are important cytological stains. One, cochineal and its derivatives, is of insect origin; the others are from plants belonging to the Caesalpiniaceae.

Brazilin.—This dye is obtained from different trees known collectively as "brazilwood," but principally from *Caesalpinia crista* or *C. echinata*. It is closely related chemically to hematoxylin but is neither so active nor so strong a stain. It has recently come into extensive use as a stain for smears, its employment for the purpose having originated with Belling, but refinements in the method have since been made (Capinpin 1930).

Brazilin is ordinarily employed as a 0.5% solution in 70% alcohol, which is allowed to ripen for about a week. Keep containers well stoppered, away from light and air. Brazilin by itself is not a dye; it reacts only after mordanting with ferric ammonium sulphate.

Hematoxylin.—Hematoxylin is a chromogen derived from logwood, *Hematoxylin campechianum* L., and is one of the most important of all stains. It is a homologue of brazilin, possessing one more hydroxyl group in its chemical constitution. The dye solution itself has little or no affinity for tissues, unless iron or aluminum is present in the latter, consequently mordanting in some form is necessary. The stain is made up in combination or in conjunction with various metallic salts, principally those of iron (always in the ferric form), aluminum, and copper. Some of the schedules are progressive; others are regressive. The color effects of hematoxylin vary with the character of the medium in which it is dissolved and according to the after treatment. In the presence of acids the color is red; in the presence of alkaline solutions it is blue. By Heidenhain's iron-alum schedule, structures such as chromosomes and pyrenoids are stained black, while the cytoplasm of sporogenous cells, for example, is stained gray. However, by exposing the sections to the action of ammonia fumes, the color is turned to blue.

The solution of the dye as used in Heidenhain's iron hematoxylin technique is simply a 0.5% solution in distilled water. It will be found to be more satisfactory to prepare a 10% solution in absolute ethyl alcohol and to dilute a portion with distilled water to the 0.5% strength when required. Formerly it was necessary to permit the solutions to stand for some time to ripen (into hematein), but the certified dyes will

often be sufficiently ripe after a few days. The process may be hastened by placing the solution in a very wide and shallow evaporating dish and exposing, at a distance of approximately 2 feet, to any rather powerful quartz mercury-vapor arc for about 45 minutes. Stir the solution frequently during exposure.

At high temperatures hydrolysis of hematoxylin solutions occurs; a metallic scum or film forms on the surface. Such solutions must be discarded. Spoilage is also indicated when the solution starts to turn brown. A relatively stable hematoxylin solution may be prepared by adding 5 cc. of a 10% absolute alcohol solution to 100 cc. methyl cellosolve, 50 cc. distilled water, and 50 cc. tap water that contains calcium compounds in solution. If the solution on shaking does not acquire a rich wine-red color, add a pinch of sodium bicarbonate and shake vigorously. Ripening usually occurs immediately, and the solution can be used at once. This solution does not spoil at high temperatures; it also retains its staining capacity for far longer periods than do simple aqueous solutions.

Most of the difficulties with hematoxylin arise during the destaining and differentiation. The same solution should not be used for both mordanting and destaining. Solutions of reagents other than ferric ammonium sulphate frequently give better results: ugly precipitates are avoided, thicker sections may be clearly stained, and disagreeable tan or brownish colors do not result (Hutner 1934).

Tuan's use of picric acid (Tuan 1930) has been extensively followed by numerous technicians. If the picric acid solutions are warmed to about 50°C., the destaining will be greatly accelerated. It is not necessary that the color effects at the end of the destaining process be in the nature of sharp black and white (or colorless) contrasts. This sometimes indicates that one has merely painted various structures black and has not really stained them in order to reveal details clearly. Sections differentiated in picric acid may be blued by washing out the acid thoroughly and by adding 1 or 2 drops of ammonium hydroxide to the 70% dehydrating alcohol.

Ferric ammonium sulphate is both an acid and an oxidizer. A fairly close approximation to the effect obtained with this reagent can be gotten with the following mixture:

0.25% solution of hydrochloric acid in 95% alcohol	2 parts
Merck's Superoxol	1 part

The solution should be freshly prepared. Destaining completed, wash the slides with 70% alcohol, and blue with ammonium hydroxide. Take care that the higher dehydrating alcohols do not become acidified by the hydrochloric acid.

Ferric chloride may be substituted for ferric ammonium sulphate, both as mordant and as differentiator. Use a 2 to 5% solution for the mordanting, for about 30 minutes. Rinse, then stain. Differentiate in a 1 to 3% solution. The stain is more blue than black, and the differentiation is more precise (Häggquist 1933).

Delafield's hematoxylin is still being made according to the original formula, in which dye and mordant are combined. To 400 cc. of a saturated aqueous solution of ammonium aluminum sulphate, add drop by drop a solution of 4 g. hematoxylin crystals in 25 cc. of 95% ethyl alcohol. Expose to light and air for four days. Then add 10 cc. c.p. glycerin and 100 cc. methyl alcohol. Allow to stand for at least two months, exposed to the air, until the color is sufficiently dark. The process may, however, be accelerated by exposure to the quartz mercury lamp, as described above for plain hematoxylin solutions, the time of exposure being about 2 hours. It is rather dangerous and unsatisfactory to follow the recommendation of some microscopists to ripen the solution with hydrogen peroxide. The ripened undiluted solution is a powerful stain; a much more precise differentiation can be obtained by diluting the staining solution with as much as twice its bulk of distilled water. The stain is differentiated in either water or 70% ethyl alcohol acidified with hydrochloric acid; the procedures are outlined in the following chapter.

Harris' hematoxylin resembles Delafield's in its color effects but is made up in an entirely different manner. It is a superb *in toto* stain. The formula is as follows:

Hematoxylin crystals	5 g.
Aluminum ammonium sulphate	3 g.
50% ethyl alcohol	1000 cc.

Dissolve the dye and the alum with the aid of heat, then add 6 g. mercuric oxide (use only the *red* powder) and boil for 30 minutes. Filter, then bring up again to the original volume with 50% alcohol. Acidify in the proportion of 1 drop hydrochloric acid to each 100 cc. of solution.

When one is in doubt as to the proper stain to use, he may safely use either Harris' or Delafield's hematoxylin. Even after they become thoroughly experienced in the use of other stains and combinations of stains, skilled technicians often encounter difficulties. Many, if not most, of them resort to either of these two hematoxylins and generally obtain the desired effects. Delafield's will sometimes stain chromatin well when the regular chromatin stains fail; it also brings out cellulose walls more sharply than most other stains. If proper care in differentiation is taken, sporogenous cells are unusually well defined by both hematoxylins.

Mayer's haemalum has been recommended for the nuclei of filamentous algae and fungi, having little or no effect upon cell walls or plastids

The improved formula is as follows: dissolve 1 g. hematoxylin crystals in 1 liter water. Then add 2 g. sodium iodate and 50 g. aluminum potassium sulphate. The solution acts much like Delafield's hematoxylin but does not keep well; add a crystal of thymol to prevent mold growth. Excellent results are secured by diluting the stain about ten times with distilled water and allowing it to act overnight. Follow any balsam infiltration method with algae or fungi; transfer to the stain from water, and wash out thoroughly with water before proceeding to the next step.

Ehrlich's hematoxylin has been extensively used with safranin on woody tissues:

Distilled water	50 cc.
Absolute ethyl alcohol	50 cc.
Glycerin, c.p.	50 cc.
Glacial acetic acid	5 cc.
Hematoxylin crystals	1 g.
Aluminum potassium sulphate	To excess

Keep in a dark place until the color becomes a deep red, or it may be ripened in 3 or 4 hours by exposure to a quartz mercury lamp as directed in the discussion of Heidenhain's hematoxylin above. Transfer sections to the stain from 35% alcohol or water, allow to remain for 5 to 30 minutes, wash off excess stain with water or 35% alcohol, and proceed with the dehydration. Orange G or erythrosin may be used as a counterstain. If safranin is used with Ehrlich's hematoxylin, stain first in the safranin.

Hematein.—Many workers consider hematein superior to hematoxylin (Kornhauser 1930) on the ground that "it is easy to prepare, easy to use, saves time, and gives good results." A MacAndrews and Forbes product should be used.

To prepare the stain, grind 0.5 g. hematein in a glass mortar with 10 cc. of 95% ethyl alcohol, and add to 500 cc. of 5% aqueous aluminum potassium sulphate. Transfer the slides to the stain from water. The stain is progressive; consequently the slides should be inspected frequently for 5 minutes or so, which is the average time required. Next rinse 1 to 3 seconds in tap water. One may counterstain 1 to 3 seconds with eosin bluish (1 part stock solution—0.5% solution in 20% alcohol—to 2 parts distilled water). Wash in several changes of tap water, dehydrate, and mount.

Cochineal and Derivatives.—Cochineal is a yellowish-red powder, obtained by grinding the dried bodies of the female cochineal insect and extracting the coloring matter. Cochineal and its derivatives are some of the most important bulk stains yet available. They are all progressive stains but may be used retrogressively. Cochineal itself is not used by botanists to any great extent.

Carmin is a bright red in color and is the lake obtained by adding alum to cochineal. It is a complicated mixture of which the essential coloring agent is carminic acid. Carmin and carminic acid are so easy to use and give such astonishingly beautiful effects that it is amazing to what a slight extent they are used by botanical technicians. Cytologists, however, have a considerable appreciation of carmin, at least, as indicated by its extensive use in the iron-acetocarmin smear technique.

Carmin is only slightly soluble in water at a neutral reaction; hence solutions must be either acid, as is the customary case, or alkaline.

Carminic acid is the active dye principle of cochineal and carmin. It is a fairly strong dibasic acid. It is best known to botanists as the principal ingredient of Mayer's carmalum.

Indigocarmin.—Indigocarmin is only indirectly a natural dye, but it is included among the natural dyes because it is based upon one. It is the sodium salt of indigodisulphonic acid, and consequently has acid properties. In color it is bluish; but when mixed with picric acid to make the picro-indigocarmin stain used in staining algae, the color becomes green. The solubility of indigocarmin at 25°C. is 1.68% in water and 0.01% in 95% alcohol.

THE COAL-TAR DYES

The older classifications of coal-tar dyes are largely artificial. The classification at present used is based upon similarity in chemical structure. Six groups have been recognized (Conn 1936):

1. The nitro dyes.
   Example: Picric acid.
2. The azo dyes.
   Examples: Orange G, Bismarck brown, Sudan IV.
3. The anthraquinone dyes.
   Example: Alizarin.
4. The quinone-imide group of dyes:
   a. Indamins: none in common use.
   b. Thiazins.
      Examples: Methylene blue, thionin.
   c. Oxazins: none in common use.
   d. Azins; subdivided into
      (1) Amido-azins. *Example:* Neutral red.
      (2) Safranins. *Examples:* Safranin 0, magdala red.
      (3) Indulins. *Example:* Nigrosin.
5. The phenyl-methane group of dyes:
   a. Diphenyl-methanes: none commonly used.
   b. Diamino-triphenyl methanes.
      Examples: Fast green, light green, malachite green.
   c. Triamino-triphenyl methanes.
      Examples: Acid fuchsin, crystal violet, anilin blue.
   d. Hydroxy-triphenyl methanes: none generally used.

6. The xanthene group of dyes:
   a. Pyronins: none used in botanical technique.
   b. Rhodamines: none used.
   c. Fluorane derivatives.
      Examples: Eosin, erythrosin.

The nomenclature of dyes is in great confusion because practically no system was used in naming them. Unscrupulous dealers have given new names to old dyes, actuated solely in adding to their profits by the introduction of a supposedly new product. The synonymy of some dyes has reached amazing proportions. The confusion in nomenclature of histological dyes has frequently misled users. One would do well to follow the recommendations of the Commission on Biological Stains as to acceptable names. This is the procedure followed in the present text.

On labels and in certain published papers one will frequently meet with numbers preceded by the initials C.I., *e.g.*, C.I. No. 841; or by the name Schultz, *e.g.*, Schultz No. 679. The first refers to a publication by the Society of Dyers and Colourists of England, known as the Colour Index. It is a very complete manual listing textile dyes, their synonymy, chemical formulae, methods of preparation, and distinctive characteristics. Schultz refers to the German equivalent, much less comprehensive, of the Colour Index, *viz.*, Schultz's Farbstofftabelln. The numbers are those given in the respective index; the two cited above refer to Safranin O as given by each. Numbers are not being cited in the present text as they are of little practical value to the average technician.

In the following list the various stains are arranged alphabetically, rather than according to the chemical classification given above, in order to give greater convenience in reference. First comes the accepted name of the dye, together with occasional citation of synonyms whenever such are in common use. Next comes an indication as to whether the dye is acidic or basic, then its chemical classification. Following this is given the solubility of the dye at 26°C. in water and in 95% ethyl alcohol, as expressed in percentages. This indicates roughly how much of any stain is needed to obtain a saturated solution in each solvent, but allowances must always be made for the presence of impurities in the dye sample, particularly if it is uncertified or of foreign manufacture. Solubility statistics are not given for dyes which are mixtures of two or more dyes. Finally, information is given concerning the customary solvents for each dye, together with occasional brief mention of treatment subsequent to staining. Detailed stain schedules are reserved for the following chapter.

Acid Fuchsin.—Acid; triamino triphenyl methane (rosanilins). Acid fuchsins are a complex mixture of sulfonated derivatives of basic fuchsin, and any given sample is probably a mixture of several different acid fuchsins. In plant technique it is used to stain the cortex, pith parenchyma, and cellulose walls and is excellent for differentiating zoosporangia and paraphyses in the Laminariales. It has some use as a mitochondrial stain.

A 1% solution in 70% alcohol is preferable, but a 0.5 to 1% solution in distilled water sometimes works equally well. The stain reacts very rapidly and is easily extracted by the higher alcohols. In staining morphological material, the stain should be allowed to react for 2 hours. If the solution in 70% alcohol is used, one may differentiate in a saturated solution of picric acid in 70% alcohol for about 1 minute and then rinse in 70% alcohol until a bright reddish-magenta replaces the yellow of the acid. Complete dehydration quickly, and mount at once.

Acid fuchsin must not be confused with basic fuchsin; when fuchsin alone is mentioned by careless writers, without specifying the acid or basic form, it is usually the basic form which is meant.

Acid Green: See Light Green, SF.

Alizarin Red S.—Acid; oxyquinone group. Solubility: 7.69% in water; 0.15% in alcohol. Used for differentiating chromatin when crystal violet has been used for staining mitochondria. It has recently been employed for staining chromosomes (Backman 1935).

Anilin Blue WS (Syn.: cotton blue, water blue, China blue).—Strongly acid; triamino-triphenyl methane group. During manufacture, the exact composition of this dye cannot be controlled, hence it is rather unsatisfactory for precise staining procedures. The dye is a mixture, and no two lots will react alike. Nevertheless, it is very valuable as a counterstain, especially to safranin when used on plant tissues. It will stain cellulose walls and the achromatic figure, and (in conjunction with erythrosin or phloxine) it is said to be excellent for the filamentous Chlorophyta.

The dye is supposed to be soluble in water only, but an aqueous solution is practically useless. A 1% solution in 90% ethyl alcohol serves well; after a brief differentiation in 95% alcohol, the anilin blue should be fixed and intensified in 95% alcohol slightly acidified with hydrochloric acid. The writer finds it most satisfactory to dissolve as much stain as possible in methyl cellosolve, and then to add clove oil and a little absolute alcohol as diluents. Use such a solution from a dropping bottle. Clear in clove oil or synthetic oil of wintergreen (methyl salicylate), wash in xylol, and mount in balsam.

Aurantia.—Acid; nitro group. Solubility: nil in water; 0.33% in alcohol. Used for the demonstration of mitochondria.

Basic Fuchsin: See Pararosanilin.

Bismarck Brown Y.—Basic; azo group. Solubility: 1.36% in water; 1.08% in alcohol. This stain was formerly much favored but has fallen into disuse. A 1% solution in 70% alcohol gives the optimum results. It works poorly on material fixed in reagents containing chromic acid. It rarely overstains and is quite permanent. Mucin and cellulose walls are brilliantly colored. Solutions of the dye should never be heated, otherwise the composition of the dye will be changed.

Bordeaux Red (Syn.: fast red B or P).—Acid; azo group. Solubility: 3.83% in water; 0.19% in alcohol. A cytoplasmic stain after iron hematoxylin. It renders centrosomes more brilliant. Use a 1% aqueous solution, and stain for 12 to 24 hours.

Congo Red.—Acid; azo group. Solubility: nil in water; 0.19% in alcohol. In plant technique it is used as a cytoplasmic stain in contrast to iron hematoxylin and other nuclear dyes. It also stains mucin and cellulose. As a cytoplasmic stain, a solution in weak alcohol may be used. Congo red should be used last, when employed in combination with any other stain, and dehydration should be as rapid as possible. In the presence of free acid, solutions of this dye become blue.

Crystal Violet (Syn.: gentian violet).—Basic; triamino-triphenyl methane group. Solubility: 1.68% in water; 13.87% in alcohol. Crystal violet is the most completely methylated of the various violets. It is one of the most useful stains and is becoming increasingly important, especially in cytology. In many cases it affords results not obtainable from safranin, iron hematoxylin, or other nuclear stains. In nearly all procedures it is now used in place of the mixture of dyes known as "gentian violet," but even if the latter dye is specified, crystal (or methyl) violet may nevertheless be safely substituted. In the Flemming triple combinations "gentian violet" is considered by some workers to react better than either crystal or methyl violet in staining the achromatic figure, but such workers have apparently never bothered to ascertain whether other violets would not work equally well. Various lots of crystal violet will be found to behave somewhat differently under a given set of circumstances.

All the violets may be used in 1% distilled water solutions. These solutions keep for some time, but in most of the critical staining procedures it is ordinarily the better part of discretion to use a freshly prepared solution. All the violets wash out so quickly in the dehydrating alcohols that one should either mordant the stain, use one of the special methods, or dissolve the stain in clove oil. The clove oil solution may be kept in a dropping bottle. Still another method is to make a saturated solution in clove oil and to add a few drops of this solution to a staining dish full of xylol. This mixture is very unstable, and when in use needs to be

brought up to strength by the addition of more stain from time to time.

The violets frequently overstain the cytoplasm of buds and root tips, thus making chromosome counts difficult. To avoid this, the sections may be transferred from water to 2% aqueous acetic acid for about 30 minutes, then washed for twice the length of time in running water.

Eosin, Bluish.—Acid; fluorane derivative. Solubility: 39.11% in water; 0.75% in alcohol. Sometimes used as a counterstain but of little value on plant materials. Erythrosin is far more satisfactory.

Eosin, Yellowish.—Acid; fluorane derivative. Solubility: 44.20% in water; 2.18% in alcohol. When eosin is mentioned, this is the type meant. Little used by botanists, but a most valuable cytoplasmic stain for animal tissues, especially after a hematoxylin stain. The eosin should be used last, and preferably in a strong alcoholic solution.

For paraffin sections a 1% solution in 95% alcohol may be used; stain for but a few seconds. For material to be mounted whole, either in glycerin jelly or by any balsam infiltration method, use a 1% aqueous solution, and stain several hours or overnight. It has been recommended that material be transferred directly from the stain to a 2% aqueous solution of acetic acid for 5 to 10 minutes, changing the acid several times, then transferring to 10% glycerin, leaving about 1 cc. of the acid to keep the whole solution slightly acid. When the glycerin is sufficiently concentrated, mount in glycerin jelly. By the balsam method wash out the glycerin with 95% alcohol slightly acidified with acetic acid, and do not drain off the last alcohol too completely before transferring to the diluted balsam. Eosin seems to keep better when the mounting media are slightly acid.

Erythrosin, Bluish.—Acid; xanthene group (fluorane derivative). Solubility varies according to the salt: 0.15 to 11.10% in water; 0.04 to 1.87% in alcohol. Used generally by botanists instead of eosin. An excellent counterstain, and fine for staining the gelatinous sheaths of algae (*e.g.*, *Nostoc*). Erythrosin is much like eosin in all respects but is an iodine rather than a bromine derivative. Use a 1% solution in 95% alcohol or in clove oil; stain for not longer than 3 minutes, although the clove oil solution stains sufficiently in about 10 seconds. Erythrosin may be substituted for magdala red or phloxine in all schedules calling for these two dyes.

Erythrosin is a tetraiodo compound corresponding to the tetrabrom compound of typical eosin (Conn 1936). It is the presence of the iodine in erythrosin which apparently makes it such an excellent counterstain to crystal violet in certain cytological staining methods (Johansen 1932).

Fast Green FCF.—Acid; diamino-triphenyl methane group. Solubility: 16.04% in water, 0.33% in alcohol. This dye is far superior to light green SF and fades practically not at all. It stains more intensely in a shorter time. Solutions should always be made up several days in advance of using and should be weak: not over 1% aqueous or 0.1% alcoholic. The clove oil-absolute alcohol solution is recommended as the one giving the optimum results: add enough dry dye to a mixture of equal parts of methyl cellosolve, absolute alcohol, and clove oil to give a dark greenish solution (about 0.5%), and try it on a sample slide. If too strong, dilute with clove oil until satisfactory. Fast green solutions can be used for differentiating safranin, but the results in the writer's experience have always been rather messy and a really sharp differentiation cannot be obtained in this fashion. Fast green turns blue in alkaline solutions. On the stems and leaves of aquatic plants and with most gymnosperm material it is generally blue to bluish-green, rarely a bright green.

Gentian Violet.—See crystal violet and methyl violet.

Iodine Green.—Basic; triamino-triphenyl methane group. Closely related to methyl green. A selective chromatin stain; also excellent for lignified cell walls. Ordinarily used in contrast to the fuchsins. Stain for 1 hour, or, if the stain washes out too rapidly, for 24 hours, in a 1% solution in 70% alcohol.

Janus Green B.—Basic; related to both the safranin and azo groups. Solubility: 5.18% in water; 1.12% in alcohol. Used in various high dilutions as a vital stain for fungi and for the flagella of the protozoa. The vital stain is prepared by dissolving from 0.001 to 0.41 g. in 100 cc. of physiological saline solution.

Light Green SF.—Acid; diamino-triphenyl methane group. Solubility: 20.35% in water; 0.82% in alcohol. An excellent cytoplasmic stain, and very extensively employed for staining cellulose walls. After certain killing fluids it will show up the achromatic figure beautifully. The stain, unfortunately, fades within a short time. Fast green FCF is an excellent substitute and far more permanent. However, on some filamentous algae, light green should be used rather than fast green as the latter tends to overstain badly even when used in very high dilutions.

The solution, in whatever medium, should not be stronger than 0.5%; a 0.2% solution is strong enough. Staining is rapid. The solution in 95% alcohol is commonly used, but many prefer to dissolve the dye in absolute alcohol and dilute with clove oil, keeping the solution in a dropping bottle. The stain will reduce other coal-tar dyes, especially safranin, consequently it should not be allowed to react too long.

If a saturated solution of light green in either water or alcohol is acidulated with hydrochloric or acetic acid, a differential stain for

lignified tissues is obtained. Simple washing with water will remove the stain from all save lignified cell walls. By combining light green with alcoholic Sudan IV, suberized and cutinized tissues are differentiated from lignified tissues.

Care should be taken not to confuse light green with malachite green, acid green, fast green, or methyl green.

Magdala Red.—Basic; safranin group. There is much doubt as to just what was meant by "magdala red" in many schedules calling for this dye. In plant technique, schedules calling for this dye give very different results with various lots of the dye. It would be better to substitute phloxine, which gives substantially identical results.

Malachite Green (Syn.: emerald green, light green E).—Weakly basic; diamino-triphenyl methane group. Now largely replaced by methylene (not methyl) green.

Malachite green may be used as a 0.5% or somewhat stronger solution in either 95% alcohol, distilled water, or clove oil. It may be applied for 1 minute when used alone or for 20 seconds following 20 minutes in safranin. The safranin should be 1% aqueous and is not differentiated before applying the green. When used alone, the green should reveal clearly all such histological elements as the cell walls, endodermis, bast, cytoplasm, nuclei, and chloroplasts. When used following safranin on plant pathological material, the host nuclei, xylem, and cutinized walls, as well as the nuclei of the infecting fungus, should appear red; the cytoplasm and cellulose walls of the host should appear green.

Martius Yellow.—Acid; nitro group. Solubility: sodium salt, 4.57% in water; 0.16% in alcohol—calcium salt, 0.05% in water; 1.90% in alcohol. Used with malachite green and acid fuchsin for staining infected plant tissues, and used alone for pollen tubes in styles.

Methyl Green (Syn.: light green).—Basic; triamino-triphenyl methane group. This is generally an impure dye, as it usually contains some methyl violet. The color thus is sometimes purple and not green, as the name might indicate. A chromatin and nuclear stain, also for lignified plant tissues. Very useful for staining chromatin in protozoa, and for the gonococcus. The dye does not usually give a good stain after fixation in a fluid that does not contain acetic acid. It does not ordinarily keep well, according to many technicians. A 1% aqueous solution is recommended.

Methyl Violet (Syn.: gentian violet).—Basic; triamino-triphenyl methane group. Solubility: 2.93% in water; 15.21% in alcohol. A mixture of dyes (methyl pararosanilins). There are many shades, designated by letters: R indicating the reddish shades and B the bluish. The bluish colors are preferable as the reddish ones usually leave a disagreeable transparency in chromosomes and other tissues. Use as indicated for crystal violet.

Many cytologists prefer methyl violet 2B to all other types of violet dye.

Methylene Blue.—Basic; thiazin group. Solubility: 3.55% in water; 1.48% in alcohol. The most valuable bacteriological stain. Used to a slight extent by zoological technicians, but rarely by botanists. It is a good stain for yeasts, but tends to overstain.

Methylene Green.—Basic; thiazin group. Solubility: 1.46% in water; 0.12% in alcohol. Derived from methylene blue. Sometimes substituted for methyl green for lignified tissues and chromatin, but otherwise is little used.

Neutral Red.—Weakly basic; azin series. Solubility: 5.64% in water; 2.45% in alcohol. Used in vital staining, especially with the protozoa, and to indicate the reactions of the contents of living plant cells.

Nigrosin (Syn.: indulin black).—Basic; composition poorly understood: a variable mixture. A 2% aqueous solution may be made up as a stock solution and a few drops added to the water containing Chlorophyta or Cyanophyta to be mounted in glycerin. Stop action when the stain seems to be right; it should look somewhat like iron hematoxylin. The stain cannot be satisfactorily used on plant material embedded in paraffin.

Orange G.—Acid; azo series. Solubility: 10.86% in water; 0.22% in alcohol. One of the most useful cytoplasmic counterstains and specified in innumerable schedules. A general rather than a selective stain. The medium in which the dye is dissolved varies according to the specific technique. The dye usually does not completely dissolve, but 1 g. of the dye to 100 cc. of solvent may be used. Action is extremely rapid. The methyl cellosolve solution is most satisfactory, and this may be diluted with other solvents.

Pararosanilin (Syn.: basic fuchsin).—Basic; triamino-triphenyl methane group. Solubility: 0.26% in water; 5.93% in alcohol. Pararosanilin is the chief constituent of the majority of samples of basic fuchsin submitted for certification as biological stains (Conn 1936); it is therefore more desirable to use the name of the specific dye rather than a generic description. Basic fuchsin has never enjoyed much of a reputation in general botanical technique, but since it is the stain involved in the Feulgen nucleal reaction, an interesting future may be predicted. It is a powerful nuclear dye and also stains mucin and bacteria. For ordinary purposes a saturated aqueous solution will be found to be satisfactory.

Phloxine B.—Acid; xanthene group. Solubility varies according to the salt: 3.75 to 50.90% in water; 0.45 to 29.10% in alcohol. Phloxine has been claimed to be a good algal stain, but the results in the writer's hands have been disappointing.

Picric Acid.—Strongly acid; nitro group. Solubility: 1.18% in water; 8.96% in alcohol. Sometimes employed as a general cytoplasmic stain. In alcoholic solutions, picric acid is coming into extensive use as a differentiator for the hematoxylins, safranin, and the violets. It is almost invariably used as a nearly saturated solution.

Resorcin Blue.—Basic; oxazin series. Used as a microchemical reagent for callose, and, combined with martius yellow under the name "lacmoid," for pollen tubes.

Safranin O.—Basic; azin (safranin) group. Solubility: 5.45% in water; 3.41% in alcohol. This is perhaps the most important stain known to botanists and is used in both morphology and cytology. It stains lignified, cutinized, suberized, and chitinized structures as well as chromosomes, nucleoli, and centrosomes.

Safranin ordinarily dissolves better in strong alcohol than in water, despite the contrary indications given by the solubility data mentioned above. A stock solution may be prepared by dissolving 2.25 g. of a certified sample in 225 cc. of 95% ethyl alcohol, and a part of this stock solution may be diluted with an equal volume of distilled water when needed for use. If this solution should prove to be too strong, it may be further diluted with 50% alcohol. Various other formulae for making safranin solutions have been published, and certain schedules also call for safranin to be dissolved in particular ways; it is best to follow such directions explicitly in order to secure the expected results.

The writer, for example, prepares safranin by dissolving 4 g. of the dye in 200 cc. methyl cellosolve; when solution is complete, 100 cc. each of 95% alcohol and distilled water are added, followed by 4 g. sodium acetate and 8 cc. formalin. The purpose of the acetate is to intensify the stain; that of the formalin is in the nature of a mordant. Slides are left in the solution for 24 to 48 hours; differentiation is exceptionally easy, giving sharp and brilliant contrasts.

Safranin invariably overstains indiscriminately and therefore requires differentiation. This, in the past, was done in 50% alcohol slightly acidulated with hydrochloric or acetic acid, but a much sharper differentiation can be obtained by adding picric acid to the 95% dehydrating alcohol. After tissues have been stained with safranin, the excess stain should always be washed away with water, otherwise there is great danger that ugly irremovable precipitates may be deposited in the tissues.

Sudan III.—Weakly acid; azo group. Now replaced by Sudan IV.

Sudan IV (Syn.: Scharlach R, fat ponceau R).—Weakly acid; azo group. Solubility: nil in water; 0.09% in alcohol. About the only specific fat stain. Use a saturated alcoholic solution for about 10 minutes, and wash rapidly in alcohol. Mount in glycerin jelly. As alcohol

is a fat solvent, avoid leaving in the alcohols any longer than necessary. Tissues should be fixed in a solution which does not extract or dissolve fats. Lecithin, resins, latex, wax, and cuticles are also stained by Sudan IV; chloroplasts are stained a dull red.

Thionin.—Basic; thiazin group. Solubility: 0.25% in both water and alcohol. This dye is little called for in botanical technique, but because of its metachromatic properties (*i.e.*, the ability to impart different colors to different cell structures) it is useful with animal tissues. To stain chromosomes, allow a saturated solution in water or rather weak alcohol to act for about 5 minutes. Following fixation in a fluid containing mercuric chloride and used diluted for about 15 minutes, it is a specific stain for mucin; the mucin is red, and everything else is blue.

Vital Red (Syn.: brilliant Congo red).—Acid; azo group. An important vital stain.

OTHER SUBSTANCES ACTING AS STAINS

Stains, frequently of a specific type, are imparted by certain reagents which act partly as color indicators of a microchemical nature.

Iodine.—Iodine is well known as a specific color indicator for starch, when made up in combination with potassium iodide. The color reactions of iodine on sections of fresh material are as follows:

Blue	Brown	Yellow
Starch	Cellulose	Pectin
Saponin	Proteins	Cutin
	Inulin deposits	Callose
	Alkaloids	Cork

Osmic Acid (Syn.: osmium tetroxide).—Osmic acid has the peculiar property of blackening certain cell inclusions. This property has been taken to be specific proof of the identity of such inclusions, but it has recently been severely questioned. It is highly probable that osmic acid is reduced to an insoluble black precipitate by so many different substances that it is rather dangerous to draw inferences from its ability to blacken various cell constituents. By using special fixatives and staining with iron hematoxylin, more precise and reliable results may be obtained.

TABLES OF STAINS FOR SPECIFIC STRUCTURES

In the following tabulated summary the structures or substances and stains are arranged alphabetically. No discrimination is being made between the different stains under any particular heading since it is clearly recognized that under varying circumstances and on different materials the same stain will not react equally well.

ACHROMATIC FIGURE
- Anilin blue
- Erythrosin
- Fast green FCF
- Methyl or crystal violet
- Light green

BULK STAINS
- Bismarck brown Y
- Carmin
- Carminic acid
- Harris' hematoxylin

CALLOSE
- Anilin blue
- Resorcin blue (specific)

CELLULOSE CELL WALLS
- Acid fuchsin
- Anilin blue
- Bismarck brown Y
- Congo red
- Delafield's hematoxylin
- Fast green FCF
- Light green

CHITIN
- Safranin

CUTINIZED CELL WALLS
- Acid fuchsin
- Crystal or methyl violet
- Erythrosin
- Methyl green
- Methylene blue
- Safranin (specific)

CYTOPLASM
- Acid fuchsin
- Anilin blue
- Eosin Y
- Erythrosin B
- Fast green FCF
- Indigocarmin
- Light green
- Malachite green
- Methyl orange
- Nigrosin
- Orange G
- Phloxine

DIVIDING CHROMATIN (CHROMOSOMES)
- Brazilin
- Carmin
- Carminic acid
- Hematoxylin
- Iodine green
- Methyl green
- Safranin

FATS
- Sudan III or IV (specific)

LIGNIFIED CELL WALLS
- Crystal violet
- Iodine green
- Methyl green
- Methylene green
- Safranin

MIDDLE LAMELLAE
- Iron hematoxylin
- Ruthenium red (specific)

MITOCHONDRIA
- Acid fuchsin
- Aurantia
- Crystal violet
- Iron hematoxylin
- Janus green B (vital)

NUCLEAR (GENERAL)
- Carmin
- Crystal and methyl violets
- Hematoxylin
- Iodine green
- Methyl green
- Methylene blue
- Pararosanilin
- Safranin
- Thionin

PLANT MUCIN
- Bismarck brown Y
- Congo red
- Pararosanilin

PLASTIDS
- Crystal or methyl violet
- Iron hematoxylin

PROTEINS
- Safranin

SUBERIZED CELL WALLS
- Safranin
- Sudan III or IV (specific)

CHAPTER VII

STAINING PROCEDURES

In this chapter the methods of using dyes will be dealt with. As was intimated in the preceding chapter, the technician has at his service some 250 different dyes. No one could possibly learn how to use correctly all these dyes, singly or in various combinations, whether according to more or less rigid specifications or empirically. This, fortunately, is a matter which need give us no concern. One would do well to follow the example set by competent technicians by selecting a very few combinations and practicing with them on all sorts of tissues until the procedure of using each of these dyes has been thoroughly mastered. The three stain combinations which are undoubtedly used by a larger number of botanical technicians than any other groups of dyes include (1) iron hematoxylin with or without a suitable counterstain; (2) safranin and fast green, and (3) a variant of Flemming's triple combination. While all schedules will be outlined in sufficient detail, the foregoing three methods will be elaborated at considerable length.

The nature of the dyeing or staining reactions which occur when tissues are immersed in a solution of a dye are still imperfectly understood. Theories to account for the reactions have been based almost exclusively upon either chemical or physical phenomena. A long discussion of these phenomena could be presented, but as it is extremely doubtful whether such an account would be of practical value it is being omitted. An exception, however, is being made in the case of hematoxylin in order to explain the necessity for the use of a mordant since the substance by itself does not stain tissues.

The question of using buffer solutions for the control of the hydrogen-ion concentration in staining procedures has received some attention (French 1930), but such solutions have been chiefly used with complicated schedules involving compound stains on other than plant tissues. Botanical technicians have generally ignored the entire subject.

The staining schedules which are given below have been divided artificially into two groups, according to whether the primary stain is produced by a natural dye or by a coal-tar dye. The artificiality occurs because the two are occasionally used together; in such instances the procedure is listed in the second group of schedules.

Solvents.—The nature of the most appropriate solvent for each dye has been cited under that dye in the preceding chapter. Summarizing,

it may be stated that the basic coal-tar dyes, as used in botanical technique, are dissolved in either water or 50% ethyl alcohol; the acid dyes employed as cytoplasmic counterstains are usually dissolved in an alcohol of high percentage or in clove oil, because they ordinarily wash out very easily; hematoxylin may be dissolved in either water or absolute alcohol but is never used without mordanting; the carmins are always in aqueous solution with the customary addition of various salts or acids.

Under certain circumstances it may be desirable to employ a solvent not ordinarily used with a particular dye. Whether such solvents can be employed depends entirely on whether the dye or dyes will dissolve in them. This can be ascertained only by trial unless one has a record of such a solvent having been used by others. There is, of course, no reason why solvents other than those specified cannot be employed, but the effects of the solvent upon the tissues themselves and upon other stains (if any are used) should be carefully watched, and the necessary adjustments made.

The basic coal-tar dyes are often dissolved in acetin (glycerin monoacetate), the acid dyes in an alcohol-ether such as methyl cellosolve or in beechwood creosote. Lactic acid or leonlinic acid (dissolve the colorless crystals in either water or alcohol) is a good solvent of the indulins ("alcohol-soluble" indulin and nigrosin).

Strength of Solutions.—In the preceding chapter the extent to which each dye will dissolve in water and alcohol has been indicated wherever known. These figures hold only for certified dyes of known dye content.

On the labels of all dyes certified by the Stain Commission is printed the total amount of soluble dye contained in each batch tested. If the dye content amounts to 80% or more, one may consider it sufficiently high for most practical purposes. It is neither necessary nor desirable to split hairs in such matters because it is the general rule to overstain tissues and then to differentiate the stain until the desired intensity or depth of staining has been acquired. This means that a dye certified as having 80% or higher dye content may be used in the proportion of 1 g. to 100 cc. of solvent to give a 1% solution. However, if the dye content is below 80%, appropriate adjustments in the weight of the dye used to make a solution of specific percentage must be made. For example, if the dye content is given as 50%, 2 g. of such dye to 100 cc. of solvent would be required to make a 1% solution. On the other hand, if a saturated solution is indicated, the problem is practically devoid of complications.

Dyes may now be purchased put up in capsules which when dissolved in the appropriate solvent will give a solution of a specified strength or percentage.

The solubility of certain dyes is accelerated by the application of heat, but some dye users have exceeded the bounds of common sense in this respect. Despite contrary recommendations, the violets—gentian, crystal and methyl—should never be boiled because the heated solutions, whether filtered or not, generally leave unsightly irremovable precipitates in the tissues.

Many dyes remain in good condition for only very short periods, no matter what the solvent. It is as a rule very risky to use spoilt solutions.

Mordants.—The carmins and hematoxylins are always used after or concurrently with various mordants, the salts of different metals serving this purpose.

The coal-tar dyes do not as a rule require mordants but many technicians have little strategems designed to improve the selectivity or intensity of certain of these dyes. Eosin and erythrosin, for instance, are supposed to be improved by the addition of a trace of acetic acid or to be differentiated in some solvent acidified with this acid. Barium chloride (2 to 4% solution) has been used as a mordant for the acid dyes; silicotungstic acid (the technical quality in a 4% aqueous solution) for the basic dyes. Other mordants include potassium permanganate (a 1% solution), ammonium chromate (to 4%) or ammonium dichromate (to 3%), aluminum hydroxide or aluminum potassium sulphate ("potash alum") (to 3 or 4%). All these are best used for 5 to 10 minutes on the slides after they have been brought down to water. Excess mordant should always be washed off with water before the slides are placed in the staining solution. The addition of a trace of lithium carbonate is claimed to improve the action of the basic dyes.

Iodine and picric acid are employed as mordants for the various violet dyes *after* the stains have been allowed to react on the tissues.

The pH of the fluid used for washing out and differentiating the coal-tar dyes determines the selective retention of the stain to a far greater extent than has been generally realized. Insufficient experimental work has been done upon this important problem. Basic dyes should as a rule be washed out with solutions which are definitely acid in reaction, while most acid dyes (which are all used as counterstains) should be differentiated in solutions which are slightly alkaline. It would be far better if the solution containing the acid dye were buffered to a specified pH, determined by trial, as affording a satisfactory stain which required no differentiating. It seems possible that the excellent results occasionally obtained with counterstains were due to one's having unintentionally gotten the optimum pH.

Effects of Fixatives on Stains.—It is not so much the chemicals of the fixatives themselves that affect the later staining of various plant materials as the chemical nature of the various tissues plus the compounds

resulting from chemical reactions involving these tissues and the reagents in the killing and fixing fluids which to a great extent determine the results that are obtained with different dyes.

Let a few examples be taken. (1) Safranin has a strong affinity for chromatin. Fluids containing chromic acid and formalin, perhaps by a sort of mordanting action, leave chromatin-containing structures such as nuclei and chromosomes in a nearly perfect condition for staining with safranin. If, however, the chromic acid reacts with substances in the cells to leave them colored dark brown, the safranin is correspondingly dulled, the dullness and brownness increasing with the intensity of the reaction between the acid and the cell contents. A point will be reached where there is no longer any differentiation between cytoplasm and other cell contents (as when the tissues are saturated with tannin compounds), and bleaching as a prelude to staining becomes necessary. (2) Some killing fluids preserve the plastin of mitotic figures perfectly; of these fluids some mordant the plastin so that it stains, but others fail to mordant it. This explains why certain staining procedures which are supposed to reveal the so-called "spindle fibers" occasionally fail: the fault is not because the staining was not carried out properly but because the structures involved were inadequately mordanted by the preservative. Navashin's fluid, for example, preserves plastin but rarely mordants it. (3) The triple combinations work as they should only on material originally fixed in a fluid that contained osmic acid or on sections that were mordanted in a weak solution of osmic acid in chromic acid. Otherwise, the chromosomes tend to appear purple rather than red, *i.e.*, they have an affinity for the violet rather than for safranin.

Bleaching.—Sections cut from materials which were excessively darkened by the fixing fluid require to be bleached before a proper stain can be obtained. One should not, however, bleach sections without being fairly certain that it should be done because bleaching frequently is more or less damaging to the tissues. Many types of material which are naturally dark (such as the mechanical tissue in fern rhizomes) do not actually require bleaching and can be beautifully stained if a proper selection of dyes is made.

Bleaching fluids have been described elsewhere (see page 21) but a few additional methods are being described below.

For the bleaching of sections to be stained in a triple combination, see under Stockwell's variation of this combination (page 85). This procedure is also excellent for removing excess phlobaphene precipitates.

If the sections appear to have suffered from too much chromate fixation, immerse the sections for 1 minute in a 1% aqueous solution of potassium permanganate, rinse, remove the permanganate in a 1% aqueous solution of oxalic acid, wash again, and proceed to the staining.

Chlorine gas fumes bleach excellently. Place enough crystals of potassium chlorate to approximate the size of a grain of wheat in a coplin and pour on a little dilute hydrochloric acid. As soon as the greenish-yellow fumes become apparent, fill the jar quickly with 50% alcohol. About 20 minutes' minimum immersion of the sections is required to effect bleaching. Sections, however, cannot be stained if commercial bleaching fluids containing chlorine are used.

Boric acid solutions have been used for bleaching as has a 2% aqueous solution of ammonium persulphate. The latter should be used with caution as it is a powerful oxidizer.

The blackening caused by osmic acid may be removed by dissolved chlorine gas or by immersing the sections in hydrogen peroxide diluted one-half with water. The process may be hastened by placing the container in the sunlight.

To restore the staining capacity of tissues, bleach if necessary, then soak for 15 minutes in a 10% solution of benzoyl peroxide in acetone; wash out in a solution of 2 parts xylol to 3 of acetone, followed by absolute alcohol.

Differential Acidification.—In order to intensify and localize stains in tissues whose elements do not present sufficient contrast in their relative degree of acidic or basic reactions, the hydrolysis phase of Feulgen's reaction may be employed to render nuclei more acid and thus to give them a greater affinity for nuclear stains. This procedure is especially adaptable to more or less thick materials which are to be mounted entire—such as fern prothallia, *Ectocarpus*, root tips, *Elodea* leaves, and similar objects. The slides or fixed materials are brought down to water, placed in cold 1/N hydrochloric acid for exactly 1 hour, rinsed with one change of distilled water, and then placed in the nuclear stain. The acid must not be heated, as is done in the regular Feulgen technique, otherwise the material is very likely to become dissociated.

Progressive and Regressive Staining.—By observing the progress of the staining under the microscope from time to time, any desired intensity may be attained. This is known as "progressive staining." A sharp differentiation usually cannot be obtained by this method. Consequently, the general practice is to overstain considerably and then to destain or otherwise to differentiate until a satisfactory optimum has been reached. This is known as "regressive staining." Practically all the schedules given in the present chapter concern regressive staining procedures.

General and Specific Stains.—A general stain is one that stains everything indiscriminately. There is little or no selective differentiation, but such may to a slight extent be obtained by regressive destaining. *In toto* staining of some classes of plant material represents this type of stain;

what differentiation occurs is usually produced by the dehydrating fluids. Botanists find it more desirable to use specific or selective stains because they need to distinguish the structural elements of tissues, *e.g.*, between lignified and nonlignified cell walls. The aftertreatment of the stain determines almost wholly the degree of selectivity that is attained.

Some dyes are highly selective. Sudan IV, for example, stains only fats or fat-containing elements such as suberin, Congo red is almost specific for plant mucins, and alkanet gives a bright red color to oils.

In plant microtechnique, dyes have occasionally been classified according to the structures for which they have the most marked affinity. Some dyes may be found in more than one category, safranin being a prominent example. Of course, the selectivity of a dye may by appropriate chemical reactions be made to reverse its usual affinities, but such procedures may be considered as being unnatural. At the end of the preceding chapter a list of dyes recommended for particular structures is given, and reference may be made to this list whenever one is uncertain what stain or combination of stains to use.

THE NATURAL DYES

Cochineal, Carmin and Carminic Acid

Mayer's Alum Cochineal.—Dissolve 12 g. aluminum potassium sulphate in 160 cc. distilled water. Bring to a boil, add 12 g. powdered cochineal, and continue the boiling for 20 minutes. Pour off the clear supernatant liquid after the mixture has cooled, add more water to the cochineal, and boil again. Decant as before, adding this liquid to the first, filter, and evaporate down to 160 cc. To prevent the growth of molds, add a small crushed crystal of thymol.

This solution is suitable for *in toto* staining. The time required is from 24 to 48 hours. Wash in water for 20 to 60 minutes to remove the alum. No differentiation is required, but to prevent the stain from becoming extracted, run up through graded alcohols to 70% alcohol. The material, if of a suitable type, may be mounted whole in balsam if first carried through hygrobutol; or the material may be embedded and the sections counterstained with Bismarck brown (in 70% alcohol) or other suitable stain.

Iron-Acetocarmin.—As used on smear preparations, this stain is fully described in the chapter on Smear Methods.

Grenacher's Alum Carmin.—Boil 1 g. powdered carmin for 10 to 20 minutes in a 5% aqueous solution of aluminum ammonium sulphate. Filter when cool. This is one of the easiest carmin stains to use since it rarely overstains. After the killing fluid has been washed out, transfer bulk material to the dye, and leave until it appears to be properly stained; this may occasionally be a matter of several days. Dehydrate (preferably

with hygrobutol, acetone, or dioxan), embed, and section. After the sections have dried to the slides, remove the paraffin with xylol, and mount in balsam.

With many materials some differentiation should be carried out. After the material has been in the stain for a day or longer, transfer to acid alcohol (2 drops hydrochloric acid to each 100 cc. of 70% alcohol) until the color changes to a clear red. The time required varies from 1 hour to 2 or 3 days. Wash in a few changes of 70% alcohol to get rid of the acid, then complete dehydration, and proceed to the embedding.

Alcoholic Borax Carmin.—Dissolve 2 g. carmin in 100 cc. of a 4% aqueous solution of borax by boiling for 30 minutes. Set aside for at least three days, then dilute with an equal volume of 70% alcohol, and filter. Leave the material in the stain until it appears to be thoroughly stained. Differentiate in acid alcohol (5 drops hydrochloric acid to each 100 cc. of 70% alcohol) until the material becomes clear and transparent. Wash out the acid with a change or two of 70% alcohol, then proceed with the dehydration. This is perhaps the best stain for bulk staining of plant materials.

Mayer's Carmalum.—Dissolve 1 g. carminic acid and 10 g. aluminum ammonium sulphate in 200 cc. distilled water, using heat if necessary. Filter, and add 0.2 g. salicylic acid or a small crushed crystal of thymol to avoid mold growth. This is an excellent *in toto* stain as one may control the differentiation satisfactorily. If it is desired that the cytoplasm remain stained, wash out the excess stain with distilled water; if a purely nuclear stain is wanted, use a 5 to 10% aqueous solution of aluminum ammonium sulphate for differentiating.

Some types of materials may be given a double stain if 1 volume of a saturated aqueous solution of picric acid be added to 10 volumes of the carmalum solution; or 1 volume of a 0.2% aqueous solution of indigo-carmin to 4 to 6 volumes of carmalum. One might also experiment with other counterstains, which should be used in high dilutions.

Lynch's Borax-Carmin.—This is one of the most superb *in toto* stains for small animals and the protozoa, and there is no reason why it cannot succeed equally well on various types of plant material which are suitable for staining with carmin. The following variation has worked well on the Volvocales:

1. After fixation, wash out the killing fluid and run up to 50% ethyl alcohol.

2. Stain in Grenacher's alcoholic borax-carmin, made up with Grübler's "rubrum opticum" carmin (other samples of carmin dye have not worked successfully), and diluted in the proportion of 12 cc. stain to 100 cc. of 70% alcohol and 1 cc. hydrochloric acid, for 2 hours or as much longer as might be required to penetrate the objects thoroughly.

3. Agitating the container constantly, drop in hydrochloric acid with a pipette until a barely perceptible brick-red precipitate is produced. Avoid excess acid. Stir well, then let remain overnight.

4. Decant the stain, and transfer material to slightly acidulated 70% alcohol. Differentiate by adding 5% or stronger hydrochloric acid, and wash with several changes of 80% alcohol when the differentiation appears to be satisfactory.

5. If a counterstain is desired, a saturated solution of indulin (Grübler) in 80% ethyl alcohol may be added to the 80% alcohol containing the material until it appears a light blue. Watch the process as the indulin is difficult to remove if allowed to overstain. A few minutes to 1 hour may be required. Stop action of the stain by pouring off the 80% alcohol and adding 95% alcohol.

6. Complete dehydration with absolute alcohol, pass through absolute alcohol-xylol, pure xylol, and mount in balsam.

Brazilin, Hematoxylin, and Hematein

Brazilin.—This dye is scarcely used in general botanical technique but is valuable on smear preparations (page 163).

Heidenhain's Iron Hematoxylin.—This is by far the most important stain in the technician's repertoire and is the one which above all others he should strive to master completely as soon as possible. For this reason the method of using this stain is being described in considerable detail. All technicians use fundamentally the same solutions, but no two follow exactly the same schedule. Some even protest that the procedure cannot be written down in precise terms since so much depends upon the individual using it. It is almost wholly a matter of personal idiosyncrasy: one should study the outline described below, striving to understand fully the reason for each step, experimenting with all sorts of properly prepared material, and noting the differences which ensue upon varying the periods in the mordant and in the staining solution itself. After that, one will soon find that he has practically formulated a schedule of his own, which can readily be adapted to fit the necessities of particular cases as they arise during the course of his work.

1. Bring sections down to water (*i.e.*, remove the paraffin with xylol, and pass through a mixture of equal parts of xylol and absolute alcohol, allowing 10 minutes in each; then pass into a mixture of equal parts of absolute alcohol and ether plus 1% celloidin for 3 minutes, remove slides, and keep in air until the sections become opaque but are not completely dried out; plunge into 70% alcohol for 5 minutes, and finally pass through 35% alcohol into water).

2. Wash thoroughly with water, and finally rinse in distilled water.

3. Mordanting is now carried out. Hematoxylin by itself does not stain, even after it has been "ripened" by oxidation into hematein. (However, if the tissues contain traces of iron, copper, or aluminum, the dye will stain such tissues without mordanting.) The chemical most commonly used for mordanting is ferric ammonium sulphate, colloquially known as "iron alum," although other substances may also be used. The crystals must be a clear violet color; any that have turned yellow should be discarded. The strength of the solution is usually 3%—some technicians use a 2%, others a 4% solution. Weaker strengths should be used on more delicate objects, such as filamentous algae. Solutions must be freshly prepared and used only once; or a quantity of the following mixture, which keeps perfectly, may be made up: to 500 cc. distilled water add 5 cc. glacial acetic acid, 0.6 cc. c.p. sulphuric acid, and 15 g. of the alum crystals. The alum may be pulverized in a mortar to facilitate solution.

The time required for mordanting should not exceed 2 hours. An hour is sufficient with thin sections, a longer time for diluted solutions or thicker sections. Sections over 12μ thick cannot be satisfactorily stained with iron hematoxylin.

4. Wash thoroughly in running water for 5 minutes.

5. Rinse in a change of distilled water. (This step is necessary if the tap water is hard. In any event, any water containing a high percentage of dissolved salts will ruin the staining solution.)

6. Stain in 0.5% aqueous hematoxylin, prepared as described in the preceding chapter (page 50). As a rule, the slides should be left in the stain at least as long as they remained in the mordant, but in most cases 24 hours is the optimum time.

7. Wash off excess stain with water.

8. Destain in 2% ferric ammonium sulphate (or use ferric chloride), using a large volume of the fluid. The time which will be required for proper destaining varies with the material, the thickness of the sections, and other variable factors. The slides may be arranged around inside a stender, taken out in order, one by one, and examined on a glass plate placed on the stage of the microscope. If a glass plate is not used, the microscope will be ruined by the iron compound. Put the slide on the plate (section side up) while dripping wet, and examine quickly to judge whether differentiation is proceeding properly. Watch chromatin-containing bodies only, rather than judge by other structures which may have been colored by reagents other than the hematoxylin. As soon as a slide seems just right, place in a flat staining dish filled with water. Remember that there is a considerable difference between the refractive indexes of water and balsam; the stain when just right looks grayish-black while in the water. When destaining bulk material, one will learn

to look for a grayish fluorescence emanating from the material: when it appears, it is a fairly safe indication that differentiation has progressed far enough.

9. Wash in running water for at least 30 minutes, but preferably for 1 hour. The washing should be as thorough as possible; if traces of the destaining solution remain in the tissues, their continued action will cause the preparation to fade completely.

10. Dehydrate in 50, 70, and 95% alcohols, allowing at least 5 minutes in each.

(11. Counterstains are sometimes undesirable, but if one seems necessary or desirable, orange G, gold orange, or fast green may be used. These are best used in methyl cellosolve-absolute alcohol-clove oil solution. Remove a slide from the 95% alcohol; with a clean cloth quickly wipe dry the underside, hold the slide level, and apply with a pipette just enough staining solution to cover the sections completely. Let remain a few moments, then pour the stain back into the bottle. Wash off with waste clove oil diluted considerably with equal parts of absolute alcohol and xylol; then clear in a mixture of equal parts of clove oil, absolute ethyl alcohol, and xylol; wash with xylol plus a trace of absolute alcohol to take care of any moisture that might be brought over, then proceed to step 13.)

12. Absolute alcohol and xylol, equal proportions, 5 minutes.

13. Xylol, two changes, 5 minutes in each.

14. Mount in balsam.

The periods noted for steps 3 and 6 are merely suggestive and need more or less to be determined for each type of material. Bryophytes, for instance, differentiate with extreme rapidity, but most root tips differentiate comparatively slowly. When the slides are taken from the stain, they should have the appearance of being badly overstained everywhere. The excess stain will come out in clouds at first, but differentiation of chromatin will proceed slowly. After a little experience in judging appearances under the microscope, it can be controlled to a nicety, and any desired intensity of stain may be obtained. A sharp black-and-white contrast is undesirable; there should be variations in color from light gray through gray to black.

Iron Hematoxylin and Bismarck Brown.—This particular combination was designed for use on the phloem tissues of woody plants and is a very useful combination for these structures (Harrar 1928).

1. If sections were cut freehand or on the sliding microtome, place in a 2% aqueous solution of ferric ammonium sulphate for 20 minutes. If materials have been killed and fixed, the time may be reduced by half.

2. Drain mordant, and wash in at least five changes of distilled water to insure removal of mordant.

3. Flood with distilled water, and add 2 or 3 drops of 1% aqueous hematoxylin. Watch progress of the stain under the microscope, and stop at the desired point by quickly changing to water.

4. Change the water two or three times, then cover the sections with a 1% aqueous solution of Bismarck brown for 3 to 4 hours, depending on the thickness of the sections.

5. Wash out excess stain with water.

6. Dehydrate gradually, and use at least two changes of absolute alcohol to remove all traces of water.

7. Clear with xylol, and mount in balsam.

Stone cells of hard woods stain a cherry red, bast fibers a brilliant orange, the ray cells and other parenchymatous tissues a chestnut brown, and the middle lamellae a dark blue. The bast fibers, parenchymatous tissues, and middle lamellae of Coniferophyta stain as indicated, but the stone cells turn a vivid burnt orange.

Iron Hematoxylin and Safranin.—As originally intended, this combination is best used after a killing fluid containing picric acid as one of its constituents, but it has been successfully used after chromic acid fixatives. The completed preparation, which should be examined with an immersion lens, is frequently of great beauty. The particular value of the combination is to enable one to differentiate between nucleoli, which take a bright red color, and other nuclear constituents in plants with large nuclei. The chromosomes during metaphase and anaphase are a bright red and stand out sharply against the dark background; during prophase and telophase the chromosomes are much darker. Trabants are easy to identify, they often being a bright red and connected to the chromosome by a black thread.

1. Bring the slides down to water (see p. 72).

2. Mordant in 3% aqueous ferric ammonium sulphate for 2 to 3 hours.

3. Wash in running water for 5 minutes, then rinse in distilled water.

4. Hematoxylin solution. (Make a 10% solution of the crystals in absolute alcohol. For use, dilute 10 cc. of this solution with 90 cc. of distilled water.) Leave the sections in the stain as long as they were left in the mordant.

5. Differentiate in 3% aqueous ferric ammonium sulphate with great care. Pay especial attention to the decolorization of the nucleoli. When they are almost colorless, stop action by transferring the slide to water.

6. Wash with running water for at least 1 hour.

7. Safranin solution. (Originally equal quantities of saturated solutions in 95% alcohol and anilin water, respectively, were specified. The older the anilin water solution, the better the action of the stain. However, any standard solution will serve.) Leave in the stain for 12 to 15 hours.

8. Differentiate either in 70% alcohol acidified with a few drops of hydrochloric acid or use 95% picro-alcohol for not longer than 10 seconds.

9. Proceed as usual to balsam.

Alcoholic Iron Hematoxylin.—Sometimes the alcoholic solution of hematoxylin affords better results than does the aqueous solution. In other cases, as with many of the marine algae which are with the utmost difficulty retained on the slides and would wash off if brought down to water, only the alcoholic hematoxylin can be used. The following schedule has consistently given a beautiful and sharp stain.

1. Bring sections down to 70% alcohol.

2. Mordant for 5 hours or longer in a solution composed of 10 parts of 50% alcohol and 1 part of a 4% aqueous solution of ferric ammonium sulphate.

3. Rinse briefly in 70% alcohol.

4. Stain for 12 to 24 hours in a hematoxylin solution made up of 10 parts of 70% alcohol and 1 part of the following hematoxylin solution:

Hematoxylin crystals	1 g.
Absolute ethyl alcohol	10 cc.
Distilled water	90 cc.
Thymol	Small crystal

Dissolve the hematoxylin in the alcohol, then add the water.

5. Differentiate in the same solution that was used for mordanting, which should finally be discarded. Watch the progress of the destaining under the microscope; it will probably take a long time.

6. Wash thoroughly in several changes of 70% alcohol to remove the mordant.

7. Dehydrate in 95% and absolute alcohol.

8. If a counterstain is desired, a solution of orange G in clove oil is preferable. Use the methyl cellosolve-absolute alcohol-clove oil solution.

9. Xylol, then mount in balsam.

Harris' Hematoxylin.—This stain was originally devised as a substitute for Delafield's hematoxylin in zoological technique but has proved to be more satisfactory than the latter stain with most plant materials, such as fern prothallia, the Rhodophyta, many fungi and similar subjects which are to be mounted whole, and for cytological staining.

1. Bring slides down to water, or wash the killing fluid out of the material with water.

2. Stain for about 20 minutes in the hematoxylin solution.

3. Rinse in distilled water until all the excess stain has been washed away.

4. Destain sections in acid water (about 5 drops or less of hydrochloric acid to each 100 cc. of water). As a rule, the time is about 5 seconds, but

one can easily stop the action of the acid, rinse the material with tap water (which will "blue" the stain), and examine under the microscope. Bulk material will require a considerably longer period.

5. Wash for a few moments in tap water. If the water is not sufficiently alkaline to "blue" the material, add 1 or 2 drops of ammonia to the water. If it is desired to clear the cytoplasm, place the slides or material in a weak solution of lithium carbonate in water. About 10 minutes suffices.

6. Rinse in water again, then proceed with the dehydration as usual.

If a counterstain is desired, erythrosin is very satisfactory for marine algae and also for many fresh-water forms (such as *Batrachospermum*), fungi, and other cryptogams, while fast green may be used on fern prothallia and bryophytes. Both dyes should be in fairly strong solution, and the sections or material should be left in the stain until completely and evenly stained.

Delafield's Hematoxylin.—A thoroughly ripened solution should always be used.

1. Transfer to the stain from either water or 50% alcohol. The length of time the sections may be left in the stain depends partly on the character of the material, partly on the nature of the killing fluid, but mainly on the thoroughness of the washing out of the latter. Some materials are well stained in 3 minutes, while others require as long as 30 minutes. As a trial, a few slides may be left in the stain for 10 minutes; if the time seems too short or too long, the duration of staining may accordingly be regulated for future batches of the same material.

2. Wash in running tap water a few minutes to remove completely all excess stain. The washing should be as thorough as possible to avoid the formation of troublesome precipitates.

3. Treat with acidulated water: add about 2 drops of hydrochloric acid to each 100 cc. of water. The sections, which should preferably be slightly overstained, are treated for a few minutes until they turn a pale pinkish-purple, but care must be taken not to extract too much of the stain. Then transfer quickly to water, and wash in running tap water until the sections acquire a rich purple color. If the tap water is not sufficiently alkaline, immerse the slides for a few moments in water to which a little sodium or lithium carbonate has been added, then complete the washing.

4. Pass through 50, 70, and 95% alcohols. A few minutes in each strength is sufficient.

5. If desired, mount directly from 95% alcohol in euparal or diaphane. These mounting media possess the property of intensifying hematoxylin stains. Otherwise pass through absolute alcohol, equal parts of absolute alcohol and xylol, then xylol, and mount in balsam.

A counterstain is ordinarily of not much value on plant tissues, but most animal tissues are improved by counterstaining with eosin Y. From step 4 in the above schedule, proceed to the counterstain dissolved in 95% alcohol; allow to react for about 3 to 5 minutes, then wash excess stain in a change of 95% alcohol. Mounting may be in euparal or diaphane, or dehydration may be completed in absolute alcohol and the slides passed through xylol to balsam.

Orange G or erythrosin B in clove oil solution may in some instances prove satisfactory counterstains. Stain on the slide after dehydrating in absolute alcohol, clear in clove oil, wash in xylol, then mount.

The clearing fluids and balsam are by most technicians required to be perfectly neutral in reaction, otherwise the rich purple color will turn red and finally disappear if these reagents are in the least acid in reaction.

Sass' Modified Mayer's Haemalum.—Primarily a histological stain.

1. Bring slides down to tap water.

2. Leave in the following staining solution until the desired depth has been reached (in about 15 to 30 minutes): Dissolve 50 g. aluminum ammonium sulphate in 1 liter boiling water. Remove from the heat, and add 1 g. hematoxylin crystals. When dissolved, add 0.2 g. sodium iodate, cool, and filter. Later filter whenever a metallic scum appears. The solution is best used fresh but will keep for a few months.

3. Wash in distilled water, then in tap water (or in $\frac{1}{100}$ sodium or lithium carbonate) and again in distilled water.

4. Dehydrate, and clear as usual.

Any desired counterstain may be used. Nuclear selectivity may be increased by the addition of acid: the alum can be increased to saturation and 2 to 5% of glacial acetic acid added. The results vary with the nature of the fixative used.

Kornhauser's Modified Mayer's Haemalum.—The customary procedure of adding sodium iodate as an ingredient of Mayer's haemalum has been criticized on the ground that the life of the solution is shortened so that it turns brown and fails to stain (Kornhauser 1930). The revised method is as follows:

1. Run the sections down to water.

2. Stain an average of 5 minutes in the hematein solution prepared thus: grind 0.5 g. hematein (of MacAndrews and Forbes manufacture) in a glass mortar with 10 cc. of 95% alcohol, and add the mixture to 500 cc. 5% aqueous aluminum potassium sulphate. The solution is ready for immediate use.

3. Rinse for a few seconds in tap water. It is claimed that, if the rinsing is quick, a little of the alum solution is carried over and serves to fix the counterstain in the tissues.

4. Counterstain with any suitable acid counterstain dissolved in distilled water. The method was originally devised for animal tissues, and eosin was therefore specified. This dye, of course, is of little value on plant tissues, consequently erythrosin B is the first substitute to be chosen. If, on washing with tap water, the counterstain comes out, dehydrate as far as 95% ethyl alcohol, and apply the desired counterstain from the usual alcoholic clove oil solution.

5. Complete dehydration, pass through xylol, and mount.

Ehrlich's Hematoxylin.—An excellent *in toto* stain for algae, fungi, small bryophytes, and similar subjects.

1. Bring slides down to 50% alcohol (or freehand sections or bulk material up to 35% alcohol).

2. Stain 5 to 30 minutes in the following solution:

Distilled water	100 cc.
Absolute alcohol	100 cc.
Glycerin	100 cc.
Glacial acetic acid	10 cc.
Hematoxylin crystals	2 g.
Aluminum ammonium sulphate	In excess

Ripen in the light until the solution acquires a dark red color. If kept well stoppered, the solution keeps for years. Hematein may be substituted for the hematoxylin; 0.4 g. is the correct amount.

3. Wash out excess stain with 50% alcohol.

4. For woody tissues counterstain with safranin, or proceed with the dehydration and counterstain with erythrosin B or orange G in clove oil.

5. Clear, and mount in balsam.

Ide-Roza Variation of Weigert's Hematoxylin.—The mordant is mixed with the stain; and an excellent general stain is produced:

Weigert's hematoxylin (dissolve 1 g. hematoxylin in 10 cc. of absolute ethyl alcohol and dilute to 100 cc. with distilled water)	15 cc.
Double distilled glycerin	5 cc.
Mordant (5 g. ferric ammonium sulphate and 6 g. aluminum chloride in 100 cc. distilled water)	5 cc.

Stain for 12 to 24 hours, and differentiate with 2 to 2.5% aqueous ferric ammonium sulphate. The staining appears much more uniform throughout a section in spite of accidental variations in thickness, a feature not obtainable with other hematoxylin schedules.

THE COAL-TAR DYES

In employing coal-tar dyes, it is important to use the basic dye first, if two stains are being used in combination. The basic dyes are generally the non-cytoplasmic stains; they stain such prominent structures as nuclei, chromosomes, and lignified cell walls. The basic dyes are usually

dissolved in alcohol of around 70%, some in water, a few in anilin water, one or two in clove oil, and rarely in other solvents. Of course, one may use a single dye, but the final result is invariably improved by the employment of a contrasting stain. Sometimes two basic dyes may be used together (as safranin and crystal violet), but the rule is for an acid cytoplasmic stain to follow a basic stain.

Most of the coal-tar dyes are used as regressive stains. A few may be employed, in high dilutions and with continual scrutiny of the sections, as progressive stains; this type of staining has most frequently been applied to counterstaining, particularly of filamentous algae for whole mounts.

Safranin and Combinations

Safranin is the most important and valuable of the coal-tar dyes to the plant technician, just as the violets are indispensable to the cytologist. It is easy to put a slide into safranin: the problem is to obtain an accurate differentiation. The custom formerly was to use a solution of hydrochloric acid in alcohol, but many of the more discriminating technicians prefer to use picric acid, which seems both to differentiate and to mordant. Advantage is taken of the fact that fast green not only provides an excellent counterstain to safranin, but serves to remove the red completely from structural elements where its presence is undesirable. In fact, a fast green solution alone may be used for differentiating safranin, but both colors finally appear too dull for this procedure to deserve recommendation.

When a safranin stain is specified, the dye known as safranin O is always meant; if a safranin of another type is required, it is indicated by the letter Y or B, as the case may be. Both safranin Y (yellowish) and safranin B (bluish) are on the market; both are worthless in botanical technique as they have never given results at all comparable in brilliancy and accuracy of differentiation with safranin O. There are two types of safranin O on the market, one which acts slowly and the other rapidly, but they can be recognized as such only by trial.

In using safranin, one should always remember the fundamental rule of washing out the excess stain with water before proceeding with the differentiation. If this precaution is not observed, an irremovable precipitate will be produced in the sections; this is particularly liable to happen if the picro-alcohol method is used for differentiating.

Safranin and Fast Green.—Long experience with this schedule leads the writer to prefer it above all others. It has proved to be wholly dependable on sections of almost every type of plant material except the algae.

1. Bring slides down to 70% alcohol (or freehand sections up to 35% alcohol), and give any special treatment which the nature of the killing fluid might render necessary.

2. Stain in a 1% solution in methyl cellosolve-50% alcohol (see page 62 for formula) for 2 to 24 or even 48 hours. Gymnosperm material should have the minimum period.

3. Wash excess stain with running water for a few moments.

4. Simultaneously differentiate and dehydrate with 95% alcohol, to which 0.5% (*i.e.*, almost to saturation) picric acid crystals are added (if the acid comes in the moist form, make allowances for the water). A stock solution should be made considerably in advance as the acid dissolves slowly. The used solutions should be kept in separate bottles and may be used until they become somewhat saturated with safranin. About 10 seconds in the 95% alcohol suffices, unless the slides have been left in the stain too long, which naturally would call for a longer washing in the picro-alcohol.

5. Stop action of the acid by immersing the slides in 95% alcohol to which is added about 4 to 5 drops of ammonia to each 100 cc. Avoid excess ammonia. The sections should appear to be a trifle overstained at this juncture. Do not leave the slides in the alcohol-ammonia solution longer than 2 minutes, as the alcohol during this period is extracting some of the stain.

6. Complete dehydration with absolute alcohol for about 10 seconds. (The writer goes only as far as 95% alcohol with purely morphological material, but absolute alcohol should be used on cytological objects.) The slides may remain in this alcohol as long as 10 minutes if a large number are being brought up and are being counterstained individually.

7. Counterstain with fast green, prepared by making a nearly saturated solution in equal parts of methyl cellosolve and absolute alcohol and adding enough of this solution to a mixture of 25 parts absolute alcohol and 75 parts clove oil to give the desired intensity. Keep the stain in a dropping bottle; it may be used over and over again for hundreds of slides. Sometimes one may prefer a weak, sometimes a deep, stain, according to the nature of the material. The fast green is such a powerful dye that its action should not be allowed to proceed for more than 15 seconds.

8. Pour the counterstain back into the dropping bottle and rinse off excess stain with used clove oil. (As a matter of economy, the clove oil clearing solution specified in the following step, after it has become too saturated with dye, is poured into a bottle, diluted one-half with equal parts of absolute alcohol and xylol, and used for washing off excess counterstain into a waste jar. See Fig. 15 for arrangement of apparatus for counterstaining.)

9. Clear in a mixture of 50 parts clove oil (U.S.P. brand serves as well as the more expensive c.p.), 25 parts absolute alcohol, and 25 parts xylol for a few moments.

10. Remove clearer by washing slide a few seconds in a xylol wash (add 3 to 4 drops absolute alcohol to take care of any moisture accidentally brought over; the slides should not show any cloudiness).

11. Two changes of pure xylol, then mount in balsam.

The safranin should appear a brilliant red in nuclei, chromosomes, and in lignified and cutinized cell walls; while the fast green should be equally brilliant in the cytoplasm and on cellulose cell walls. In some cell walls at certain developmental or formative stages, portions will be more or less sharply stained by the safranin, other portions weakly by the green. Safranin and fast green are both durable and should show no signs of fading after six years.

Safranin and Anilin Blue.—Anilin blue may be substituted for fast green in the preceding schedule. As the blue does not affect the safranin to any appreciable extent, the latter must be somewhat more accurately differentiated than is necessary when fast green is used as the counterstain. The anilin blue solution is made by preparing a saturated solution in equal parts of methyl cellosolve and absolute alcohol and later diluting with an equal volume of clove oil. The stain should be allowed to react for about 1 minute; it scarcely ever overstains and requires no differentiating. To avoid extracting too much of the blue, clearing is usually carried out in pure methyl salicylate (synthetic oil of wintergreen) instead of in clove oil.

Anilin blue is a little more precise than fast green on many types of plant materials. If the effects given by the fast green are too strong or too gaudy, substitute anilin blue. The combination is superb on gymnosperm ovules, archegonia, and embryos and on angiosperm stems and roots. The anilin blue is occasionally a little fugitive, especially in acid balsam.

Safranin and Picro-anilin Blue.—The combination is intended for use on freehand sections of woody tissues. First stain the sections in safranin as usual, the optimum time being 2 hours. Wash out the excess stain with water and destain with either 95% alcohol saturated with picric acid or with a mere trace of hydrochloric acid in 50% alcohol. Stain for 2 hours in a picro-anilin blue stain (make up saturated solutions of picric acid and anilin blue in 95% alcohol, and when ready to use, mix in the ratio of 78% picric acid to 22% of anilin blue). Wash for 10 seconds in absolute alcohol, clear in clove oil, and mount in balsam after going through xylol.

Safranin and Crystal Violet.—A rapid schedule involving these two stains has given very good results on various gymnosperm tissues. It

has been successfully used on various stages in the development of the ovule and associated structures of *Pinus*, *Cedrus*, *Ephedra*, and *Ginkgo*.

1. Stain in safranin as usual and differentiate with 95% alcohol saturated with picric acid, then complete dehydration with a change of absolute alcohol.

2. Dip into a solution of equal parts of absolute alcohol and xylol for several seconds, then into another composed of 25 parts of absolute alcohol and 75 parts of xylol.

3. Stain in a crystal violet solution made by putting 6 to 8 drops of a saturated solution of the dye in equal parts of absolute alcohol and clove oil into a coplin of xylol. This staining solution is very unstable and should be freshly made up for each batch of slides. The strength of the violet needs to be renewed after every 30 or so slides by the addition of another drop of stock solution. The stock solution keeps indefinitely. Leave the slide in the stain while a second one is being taken through the picro-alcohol, and differentiate while the second is staining.

4. Differentiate the crystal violet in a mixture of equal parts of clove oil and xylol. A few seconds generally suffice.

5. Wash thoroughly in xylol, pass through a change of pure xylol, and mount in balsam.

Safranin and Harris' (or Delafield's) Hematoxylin.—The following schedule may be used either for freehand sections or for paraffin sections. It is especially serviceable for semiwoody tissues.

1. Bring freehand sections up to 35% alcohol, or paraffin sections down to 70% alcohol.

2. Stain in safranin (use the 1% methyl cellosolve-50% alcohol solution) for 18 hours.

3. Wash out excess stain with water.

4. Differentiate carefully in 50% alcohol slightly acidulated with hydrochloric acid. When xylem appears bright red and cellulose walls are a deep pinkish color, the stain is about right. Avoid too much destaining since more of the safranin will be removed during subsequent steps.

5. Wash thoroughly in water for 5 minutes.

6. Transfer to the hematoxylin for about 15 or 20 minutes. After removing from the stain, the color of the sections should be a deep purple all over.

7. Treat with water slightly acidulated with hydrochloric acid for a very few seconds. (Slides should be handled individually.) As soon as the sections appear reddish, transfer to tap water, and wash thoroughly. It is vitally important to remove every trace of acid, hence the washing process should be continued for at least 20 minutes. If the

hematoxylin is not sufficiently blued by the tap water, dip momentarily in a stender of water plus a few drops of ammonia.

8. In the case of paraffin sections, run up through 50 and 70% alcohols to 95%, allowing 3 to 5 minutes in each. Mounting may be in euparal or diaphane, or one may proceed to absolute alcohol, and pass through xylol to balsam. With freehand sections dehydrate by gradually substituting hygrobutol for the water, finally infiltrate with balsam, and mount.

Safranin, Crystal Violet, and Orange G (Flemming's triple stain).—Botanists, and biologists in general as well, seem to be divided on the merits of this combination. Nevertheless, it has its uses and its value, but by some it is underrated, by others overrated, while still others have flagrantly abused it. By this it is meant that it was used when other and more appropriate stain combinations should have been chosen.

In cytological work, Flemming's triple stain may be routinely employed to supplement and corroborate the results obtained with iron hematoxylin. The contrasting colors are at times a pronounced advantage. In a properly stained cytological preparation, the chromatin of the metaphase and anaphase chromosomes should show the safranin predominating, while the chromatin during prophase stages should retain the violet. In the chromosomes the condensed chromatin should be red, while the parts in which little or no chromatin is present should be violet. In cytological terminology, in other words, the chromonemata should be red and the chromosome matrix purplish, but it is extremely rare to obtain such an accurate differentiation. The nucleoli should be red, the spindle fibers and plastids violet, and the cytoplasm a buff-gray color. The orange G is used not to provide a third (cytoplasmic) stain—it is in this respect that most technicians are in error—but to differentiate between the other two dyes.

In general only tissues fixed in a mixture containing chromic and osmic acids can be successfully stained by a Flemming combination. Material killed in other fluids should first be run down to water and mordanted for a day or so in a 1% solution of osmic acid in 2% chromic acid, or as directed under Stockwell's variation described below.

A. A *typical schedule*, which many prefer, is as follows:

1. Slides in 70% alcohol (or, if they have been mordanted, first place in 35% alcohol for 5 minutes).

2. Stain in a standard safranin solution. The time varies according to the material; in any case, it should not be less than 2 hours. For most plant materials 6 hours is sufficient, but there is no harm leaving in the stain for 24 hours.

3. Rinse thoroughly in water.

4. Stain in a 1% aqueous solution of crystal violet. The time here must be determined experimentally. Fifteen minutes to 1 hour or more are required.

5. Rinse in water to remove surplus stain.

6. Dip twice in 95% alcohol and three or four times in absolute alcohol.

7. Remove slide, wipe off underside, and put on just enough orange G (saturated solution in clove oil) to cover the sections. The time should not exceed 10 seconds.

8. Pour the orange back into the bottle, wash off the excess stain with used diluted clove oil, and drop slide into a stender of pure clove oil. In a few moments put the slide on a glass plate, and examine under the microscope. When the violet is satisfactory, wash in xylol to stop action of the clove oil.

9. Two changes of xylol, then mount in balsam.

B. A *variation of* the preceding schedule followed by some technicians may be outlined, but the result is frequently sloppy in appearance.

1. Proceed to the end of step 4 in the preceding schedule.

2. Remove slide from staining jar, wipe off the underside, and flood with a saturated aqueous solution of orange G. About 30 seconds suffice.

3. Drop 95% alcohol on the slide until clouds of color no longer arise from the sections.

4. Wash with used clove oil, then differentiate as before with pure clove oil.

C. Stockwell's Variation.—If the sections are too dark, overchromated or contain too much phlobaphene, they should first be run down to water and bleached overnight in the following solution, which prepares the sections for very sharp staining:

Water	90 cc.
Potassium bichromate	1 g.
Glacial acetic acid	10 cc.
Chromic acid	1 g.

The theory underlying the use of this fluid, taking as examples tissues such as the root tips of *Quercus*, buds of *Dudleya* and other Crassulaceae containing much phlobaphene, is that the chromic acid renders the precipitated tannins soluble, the acetic acid then removes them, and the dichromate thereupon catalyzes the tissues.

If the material was not fixed in a solution containing sufficient chromic acid to mordant it, the slides should first be mordanted in a 1% aqueous chromic solution for at least 1 hour, but preferably overnight. Wash out the acid thoroughly before proceeding to the stain.

Stain 1 to 24 hours in the following solution:

1% aqueous solution of gentian (crystal) violet	1 part
1% aqueous solution of safranin	2 parts
Distilled water	1–4 parts

For most plant tissues an hour is sufficiently long, but a dilute stain acting over a period of 24 hours presumably gives superior results. Wash off excess stain with tap water, place for 30 seconds in 1% potassium iodide plus 1% iodine in 70% alcohol, then pass through the following fluids, allowing but a few seconds in each: 50 and 70% alcohols, 95% alcohol plus picric acid (about 1 g. per 100 cc.), 95% alcohol plus ammonia (8 to 10 drops per 100 cc.), pure 95% alcohol, absolute alcohol, clove oil plus orange G (0.2 g. per 100 cc.), pure clove oil, and finally through three jars of xylol to make certain that all traces of clove oil are removed before mounting in balsam.

The slides may be examined in the first xylol after the staining has been completed. If there seems to be too much safranin, return the slide to absolute alcohol, then back to xylol. Excess violet may be reduced by returning the slide to clove oil, then back to xylol.

Chromosomes are stained varying shades of light to dark purple; the spindle fibers are purplish, the nucleoli red, and the cytoplasm orange. With morphological and anatomical material, the final effect is the same as with the usual versions of the triple combination.

D. An abbreviated form of the preceding schedule, for use on non-cytological materials, is as follows:

1. Bring slides down to water, then stain for 1 hour in the staining solution.
2. Rinse thoroughly in water.
3. Rinse in 95% alcohol for a few minutes. This serves to remove most of the surplus safranin.
4. Rinse in absolute alcohol.
5. Differentiate the violet in the clove oil-orange G solution for a few seconds.
6. Wash in xylol, and examine under the microscope to determine if the violet is satisfactory. If not, go back to a plain clove oil, and differentiate until satisfactory.
7. Wash thoroughly in xylol, then mount in balsam.

E. Craigie's Modification.

1. Bring slides down to water and mordant in 0.5% aqueous osmic acid for 30 minutes to an hour. This step is omitted for material which has been fixed in a fluid containing osmic acid.
2. Wash thoroughly in water and stain 1 to 3 hours (or longer if necessary) in 1% aqueous safranin.

3. Rinse in water and differentiate the safranin in 0.025/N hydrochloric acid. Allow the acidulated water to react until the outline of the nucleus is definite and chromatin granules are discernible. Do not remove too much stain at this point. After differentiation, rinse thoroughly in water.

4. Stain in a 0.27 to 0.3% solution of crystal violet in 7% alcohol for about 30 minutes.

5. Rinse in water and differentiate in a 0.025/N solution of hydrochloric acid. Differentiation is far more important than the time of staining; it should be stopped when the nuclei take a definite outline.

6. Rinse in water and put in Gram's solution (1 g. iodine and 3 g. potassium iodide to 300 cc. water) for 1 to 3 minutes, or until the slides have turned a deep black.

7. Wash away excess iodine solution thoroughly and immerse slides in a 1% solution of mercuric chloride for 1 to 3 minutes, or until the sections have turned a bright blue.

8. Wash in water and blot excess cautiously, not letting the sections become completely dry.

9. Immerse in 95% alcohol for 4 to 6 seconds and transfer to carbol-xylol (25 parts phenol to 75 parts xylol) while the violet is still coming out in clouds.

10. Leave in carbol-xylol from 15 seconds to several minutes or until proper differentiation has occurred, which will be when prophase stages are deep blue and chromosomes and nucleoli are definitely red.

11. Wash in xylol, and stain in a 1% solution of orange G in clove oil for not longer than a minute.

12. Clear briefly in pure clove oil, wash thoroughly in xylol, and mount in balsam.

If it is desired that the violet be intensified in the spindles, sections should first be mordanted with potassium permanganate.

Quadruple Combinations

Safranin, crystal violet, fast green, and orange G may be combined for use on tissues in which there is either a variety of cell types or considerable chemical difference in the structure of the cells. The combination is not suitable for tissues with little differentiation, as in meristematic regions.

Conant's Quadruple Stain.—An excellent combination, giving clear results.

1. Bring slides down to 70% alcohol.
2. Stain 2 to 24 hours in 1% safranin in 50% alcohol.
3. Rinse thoroughly in water.

4. Stain in a saturated aqueous solution of crystal violet for about a minute.

5. Rinse in water.

6. Dehydrate through two changes of absolute alcohol.

7. Dip slides rapidly, for 5 to 10 dips, in 1% fast green in absolute alcohol, then transfer quickly to a saturated solution of gold orange (orange G may be substituted) in clove oil, agitating the slide until the adhering alcohol is completely diffused through the clove oil.

8. Transfer through three more jars of orange-clove oil solution, allowing several minutes in each, for further differentiation and clearing of the background.

9. Rinse thoroughly in xylol, then mount in balsam.

Johansen's Quadruple Stain.—The preceding method is not intended to correlate stain affinity with specific structures. To achieve as closely as possible this effect, the following procedure has been devised, employing the newer stain solvents. It is a simple procedure, even if the mixtures are rather complicated, inasmuch as differentiation is automatic and little need be left to personal judgment.

1. Bring slides down to 70% alcohol.

2. Stain in the methyl cellosolve-50% alcohol safranin solution (page 62) for 24 to 48 hours. Overstaining is not possible.

3. Rinse in tap water.

4. Stain in 1% aqueous methyl violet 2B for 10 to 15 minutes.

5. Rinse in tap water.

6. Rinse for 15 seconds in a mixture of equal parts 95% alcohol, methyl cellosolve, and tertiary butyl alcohol.

7. Immerse for 10 to 15 minutes in a fast green FCF solution prepared thus: take 1 part of a saturated solution of the dye in equal parts of clove oil and methyl cellosolve, 3 parts 95% alcohol, 3 parts tertiary butyl alcohol and 1% glacial acetic acid. The time may require some experimental determination according to the tissues and the fixing fluid. (The time cited is for formalin-aceto- (or propiono-) alcohol; tissues fixed in chrom-acetic fluids may require more time.)

8. Rinse briefly in a mixture of equal parts of 95% alcohol and tertiary butyl alcohol plus about 0.5% glacial acetic acid.

9. Immerse for about 3 minutes in an orange G solution prepared as follows: 1 part each of a saturated solution of the dye in methyl cellosolve, pure methyl cellosolve, and 95% alcohol.

10. Rinse briefly in a wash composed of 1 part each of clove oil, methyl cellosolve, and 95% alcohol.

11. Rinse in a wash made up of equal parts of clove oil, absolute alcohol, and xylol.

12. Rinse in two changes of xylol, then mount in balsam.

The staining solutions can accommodate a large number of slides before replacement is required, but the washing solutions in steps 6, 8, and 10 will need replacement frequently. As soon as one of them becomes saturated with dye, it should be renewed, otherwise the sharpness of the stains will be diminished.

The staining effects should be as follows: dividing chromatin red, resting chromatin purplish, nucleoli red (occasionally violet), nucleoplasm colorless or greenish, lignified cell walls bright red, cutinized cell walls reddish-purple, suberized walls red, cellulose cell walls greenish-orange, cytoplasm bright orange, middle lamellae green, starch grains purple with green or orange halos (the color of the halos soon becomes replaced by the purple in some types of material), plastids purplish to greenish, invading fungal mycelium green, the callose portion of the guard cells of stomata bright red and the remainder purple, and Casparian strips red and the remainder of the cell wall of the endodermis yellow. In sections of roots for origin of the lateral roots, the cytoplasm of the latter should be stained green, with purplish nuclei, while the cytoplasm elsewhere should be orange with red nuclei. The combination is exceptionally good for sections of lichens, as the algae are well differentiated, and also for *Puccinia graminis* telia and uredinia.

Other Coal-tar Dyes

Crystal Violet and Erythrosin.—This method is especially valuable where a sharp differentiation between weakly lignified and nonlignified tissues is desired and is also particularly useful for general differentiation as vascular structures are rendered readily distinguishable from neighboring tissues (Jackson 1926). The schedule must be adapted for different types and lots of material and often also according to the nature of the fixing fluid; in some materials the xylem elements stain satisfactorily in a few minutes, but in other types 30 minutes or longer may be required. The action of the erythrosin must be closely watched as it tends to replace the violet in lignified cell walls.

1. Bring slides down to water.
2. Stain in 1% aqueous crystal violet, 15 minutes as a trial.
3. Rinse quickly in tap water.
4. Dehydrate quickly but thoroughly in 95% and absolute alcohol.
5. Immerse in a saturated solution of erythrosin B in clove oil for 3 to 5 minutes.
6. Absolute alcohol and xylol, equal portions, 2 minutes or less.
7. Wash thoroughly in xylol, then mount in balsam.

Cooper's Triple Combination.—Primarily a cytological stain. Stain sections in 1% aqueous methyl green (use a strictly fresh dye for making

up the solution) for 1 hour, counterstain in 1% aqueous acid fuchsin for 1 minute, then stain in 1% aqueous erythrosin B for 2 to 3 seconds. Chromatin granules and nucleoli in early prophase stages of microsporogenesis are stained green, and the linin threads are stained red. In later stages the chromosomes are stained a brilliant green (Cooper 1931).

Smith's Picric Acid-Gram Stain.—This is likewise primarily a cytological stain (F. S. Smith 1934).

1. Bring sections down to 95% alcohol, or smears up to 70%.
2. Mordant 10 to 20 minutes in a solution composed of 1 g. each of iodine and potassium iodide to each 100 cc. of 80% alcohol.
3. Rinse in water, then stain in 1% aqueous crystal violet for about 15 minutes.
4. Rinse in water again, then place into a second iodine solution, of the same proportions as that noted in step 2, for a few minutes.
5. Rinse in 95% alcohol.
6. Flood slide quickly with a saturated solution of picric acid in absolute alcohol, then wash immediately with plain absolute alcohol for only a few seconds. The purpose of the picric acid is to fix the violet in chromatic materials.
7. Differentiate in clove oil. The violet will be extracted from the cytoplasm but not from the chromosomes.
8. Wash in xylol, then pass through two changes of pure xylol, allowing the slides to remain in one of them for about 1 hour before finally mounting in balsam.

Johansen's Methyl Violet-Erythrosin Stain.—This is a beautiful combination for mitoses in root tips (Johansen 1932).

1. Bring slides down to water.
2. Stain 15 to 30 minutes in a 1% solution of methyl violet 2B in distilled water.
3. Rinse off excess stain with water, then simultaneously differentiate and dehydrate with a saturated solution of picric acid in 95% alcohol. About 10 to 15 seconds suffice.
4. Stop action of the acid by placing the slide for 15 seconds in 95% alcohol plus 2 to 3 drops of ammonia per 100 cc.
5. Wash in pure 95% alcohol, for about 15 seconds.
6. Counterstain with a nearly saturated solution of erythrosin in equal parts of absolute alcohol and clove oil for 5 to 10 seconds.
7. Clear in clove oil for about 30 seconds, then wash thoroughly in two changes of xylol, and mount in balsam.

Resting and dividing chromatin is stained a brilliant purple; plastin dark red, cell walls red, and the cytoplasm pinkish.

Newton's Gentian Violet-Iodine Method.—For this method, see the chapter on Smear Methods (page 155).

Modified Cajal Basic Fuchsin-Indigocarmin Stain.—A cytological stain valuable on root tips (Hruby 1933).

1. Bring slides down to water.
2. Stain 5 to 20 minutes in a saturated aqueous solution of pararosanilin (basic fuchsin). (Do not use a dye labeled with the synonym "diamond fuchsin" or with the German "Diamantfuchsin." Neither of these dyes can be differentiated properly.)
3. Rinse in water until free stain no longer comes out.
4. Stain 5 to 15 minutes in a mixture of equal portions of saturated aqueous solutions of picric acid and indigocarmin.
5. Pass rapidly through 70% alcohol, in which the sections will appear red, then through 95% and absolute alcohol until the sections appear greenish.
6. Clear in xylol, and mount in balsam.

If acid instead of basic fuchsin is used, the procedure varies slightly:

1. Proceed to the end of step 4 in the above schedule.
2. Wash in water to which a trace of acetic acid has been added.
3. Wash rapidly in 70, 95%, and absolute alcohol, as indicated in step 5 above, then clear in xylol, and mount in balsam.

With onion root tips as an example, the coloration should be as follows: chromosomes and late prophase stages various shades of bright or dark red, early prophases bluish-red, nucleoli clear blue, spindle fibers and cell walls a dark blue against a light blue cytoplasm.

Foster's Tannic Acid-Iron Chloride Method.—This method was primarily designed for the cell walls in meristematic tissues but is capable of wider application, as noted in the Northen methods cited below.

1. Bring slides down to water.
2. Mordant in 1% aqueous solution of c.p. tannic acid for 10 minutes. (Add 1 g. sodium salicylate to each 100 cc. of solution to prevent growth of molds.)
3. Wash thoroughly in water.
4. Bring out stain by placing slides in 3% aqueous c.p. ferric chloride for several minutes. Then examine under the microscope: if the cell walls of the meristem appear black or dark blue and the nuclei and cytoplasm gray, the stain is correct. If the stain seems to be too weak, wash the slide thoroughly in water, and return to the tannic acid solution. Alternate between steps 2 and 4 until differentiation is satisfactory, taking care to wash the slide thoroughly in water each time a transfer is made.
5. Immerse in 50% alcohol for a few minutes.
6. Stain for 48 hours in a 1% solution of safranin in 50% alcohol.
7. Rinse in water, and destain cautiously in 70% alcohol very weakly acidulated with hydrochloric acid.

8. Pass through 70% and higher alcohols to xylol, then mount in balsam.

Cell walls should be colored an intense black, the cytoplasm violet or pink, plastin blue, and the nucleoli and chromosomes red. Other cytoplasmic or nuclear stains may also be used.

Northen's Variations of Foster's Method.—These procedures add a third stain to those given by the preceding method (Northen 1936) and are especially adapted to the staining of stems and of roots showing stages in the development of lateral roots.

A. If the safranin is retained by the tissues, the safranin may be applied first:

1. Stain 24 hours in 1% safranin in 50% alcohol.
2. Rinse for 10 seconds in 50% alcohol.
3. Immerse for 15 to 30 seconds in 0.5% tannic acid in 50% alcohol (use c.p. Merck or Mallinckrodt tannic acid).
4. Pass through two coplins of 70% alcohol, allowing 10 to 15 seconds in each.
5. Immerse for 10 to 20 seconds in 1% ferric chloride in 70% alcohol.
6. Place in 80% alcohol for 10 seconds, in 95% alcohol for 20 seconds, and in absolute alcohol for 30 to 40 seconds.
7. Stain for 30 to 60 seconds in 0.5% crystal violet in clove oil.
8. Rinse in xylol (plus a trace of absolute alcohol), pass through pure xylol, then mount in balsam.

When properly applied, the procedure should stain parenchyma and collenchyma cell walls purple, fiber walls red or purple, lignified cell walls red, walls of the cambial cells black, nucleoli red, and cytoplasm purple. If too much safranin has been extracted, reduce the time in the alcohols; if the safranin has been insufficiently differentiated, allow more time in the alcohols or add a drop of hydrochloric acid to one of the 70% wash alcohols.

B. If the safranin is easily extracted, the tannic acid-ferric chloride part should be applied first.

1. Bring slides down to 70% alcohol, then leave for 2 to 3 minutes in 1% tannic acid in 50% alcohol.
2. Rinse for 15 to 20 seconds in each of two coplins of 50% alcohol.
3. Immerse in 3% ferric chloride in 50% alcohol for 30 to 60 seconds. The slides will become blackened, but this is of no moment.
4. Rinse in 50% alcohol.
5. Stain for 24 hours in 1% safranin in 50% alcohol.
6. Rinse in 50% alcohol.
7. Destain partially in 70% alcohol; since more safranin will be removed during the following steps, the differentiation should not be carried too far.

8. Dehydrate in 80, 95%, and absolute alcohol for 10 to 30 seconds in each.

9. Stain for 1 to 3 minutes in 0.5% light green (or substitute fast green) in clove oil plus 7 cc. absolute alcohol to each 100 cc. clove oil.

10. Rinse in absolute alcohol, then in xylol, and mount in balsam.

By this procedure, cambial cell walls and parenchyma cell walls are black, lignified walls are red, collenchyma walls are a blackish-red, nuclei are red, and the cytoplasm a bluish-green.

Acid Fuchsin and Fast Green.—This histological combination is suggested for the marine Phaeophyta as a quick and easy method giving good differentiation.

1. Bring slides down to 95% alcohol.
2. Stain 20 minutes in 1% acid fuchsin in 70% alcohol.
3. Rinse off excess stain by slowly plunging slide 2 to 3 times into a large dish of tap water.
4. Rinse in 95% alcohol for a few seconds.
5. Wipe off underside of slide, and cover sections with the usual solution of fast green in methyl cellosolve-absolute alcohol-clove oil for a few seconds.
6. Clear in absolute alcohol-clove oil-xylol mixture.
7. Wash in xylol, and mount in balsam.

The sections should, as with all Phaeophyta, have been covered with celloidin, either before or after removing the paraffin with xylol. When used on sections of the fruiting frond of any of the Laminariales, for example, the zoospores are stained a brilliant magenta, the nuclei and pigment bodies various shades of red, the cell walls light green, and the layers of the slime cap are differentiated by the green. This is the only known combination which will reveal clearly the internal structure of the paraphyses.

Iodine Green or Methyl Green and Acid Fuchsin.—If one wishes to stain lignified tissues with a green dye, iodine or methyl green (not methylene green) works admirably. These two dyes stain similarly to safranin. Methyl green is probably easier to differentiate than iodine green, but is extracted more rapidly.

1. Bring slides down to water (or have freehand sections in water).
2. Stain 12 hours or longer in 1% aqueous iodine or methyl green.
3. Wash in water until the stain is almost but not entirely removed from nonlignified elements.
4. Counterstain about 3 to 8 minutes in 1% aqueous acid fuchsin. The stain should not be allowed to react long enough to extract the green from the lignified tissues. The point may be determined by removing a slide from the staining solution and examining under the microscope.

5. Rinse rapidly in 95% and absolute alcohol.

6. Clear in clove oil solution, pass through xylol, and mount in balsam.

Both stains may be used as 1% solutions in 70% alcohol if desired; washing may be in 70% alcohol. This method may be better than using the stains in aqueous solutions, but the use of an alcoholic wash naturally makes it somewhat more expensive. The combination may be used as a cytological stain, but the washings must be made very quickly. Methyl green is sensitive to alkalies, so that all solutions should have a trace of acetic acid added to them. If there was no acetic acid in the killing fluid, a chromatin stain is unobtainable. The resistance of the stain to alcohol may be increased by mordanting the sections for 5 minutes previous to staining with 1% aqueous iodine solution. The green stains, unfortunately, do not keep any too well. Chromosomes and nuclei are stained green, plastin and cytoplasm light red.

Feulgen's Nucleal Reaction.—This is not so much a staining procedure as a chemical reaction for chromatin; consequently it is being described elsewhere (page 95).

CHAPTER VIII

SPECIAL METHODS

Feulgen Nucleal Reaction.—This procedure was originally devised as a microchemical test to distinguish the particular type of nucleic acid found in chromatin from similar types. The essential feature of the method is the production of a specific purple (or occasionally magenta) color when a reduced, or colorless, form of pararosanilin (basic fuchsin) is brought into contact with an aldehyde in the presence of the nucleic acid peculiar to chromatin.

The original version (Feulgen and Rossenbeck 1924) has undergone innumerable variations. The majority of the proposed changes have since been demonstrated to be of little or no value (de Tomasi 1936). Botanical technicians who have experimented with the technique have demonstrated beyond all doubt its great utility on all types of material (*e.g.*, Margolena 1932*a*, Westbrook 1930), and it has been of especial service where it is necessary to differentiate between true chromatin and other substances which give a staining reaction similar to that of chromatin with ordinary coal-tar dyes.

The staining solution is prepared as follows (de Tomasi 1936): Dissolve 0.5 g. basic fuchsin [use a Stain Commission type especially certified for use in the Feulgen technique (Scanlan and Melin 1937)], by pouring over it 100 cc. boiling distilled water and shaking thoroughly. Cool to 50°C., and filter. To the filtrate add 10 cc. 1/N hydrochloric acid, then 0.5 g. potassium metabisulphite. Shake thoroughly, stopper tightly, and place in the dark for about 18 hours.

All substances should be of the highest purity: the water should be glass-distilled and the chemicals of reagent quality. If a satisfactory certified basic fuchsin sample is not available, Grübler's Diamantfuchsin, as specified by the originators, almost always works well. The 1/N hydrochloric acid may be prepared with sufficiently close accuracy by adding 82.5 cc. reagent hydrochloric acid to 1 liter of glass-distilled water.

The staining is carried out as follows: Bring the slides down to distilled water, and rinse in cold 1/N hydrochloric acid; place in fresh 1/N hydrochloric acid, and heat the solution quickly to 60°C. (but not above that temperature), allowing the slides to remain for 4 to 5 minutes. Next rinse in cold 1/N hydrochloric acid, then in distilled water. Trans-

fer the slides to the staining solution, which should be either colorless or of a light straw color. The optimum time for animal tissues is 2 hours, but most plant tissues require from 3 to 5 hours. Remove a slide from the stain, touch the lower edge to absorbent paper, then pass quickly to the first of three closed coplins, each containing the following differentiating solution: 1/N hydrochloric acid, 5 cc.; 10% aqueous potassium metabisulphite, 5 cc.; distilled water, 100 cc. Wash the slides for 10 minutes in each of the three jars. Then rinse in distilled water and dehydrate. Plant materials may be counterstained momentarily with 0.05% fast green in 95% alcohol. Mount preferably in dammar balsam, although strictly neutral Canada balsam serves equally well.

Solutions should be made up only as required, since none of them keeps well. In time the staining solution will become colored and should then be discarded.

Various methods have been devised to secure satisfactory samples of the basic fuchsin dye or to purify such samples (Scanlan and Melin 1937), but such treatments are either unnecessarily cumbersome or call for more apparatus and chemical knowledge than the average technician possesses. Given a certified dye sample, the staining solution can be rendered practically colorless by adding a decolorizing carbon to the solution after it has been allowed to bleach for 24 hours (Coleman 1938). To each 200 cc. of the basic fuchsin solution prepared as directed above, add 2 g. potassium metabisulphite and 10 cc. 1/N hydrochloric acid. After bleaching for 24 hours, add 0.5 g. of a powdered decolorizing carbon or charcoal (the brand known as Supra-neutral Norit, obtainable from L. A. Salomon, 216 Pearl Street, New York, N. Y., has been recommended). Shake for a few minutes, then filter rapidly through coarse filter paper.

A procedure has recently been devised (Semmens and Bhaduri 1939) for the differential staining of the nucleoli following the application of the Feulgen technique. The originators claim that the procedure does not affect the chromatin stain, but experience has shown that the fuchsin is often completely removed. The method may be briefly outlined:

1. Mordant for 1 hour in filtered 5% aqueous sodium carbonate.
2. Wash for 30 minutes in distilled water.
3. Stain for 10 minutes or longer in a 0.5% solution of light green SF yellowish in 100 cc. of 90% alcohol plus a few drops of anilin oil.
4. Rinse with 70% alcohol saturated with sodium carbonate. This step has been the most troublesome one. The amount of carbonate that dissolves in the alcohol is highly variable: if too little dissolves, differentiation is poor, whereas, if the proportion is too great, complete discoloration results.

5. Transfer to 95% alcohol, and leave until the green dye has been removed from the cytoplasm. At least 10 minutes will be required.

6. Immerse in absolute alcohol for 10 minutes or longer.

7. Place in absolute alcohol and xylol, equal parts of each, for 10 minutes.

8. Clear through xylol, and mount.

Osmium Impregnation Methods.—All methods of osmic acid impregnation are in general tedious, variable, and frequently unsuccessful (Bowen 1929). The following rules have been laid down:

1. Material should be absolutely fresh. Do not use plants which have lingered about the laboratory or greenhouse under unfavorable conditions. Use pieces of material cut into as small portions as possible. Large root tips and other massive parts will probably not react successfully unless subdivided.

2. Use osmic solutions, both for fixing fluids and for the osmication process, which are positively fresh—not over one or two weeks old—and which have been kept properly clean and preserved from the action of light.

3. Keep the ingredients of fixing fluids in separate stock solutions, and mix them just before using.

4. All fixation, and especially all osmication, steps must positively be conducted in glass-stoppered bottles. If such bottles are not available, it is not worth wasting time and supplies attempting osmication.

5. After the material is finally washed out in running water after osmication, do not spend more than five or six days at the most in the alcohols before embedding in paraffin. For these post-osmication processes, the use of corked shell vials is permissible.

The Kolatchev Method (Bowen's Schedule).

1. Fix for 24 hours in Champy's fluid:

1% aqueous chromic acid	7 cc.
3% aqueous potassium bichromate	7 cc.
2% aqueous osmic acid	4 cc.

Do not attempt to work up too much material in one bottle. Gently agitate the bottle occasionally.

2. Wash in running water for 24 hours. It is very important to remove all traces of the chromium.

3. Place the material in new glass-stoppered bottles and commence the osmication process. This may be carried out by one or all of several methods:

a. Place the material in 1% osmic acid solution, and then

(1) Put the bottles in an incubator at 35°C. for periods of 4–9 days.

(2) Put the bottles in an incubator at 40°C. for 8 hours. Then transfer the bottles to an incubator at 35°C. without changing the osmic solution. Leave in this second incubator for periods such that the total time in the acid is from 4 to 9 days.

b. Place the material in 2% osmic acid solution, and then proceed:

(1) As in method *a*(1).

(2) As in method *a*(2).

It is generally advisable, to obtain a greater chance of success, to divide each lot of material into four portions and to try all four methods simultaneously. Otherwise, method *b*(2) is most likely to afford successful impregnation. In all methods, a volume of osmic acid solution at least equal to twice the bulk of the material should be used.

The osmic solutions will soon begin to become blackened. When the blackening has become pronounced, rinse out with distilled water, transfer to a clean bottle, and cover with fresh osmic solution.

4. Wash in running water for 24 hours. It requires considerable experience to determine when to terminate the osmication process. There is no way of knowing when anything whatever has been successfully impregnated. Trial by sampling is the only method. If the osmication period was too short, nothing will be blackened; if too long, the material may be too overblackened for critical investigation. Each day after the four-day minimal period a few samples may be removed from the bottle with a pipette (take every precaution not to let the pipette be contaminated by other substances, particularly chromium compounds), dehydrated, and embedded.

5. Dehydrate by any rapid process, and embed in paraffin. The impregnation is relatively stable, and no unusual precautions are necessary.

6. Cut sections not over 6μ thick and mount on slides as usual. After the sections have dried down, remove the paraffin with xylol, pass through a change of absolute alcohol to ensure complete dehydration, then pass through another jar of xylol, and mount in balsam. Or one may bleach cautiously by the potassium permanganate-oxalic acid method after taking the slides down to water. Counterstaining, if desired, may be with eosin, erythrosin, orange G, fast green, or a similar dye, made up with 95% alcohol.

The Weigert (Mann-Kopsch) Method.

1. Fix in corrosive-osmic:

1% aqueous osmic acid	1 part
Saturated solution of mercuric chloride in 0.75% aqueous sodium chloride	1 part

As soon as the material has become blackened, which with some plants may be in 15 minutes, pour off the fluid, and add fresh fixative, repeating

if necessary later on. The time of fixation is variable and can be determined only by trial. Periods of 1, 2, or 4 hours may be tried.

2. After fixation, wash in running water for 30 minutes.

3. Wash in distilled water, and begin the osmication with 2% aqueous osmic acid. It would be best to place the material in an incubator at 25°C. if the room temperature fluctuates too much. As in the Kolatchev method, the osmication step is subject to wide variables, which should be determined as described for that method.

4. Wash material for 24 hours in running water.

5. Dehydrate, embed, section, deparaffin, and mount, as described for the preceding method.

The results are so variable and interpretation so problematical that one should proceed with great caution when studying the resulting preparations. Reference should be made to the papers of Bowen (1929, for bibliography) and other reliable investigators.

Mitochondria.—Far more work has been done on the mitochondria of animal tissues than on those occurring in plants, but the latter, despite inherent difficulties, can be easily manipulated if the requisite attention to details is exercised.

Ordinary technical methods do not reveal the mitochondria, since the acids dissolve them. Special chemicals are required in the fixing fluids, which should always be ones that give basic fixation images.

One of the most successful killing fluids consists of:

Cupric bichromate	5 g.
Cupric oxide	1 g.
10% aqueous acetic acid	1 cc.
Distilled water	100 cc.

The fluid should be made up at least a day before being used, and it should be shaken frequently during that period. Fix from 36 hours to six days, and wash out with two changes of 70% alcohol within ½ hour; immerse for the same length of time in 80 and 95% alcohol. Complete dehydration with two changes of absolute alcohol, allowing 1 hour in each, clear in xylol or tertiary butyl alcohol, and embed. Cut sections not over 10μ in thickness. Stain with iron hematoxylin; as the mitochondria do not retain the stain so well as do chromatin-containing structures, destaining should not be carried so far as is ordinarily done when nuclei or chromosomes are being studied (Fig. 1).

Another formula, which gives a similar fixation image, is

Chromic acid	5 g.
Glucinum carbonate	3 g.
Distilled water	200 cc.

If the glucinum carbonate is not in excess, a little more should be added. Dehydrate as described above.

Still another formula also gives beautiful results on occasion:

10% aqueous chromium sulphate.....	1 part
8% aqueous formalin neutralized with an excess of calcium or lithium carbonate.....	1 part

If the washing is with 70% alcohol and the dehydration is carried through rather rapidly, the cytoplasm is granular, and the mitochondria

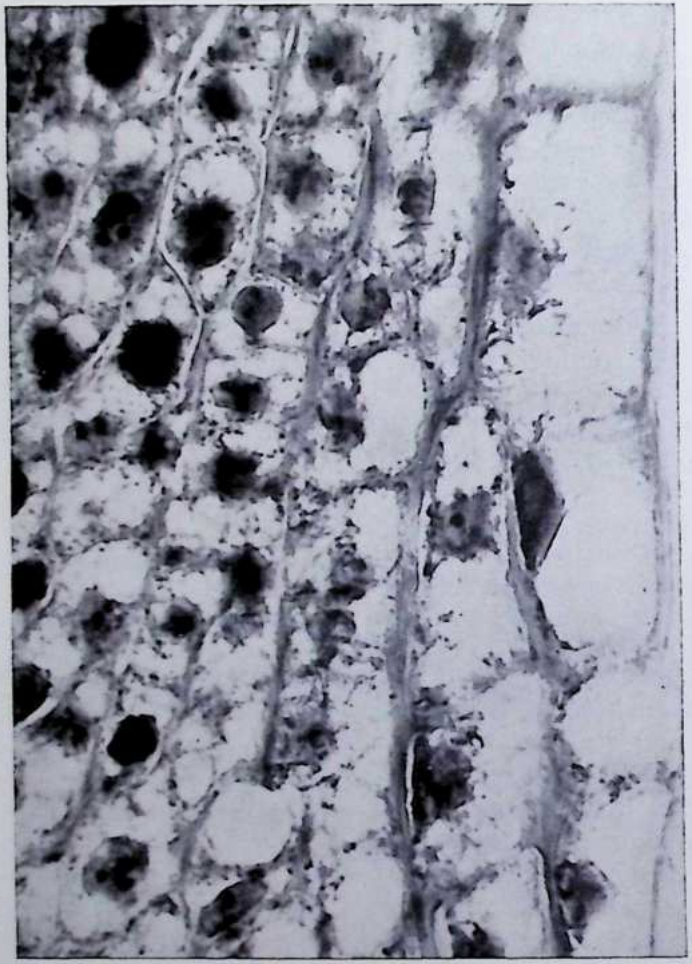


FIG. 1.—*Allium cepa:* portion of a longitudinal section of a root tip fixed and stained for mitochondria. Fixed with Zirkle's reduced chromic fluid; stained with iron hematoxylin.

are preserved in nearly every part of each piece of tissue. If washing is with water and dehydration proceeds slowly, the mitochondria are dissolved from the epidermis and cortex, remaining only in the central

portions. Dividing nuclei and the cytoplasm are well fixed (the cytoplasm is less granular than after alcohol washing), and in the cytoplasm the vacuoles are so sharply delineated that their growth as well as their behavior during mitosis can be studied.

Licent's fluid also gives good results:

2% aqueous chromic acid	50 cc.
Neutralized formalin	30 cc.
Glacial acetic acid	A few drops
10% aqueous nickel acetate	10 cc.

To this mixture 10 cc. of a saturated aqueous solution of mercuric chloride may be added. Make up the fluid only as required.

Mitochondria are perfectly preserved by quinone (parabenzoquinone). Treat tissues for 1 hour in quinone dissolved in normal saline solution, then transfer to any other fixative (Bouin's or Allen's B-15 Bouin). The quinone may be at any concentration: 0.05% for delicate tissues and 0.5% for tougher ones. Stain with iron hematoxylin.

The complicated staining schedules used by animal technicians are practically useless on plant materials and fade quickly.

Milovidnov's Method of Differentiating between Bacteria and Mitochondria.—It is frequently necessary to distinguish between bacteria and mitochondria in sections of infected tissue: the following method affords a means of differentiating between the two (Dufrenoy 1928).

1. Kill and fix for 24 hours in

1% aqueous chromic acid	50 cc.
1% aqueous potassium bichromate	50 cc.
Formalin, neutralized with powdered calcium carbonate	8 cc.

2. Wash for 24 hours in running water, then dehydrate and embed by the tertiary butyl alcohol method. Cut sections as thin as possible, preferably at 4μ.

3. Dissolve paraffin from slides, dip slides into a very thin solution of celloidin in equal parts of absolute alcohol and ether, then run down to water.

4. Stain in the following solution, heated to about 80°C.

Acid fuchsin	2 g.
Anilin water	10 cc.

(Anilin water consists of about 2 parts anilin oil to 100 of water, very thoroughly shaken.)

5. Wash in running water.

6. Destain for a few seconds in

Aurantia	0.5 g.
70% alcohol	100 cc.

7. Wash again in water.
8. Treat for a few minutes in

Phosphomolybdic acid	1 g.
1% aqueous sodium hydroxide	10 g.
Distilled water	100 cc.

9. Rinse in water.
10. Stain for a few minutes in Unna's polychrome methylene blue, prepared as follows: a solution of 1 part methylene blue dye and 1 part potassium carbonate in 20 parts 95% alcohol and 100 parts distilled water is evaporated down to 100 parts. It may be used at once or diluted with an equal part of anilin water for sections.
11. Rinse again, dehydrate very quickly with 95% and absolute alcohol, pass through xylol to balsam.

Bacteria stain a deep violet blue, mitochondria and plastids red.

Freehand Sections.—The term "freehand sections" was originally applied to sections which were cut by means of a razor from material held in the hands or placed against a length of elder pith. Later it came to include sections cut from live or preserved, but not embedded, material by means of a sliding microtome. The present usage includes all sections, no matter how cut and whether embedded or not, which are handled loosely and not attached to slides by means of an adhesive.

Few, if any, technicians nowadays use the primitive sectioning methods employed by the early botanical technicians, since the availability of all types of sliding microtomes greatly facilitates sectioning.

To say the best for them, freehand sections of nonembedded materials are rarely perfect insofar as the sectioning goes. The thickness, for example, is generally very uneven, but when microtomed at 25μ or over, it becomes better. The material must be fairly rigid, not soft or succulent or composed of mixed soft and hard tissues. The most difficult problem is to clamp the piece of material tightly enough to prevent it from bending when struck by the knife yet not so hard that it is crushed. Pieces of wood or woody stems give comparatively little difficulty unless they are very small.

Any type of sliding microtome may be used for cutting freehand sections. None, however, has a really satisfactory clamp, consequently one must exercise some ingenuity to devise supplementary aids. For instance, one may obtain a suitable cork, cut away sectors from opposite sides, then bisect parallel to the flat sides and cut longitudinal U shaped grooves down the center of the bisected sides. The depth of the groove should be shallower than the radius of the piece of material. (Or use two pieces of thick cork linoleum and cut a groove in each.) The material is placed in the grooves and the whole placed in the clamp.

The operation of the sliding microtome should be carefully studied. Most such microtomes require hand adjustment of the feed, although a few models resemble all rotary microtomes in having an automatic feed. It is impossible to cut good freehand sections thinner than about 16μ; most technicians cut at about 30μ. The knife should be oriented at a wide oblique angle in order that as much of the length of the blade as possible may be used for cutting purposes. The knife must, obviously, be kept sharpened and free from nicks. With the material clamped, adjust the feed until the material almost touches the underside of the knife. If the material is fresh, moisten it and the knife with water by means of a camel's-hair brush; if preserved, use the preservative or 50% alcohol. In some instances, a mixture of equal parts of 95% alcohol and glycerin affords a better lubricant. As the sections are cut, move them toward the back of the knife with the brush and leave there for a few minutes to lessen danger of curling. Many technicians hold the brush over the piece of material as a further precaution against curling. Fresh material should then be transferred to watch glasses containing formalin-aceto-alcohol or other desired fixing fluid; sections of preserved material should be placed in 50% alcohol. One should cut about twice as many sections as might actually be required, so that the best ones may be picked out for mounting after the staining and dehydration are completed.

Hard woods should be sectioned as described in the following section. Celloidin sections are commonly treated as if they were freehand sections (see Chap. XI). Paraffin sections of woody stems and other objects from which essential portions will not drop out and become lost may also be treated as if they were freehand sections. Sections of old *Aristolochia* stems may, for instance, be placed in a watch glass of xylol to remove the paraffin and then carried down to weak alcohol or water for staining.

The staining and dehydration may be carried out in watch glasses; the liquids are removed by means of pipettes. Useful stain combinations include: safranin and either Harris' hematoxylin, fast green, anilin blue or crystal violet (the last dye especially for gymnosperm tissues), iodine or methyl green with acid fuchsin, iron hematoxylin and orange G, or any other stain recommended for lignified cell walls (see page 64), plus a contrasting cytoplasmic or cellulose cell-wall stain. Wide changes between water and all alcoholic percentages can be made without much damage to the tissues. When the staining is completed, commence rapid dehydration according to the hygrobutol method (page 110), no matter in what solvent the sections might lie. Give two changes of pure hygrobutol. Mounting may be done directly in balsam, but it is preferable to place first in dilute balsam and partially evaporate the solvent. Xylol and similar hydrocarbons should be avoided since they

desiccate the sections and cause them to become very brittle and to curl up.

Hard Wood Sectioning.—Hard woody stems have long been exasperatingly difficult to microtome. It is now possible to cut relatively thin sections in a good sliding microtome by a simple expedient (Crowell 1930). Arrange a large Erlenmeyer flask over a gas flame near the microtome; from the flask run a narrow-bore aluminum or monel metal (not copper) tube, in which a few coils have been turned, to a position above the block holder in the microtome. Adjust the heat under the flask so that the water boils slowly. Below the coils in the tubing place another burner (preferably with a fishtail attachment) in order to vaporize all the water before it leaves the open end of the tube. No water of condensation should leave the tube, but the steam should condense upon the wood, keeping it hot and wet. Sections as thin as 10μ, free from air and curling, may now be cut, and thereafter treated as freehand sections.

Maceration of Tissues.—Sections of plant stems, roots, barks, and other organs rarely convey an accurate conception of the real nature of the cells of which they are composed. The only method which reveals cells in their entirety is the dissociation method. By this procedure the stem or other organ is treated with chemicals which dissolve the middle lamellae and allow the cells to become separated from one another. Thickenings, pores, and other distinguishing characteristics of the cells are clearly brought out.

Jeffrey's Method.—First cut the material (either fresh or dry) into small slices or slivers about 300μ thick. Either boil and cool repeatedly until free from air, or use the suction pump for the same purpose. Then macerate in a solution of equal parts of 10% aqueous nitric acid and 10% aqueous chromic acid. The solution may be heated in the paraffin oven for woody tissues, but not for herbaceous stems. The time varies according to the material, but cells should begin separating in about 24 hours. A thick glass rod with rounded end may be used to crush the material very gently. If it does not crumble easily, replace the macerating mixture with fresh fluid and continue action. Wash very thoroughly with water to remove the acids. The use of a centrifuge is advisable in order to speed up the process. The material may now be stained with any suitable basic stain; safranin may be recommended. Leave in 1% safranin for about 6 hours; rinse thoroughly in water, then dehydrate by the fairly rapid addition of hygrobutol. Give two changes of pure hygrobutol, then infiltrate with balsam highly diluted with hygrobutol, and evaporate down to a mounting consistency. Take care not to put too much material on the slide (see Frontispiece).

Various other methods have been proposed (*e.g.*, Harlow 1928), but Jeffrey's method is the simplest and has been found to be entirely adequate for all maceration purposes.

Preservation of Plant Materials.—The permanent preservation of all types of plant materials in their original colors has long been a perplexing problem. Considerable success has recently been attained with many types of material, but others, such as the Rhodophyta, still give plenty of trouble. In the second section of the present text, methods are cited for many of the plants therein discussed, but the following general methods may be utilized.

1. *A saturated solution of boric acid in glycerin* is said to be excellent.

2. *Conant's Hot Method.*—Prepare a stock solution of 50% acetic acid saturated with cupric acetate. For use, dilute 1 part stock solution with 4 parts water, place the material in this diluted solution, and boil until the permanent "copper chlorophyll" develops in the material.

3. *Conant's "Cold Pack" Method.*—Dilute 1 part of the stock solution described in the preceding paragraph with 10 parts of 5% aqueous formalin. Place specimens in the fluid, and leave until the proper chemical changes occur. This may require several weeks, but a more natural green than that imparted by the hot method usually results.

For more delicate material, such as the Chlorophyta, add a few drops of the stock solution to the following formalin-aceto-alcohol variation:

Formalin	6.5 cc.
Glacial acetic acid	2.5 cc.
50% ethyl alcohol	91.0 cc.

4. *The standard formalin-aceto-alcohol solution* may also be used, adding 0.2% cupric sulphate (Blaydes 1937). Or the proportions of the formalin-aceto-alcohol may be changed as follows, adding the same percentage of cupric sulphate:

Formalin	10 cc.
Glacial acetic acid	5 cc.
70% ethyl alcohol	85 cc.

Material preserved in these mixtures may, if necessary, be embedded for sectioning. Preliminary experiments show that formalin-propiono-alcohol is even better than formalin-aceto-alcohol, since the acetic acid of the latter occasionally causes artifacts.

5. *Dissolve cupric acetate crystals in 50% acetic acid* until no more are taken up. To 1 part of the solution, add 4 parts water. Place the material or specimens into this solution, and bring the latter to boiling over a flame. The green color of the material will change first to yellowish-green, then back to green. The boiling may take from 3 to 15 minutes. When the color appears satisfactory, remove the material, wash in water, and store in 70% alcohol plus about 5% glycerin to prevent excessive evaporation.

6. *Keefe's Preserving Fluid.*—This solution is supposed both to kill and to preserve the green color. In the experience of most users the results are very inferior to Conant's methods. Gymnosperm material and fruits, particularly, are badly preserved. The color of flowers is not preserved, but the fluid, if 10 g. copper acetate is substituted for the copper chloride and uranium nitrate and if it is diluted somewhat with water, is excellent for the Cyanophyta.

50% ethyl alcohol	90 cc.
Formalin	5 cc.
Glycerin	2.5 cc.
Glacial acetic acid	2.5 cc.
Copper chloride	10 g.
Uranium nitrate	1.5 g.

Frozen Sections.—The freezing microtome, so commonly used in clinical laboratories, has scarcely been employed in botanical microtechnique because plant materials do not lend themselves readily to the customary freezing methods.

Many types of plant materials containing both soft and hard tissues may, however, be embedded in an agar matrix and excellent sections may then be cut on a freezing microtome (Evenden and Schuster 1938).

Fix with formalin-aceto- (or propiono-) alcohol, rinse with water and immerse in 5% agar in distilled water, and pour into heavy paper trays of the sort used for paraffin embedding. Allow the agar to solidify. Block and place in the freezing microtome.

Some practice is required in judging the degree of freezing of the material. Before each section is made, the material should be partially thawed to a depth approximating the thickness of the section to be cut. This may be done by rubbing the finger gently over the face of the block or moistening with 50% ethyl alcohol. The directions for operation of the freezing microtome, which come with each machine or which can be obtained from the manufacturer, should be explicitly followed, particularly with regard to the attachment of the cylinder of freezing gas.

The solidifying qualities of the agar decrease with age and remelting. The stock solution should not be remelted more than three or four times. If necessary to preserve the blocks, wrap in wet paper toweling arranged with a wick and water reservoir to insure a constant moisture supply, then place the whole under a bell jar. The blocks will keep, in this fashion for two or three weeks.

Those who wish to experiment further with freezing microtome methods should consult texts on histological technique, clinical laboratory methods, or recent research papers (*e.g.*, Geschichter, *et al.*, 1931; Hjort and Moulton 1931).

Special Fixation-staining Methods

Tannins.—To show the distribution and nature of tannin-containing vacuoles, fix in

Ferrous sulphate	2 g.
Formalin	10 cc.
Distilled water	90 cc.

Wash in water after 48 hours' immersion, embed, section at not over 10μ, remove paraffin from sections, and mount in balsam without staining. Root tips of *Pinus* are exceptionally favorable material.

Nucleoli.—After fixation with a fluid giving an acid fixation image, the resting nucleus is a hollow body with a centrally located, darkly staining, spherical nucleolus, and a periphery composed of the chromatin reticulum. The colorless halo surrounding the nucleolus is thus quite evident. The halo surrounding the nucleolus has been reported by some cytologists to be a distinct structure in the living cell, but others more correctly consider the halo to be an artifact. After fixation in a fluid giving a basic fixation image, the nucleus is a solid body composed of fixed nuclear lymph in intimate contact with a centrally located nucleolus. In this type of fixation image there is no halo.

The best fixing fluids for nucleoli are mixtures of bichromates and acetates. The acetates seem to penetrate the tissues more rapidly and thus primarily determine the fixation image. The bichromates harden the image and enable it to pass through the dehydration stages relatively unaltered. Most of the fixing fluids designed to fix chromatin have no cation except hydrogen. These solutions are satisfactory preservatives of chromatin but are quite erratic in the fixation of nucleoli. When the cation of a chrom-acetic mixture is one of the group of heavy metals (*e.g.*, copper, mercury, uranium, or lead), both chromatin and nucleoli are well fixed and mordanted (Zirkle 1928).

In the chapter on Staining Procedures the effects of different dyes on nucleoli have been noted. To distinguish the nucleolus during mitoses, staining in the following mixture for about 12 hours has been recommended:

Distilled water	100 cc.
Iodine green	0.15 g.
Acid fuchsin	0.01 g.

Dehydration should be rapid to prevent excessive removal of stain.

Mycorrhiza.—Mycorrhizas of forest trees occur on the smallest rootlets and in the more superficial layers of the soil. *Alnus* growing on the banks of streams furnishes an excellent source of material, as the

mycorrhizas are easily recognized on roots growing in the water or along the banks. Fixation of such types may be in 1% chrom-acetic or in formalin-propiono-alcohol.

Another fixing fluid which has been recommended is 5% chromic sulphate in 4% formalin saturated with picric acid or preferably with salicylic acid (Cohen and Doak 1935). The ectotropic type of mycorrhiza is more difficult to recognize; the roots of the orchid *Corallorhiza* furnish the best material. Portions of the root may be fixed with the fluid just mentioned or with chrom-acetic.

The ordinary safranin-fast green schedule gives a good stain on *Alnus;* a triple combination is usually excellent on *Corallorhiza;* or the following method might be tried. Stain sections for 30 minutes in 3% aqueous acetic acid saturated with orsellin BB (a Grübler dye), rinse with water, pass through the alcohols to 95%, counterstain with 1% crystal violet in clove oil (prepare by adding enough alcohol to 1 g. of the dry dye in a flask to form a paste, heat the flask gently to drive off excess alcohol, then add 100 cc. clove oil) for 5 minutes, rinse with xylol, then clear in another jar of xylol, and mount in balsam.

If it is desired to cultivate the mycorrhiza-forming organism, Arculariusʼ solution may be utilized:

Malt extract	30 g.
Dibasic potassium phosphate	8 g.
Potassium carbonate	6 g.
Agar	25 g.
Distilled water	1000 cc.

Plasmodesma.—None of the usual embedding methods gives as good or as striking pictures of plasmodesma as do temporary mounts of freshly stained tissue. Although the mounts are only temporary, the preparations give a clear and vivid picture of protoplasmic continuity between cells (Crafts 1931). The following solutions should be made up in advance:

Killing Solution: Potassium iodide, 0.75 g.; iodine, 1.50 g.; water, 100 cc.

Mordanting Solution: Potassium iodide, 1.25 g.; iodine, 1.0 g.; 5% aqueous sulphuric acid, 100 cc.

Staining Solution: (1) 5% aqueous sulphuric acid; (2) crystal violet, 0.5 g.; distilled water, 100 cc.

Mounting Solution: Glycerin, 30 cc.; distilled water, 60 cc.; zinc chloride, 2 g.; potassium iodide, a trace; iodine, 0.2 g.

The killing and mordanting solutions should be mixed and warmed until the iodine has dissolved to the point of saturation. The mounting solution should also be warmed a little and a small crystal of potassium iodide added, which dissolves some of the excess iodine. The resulting

solution should be of a light tan color. All solutions containing iodine should be kept in brown bottles in the dark when not in use.

Place fresh sections in the killing solution for 5 minutes; then swell for 5 minutes in 10% sulphuric acid. Transfer to the mordanting solution for 5 minutes, followed by washing in the 5% sulphuric acid until the iodine starts to fade. The staining solution is prepared by placing 1 cc. of the sulphuric acid in a small watch glass and adding the violet solution drop by drop until a deep green color is obtained. This unstable stain solution should be used at once, and discarded when it starts to fade. Transfer sections to the stain and allow to remain until darkened. Remove sections to a slide, brush with a camel's-hair brush on both surfaces to remove any adhering precipitates, then mount in the mounting solution. The sections are good for several weeks, and may even be mounted in glycerin jelly for longer preservation.

The most satisfactory and easily available material in which to demonstrate plasmodesma is the pith tissue from the center of a potato tuber. Cross sections of the leaflets of *Cycas* are also excellent.

Statoliths.—The name is given to the starch grains found in the basal region of the cells of the root cap. They are rarely found in root tips grown in nutrient solution, consequently tips grown in soil should be used. Most fixing fluids so hydrolyze the statoliths that they are not revealed by the subsequent staining. LaCour's fluids have given good fixation. When the sections are stained by a triple combination, the statoliths usually but not always take up the violet dye; when a quadruple combination is used, they absorb the green.

Cilia.—Drop material into a concentrated aqueous solution of mercuric chloride; after 10 to 20 minutes, wash in water; mordant 10 to 15 minutes in 0.33 to 1.65% phosphotungstic acid (discard the mordant after using once); wash well in water; stain in toluidin blue (a 1:1000 to a 1:5000 dilution) for 30 seconds to several minutes, depending on the form, at 50 to 60°C.; dehydrate through the alcohols, clear in xylol, and mount in balsam. Use the centrifuge whenever necessary. Cytoplasm should be light blue, cilia and basal bodies dark blue.

Antherozoids.—Put 1 drop of water containing the antherozoids on a chemically clean slide, and fix by inverting the drop over the mouth of a bottle containing osmic acid. About 2 minutes is required for fixation. Then set aside to dry. Place slides in a saturated aqueous solution of tannic acid for several hours (Steil 1933). Remove and wash the slides thoroughly. Almost any brilliant coal-tar dye may now be used: safranin, the violets, and fast green all give excellent results. Iron-acetocarmin is good for the sperms of all Pteridophyta. The stain may be allowed to react for 10 minutes to several hours. After staining, rinse the slide in water, dehydrate with two changes of absolute alcohol, clear with clove oil, pass through xylol, and mount in balsam.

CHAPTER IX

WHOLE-MOUNT METHODS

The present chapter deals with methods of making whole mounts of entire small or more or less flat objects. There are many types of material which are most satisfactorily mounted entire, *i.e.*, without sectioning, although some trimming of excess tissue, or reduction of large pieces to a more convenient size may often be required. Among the subjects commonly mounted whole may be mentioned filamentous algae and fungi, flat thalloid algae, fern prothallia, delicate bryophytes and mosses, pieces of leaf epidermis with stomata and occasionally trichomes or glands, sori of ferns, small flowers, and pollen grains.

Whole mounts display many features not revealed by sectioning methods and frequently permit a better comprehension of the nature of certain organs than do sections. In well-prepared fern prothallia, for instance, the development of the antheridia and archegonia can be followed out far more easily than in sections. Again, whole mounts are the only means by which the surpassing beauty of some of the most fragile of plants can be preserved permanently. The red algae *Antithamnion* and *Ceramium* are examples.

Certain types of preparations are actually whole mounts but are commonly included under other classifications. Smears of pollen mother cells are really whole mounts, but since they are objects intended for specialized cytological investigation, rather than for general morphological purposes, they are classified and described elsewhere.

Freehand sections of woods or stems might also be included as whole mounts, but their treatment is so different that they must be excluded.

Various staining methods applicable to the classes of material mentioned above are outlined in a special section at the conclusion of the descriptions of the different procedures.

PROCEDURES

The Hygrobutol Method.—This method is probably the one most certain to afford satisfactory results. Even beginners in microtechnique have had complete success with the schedule, as it works well even with such a supposedly difficult subject as *Spirogyra*. It is the least expensive of all.

It must be emphasized that each step, in its logical sequence, must be carried out. Variations in or departures from any of the steps are likely to result in disaster.

1*a*. A weak or medium chrom-acetic killing fluid will probably, in most cases, allow the most brilliant staining effects. The filamentous Chlorophyta will come out better in such a fluid than from one containing formalin. Wash out the fluid thoroughly with running water.

b. The marine algae of a filamentous or sufficiently thin thalloid type may be fixed in a 1% chrom-acetic fluid, or in 10% formalin, both made up with sea water; washed out thoroughly with sea water; and the transfer from sea to distilled water carried out by gradual stages.

c. Formalin-aceto-alcohol works satisfactorily with all types of material except those from aquatic habitats. Wash out the fluid with distilled water instead of the customary 50 or 70% alcohol.

The material should be kept in deep solid watch glasses or low stender or crystallizing dishes for this and all the succeeding steps. Fluids may be removed with a giant pipette. All the steps may be carried out in the one container, which procedure obviously lessens the danger of damage resulting from too much handling of the objects.

2. Stain with an aqueous stain or preferably some hematoxylin or carmin mixture. Harris' or iron hematoxylin or Mayer's carmalum are strongly recommended, the first and last particularly so. Differentiate the dye sharply, taking care not to destain too far since the following steps may sometimes carry the differentiation a little further. Wash out the differentiator thoroughly.

3. Dehydrate in 15, 30, 50, and 70% ethyl alcohol, allowing at least 20 minutes in each. Always keep the container covered between changes of fluids.

4. Leave overnight or for at least 18 hours in 85% ethyl alcohol. The purpose of the long immersion is to harden the tissues; no change will be actually apparent, but the hardening nevertheless occurs. The material does not become rigid or brittle; in fact the apparent lack of change during this period may lead incautious technicians astray. If the step is shortened, the material will become excessively brittle immediately upon the addition of a stronger alcohol, and it generally will not be amenable to further treatment.

5. Counterstain with any desired cytoplasmic dye dissolved in equal parts of 95% alcohol and methyl cellosolve. After a hematoxylin, erythrosin B is recommended for the Rhodophyta, orange G for the Phaeophyta, and fast green for the Chlorophyta, since in each instance the material will be given a more or less naturalistic coloration. On some materials stained basically with a carmin stain, a counterstain may be undesirable, but on others one will reveal otherwise obscured or

unstained details. With leaves or portions of epidermis, for example, anilin blue or fast green provides a good contrast for Mayer's carmalum. The counterstain may be used in fairly strong concentration as there is little chance of overstaining. The time should be about 15 minutes, but one should make certain that the stain has thoroughly penetrated the material. Differentiation of the counterstain is rarely required. The hygrobutol, to follow in the next step, exerts a certain differentiating effect at the same time that it prevents the 95% alcohol from extracting too much of the stain.

6. Pour off the counterstain, and rapidly wash out excess dye with 95% ethyl alcohol. Replace with fresh 95% alcohol, and immediately (even if the second 95% alcohol is tinged with the counterstain) begin the addition of hygrobutol by the gradual substitution method. This consists in adding a small amount of the hygrobutol every 30 seconds or so, avoiding strong diffusion currents, and mixing the fluids by tilting the container back and forth. Between every third or fourth addition, discard some of the mixture. Continue in this fashion until the proportions are about 90 parts of hygrobutol and 10 parts ethyl alcohol. It is unnecessary and probably undesirable to attempt to get rid of all the ethyl alcohol, unless a very delicate counterstain which might be injured by the ethyl alcohol has been used.

7. Pour off most of the mixture of butyl and ethyl alcohols, and replace immediately with balsam (thick), diluted at least ten times with hygrobutol. Use many times as much diluted balsam as the bulk of the material. Set the container aside, exposed to the air, but in a warm, dust-free place, until the solvent has evaporated sufficiently to leave the balsam of a mountable consistency.

The evaporation should not proceed too rapidly on delicate materials; 2 hours is probably the safest minimum for the process. Watch the progress of the evaporation; if the material should be in danger of becoming exposed owing to an insufficient amount of diluted balsam having been used at the beginning, add more of the diluted balsam. Never add thicker balsam to a diluted mixture.

8. Mount in balsam. Circular cover glasses should preferably be used since a more accurate judgment of the required amount of balsam can be made.

Objects which are to be mounted singly can be manipulated without difficulty. With filamentous algae or fungi, fine scissors should be used freely. One should never attempt to pull filaments of *Spirogyra*, for example, out of the mass of material, but the entire mass should be cut into portions not over 5 mm. long. The mass can then be easily separated and the individual filaments spread out in the drop of balsam on the slide.

To make the mount, put a fairly large drop (about the size of two grains of wheat) in the center of a clean slide. Take up some of the material with a needle or tiny spatula hammered out of a needle, and place in the drop of balsam, taking care that the balsam covers the material. Carefully spread the material to avoid overlapping. It would be a good idea to trace the outline of a slide on a 3 × 5 piece of cardboard, then within the outline to trace around the size of coverslip being used, allowing sufficient room at the left for the label (note the first two slides in Fig. 13). Pick up the cleaned coverslip with fine-pointed forceps, pass once through an alcohol flame to remove moisture, then apply to the balsam and lower in a slanting position so as to avoid trapping air bubbles. If the amount of balsam has been properly judged, it should just come up to the edges of the coverslip. Slight pressure on top of the cover with the forceps may be required if the amount of balsam was somewhat insufficient, or if the material is curled up a little. Finally place the slide on a warming plate for a day or two to solidify the balsam.

The Dioxan Method.—The dioxan method is also easy but is a rather expensive method. It has one serious disadvantage, *viz.*, that only aqueous or weakly alcoholic stains can be employed. But even this disadvantage may easily be circumvented if a thorough knowledge of the manipulation of the various dyes is already possessed by the technician.

Only pure anhydrous dioxan can be employed in this method; the practical and ordinary commercial types are useless.

1. Kill, fix, wash, and stain the material with aqueous stains. Carefully wash out all surplus dye with water; if unattached dye particles remain, they will be precipitated by the dioxan.

2. Dehydrate with the following mixtures of dioxan and distilled water, allowing about 30 minutes in each: 50, 75, 80, 90, 95%, then leave overnight in pure dioxan and follow by two changes during a ½-hour period the next morning. The critical period is between the 90% and pure dioxan. If any plasmolysis (apart from any produced by the killing fluid) occurs, return to the next lower percentage, and leave until turgor is restored and upgrade again by a more gradual series.

3. Infiltrate with balsam highly diluted with dioxan, in the same manner as described above in the hygrobutol method. The evaporation of the dioxan will proceed somewhat rapidly, consequently progress should be checked at frequent intervals. In mounting, do not use too much balsam because a small amount of dioxan-diluted balsam will be carried over with the material, and it will dilute the balsam somewhat but not enough to cause bubbles to form under the edges of the coverslip later.

The Creosote Method.—While the two preceding methods should prove satisfactory with most materials, one may occasionally prefer to use for bulkier materials, such as fern prothallia with young sporophytes attached, *Nemalion* and similar Rhodophyta, thick pieces of leaf epidermis or styles with microgametophytes, a clearing agent which renders them more transparent than does hygrobutol or dioxan.

Beechwood creosote is excellent for the purpose. The brand employed should be Hartmann and Hauer's, as all other brands, most of which are synthetic compounds, have failed completely to give even passable results. Great care must be taken to avoid getting any creosote on the skin as it produces extremely irritating blisters.

Kill, fix, wash, and stain the material as usual. One may freely transfer the material, if necessary, from water to 50% ethyl alcohol, and vice versa, without appreciable harm. Pass through 70 and 80% ethyl alcohol, allowing at least 10 minutes in each. Any counterstains which are best employed in strong alcoholic solutions may be used at this juncture. Next pass through the following mixtures of creosote and 85% ethyl alcohol, allowing 15 to 20 minutes in each: 1 part creosote to 4 parts alcohol, equal portions of the two reagents, 4 parts creosote to 1 part alcohol, followed by two changes of pure creosote. Since creosote and thick balsam are perfectly miscible, one could theoretically go directly into balsam diluted with creosote. The practical difficulty, however, is that it would be many months or even a year before the creosote would evaporate sufficiently to permit mounting the material, and many more months would elapse before the balsam mounts hardened. It is therefore better to remove most of the creosote with a more volatile solvent which is perfectly miscible with both creosote and balsam. Either hygrobutol or dioxan may be used, in the following proportions of solvent and creosote: 1:5, 2:5, 3:5, 4:5, then through a change of the pure solvent. About 15 minutes in each change suffices. Next place the material in balsam highly diluted with the solvent, then set aside to concentrate as usual.

The Glycerin-Xylol Method.

1. Kill, fix, and wash as usual. Stain with an aqueous basic stain, such as one of the hematoxylins.

2. Put the material in 10% aqueous glycerin, place the container where dust will not settle in (but do not cover), and leave for several days until the diluted glycerin attains the consistency of pure glycerin. The process may be accelerated by placing the container in the paraffin oven on an upper shelf. Do not allow the material to become exposed; add more of the diluted glycerin if necessary.

3. Remove the glycerin with several changes of 95% alcohol. The thicker the mass of material, the more the number of changes of alcohol

required to remove the glycerin, as it sticks to the mass rather closely. If the material is placed in a wire strainer and the latter placed in a suitable dish so that about 1 cm. of space is left clear below the strainer, the heavier glycerin will soon settle down.

4. Counterstain, if desired, with any suitable acid dye dissolved in 95% alcohol.

5. Complete dehydration in a change of absolute alcohol.

6. Dealcoholize in a series of about six to eight intermediates between absolute alcohol and xylol; a longer series is preferable to a short one. Start with a proportion of 9:1, then 8:2, etc. The time in each mixture may be between 5 and 10 minutes. It is not necessary to get rid of all absolute alcohol as it will evaporate during the next step, but if there seems to be danger that the alcohol may extract any stain, give two changes of pure xylol.

Hygrobutol may be substituted for the xylol, and there is less danger of the material becoming hardened.

7. Transfer to balsam highly diluted with xylol (or with hygrobutol if that reagent has been substituted), then set aside to evaporate to a mounting consistency.

The Venetian Turpentine Method.—The following schedule represents a modernization and simplification of the old Venetian turpentine method, long a favorite with the older botanists.

1. Kill, stain, and dehydrate gradually with ethyl alcohol until absolute alcohol is reached. Give two changes of the absolute alcohol.

Make a 10% solution of Venetian turpentine in absolute alcohol, keeping it in a tightly stoppered bottle. If the turpentine is too thick to flow easily, place the container in a hot-water bath until it flows easily. Prepare an exsiccator. A specially constructed exsiccator should be available in most laboratories, but if one cannot be found, it is not at all difficult to arrange a satisfactory substitute. In the regular exsiccator, fill the bottom compartment half full of a mixture of equal portions of sodium hydroxide (white sticks) and calcium chloride. Or fused calcium chloride may be used alone. In lieu of an exsiccator, secure a piece of plate glass and a bell jar of as low a height as possible but wide enough to cover a saucer and one or more watch glasses. Place the hydroxide-lime (or chloride) in the saucer. Cover the top of the exsiccator or the bottom of the bell jar with a thin but even layer of vaseline or petrolatum. The transfer is now ready to be made, and it should be performed as expeditiously as possible in damp climates. Pour off the absolute alcohol, place the watch glass or other container in the exsiccator, and fill with the 10% turpentine. Then place the cover or bell jar in position, and see that the vaseline has spread evenly, leaving no air leaks. Be careful not to let any of the drier get into the turpentine. The concentration

of the turpentine should not proceed too rapidly. The amount of drier can be regulated so that the process takes four or five days. Do not try to open the exsiccator until you are fairly certain that the turpentine has become thick enough for mounting, which will be when it has the consistency of pure glycerin. If the cover is taken off too soon, the turpentine is practically certain to become cloudy.

After the drier absorbs sufficient moisture to become nearly saturated, it is best to remove it, heat until dry and then replace; or fresh drier may be used. If the turpentine shows any sign of cloudiness, it is spoiled. If the material is too valuable to be thrown away, wash in absolute alcohol until the cloudiness disappears, then begin again with the 10% turpentine.

To mount, first cut the material into suitable portions with fine scissors, if this should be necessary. Gather up a small quantity of the material with a needle, and place on a clean slide. Add the requisite amount of xylol-balsam, then the coverslip (circles are preferable). The material may be mounted in the turpentine, of course, but experience has shown that it is far better to mount in balsam as the turpentine is rather prone to crystallize, and one cannot be perfectly certain of its quality.

If it is not convenient to mount all of the material at once, it may be stored in vials which must be securely corked; the cork and upper part of the vial should be sealed with melted paraffin as a precaution against the turpentine becoming too thick.

2. Proceed as far as the end of step 2 of the dioxan method. Make a 10% solution of Venetian turpentine in dioxan and transfer the material to this mixture. The solvent can be evaporated in the open air without danger of the absorption of moisture. Mount in balsam as usual after the turpentine has become sufficiently thickened.

STAINS AND STAIN COMBINATIONS FOR WHOLE-MOUNT MATERIALS

The selection of the stain or stain combination to be used on objects intended for mounting whole depends entirely on the nature of the material itself. The greater the clarity of internal detail that may be required, the more transparent the stain or stains must be. Some dyes give an opaque stain, which obviously tends to obscure finer internal details. Most of the coal-tar dyes are in this category, but with certain types of material there frequently results more differentiation than might be expected. One can only try the stain or combination on a small portion of the material and observe the result under the microscope. Harris' hematoxylin is an unusually transparent stain eminently suited for bulky materials and is unsurpassed for many types.

The basic or nuclear stain should be used in rather dilute concentration. Differentiation is not always easy.

Iron Hematoxylin.—For filamentous algae and many fungi of a similar structure, this is particularly suitable. It is, unfortunately, very difficult to apply to the Rhodophyta as these plants usually break up in either the mordant or the stain. Nor is it suitable for pieces of leaf epidermis, for Bryophyta, or for fern prothallia.

After the fixing fluid has been thoroughly washed out, mordant in 2% aqueous ferric ammonium sulphate for 2 hours, no longer. Then wash thoroughly in running water for about 20 minutes, and rinse in two changes of distilled water. Stain for at least 2 hours or overnight in 0.2% aqueous hematoxylin, well ripened. Wash out all excess stain with distilled water. Destain in 2% aqueous ferric ammonium sulphate or ferric chloride until the stain, as observed under the microscope, seems to be satisfactory. When the nuclei appear dark grayish in water, they will look black or bluish-black when finally mounted in balsam. One soon learns to recognize a grayish fluorescence in the material, which is generally an accurate criterion of proper differentiation. Wash the differentiator out *very thoroughly* with running water.

Iron Hematoxylin with Counterstain.—Many excellent counterstains for iron hematoxylin are available, the one to be selected being governed principally by the nature of the material. Sometimes one dye works better than another; only experimenting can determine this point. Erythrosin, fast green, orange G, anilin blue, and safranin may all be used in 1% solutions in 95% alcohol. In the case of the safranin add a few drops of the 1% solution in the 95% alcohol in which the material lies, and watch the material critically for the next 20 minutes or so. When the stain is just right, wash out the excess stain in a change of 95% alcohol.

Delafield's Hematoxylin with Iron Hematoxylin and Fast Green.—After washing out the ferric destaining solution, counterstain with Delafield's hematoxylin. With some materials this solution (which should be used in highly diluted form) imparts a violet stain, with others it merely acts as a sort of mordant for the fast green and causes the otherwise faint or colorless cell walls to stain brilliantly. Apply the fast green in 95% alcohol as usual. The combination is most suitable for filamentous Chlorophyta.

Harris' Hematoxylin with Counterstain.—Harris' hematoxylin is really superb for the Rhodophyta and for fern prothallia with sex organs. The stain is decidedly transparent and clearly reveals the internal cellular organization of even thick objects. The color imparted by Harris' hematoxylin is similar to that of Delafield's but is less variable and more precise.

For the Rhodophyta the best counterstain is erythrosin B; it may be used in 95% ethyl alcohol as usual. If the dioxan method is used, a 1% aqueous solution of the erythrosin may be employed with safety since the dioxan scarcely extracts the stain. Fast green should be substituted for the erythrosin in the case of fern prothallia, leaf epidermis, and similar subjects; the erythrosin will stain such material more evenly, but the red color is too unnatural.

Safranin and Fast Green.—This combination is excellent for many types of plant material, such as powdery mildews and similar fungi on leaves, fern leaves with sori, and thick epidermis peelings. It would be best to follow the glycerin dehydration method. When the dehydration with glycerin is begun, add a few drops of a 1% aqueous solution of safranin O. As the glycerin concentrates, those structures possessing an affinity for safranin become red. Wash out the glycerin as usual. To the last change of absolute alcohol add a few drops of 0.2% fast green in 95% alcohol. Proceed to Venetian turpentine. (Unpublished method of Dr. G. H. Conant.)

Anilin blue may be used in place of the fast green, but a saturated solution in 95% alcohol is required.

If the dioxan or creosote dehydrating schedule is used, the fast green or anilin blue should obviously, and as a rule, be used in aqueous solution. However, the fast green may be dissolved in 50% dioxan or in pure creosote and used at the appropriate stage in the respective schedule. It scarcely overstains, and only the surplus stain needs to be washed out (with 50% dioxan or with pure creosote, as the case may be).

Fast Green.—The dye alone suffices for many classes of material, especially those which are naturally dark colored and do not lose the color during the various processes. Maturing zygotes of *Rhizopus* or ripe sporangia of many ferns are cases in point. The stain will also reveal clearly the embedded foot of the sporophyte in whole mounts of *Anthoceros*.

CHAPTER X

THE GLYCERIN METHOD

Among the older botanists, and especially with the algologists, the glycerin method was the only one followed in the mounting of algae and similar plants. The method has fallen into disuse since the introduction of more permanent methods, but it still has its occasional uses, and for this reason it is being presented. When first proposed, the schedule called for mounting in pure glycerin, but later improvements included final mounting in some form of glycerin jelly, which added to the permanence of the preparations.

Unstained Preparations.—Glycerin will frequently preserve the natural colors of various plants, such as unicellular and colonial Chlorophyta, moss protonema, spores of fungi, *Equisetum*, and ferns. Delicate objects which might easily plasmolyze may be placed in a drop of 10% aqueous glycerin on a chemically clean slide and the glycerin allowed to concentrate. When this has occurred, add a coverslip very cautiously so as not to allow any of the glycerin to ooze beyond the edge, and later seal as described below. If only temporary sealing is required, vaseline will serve.

Stained Preparations.—Kill and fix the material as usual. Only aqueous stains may be used on material intended for mounting in glycerin jelly. Iron hematoxylin with or without a counterstain will generally be found wholly satisfactory. The staining completed, place the material in 10% aqueous glycerin in a dish which permits the exposure of as much surface as possible. Put the container where evaporation of the water may take place. It may be put on an upper shelf or cooler part of the paraffin oven, but care should be taken to see that evaporation does not proceed too rapidly; otherwise some shrinkage may result.

When the diluted glycerin has become about as thick as pure glycerin, the material is ready to be mounted. The best mounting medium is Kaiser's glycerin-gelatin. To make it, place 1 part by weight of Knox's or other pure gelatin in 6 parts by weight of water, and allow to soak for at least 2 hours. Add 7 parts by weight of glycerin, and for every 100 g. of the mixture add 1 g. phenol crystals. Warm for about 15 minutes, stirring constantly and vigorously until all the flakes produced by the phenol have disappeared. Filter through cotton while still warm. The mixture is best stored in a small bottle that can conveniently be

warmed in a hot-water bath. When ready to mount, put some of the material, freed from as much of the excess glycerin as possible by touching to absorbent paper, on a chemically clean slide, and add just enough of the warmed jelly mixture to come to the edge of the round coverslip after the latter has been applied. If any of the mixture has oozed beyond the coverslip, it is impossible to seal the mount properly. The mounts may be sealed either as soon as the jelly has solidified, or they may first be set aside for a week or 10 days.

Various cements and other media have been used for sealing the mounts. Gold size has been most commonly used, Canada balsam coming next. Shellac, asphaltum, King's cement, Duco cement and plain Duco, Hazen's cement (8 parts rosin and 2 parts anhydrous lanolin: melt the rosin first, then add the lanolin; apply hot), and different kinds of varnishes have all been employed.

Sealing may be done freehand or with the aid of a turntable. In either case considerable practice is required to turn out a satisfactory ring. Brush a thin coating of the cement over the margin of the coverslip and the adjacent slide. Wait for one coat to dry thoroughly before applying the next. The number of coats required depends mostly upon the character of the cement used, but two should suffice. In using the turntable, place the slide in position, and center accurately. Dip a fine-pointed brush in the cement, give the table a spin and touch the tip of the brush to the slide as far from the coverslip as it is desired that the ring shall extend. Approach the coverslip gradually but without actually touching it. Dip the brush in the cement again, and give the table another spin. Bring down the brush so as to touch the coverslip gently, and bring the ring of cement into contact with the earlier application. The cement should be extended over the coverslip about 1.5 mm. from the periphery.

Glycerin or glycerin jelly preparations should always be stored flat, never on edge, otherwise the material will sink to the lower edge.

CHAPTER XI

CELLOIDIN METHODS

To some schools of microtechnical philosophy, the celloidin method is one that has lapsed into desuetude; to others it is the only procedure worth following. The one considers the smear and paraffin methods adequate for practically all purposes, while the other regards them as distinctly inferior. The beginning technician will very soon find himself confronted with this very sharp conflict of opinion and will wonder why it should exist. The celloidin method as applied to plant materials is practically the procedure developed by a single man and his students, while the other methods were built up by numerous workers. For plants one method is scarcely better than another, intrinsically; it all depends upon the individual technician and his manipulative ability, whether time is an important factor and cost no great object.

Despite the claims of its proponents, the celloidin method is a difficult one to master in its details. It is also the most expensive one. Nevertheless, anyone who has had sufficient experience with both the paraffin and the celloidin methods will readily admit that the celloidin schedule will afford better results with certain types of materials. For example, perfect sections of woody stems cannot always be cut in paraffin, but really remarkable results are obtainable with celloidin. On the other hand, root tips and buds for chromosome studies are better in paraffin than in any other medium, if sections rather than smears are indicated.

Almost any sort of plant material may be embedded in celloidin, if the nature of the material is carefully considered.

The treatment of the material preliminary to embedding is the same as for the paraffin method. After the killing fluid has been washed out, the material is gradually brought up to absolute ethyl alcohol, of which at least two changes should be used to insure the removal of all water. The material is then ready for infiltration.

By another method the material may be dehydrated with tertiary butyl alcohol in the regular manner but carried only as far as the mixture of 75% tertiary butyl alcohol and 25% absolute ethyl alcohol. From this it is transferred to a mixture of equal parts of tertiary butyl alcohol, absolute alcohol, and ether.

The pieces of celloidin should be removed from the bottle in which they are stored in water, washed with water, then with 95% alcohol, and finally spread on paper toweling to dry thoroughly. Equal parts of

ether and absolute alcohol is the solvent almost universally employed. Make up five solutions of solvent and celloidin, each in a separate, tightly stoppered bottle: 2, 4, 6, 8, and 10%.

From absolute alcohol the material is passed through an intermediate stage of equal parts of ether and absolute alcohol, then transferred to a wide-mouthed bottle and covered with 2% celloidin. Subsequent treatment may follow either of two methods, in one of which (1) the celloidin is used cold, in the other (2) heat is employed.

1. From the 2% celloidin transfer to the 4% and so on to the 10% solution, allowing from one to many days for each percentage. The length of time required depends on the nature of the material and can be easily judged. Or one may partially expose the material in 2% celloidin to the air and allow the solvent to evaporate slowly. In either case, weeks are often required for the proper impregnation and embedding of the material.

2. If heat is to be used to hasten the impregnation process, wide-mouthed flanged bottles are needed. After placing the material in the bottle and covering with 2% celloidin, insert the cork stopper securely, and either wire or otherwise clamp the cork in firmly so that it will not be blown out by the pressure exerted through the vaporization of the solvent. Place the bottle on its side in a constant temperature oven, with the temperature between 45 and 55°C. The change from one concentration of celloidin to the next higher one may be made about every 24 hours. Always cool the bottle before attempting to open, and clamp the cork tightly, as before.

After the 10% celloidin has been reached, it is impracticable to use higher percentages because of the difficulty of holding back the material when making transfers. The 10% solution may be thickened by the occasional addition of small chips of dry celloidin. The thickening process should be carried on until the celloidin mass appears more or less stringy as it runs down the sides of the bottle when the latter is quickly inverted.

Embedding is accomplished by removing the individual pieces of material from the thickened celloidin with a pair of fine forceps and holding in chloroform to harden. Care should be taken to see that each piece of material is surrounded by an ample coating of celloidin before being immersed in the chloroform. The mass will in a few moments be sufficiently hardened on the surface to be cut from the forceps with a scalpel. After 24 hours' immersion in the chloroform, transfer the pieces of material to a mixture of equal parts of glycerin and 95% alcohol, in which they may be left indefinitely.

A sliding microtome is required for sectioning materials embedded in celloidin, although the new Spencer No. 820 rotary microtome may be

equipped with a special holder in which the knife may be oriented at an oblique angle. The knives used should be heavier in style than those employed for paraffin sectioning and a keen edge must be maintained.

After the embedded material has remained in the glycerin- 95% alcohol mixture for several days, it is ready for mounting and sectioning. One should not attempt microtoming the material without first treating with the glycerin-alcohol mixture. One end of a block of wood, cut to a convenient size, is dipped in 6% celloidin, then allowed to dry. Remove excess celloidin from the embedded piece of material which is to be sectioned. Then immerse both the wood block and the piece of material in 6% celloidin, place the object in position upon the end of the block, and set the whole aside to dry for about 10 minutes.

Place the mounted block in the object holder of the microtome. Insert the knife, and clamp tightly. The edge of the knife and the object are both kept wet with 95% alcohol, which may be applied with a fine-pointed camel's-hair brush. Hold the brush on top of the object (as is done when cutting freehand sections), taking care not to move it or to allow it to project between the object and the knife, then move the knife carriage forward with a steady stroke to cut a section. The section may be moved toward the upper edge of the knife blade and left there for awhile. If removed from the knife immediately, there is a strong tendency for the sections to curl. Serial ribbon sections, such as occur when one microtomes material embedded in paraffin, cannot be obtained from celloidin material. The sections may presently be transferred to a watch glass of 95% alcohol. After a section is cut, if the sliding microtome is used, move the knife carriage back, and if the feed is not automatic, adjust the object holder to the proper thickness for the next section. The sections should be examined from time to time by placing one on a clean slide and placing this under a low-power microscope objective, paying attention to the proper thickness of the material and the smoothness of the sectioning.

The angle at which the knife is oriented is a matter of importance and is best determined by experience. As a rule, the softer the material to be sectioned, the more obliquely the knife should be placed to the line of movement of the knife carriage. For hard materials, for example, such as tough stems or pieces of coal, the knife should be at a right angle.

Almost any desired combination of stains can be used on celloidin sections. The celloidin matrix is usually not removed from the sections; if it seems desirable to remove it, it is best to do so during the later stages of dehydration or just before mounting. The matrix sometimes prevents the dyes from being as brilliant as they are with freehand or paraffin sections; the tones, in other words, appear flat. The matrix itself is usually stained more or less but this does not interfere to any appreci-

able extent when the completed slides are examined under the microscope. For woody sections iron hematoxylin and safranin form an ideal combination.

To effect dehydration, transfer the sections to 95% alcohol for about 1 minute, then pass through two changes of absolute alcohol to which about 5% of chloroform is added to prevent the alcohol from dissolving the matrix. Clearing may be effected with either xylol or preferably benzol. If it is desired that the matrix be removed, this can be done by interpolating ether or a mixture of equal parts of ether and absolute alcohol between the second absolute alcohol and the clearer. The 95% and the first absolute alcohol will remove the excess stain, in case coal-tar dyes have been used, from both sections and matrix. Dehydration may also be effected by the use of beechwood creosote; the creosote softens the celloidin just enough to prevent the sections from curling when mounting and also removes excess stain from the matrix. Wash out the creosote with xylol. When ready for mounting, the sections may be transferred to a slide and arranged as desired. Place a few drops of balsam, preferably dissolved in benzol and somewhat thicker than that used for paraffin sections, on the sections, and add the coverslip. If the sections appear not to be sufficiently flattened, a lead weight may be placed on top of the coverslip to press out the excess balsam. Dry the mounts on the drying table as usual.

DOUBLE EMBEDDING

Suitable materials may first be embedded in celloidin and then in paraffin. Various methods have been proposed for accomplishing the result, but all differ solely in the nature of the reagents employed for making the transfer into paraffin. In any case, the infiltration with celloidin is carried out as usual, and the block is hardened in chloroform.

From the chloroform the celloidin-embedded material may be transferred to castor-xylol (1 part castor oil and 3 parts xylol) in order to clarify and to complete hardening. Then place in melted paraffin in the oven for a day or two. If desired, the material may be placed in either pure xylol or pure cedarwood oil for 12 hours before going into the paraffin. Finally embed, and section as for ordinary paraffin materials.

Another method is to transfer the material to 4% celloidin in clove oil (an 8% solution of celloidin in absolute alcohol-ether diluted with an equal volume of clove oil) for 2 to 24 hours (Tschernyachinsky 1930). Place in chloroform for 30 minutes, then transfer to chloroform-paraffin in the oven, and leave until the material sinks. Next transfer through several changes of pure paraffin and finally embed.

A third method, which involves the chance of considerable danger from explosion if ovens with exposed heating elements are used, utilizes

benzene (not benzine). From the chloroform, transfer to equal parts of chloroform and benzene, then to pure benzene, next to a mixture of benzene and melted paraffin, and finally to pure paraffin.

Sections may be cut on a rotary microtome, exactly as plain paraffin sections are made, and a ribbon will be formed. To mount the sections on slides, prepare the following adhesive: mix 2 cc. acetone with 1 drop methyl benzoate, and add 8 cc. distilled water. Two or three drops of Mayer's adhesive may also be added and the whole mixed thoroughly. Flood the slide with this mixture, and place the sections thereon. Use little heat for spreading, as the sections begin to straighten out as soon as they are placed on the mixture.

CHAPTER XII

PARAFFIN METHODS

The paraffin method is still the most popular one, and there is little reason to suppose that it will ever become entirely supplanted unless some as yet unknown substance can be found which has all the properties of paraffin except that it does not require heat to transform it to the fluid state and that it is miscible with all solvents. There is scarcely any danger that either the smear or the celloidin method will supersede the paraffin method since both have inherent faults not found in the paraffin method, although it is freely admitted that the paraffin method has its occasional difficulties. The different methods should, as a matter of fact, be considered as supplementary to one another.

The older paraffin infiltration procedures called for the use of bergamot oil, cedar oil, or xylol as a "clearing agent" but the recent introduction of tertiary butyl alcohol as a substitute for these reagents has permitted considerable refinement, shortened the time, allowed greater latitude in the selection of materials to be embedded for microtoming, eliminated excessive hardening of the material, and greatly lessened the probability of failure, as well as making the method far less tedious and expensive. The advantages of tertiary butyl alcohol over other fluids are numerous, its disadvantages so few as to be negligible. However, for the benefit of those who might wish to use them, several of the older schedules will be described in sufficient detail.

Whether one uses xylol, tertiary butyl alcohol, chloroform, acetone, or whatever other clearing agent, the preliminary stages of manipulating the material are identical and may be discussed independently.

COLLECTING OF MATERIAL, KILLING AND FIXING

Collection as well as preparation of the material to be embedded is a far more important step than it has usually been regarded. Indeed, with the newer and highly refined schedules, the preliminary stage becomes the one upon which everything else depends. If the material is not selected with sufficient care and properly prepared at the beginning, none of the succeeding steps will correct the initial errors.

In a single collection of material there should be included only one species or form, and only some particular organ or portions of this organ, such as anthers or sections of stem or leaf. If, for example, one wishes to study meiosis in the anthers of *Lilium*, the anthers should be dissected

out of the buds and partitioned into sections not over 8 mm. in length. An entire series of stages in the development of the anthers should be included in the first collection since one can learn only from personal experience or by experiment the exact stage in growth during which a particular phase, such as diaphase, occurs. Then, in later collections, any desired stage may alone be collected. Buds, ovaries, and similar organs with enclosed air spaces of considerable size should be opened in some innocuous way to facilitate penetration of the various reagents. When there is only one kind or type of material in a collection, far more uniform fixation, dehydration, and infiltration are secured.

It is presumed that the material to be embedded has been accurately identified. It goes without saying that anything whose source and exact identity are unknown is not worth bothering about.

Some permanent record of the nature of the material, the procedures followed, and other pertinent data, is necessary. A series of numbers or other symbols may be written on fairly stiff white paper with waterproof India ink, and the corresponding symbols on ordinary 3 × 5-inch index cards or in some sort of record book. A number is cut off and placed in the vial or bottle with the material and embedded with the latter (note Fig. 4, *A*, *C*, and *D*), while all requisite information is written on the card or in the record book. In a large research laboratory it is necessary to keep rather accurate and detailed records; a printed form will then be found most satisfactory.

The various killing and fixing fluids have been described in an earlier chapter, together with notes regarding their suitability for various kinds of material and directions concerning aftertreatment. In the second section further suggestions with respect to the different groups or families of plants are offered. It is well to bear in mind the fact that, simply because a certain fluid gave excellent results with some particular material, it does not follow that equally good results will be obtained with almost anything else. To use the first available fluid and to expect the material to be properly fixed is a common practice and one severely to be condemned. Some study should be made of the nature of the material to be fixed, with special reference to exactly which structures one particularly wishes to study, at the same time giving consideration to suitable killing and fixing fluids. Formalin-aceto- (or propiono-) alcohol, for example, gives perfect fixation of such diversified structures as filamentous algae and woody stems, but usually gives atrocious fixation of chromosomes and dissolves mitochondria.

For ordinary purposes shell vials about 2.5 cm. in diameter and 7 cm. in length, with tightly fitting corks, are wholly satisfactory. For collecting in the field or on long trips, where there is danger of crushing the rather thin shell vials, homeopathic vials of about the same size are

preferable. If the collections are bulky, corked or screw-cap bottles of an appropriate size may be used. In general, all the processes of killing, fixing, washing, dehydrating, and clearing can be carried out in the one vial or bottle. For infiltration other vials or bottles should be used, since it is very difficult to remove the paraffin. Vials which have once contained paraffin may be used for this purpose over and over again.

The amount of killing and fixing fluid should always be at least ten times the volume of the material. A much greater volume, even to fifty times, is necessary with material containing a high percentage of

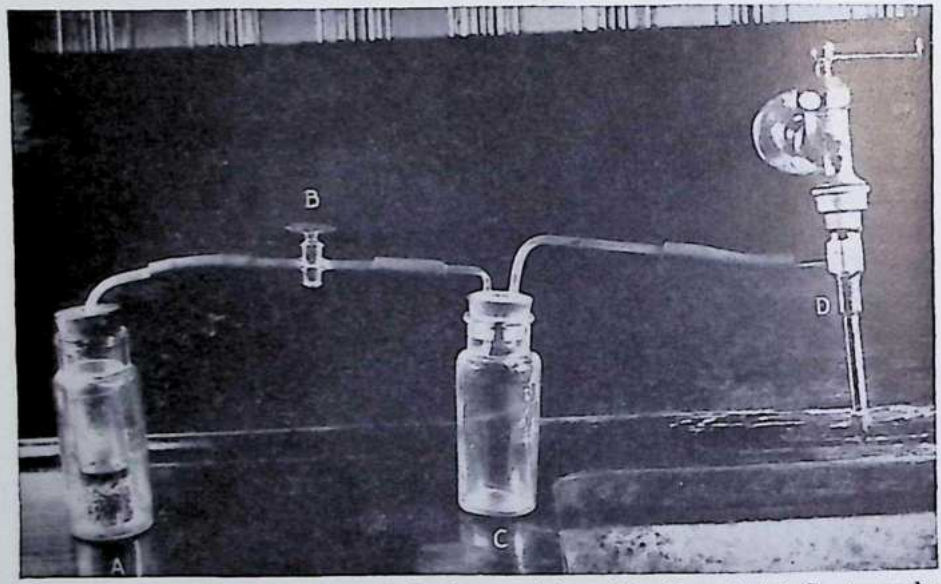


FIG. 2.—Suction pump setup: *A*, bottle containing vial with material; *B*, stopcock; *C*, safety bottle; *D*, pump attached to faucet. The glass tubing at the right-hand side of the safety bottle *C* should extend to within 1 cm. of the bottom of the bottle.

water. The water, obviously, dilutes the killing fluid, and allowances should be made for this fact. With the dehydrating and clearing reagents one need use only a little more than enough to cover the material. All fluids should be discarded once they have been used.

Some classes of material contain too much air for them to sink into aqueous killing fluids; this also occurs in some alcoholic fluids as well. The Bryophyta are of this type. The use of a motor-driven suction pump for long periods, as advocated by a few technicians, can hardly be countenanced because of the artifacts which such a violent procedure demonstrably produces. A pump attached to a water faucet can be used safely (Fig. 2). The apparatus is rigged up as follows: connect the side tube of the pump by means of thick rubber tubing and a right-

angled glass tube to a safety bottle fitted with a two-holed rubber stopper; insert another right-angled glass tube in the other hole, then attach a short piece of thick rubber tubing to this, followed by a stopcock and another piece of rubber tubing attached to a right-angled glass tube inserted in a one-hole rubber stopper placed in a bottle whose mouth is wide enough to allow for easy insertion of the vial containing the material. The rubber tubing should be inserted well over the ends of each piece of glass tubing, and all connections should be airtight. Insert the vial containing the material (or even several vials, if of a sufficiently small size), plug the stopper tight and then turn on the water almost to full force. Adjust the stopcock to prevent too rapid extraction of the air. Exhaustion complete, close the stopcock, then turn off the faucet, and wait a few minutes before opening the stopcock and then the bottle containing the material. The safety bottle should be emptied of its water occasionally.

Material which is heavily cutinized or suberized may take several hours to sink. In fluids containing at least 50% alcohol, most materials will sink either immediately or within a short time, but the air should nevertheless be exhausted, or one might find the material floating when put in the paraffin oven.

WASHING

It is almost always necessary to wash out the killing fluid thoroughly before proceeding with the dehydration and infiltration. Some reagents inhibit proper staining, others leave precipitates which will later cause trouble and annoyance, not to mention their being a source of error in interpretation when the completed slides are examined, while still others must be removed lest their continued action damage the material.

In the chapter on killing and fixing fluids the nature of the washing and the approximate time required for the process have been noted for most of the solutions. The following general rules are, for convenience, summarized here. Practically all aqueous fluids, particularly those containing chromic acid, are washed out with water; if an arrangement to utilize running water cannot be set up, then the water should be used in large volumes and changed as often as possible. In some sections of the country the water frequently contains so much air that it would be advisable to exhaust the air first by boiling or by using a suction pump, in order to prevent the air from driving the material to the surface. Alcoholic solutions should be washed out with plain alcohol of approximately the same percentage as that in the solution. Reagents containing picric acid, whether in aqueous or alcoholic solution, should always be washed out with alcohol, never with water unless the fluid also contained some substance which fixes chromatin indissolubly. If mercuric chloride

is used in an aqueous solution, it should be washed out with water, but if employed in an alcoholic solution, it should be washed out with 70% alcohol. The deposits of mercury are best removed from the sections as described elsewhere (p. 38).

DEHYDRATION

The washing completed, the next step is to remove the water from the tissues. When xylol, chloroform, or an essential oil is employed as clearer or paraffin solvent, it is necessary to insure removal of every trace of water before beginning replacement of the absolute alcohol with the clearer or solvent. With the introduction of the tertiary butyl alcohol method, this necessity was removed: in other words, one may begin the transfer to the butyl alcohol at a point where the dehydration process is barely half completed. In any event, the transfer from one step to another in the dehydrating process must be very gradual, otherwise plasmolysis is certain to result. The process should never be rushed, but, on the other hand, tissues become brittle if left too long in the higher alcohols beyond 70%.

Dehydration with Tertiary Butyl Alcohol.—From the writer's experience, the tertiary butyl alcohol method is the most satisfactory of all (Johansen 1935, 1937–1938).

If the washing was with water, commence the dehydration process with 5% ethyl alcohol in distilled water, followed by 11, 18, and 30% ethyl alcohol. Two hours in each percentage is ordinarily sufficient, but bulkier or tougher materials such as woody stems should be immersed for a longer period. Material which has been washed with either 50 or 70% alcohol has already received sufficient preliminary dehydration. However, if the washing has been with an alcohol of a percentage above 70%, it will be necessary to use the xylol, chloroform, or an essential oil schedule after first dehydrating the material thoroughly with absolute alcohol.

The following series of solutions of water, ethyl and tertiary butyl alcohols should be prepared. The volumes are in cubic centimeters; the various solutions may, for convenience, be designated by the approximate total percentage of alcohol:

Approximate total percentage of alcohol.....	50	70	85	95	100
Distilled water.....	50	30	15		
95% ethyl alcohol.....	40	50	50	45	
Tertiary butyl alcohol.....	10	20	35	55	75
100% ethyl alcohol.....	..	..	..	..	25

In the last solution put enough dry erythrosin dye to give a red tinge; this will stain the material superficially so that it can be easily oriented during embedding and microtoming. The dye will come out of the sections readily after they have been brought down to alcohol when the staining is being carried out.

Transfer to the 50% solution from either 30, 50, or 70% ethyl alcohol, depending upon the circumstances of the previous washing. After 2 hours or longer, pour off, and replace with the 70% solution, which should be allowed to remain overnight. If the killing reagent contained chromic acid, the longer immersion in the 70% solution tends to correct certain irregularities in the fixation image. After the 70% solution, an hour in each of the remaining percentages will suffice as a minimum period for most materials. Following the 100% solution, there should be three changes of pure tertiary butyl alcohol (one of which should be allowed to remain overnight), by which time every trace of unbound water should have been removed from the tissues.

The solutions should be used only once because the stock solutions are readily contaminated by the many substances dissolved out of the tissues by the two alcohols. If strict economy must be observed, the used solutions should be kept in separate containers and not poured back into the original bottles.

The material is now ready for infiltration. The transfer from the butyl alcohol to paraffin should be gradual, but fortunately there is a simple method of doing this. Transfer the material to a mixture of equal parts of paraffin oil and tertiary butyl alcohol, in which it should remain for at least 1 hour or somewhat longer for bulkier or tougher materials. (Vials which have contained paraffin oil may be cleaned with waste ethyl alcohol, then washed with water.)

Fill a vial or other suitable container three-fourths full of melted Parowax (preferably Standard Oil Company of Indiana brand), and let stand until the Parowax has solidified but not cooled completely. If the Parowax is allowed to become cold and hard, the glass will break shortly after the vial has been placed in the oven. Put the material on top of the solidified Parowax, just cover with the butyl alcohol-paraffin oil mixture, and place the container in the paraffin oven at once. Do not place where the Parowax will melt too quickly; an upper shelf is preferable to a lower shelf which is apt to be directly over the heating elements. It is important that the oven be well ventilated in order that the evaporating alcohol will be carried away; if there is no air circulation, there will be no evaporation. The material will slowly sink through the melting Parowax until it rests on the bottom of the container. Infiltration is thus really a gradual process. The paraffin oil both prevents any actual damage to the tissues by the heat of the oven, and allows

the Parowax to diffuse in gradually. About 1 hour (minimum period) after the material has sunk to the bottom of the container, pour off the entire mixture of Parowax, oil and what traces of alcohol remain, and replace with pure melted Parowax. (A large beaker of melted Parowax should always be kept ready in the oven, as well as another beaker of the final embedding mixture.) Repeat the process twice during the next 6 hours or so, discarding each change of Parowax. Finally replace with a rubber-Parowax mixture (such as that described on page 22) or with a good quality of paraffin melting at around 56°C., and the material will be ready for embedding within the next 30 minutes.

Small, delicate objects require much less time for infiltration than do large and tough objects. Aquatic plants are more easily infiltrated than xerophytic material. The heat of the oven does not do much damage to plant tissues if paraffin oil precedes infiltration; consequently the time element may to some extent be disregarded. The important matter is to be rid of all the tertiary butyl alcohol and paraffin oil. Just before making the final change of Parowax, there should be no discernible odor of butyl alcohol. The customary practice is to place materials in the oven the last thing before leaving for the day, to make the first change as soon as one arrives the next morning, and to get the embedding completed by late afternoon.

Normal butyl alcohol may be used in place of tertiary butyl alcohol, but is far less satisfactory. It causes some hardening. It is miscible with water to the extent of about 8% by volume.

Dehydration with Xylol, Chloroform, Secondary Butyl Alcohol, or an Essential Oil.—With all these reagents, the material must first be thoroughly dehydrated through a graduated series of ethyl alcohol and distilled water.

If the fixing fluid was washed out with water, a close series of dilutions of ethyl alcohol (based on 95% alcohol) should be used: 2½, 5, 7½, 10, 15, 20, 30, 50, 70, 85, 95%, and absolute alcohol. In case the fluid was an alcoholic one, begin with the alcohol next above the one used for washing. Several hours' immersion in each percentage should be allowed; the last three percentages should be used for at least 12 hours each. The absolute alcohol should be changed two or three times. If it is necessary to store the material for any length of time, it should be left in the 70% alcohol.

The clearing should be as gradual as the dehydration. There should be a series of mixtures of clearing agent in absolute alcohol in the following percentages: 2½, 5, 10, 15, 25, 50, 75, and the pure clearing agent in at least two changes. To either of the last two solutions add enough erythrosin dye to impart a red coloration. The solutions may be used

again two or three times. The vials or other containers must always be kept tightly stoppered except when solutions are being changed.

Xylol hardens nearly all plant tissues more or less and causes some shrinkage or plasmolysis; chloroform and secondary butyl alcohol have only a slight hardening action. Bergamot and cedar oils have none, and these two oils furthermore clear bulkier pieces of tissue better than most other reagents; it is obvious that the different grades should be allowed to react far longer than in the case of the more volatile fluids. For the latter, 2 or 3 hours in each fluid is ordinarily long enough.

After the pure clearing reagent has been reached, the material is ready for infiltration with paraffin. This process must also be gradual. There are three methods from which to choose. The easiest, but one which can be dangerous to the material if one tries to rush things, is to add very small paraffin shavings from time to time to the clearing reageat containing the material until it is finally saturated with partially dissolved paraffin. Wait until the first chips have dissolved before adding the next one. A second method is to carve a small block of Parowax into the form of a long, narrow cone and place it in the vial point end down and resting at the bottom of the container. It will sink into the reagent slowly as it dissolves. The third method is to cut coarse wire gauze into squares just wide enough to be a fraction less than the diameter of the vial or bottles. Bend the corners down to serve as legs and insert into the vial in such a position that the gauze serves as a table above the material. Place chips of Parowax on the table; as they dissolve, the paraffin settles down upon the material. Whatever the method that has been used, the preliminary steps should be carried out at room temperature. As soon as the clearing agent has become saturated with paraffin, the corks may be removed from the vials and the latter placed on top of the paraffin oven or in a warm place. Shake or stir the contents occasionally, and add more paraffin chips. In about 24 hours, the vials may be placed inside the paraffin oven. After the vials have been in the oven about 3 hours, pour off the mixture of xylol and paraffin and immediately replace with pure melted paraffin. It is of no use to attempt to salvage the paraffin from the first change as there is too much xylol present; Parowax is so cheap that it can be freely used. Within the next 12 hours or so make at least two more changes; it is necessary to get rid of all traces of xylol or chloroform, whichever has been used, otherwise the paraffin will crystallize after embedding. Crystallization can to a great extent be prevented by embedding in rubber-Parowax. It is not so imperative that all traces of the essential oils be removed before embedding. In the case of bergamot oil, the characteristic odor can be detected in paraffin blocks that have not been

cut for as long as 25 years, consequently it is probably impossible to remove every trace of the oil.

If desired, one may go from the last change of xylol, chloroform, or secondary butyl alcohol to a mixture of any of these fluids and paraffin oil and carry out the infiltration process as described for tertiary butyl alcohol.

Dehydration with Benzol.—Benzol may be satisfactorily substituted in case absolute ethyl alcohol is unavailable (Kisser 1929). Dehydrate the material as described in the preceding section. After 95% ethyl alcohol, pass through each of the following mixtures of 95% ethyl alcohol and c.p. benzol (benzene, water-free), allowing several hours in each:

3 parts 95% alcohol and 1 part benzol
2 parts 95% alcohol and 2 parts benzol
1 part 95% alcohol and 3 parts benzol
Pure benzol, changing at least once.

Infiltrate as described in the preceding section.

Dehydration with Acetone.—Acetone constitutes a useful substitute for ethyl alcohol when supplies of the latter cannot be secured (Sass 1932). The series of acetone in distilled water should be fairly close: begin with 7½%, thence to 10% and by 5% stages to 40% and 10% stages thereafter until pure acetone is reached. The intervals can be less than 1 hour in each fluid. Clear in four grades of chloroform and acetone, and infiltrate gradually.

Dehydration with Dioxan.—Transfer the material from water directly into 60% dioxan in distilled water, thence to 70, 95%, and at least two changes of pure dioxan. Or one may transfer directly to pure dioxan, then give two changes of the reagent. Add little chips of Parowax gradually to the pure dioxan, and place the container in a warm place to accelerate the solution of the Parowax, as dioxan is a very poor paraffin solvent. After the dioxan becomes saturated, place the container in the paraffin oven, give at least four changes of pure Parowax, then embed in rubber-Parowax.

Dioxan is irrational in its action and apparently works far better on animal tissues than with plant materials, but it is worth trial. It should always be borne in mind that dioxan is heavier than melted paraffin and consequently is difficult to get out of tissues during the infiltration process.

EMBEDDING

The infiltration of the material completed, the next step is that of embedding. This consists of pouring the contents of the vial or other container (with the paraffin or rubber-Parowax in the liquid or melted condition) into suitable receptacles, arranging the material in proper order and then quickly cooling the entire mass.

There are many kinds of embedding receptacles, or "trays" as they are sometimes called, on the market. None of these, however, has any great advantage over a simple folded paper tray. Use a fairly stiff paper with slightly glazed surface (if a porous paper is used, the paraffin will penetrate it). The size of the paper may be regulated to any dimensions, and the side walls may be made as shallow or as deep as required. Using the diagram (Fig. 3) as a guide, first fold over along *CC'* and *DD'*, the width of the fold being governed by the thickness of the material to be embedded. This should be about 2 or 3 mm. more than actually needed because the layer of paraffin, when cooled, is somewhat thicker along the periphery than in the center. Next fold over on *AA'* and *BB'*, the width being twice that of *CC'* or *DD'*. Then fold back along the middle of each of these two flaps, as indicated by *aa'* and *bb'*. Hold the paper in the fingers, and, by using the nail of a thumb, make the short diagonal creases. To complete the folding, bring one end and one side perpendicular, with the fold at the short diagonal crease, and turn the resulting flap back of the end wall. Bring up the opposite side wall, and fold its flap back. Fold down, backward, the upper flap of the end wall, thus securely locking the entire end. Follow the same procedure for the opposite end. The size of the central area should be so regulated as to allow a little more room than may be actually needed for the proper disposition of the pieces of material. These trays are easily removed after the paraffin has cooled and may be used over and over again if dried under pressure to prevent curling. In fact, they actually improve with use. Trays may also be made from thick cellophane (No. 450 M.T. cellophane). Or one may use small porcelain trays or even solid watch glasses, first coating the inside with a thin smear of glycerin.

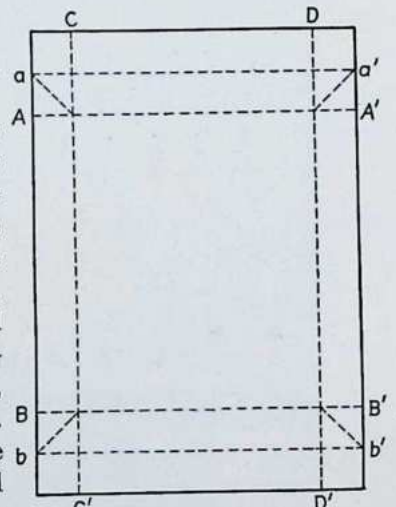


FIG. 3.—Paper embedding tray. Description in text.

Place the embedding dish at one side of the paraffin oven. A gas flame should be placed near-by for heating the needle used for arranging the material. Remove the vial or container from the oven, shake the material to get it off the bottom and quickly pour into the tray. Add more melted paraffin from the stock container if necessary; there should be just enough to cover the material adequately. A superfluity of paraffin prevents rapid cooling of the mass. With a needle, heated

slightly in the flame, quickly dispose the pieces of material into an orderly arrangement; avoid overlapping and leave plenty of space between neighboring objects. Many workers place the embedding tray on some form of hot plate while arranging the material. This procedure is scarcely to be recommended (except when the temperature of the room

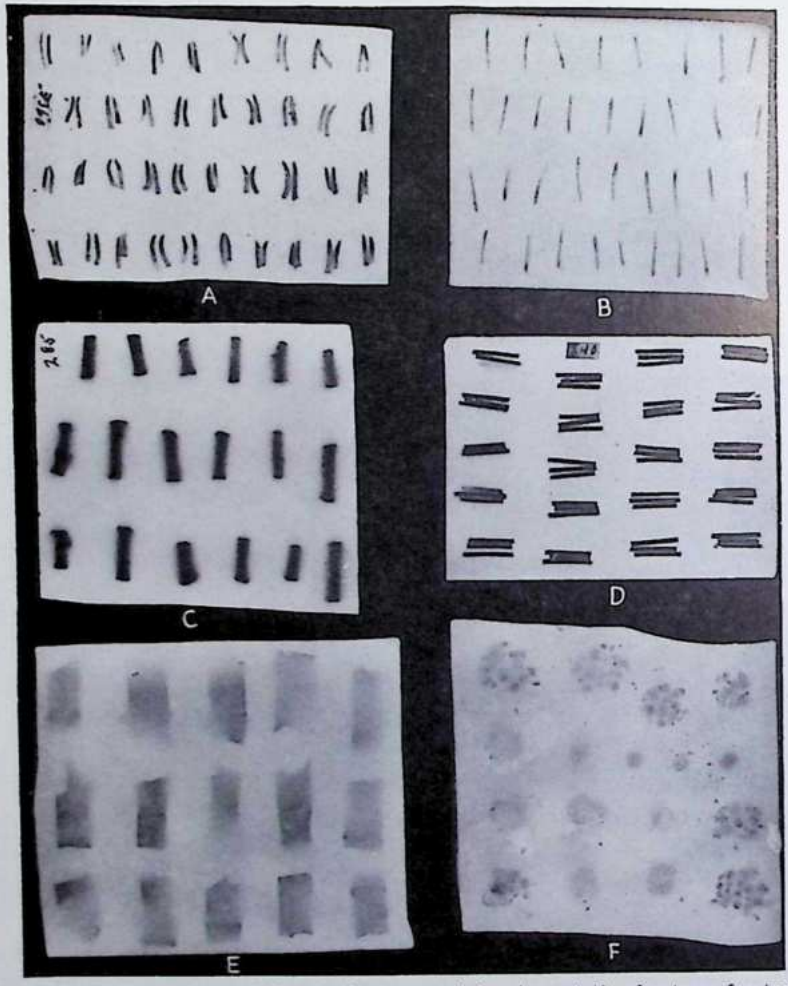


FIG. 4.—Methods of embedding various materials: *A*, root-tips by twos for transverse sectioning; *B*, root tips (onion) for longitudinal sectioning; *C*, single stems; *D*, leaves (pine) in bunches of threes; *E*, single leaf portions; *F*, small buds in bunches, large ones singly.

is below 50°F.) because of the danger of overheating and the unnecessary prolongation of the cooling process. Quick work and skillful manipulation, gained after a little practice, are all that one requires. If the tray is too cold, it may be warmed by leaving in the paraffin oven for a short time or by passing through the flame.

The accompanying illustrations (Fig. 4) indicate the correct methods of arranging various types of plant material during embedding. The arrangement of the material depends upon (1) the purpose for which the material is to be used, (2) the size of the pieces, and (3) their nature. Leaves should be arranged individually, but small ones, as well as needles of conifers like *Pinus*, can be embedded in bunches of three or more. Root tips which are to be sectioned longitudinally, such as those of *Allium cepa*, are likewise embedded separately, but tips which are for cytological purposes should be embedded in bunches for transverse microtoming. Buds are in practically the same category as root tips. For studies of the meiotic chromosomes, buds should be embedded and cut in bunches until they become of a size too large for this method; the chances of getting the reduction divisions are thus increased and the amount of work as well as the quantity of slides, covers, and reagents is lessened. Some types of ovaries may be treated similarly, but it is safer to embed and section such organs singly if critical studies of megagametogenesis are being undertaken. If soft enough to section easily, stems may be embedded in bunches, but most stems should be treated individually.

The paraffin usually begins to cool first at the bottom of the receptacle. As soon as the tray can be moved without disturbing the arrangement of the pieces of material, transfer to a vessel of cold water. (Do not leave the faucet running, if the tray is in the sink, and avoid splashing water in from above. Attach a piece of rubber tubing to the faucet, and run it to the bottom of the dish: in this manner running cold water may be utilized.) Let the tray float until the surface of the paraffin becomes sufficiently firm to permit plunging the tray slowly beneath the surface of the water. The tray may be weighed down by placing a heavy steel scalpel or similar object across the ends. As soon as the paraffin is firm enough, it may be removed from the paper tray by unfolding the latter. The paraffin blocks will usually float out of watch glasses and similar dishes after a while, but may need to be loosened by inserting the point of a scalpel at some place where there is no material that might be damaged. Always leave the blocks in the water for ½ hour or until thoroughly cooled.

The rapid cooling of the paraffin is a point which needs to be emphasized. If the paraffin cools too slowly it may crystallize: the blocks become full of fluffy white spots and patches. Such crystallized paraffin cannot be microtomed. The only remedy is to cut the material out and reembed it. In warm or hot weather ice water should be used for cooling the paraffin.

The finished blocks should be stored and not allowed to remain about to become covered with dust. The small boxes in which slides come are very convenient for storage purposes. Do not store one block upon

another without inserting a piece of stiff paper or thin cardboard between them. Excess paraffin should be trimmed away first; it may be melted down and used over again. If scraps of tissue are present in used paraffin, the latter should be filtered through cotton before being reused.

MICROTOMING

The material is ready for sectioning as soon as the paraffin has thoroughly cooled. Some materials seem to section better if cut at

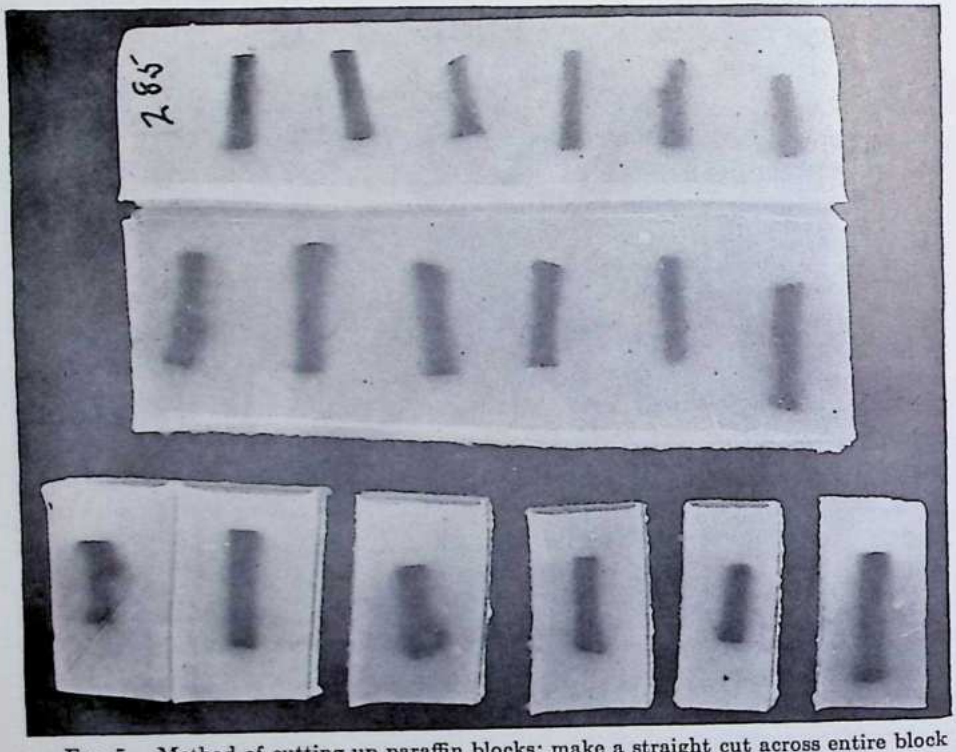


FIG. 5.—Method of cutting up paraffin blocks: make a straight cut across entire block with tip of scalpel, then break apart. Next make similar cuts between individual pieces of material and break up.

once, while others cut badly under the same circumstances. Little or no definite information concerning this phenomenon has been collected.

A piece of tissue may be removed from the block of material by inserting the point of a sharp, narrow scalpel and cutting a nearly straight furrow across the block (Fig. 5). The furrow should be at least 2.5 mm. deep, or deeper for thick blocks. Hold the block firmly in the hands and break apart along the cut. Trim down the piece until surrounded by about 3 mm. of paraffin except on the bottom, where at least 5 mm. should remain. The photographic diagrams show the proper method of trimming down the material (Fig. 6).

The round metal holders which are furnished with most microtomes are cumbersome, too large, and generally unsatisfactory. Hardwood blocks are far better and can be made in any desired end dimensions and in any quantity (Fig. 6). The wood should be of a kind not readily crushed by the pressure exerted by the holder of the microtome. The length should be at least 2 cm., while the ends may be either square or rectangular and of different dimensions to receive paraffin blocks of various sizes and shapes. Make a large and assorted quantity of such

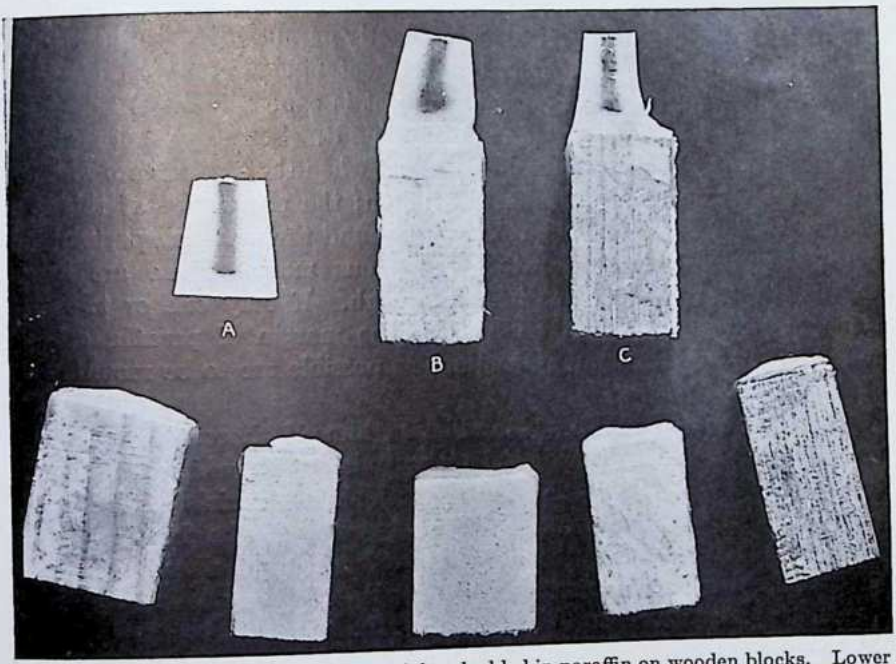


FIG. 6.—Method of mounting material embedded in paraffin on wooden blocks. Lower row shows hardwood blocks of various widths and thicknesses, with mound of paraffin on top. *A*, paraffin material roughly trimmed and ready for mounting; *B*, material mounted; *C*, material trimmed down, ready for microtoming.

blocks if only a few sections are to be cut at first; the whole can be set aside and there is no need of removing the pieces of embedded material, as is the case with the metal holders.

Heat waste paraffin very hot, immerse one end of the wooden block for a depth of 2 mm. until saturated with paraffin, then stand on the other end until all the blocks have been dipped. With a pipette, build up a mound of paraffin on top of each block, using paraffin that is barely liquid. When ready to attach a piece of embedded material to such a block, heat an old scalpel or a similar flat instrument with nonmetallic handle in a gas flame, and touch first to the paraffin on the wooden block then to the bottom of the embedded piece, bringing the two together

while the paraffin is still more or less melted. Reheat the instrument and cautiously melt together the two paraffins so that they form a single mass on the wooden block. Avoid touching the plant tissue with the hot instrument. Work quickly. Next stick a needle (inserted in a holder) in the unused end of the wooden block, and put the latter in a dish of cold water to cool the paraffin.

The next step is to trim away the excess paraffin (Fig. 6). Use a sharp scalpel with a long, straight blade, not a curved one. It is absolutely essential, if perfect sections are to be cut and the ribbon is to be straight, that the two longest edges be exactly parallel to each other. The face may be square, of course, but the rectangular form is preferable. Do not cut away paraffin until the object is exposed on the sides, but leave at least 1 mm. of paraffin surrounding it. Some types of material are very liable to swell up and break apart when the sections are warmed to straighten out the kinks in the ribbon; the preventive of this sort of disaster is to leave enough paraffin around the material, particularly at the short ends. The rather gelatinous marine algae are likely to swell if fixation has not been thorough. If only one or two sections are to be mounted on a slide, the margin of paraffin can be much wider than if as many sections as possible are to be mounted on each slide.

The student should now familiarize himself thoroughly with the mechanism and operation of the rotary microtome, which is preferred by the majority of technicians for sectioning materials embedded in paraffin. Note particularly the following points: (1) the clamp or holder for the wooden block and the method of orienting it at all angles; (2) the feed mechanism and the method of setting it to give any desired thickness (the scale is in microns); (3) how to reset the mechanism after it has run its course; (4) the points at which oiling should occasionally be done; (5) the knife holder and the method of adjusting both the holder as a whole and the knife itself. Note that the holder is adjustable so that the knife can be oriented at different angles to the vertical. This is to allow for differences in the bevels of different knives. An ordinary knife should be inclined at an angle of about 8° from the vertical, which will give ample clearance. Safety razor blades in holders should be set nearer to the vertical, but take care to see that there is nothing in the way which might scrape or be struck by the paraffin block. The blade should never scrape against the paraffin at any point; if it is almost vertical it will scrape rather than cut.

For one's own peace of mind, one should possess his own knife, or furnish his own safety razor blades if a holder is available, rather than depend upon knives in common use. The latter are too apt to have an edge like that of a saw.

It is of the utmost importance that microtome knives be *sharp*. Never begrudge time spent in sharpening knives, for it is time well spent. A regular microtome knife, kept in perfect condition, provides the best of all cutting edges, no matter what the claims of those who favor safety razor blades. Begin cutting at one end of the blade and gradually work

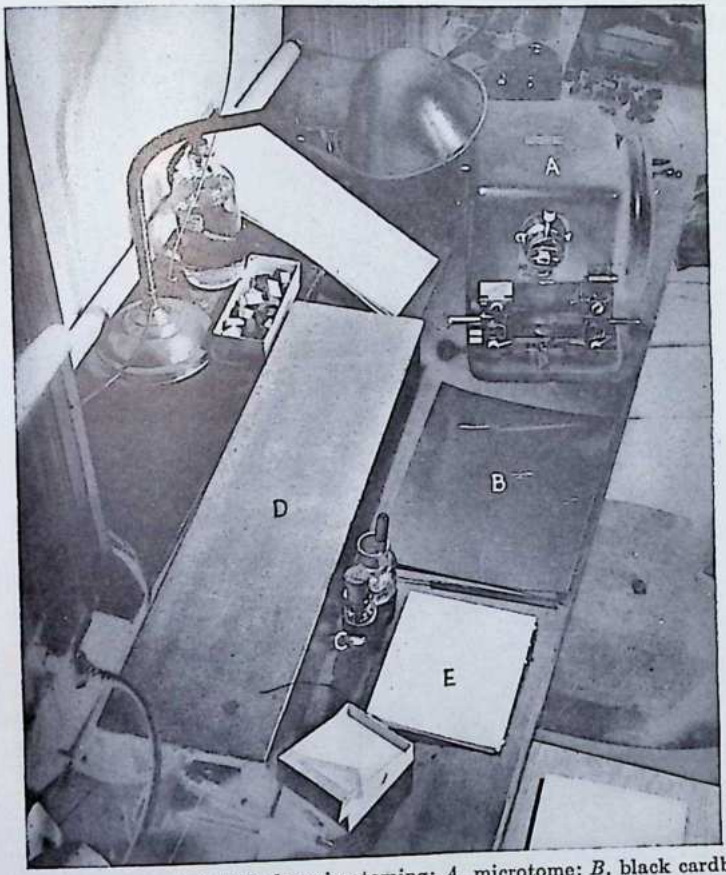


FIG. 7.—Arrangement of table for microtoming: *A*, microtome; *B*, black cardboard on which to place ribbons; *C*, bottles with adhesive and flooding solution; *D*, warming plate; *E*, absorbent paper towel folded over, for draining excess flooding solution. Box of slides is at one end of the warming plate. Lamp should be so placed that the light is directed in front of the microtome knife.

over to the opposite end. Trim down unwanted parts of the material on a used part of the knife, then move the latter forward to a new place when the desired sections have been reached. When the whole of the edge has been used, remove the blade, and strop, or resharpen and strop.

Insert the wooden holder in the clamp and screw down securely (see Fig. 8). The part of the wood projecting from the clamp should not be more than 3 mm. in length. First look down upon the paraffin block

from above, and adjust so that the face of the block is parallel with the blade; move the carriage forward until the blade barely touches the paraffin at its lower edge. (If the object is not oriented so that it is perfectly perpendicular to the blade edge when examined from above, adjust properly, cutting away any excess paraffin with a sharp scalpel.) Then cautiously bring the block down toward the knife by turning the

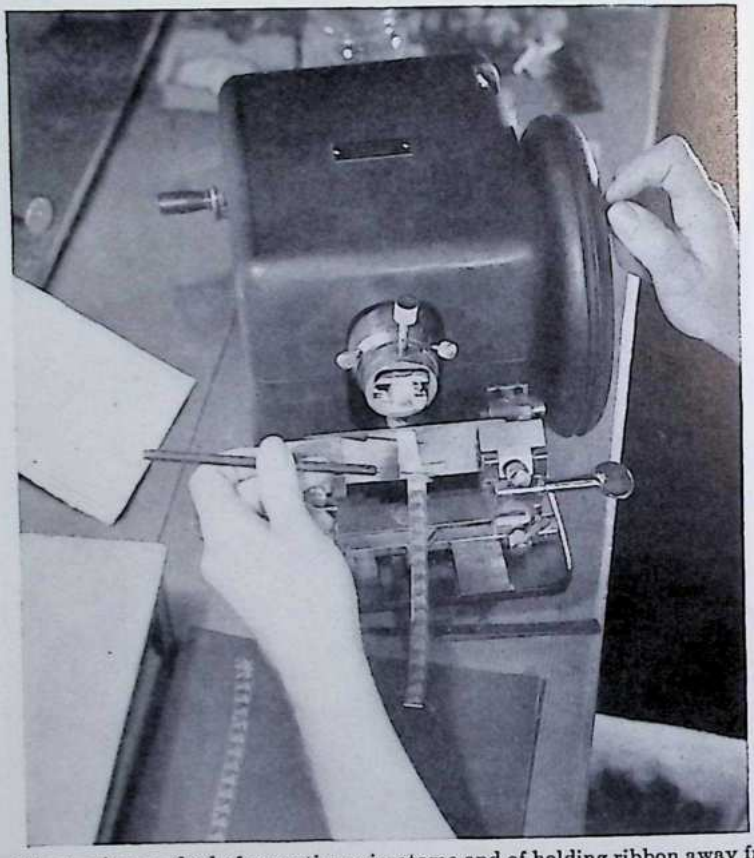


FIG. 8.—Illustrating method of operating microtome and of holding ribbon away from knife by means of a needle in order to prevent telescoping of sections.

wheel, and examine from directly in front. If the lower (longer) edge of the paraffin block is not parallel horizontally with the edge of the knife, adjust the holder until the edges of block and knife coincide. The vertical adjustment should be permanently fixed; there is no excuse for not trimming down the front of the paraffin block so that when it is placed in the holder the object is perpendicular to the knife when viewed from either side. If the embedded object is nearer to one long edge of the paraffin block than the other, orient with the nearer edge toward the knife

to avoid knocking the object out of the paraffin or tearing the upper edge of the sections.

Set the micron scale at the desired thickness. On most modern rotary microtomes a single notch in the geared wheel is equivalent to 1μ in thickness, but on many sliding and a few older rotary microtomes each notch is equal to 2μ. One can easily determine which is the case by studying the indicator scale. The scale should be carefully adjusted. Do not leave the indicator between points on the scale; this will cause sections of very uneven thickness to be cut and will wear down the

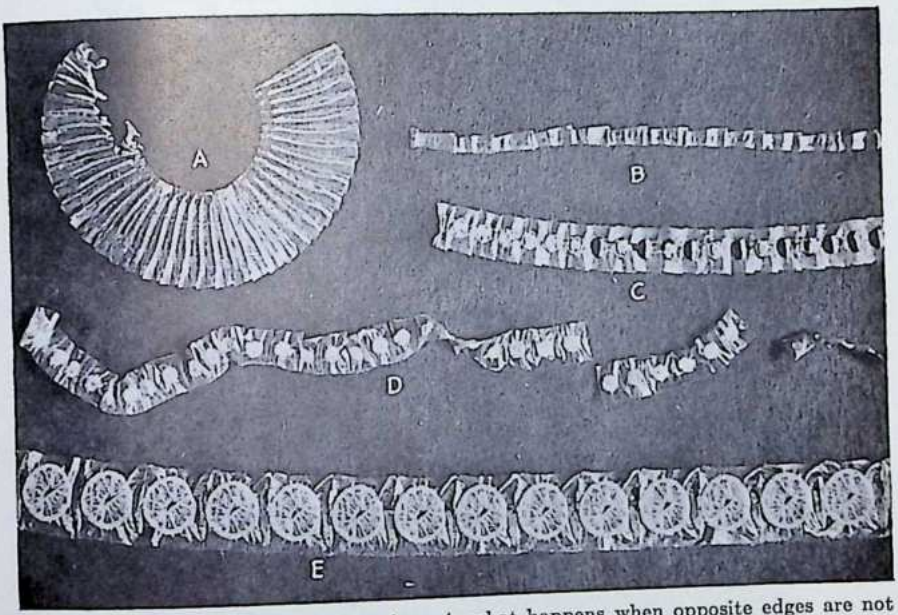


FIG. 9.—Good and bad microtoming: *A*, what happens when opposite edges are not parallel; *B*, when the narrow edge is toward the knife—an example of careless trimming and orientation of the block; *C*, result of leaving a hard stem too long under water—all sections torn, some curled; *D*, mediocre ribbon from a woody stem, but some of the sections can be salvaged; *E*, perfect ribbon of old *Aristolochia* stem.

mechanism quickly. There is no such thing as fractional microns; however, specially constructed machines which will cut at half-microns are available. (If one is uncertain at which thickness to cut, run off a few sections at 12μ, mount on a slip and examine under the low power of the microscope to see whether they are too thick or too thin.)

One is now ready to commence cutting (Fig. 8). Move the wheel with a steady, even stroke. The first few sections are invariably worthless and should be discarded, except in the case of materials of which complete serial sections are required. If the material was such that it could not be trimmed down sufficiently before being killed and fixed, the excess tissue is easily cut away; use for this purpose any part of the knife

that has already been used. On the rotary microtomes the sections form a ribbon as they are cut one by one. There is a very slight heating of the lower edge of the block as it strikes the knife; this is what makes the sections stick together to form a ribbon. If the cutting is stopped, the ribbon is likely to become broken. The ribbon sometimes exhibits an annoying tendency to curl up and under the paraffin block. To avoid

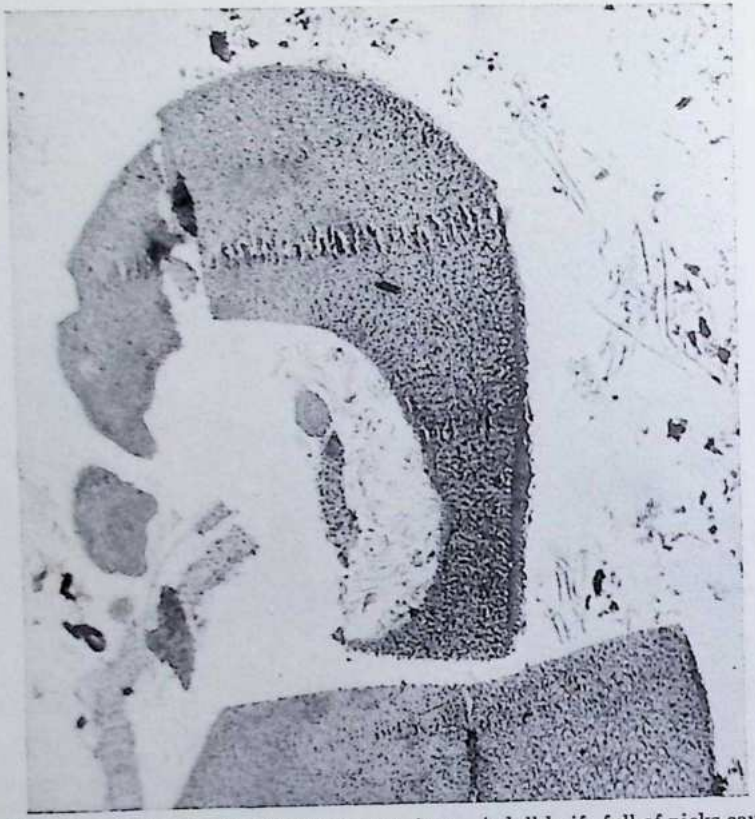


FIG. 10.—A perfect example of bad microtoming. A dull knife full of nicks caused both sharp cuts (near bottom) and corrugated tears (above middle and elsewhere), while there was so much compression that breaks parallel to the plane of sectioning were produced (upper left) and portions of the section were also moved out of position. (Material is bud of *Botrychium*.)

cutting it, learn to hold a needle or small camel's-hair brush in one hand and keep the ribbon from getting under the block or from becoming telescoped on the knife (Fig. 8). The ribbon should be straight (Fig. 9*E*). If it is not, the opposite edges of the paraffin block are not parallel; less often, the object is of uneven hardness, or the paraffin may not have solidified homogeneously. In any case, the remedy is to trim away carefully a tiny wedge from the thick edge, cutting a few sections to ascertain if the ribbon has become straightened or if not cutting away a little more

paraffin. Of course, if the sections are to be mounted singly, it does not matter if the ribbon is not strictly straight. It will be observed that the side of the ribbon toward the knife is smooth and glossy while the other side is dull and slightly rough; keep the glossy side down and mount likewise in order to secure greater adherence to the glass slide.

MICROTOMING OF REFRACTORY MATERIALS

Many plant tissues, although soft and easily cut with a scalpel at the time of collecting, are rather tough and difficult to section easily after having been embedded.

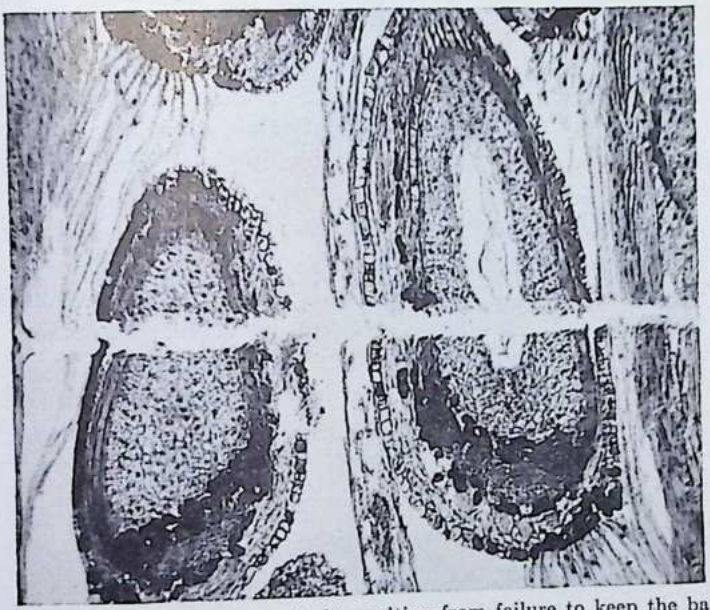


FIG. 11.—Illustrating a common fault resulting from failure to keep the back of the microtome knife free from debris. The tear extends across an ovule, rendering the megagametophyte useless for study.

The remedy is easy of application. Cut away enough of one end of the paraffin block and through the piece of material to be cut so that the latter is exposed. Place the material in a vial or bottle full of sterile water, cork tightly, and set aside overnight. Of course, several pieces of material may be softened simultaneously in the one container. The water will penetrate the cut end and soften the material. Some woody stems may require a month or longer to become sufficiently softened, but no specimen should be left in the water any longer than the time required to soften it, otherwise maceration will set in. The rate of penetration may be gauged by the increased opacity of the paraffin around the material. If a number of sections are cut and then trouble is encountered,

return the material for further soaking. Once material has been placed under water, it cannot be dried and then resoaked; it should all be sectioned and the ribbons stored if all are not to be mounted.

Some objects, such as root tips, which have little paraffin in them, cut better and one also obtains less wrinkled sections if the block with its mounted object is immersed in ice water for a few minutes before being microtomed. Do not leave in the ice water too long, otherwise the sections will fail to ribbon. If this occurs, warm the knife on the drying plate, but do not attempt to warm the paraffin itself.

It has frequently been claimed that better microtoming can be done during rainy than during dry weather; this may be true for some geographical regions but is otherwise merely a superstition. One should easily be able to arrange things so that microtoming can be done at any time in any sort of weather. If the atmosphere is very dry and the sections become so full of static electricity that they stick to everything, the remedy is to keep a beaker of water boiling in the immediate vicinity of the microtome.

The newer safety razor blade holders are so constructed that either ice or lukewarm water may be run through them. For ordinary purposes and under average weather conditions, let ice water drip through somewhat slowly; for very thin sections or during very hot weather, run the ice water through rapidly; during very cold weather or for very thick sections, which are prone to curl and refuse to form ribbons, lukewarm water may be run through the holder. A little experimenting will reveal the optimum temperature for different sorts of material under various atmospheric conditions.

MOUNTING

Place the length of ribbon on a piece of stiff paper or thin cardboard with a smooth, more or less glossy surface. A piece of black poster board, as used by artists and sign painters, is excellent for the purpose, as the sections are more readily detected against the dark background. Do not put the ribbons on paper towels or similar soft papers. Be careful to avoid drafts and not to blow or to breathe heavily on the fragile ribbons. The ribbon may be cut into sections of a convenient length; use a sharp scalpel for cutting, and cut by drawing the scalpel rather than by pressing down.

The number of sections to be mounted on each slide is governed by the nature of the material. Sections of stems (if not too wide), leaves, roots, and similar subjects are best as a series of not more than three, since one section is about identical with the others. Longitudinal sections of root tips are usually mounted five to a slide, but cross sections of root tips are usually all mounted on one slide (see Figs. 12–14). In

the case of ovaries the sections should be examined with a hand lens; th[illegible] showing ovules or ovules with clear spaces indicating the presence

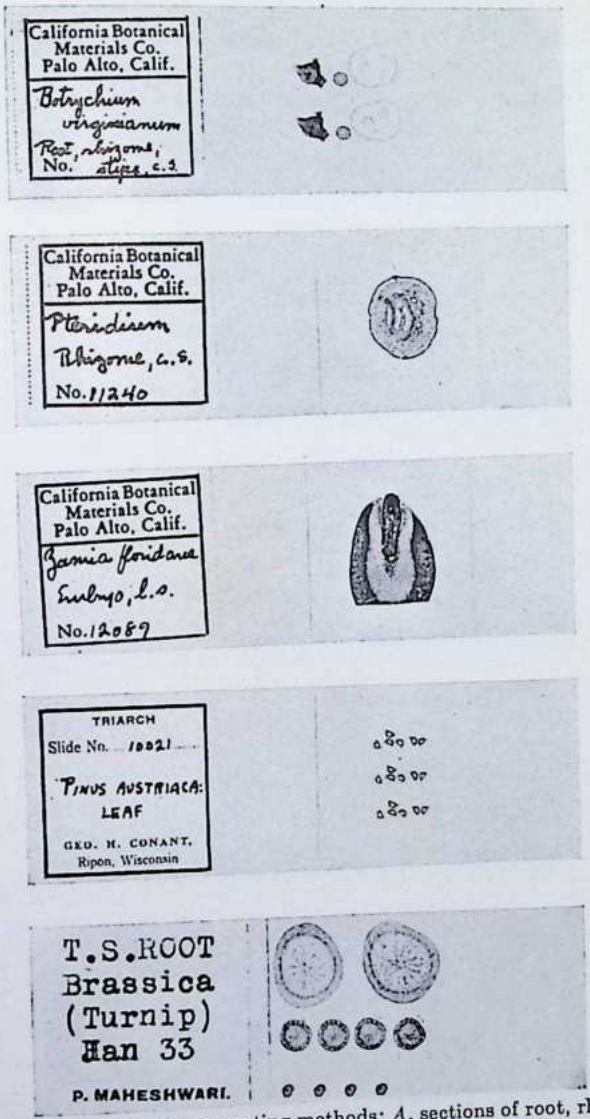


FIG. 12.—Illustrating various mounting methods· *A*, sections of root, rhizome and stipe of the same plant mounted together; *B* and *C*, single large sections; *D*, three serial sections of material embedded in bunches; *E*, three different growth stages of the samo type of material.

of megagametophytes should be selected and the rest of the sections discarded. With such materials there are invariably a large number of worthless sections, and it is only a waste of time and reagents to run all

of them up. Always make allowances for the fact that the sections expand when warmed to flatten them out.

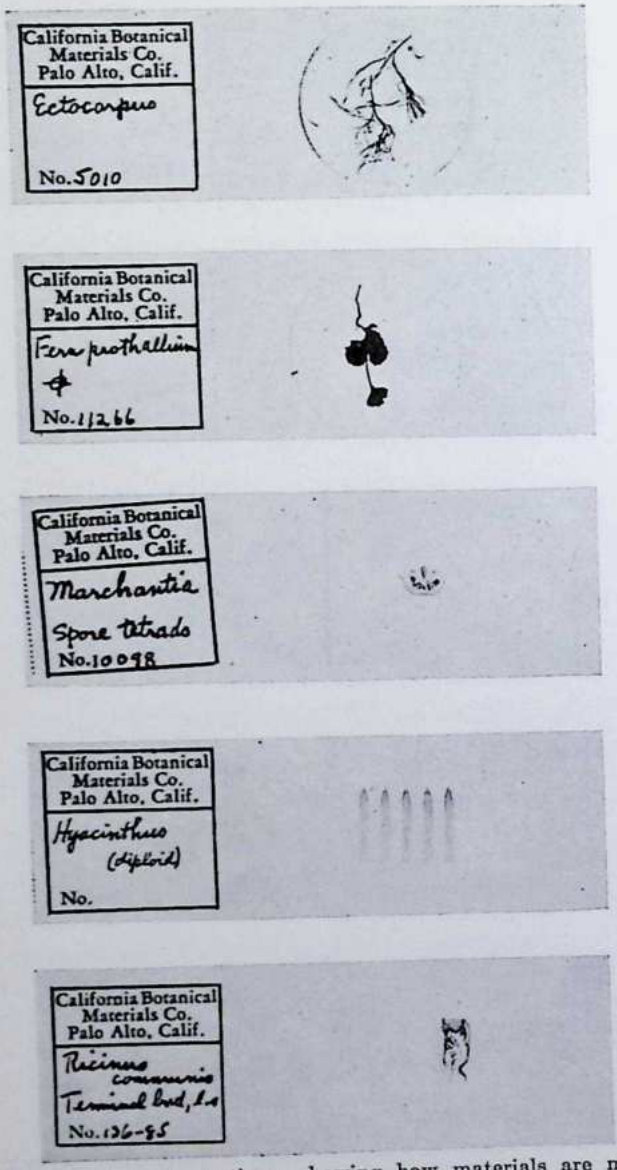


FIG. 13.—Commercial preparations, showing how materials are mounted "upside down," in the case of both whole mounts and sections, so that they will appear in the natural position when observed under the microscope.

In still other cases, as in *Ricciocarpus*, *Anthoceros*, and other Hepaticae, only certain sections are really worth anything. The difficulty is to locate such sections. Fill either half of a large Petri dish half full of

distilled (or air-free) water; cut the ribbon into sections about 1.5 or 2 cm. less than the length of a slide (*i.e.*, about 5 cm. in length), and

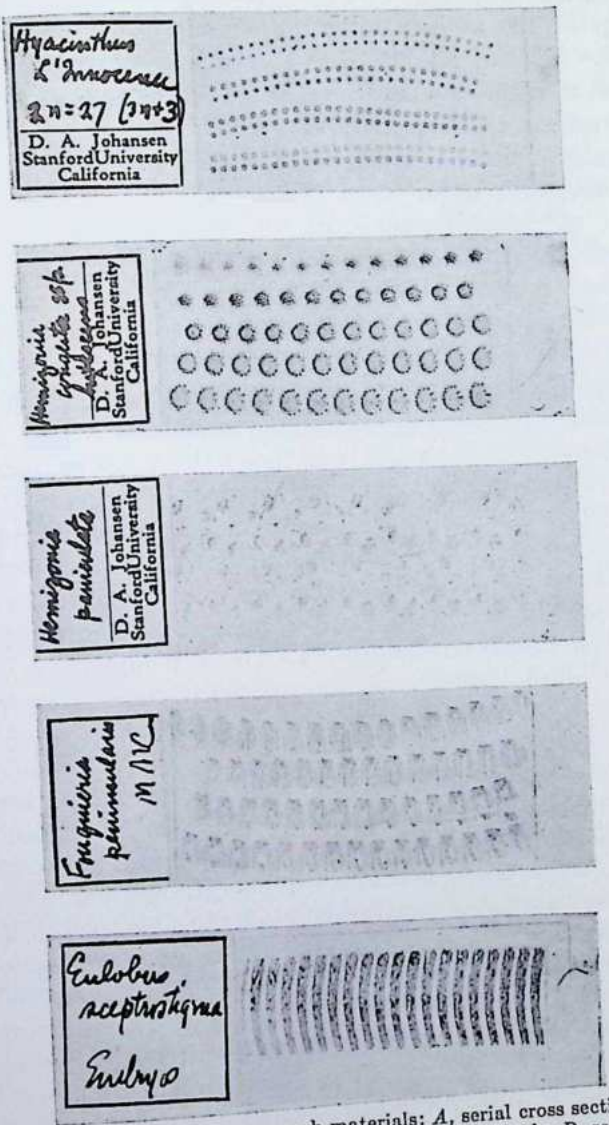


FIG. 14.—Methods of mounting research materials: *A*, serial cross sections of root tips; *B*, serial sections of a single bud; *C*, serial sections of bunched buds; *D*, serial sections of a single bud for megasporogenesis; *E*, serial longitudinal sections of a single ovary for embryogenesis.

place the sections on the water in the Petri dish. Place the dish on the warming plate, and leave until the sections have wholly straightened out, whereupon the dish should be removed from the heat and set aside for

the water to cool since the ribbons would be irreparably damaged if handled while still warm. Smear adhesive over the entire surface of a slide, flood with water, take up a length of ribbon with a scalpel and place on the slide. Drain off excess water, then examine the sections under the microscope. Do not let all the water evaporate under the ribbons. The desired sections may then be selected and cut away with a scalpel, and the worthless ones rejected; then reflood the slide with water, pick up the sections with a scalpel, transfer to other slides smeared with adhesive, and warm again.

Haupt's adhesive is unexcelled for affixing sections to the slides. Place a small drop of the adhesive on the slide, smear it evenly over the surface with a finger, and wipe off any excess adhesive; only a barely perceptible film should remain. Place the slide on the table, or better still on the bottom half of the box in which 24 × 50 mm. coverslips come, and immediately flood with 3% formalin in distilled water. The adhesive must not be allowed to get dry before the formalin is added. Touch the point of the scalpel in the water, and insert the tip of the blade under a section or portion of the ribbon. The drop of water will cause the section to stick to the scalpel so that it can be lifted. Place the section on the flood, then put the slip on the warm plate to flatten out the wrinkles in the paraffin and incidentally to generate fumes of formalin which will serve to coagulate the gelatin of the adhesive. The temperature of the warm plate should not be over 43°C., as it is important not to melt the paraffin. If a thermostatically controlled electric warm plate or slide warmer is not available, hold the slide cautiously over an alcohol flame and watch the sections to avoid melting the paraffin. After the sections have stretched out, take the slide off the warm plate and set aside for a few minutes until the water cools. Do not try to move the sections while the water is still warm, as the paraffin is easily damaged at this stage. After the slide has cooled, hold the sections in place with a needle, drain off the excess water, and touch the slide to absorbent paper to get rid of as much water as possible. Then move the sections to the position it is desired that they occupy, and set the slide aside to dry completely. If the sections show a tendency to move around, watch them, and restore to the proper position before the slide gets completely dry. Do not attempt to move the sections from a wet to a dry place on the slide: this is usually impossible without damage to the sections.

Set the mounted slide away in a dustproof place for several hours or a day for the drying to be completed. The slides should not be left on the warm plate to dry as untidy stained backgrounds frequently result from this practice. Mounted slides may be stored for as long as several years if it is not convenient to stain them within a few days. As soon as the slides have dried thoroughly, they are ready for staining.

STAINING

Before the sections can be stained the paraffin must be removed. Xylol is used for the purpose by the majority of technicians. Place the slides, either singly or as a series in a rack, in a jar of xylol for about 5 minutes or somewhat longer. There should be sufficient xylol, as well as enough of all reagents subsequently to be used, to cover the slides. There is no economy in using as small quantities as possible since they will soon become so badly contaminated that replacement becomes necessary more frequently than is the case when ample quantities are used.

From the xylol withdraw the slide slowly, and transfer to a jar containing equal parts of absolute ethyl alcohol and xylol. The slides should not be pulled out of solutions quickly as this procedure causes them to carry over a large amount of fluid, which contaminates the next solution. Withdraw the slide somewhat slowly, thus carrying over a minimum amount of fluid, but not so slowly as to permit drying, or absorption of moisture from the air.

Thin sections and the average nonwoody tissues will stick safely to the slides, but sections of woody tissues, those containing mucilage, fats, and waxes, as well as those which are overchromated, are retained only by the exercise of great caution in avoiding diffusion currents. Sections may stick until water is reached, whereupon they float right off the slide. It is therefore advisable to play safe at all times by covering the sections and slides with a protective coating of thin celloidin. The carmin dyes are the only ones which have any tendency to stain the celloidin, but, fortunately, these dyes are scarcely ever used on sections of plant material.

After 5 minutes in the absolute alcohol-xylol mixture, transfer the slides to a mixture of equal parts of absolute alcohol and ether (anesthetic ether should be used) to which has been added sufficient of any celloidin solution to make the strength about, but not over, 1%. Between 5 and 10 minutes in this fluid is long enough.

Withdraw the slides, and allow them to remain in the air (which should be warm and dry) until the sections show signs of becoming whitish-opaque, which indicates that the celloidin film is nearly dry. Then plunge the slides into either 95 or 70% ethyl alcohol for 5 minutes to harden the celloidin. Next transfer to 70% alcohol if 95% was used, otherwise to 35%, for 5 minutes. If an alcoholic stain is to be used, pass to this stain from the 70% alcohol; if the stain is aqueous, go through 35% alcohol to water before placing in the aqueous stain.

Staining procedures are outlined in Chap. VII.

DEHYDRATING AND MOUNTING

After the sections have been stained, they must be dehydrated preparatory to mounting in balsam. More or less of the dehydration may be accomplished during the staining procedure. If aqueous staining fluids alone have been employed, proceed to 35% alcohol, thence to 70 and 95%. After alcoholic stains the slides are usually in 95% ethyl alcohol, or may be transferred to that percentage. Counterstaining of most plant materials is done with the dye dissolved in clove

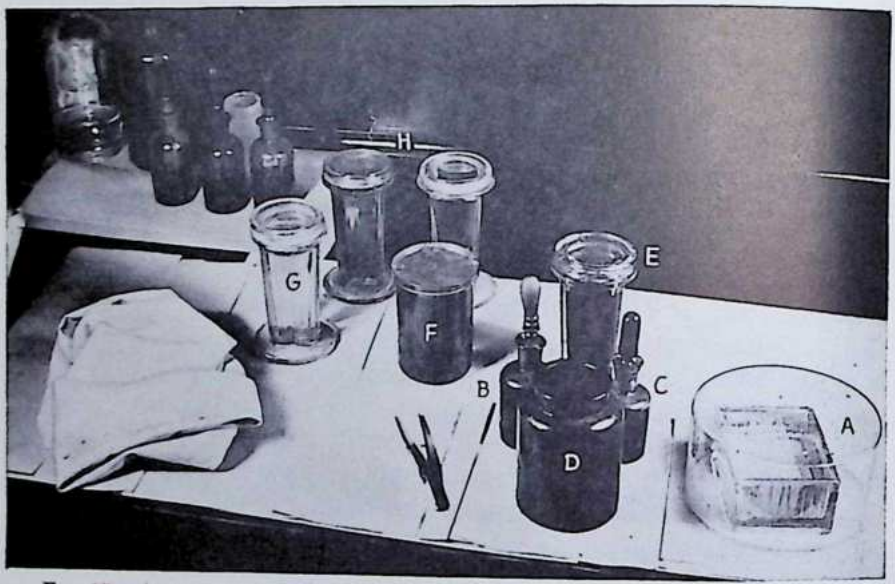


FIG. 15.—Arrangement for counterstaining with clove oil solutions: *A*, slides in 95% alcohol in flat dish (covered with large crystallizing dish or similar cover); *B*, dropping bottle with counterstaining solution; *C*, dropping bottle containing used clove oil solution for washing off excess stain into waste jar *D*; *E*, jar containing differentiating solution; *F*, xylol wash; *G*, first xylol jar; *H*, second xylol jars. Also shown are forceps and cloth for wiping off underside of slides.

oil diluted with absolute alcohol, and frequently also with methyl cellosolve (Fig. 15). The slides, in such cases, are taken from the 95% alcohol, the lower side swiftly wiped dry with a clean cloth, the counterstain applied from a small bottle equipped with a pipette stopper, allowed to remain for a few seconds, and then poured back into the bottle. Used clove oil solutions should be saved up; put a portion in a bottle with a pipette stopper, and dilute with one-fourth its volume of a mixture of equal parts of absolute alcohol and xylol (if the slides were too heavily coated with celloidin and it becomes stained, add a little ether to the mixture). Use this diluted clove oil mixture to wash off excess stain into a waste bottle, taking care not to squirt the fluid directly against

the sections with the pipette, and thus avoiding too rapid contamination of the clove oil differentiator with stains. The differentiator is made by mixing 1 part U.S.P. clove oil and 1 part of a mixture of equal parts of absolute alcohol and xylol. Move the slide slowly back and forth in the differentiator for about 10 seconds, then transfer to a jar of xylol to which a trace of absolute alcohol has been added to take up any moisture inadvertently brought over. (Always keep the clove oil mixture covered except when actually in use, as it absorbs moisture readily.)

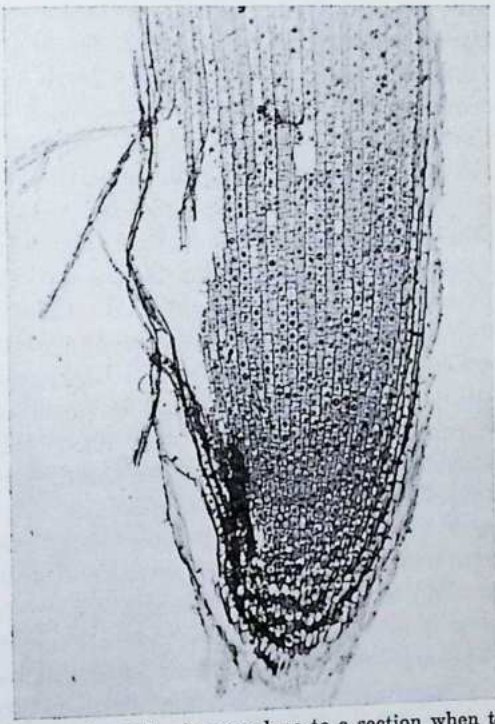


FIG. 16.—A typical example of the damage done to a section when there is little balsam under the coverslip and the latter is moved around.

Move back and forth for a few seconds, then place the slide in a jar of pure xylol. A large number of slides can be stained and left in pure xylol for some time or until convenient to mount in balsam. Just before mounting, place for a short time in a third jar of xylol.

Remove a slide from the xylol, wipe the xylol from the underside with a clean, dry, lint-free cloth and also wipe it from around the sections (taking great care not to come too close to them) in order that, if too much balsam should inadvertently be applied, it will not become dissolved and run over the slide. Next place a small drop of thin balsam on top of or at the left of the sections. Pick up a cleaned coverslip (an entire box of coverslips should be cleaned at one time and placed

in an empty slide box: to clean them, dip in a mixture of equal parts of 95% alcohol and xylol, touch to paper toweling to absorb excess fluid, then polish quickly with a scrupulously clean cloth), with fine-pointed forceps, pass quickly through a clean alcohol flame to remove surplus moisture, hold so that the forceps are at the right, and bring the left edge of the coverslip down on the slide, still holding up the opposite edge of the coverslip with the forceps. Let the coverslip come down gradually, allowing the balsam to spread to the opposite edges and then to be pushed ahead as the coverslip is brought lower and lower. At times a little pressure exerted with the nail of the left index finger on top of the coverslip will be helpful, especially if bubbles have become trapped. Do not lift the coverslip up once it has balsam under half of its area. After three-fourths of the area under the coverslip has been covered with balsam, withdraw the forceps (also the finger if it has been used), and allow the uncovered area to become covered with balsam by the weight of the coverslip. The size of the drop of balsam must be learned by experience; it is determined first by the thickness of the balsam itself and secondarily by the thickness of the sections. Thin sections require less, as well as thinner, balsam. The area of the coverslip does not enter into calculations to the extent that might be imagined, unless the balsam is quite thin. Thick balsam should be used on sections, no matter what their number or size, that are over 20μ in thickness. Some kinds of material tend to lift the cover during the drying process, thus admitting air bubbles or canals. Sometimes these can be forced out by gentle pressure on the coverslip, but in other cases one can only run in more balsam from one edge of the cover, or put the slides into xylol to dissolve the balsam and to loosen the coverslips, later remounting.

When the coverslip is first applied, it should be held in the exact position desired, and after coming down completely, it should never be moved sidewise in any direction, otherwise there is great danger of damaging the sections (Fig. 16). The less balsam there is under the coverslip, the greater the chance of damage. Sections containing loose cell contents, such as very starchy tissues, are more liable to distortion.

The slides are now ready for drying on a warming table or in an incubator heated to not over 60°C. The balsam does not become completely solidified throughout the area under the coverslip for several months; consequently the slides should be removed from the warming plate after a day or two (by which time the balsam will have become sufficiently hard around the edges of the coverslip), lest the stains become damaged by heat. The slides may then be placed in slide boxes, which come in various types and capacities.

CHAPTER XIII

SMEAR METHODS

The more refined of the various smear methods afford, in a relatively short time, preparations which are of the greatest value in counting the monoploid and diploid chromosomes and in studying the chromosomes themselves. In fact, smear preparations are so useful that at the present time many critical cytological studies are based principally upon such slides. The student consequently would do well to master as soon as possible the technique of smearing either plant or animal material or both, to gain some familiarity with the different methods of staining smears, and to judge correctly what finally results.

The smear method is limited to some extent in applicability to cells which are not firmly united to one another, as by middle lamellae. In the higher plants this refers to the microsporocytes after they have begun to round up, and in animals to the cells of the testis at the corresponding stages. The microsporocytes of some plants (*e.g.*, those of *Acacia* and some orchids, which are collected together into pollinia) and the testis cells of many animals cannot be satisfactorily smeared but should be prepared by other methods. In the present chapter only plant material will be considered.

The essential idea underlying all smear methods is to spread the cells out into a single layer in order that they may be killed instantly and fixed evenly and uniformly, without distortion or the production of artifacts. Practically all cells which can be smeared will adhere to the slide; hence the necessity of using a cementing agent (which might itself be stained) is obviated. The slips can be carried through the various staining and dehydration processes with the loss of only a few cells, provided, of course, that the changes are not too violent. Special precautions will occasionally be noted in the procedures listed below.

Technique of Smearing.—The slides upon which the smears are to be made must be chemically clean. New slides should be given a long immersion in the sulphuric acid-potassium bichromate mixture (page 13), rinsed in running water, placed for a short time in strong alcohol to which a little ammonia is added, rinsed again, and finally dried with an absolutely clean cloth free from starch and lint. It will save time if an entire box or two of slides are cleaned at one time. Slides which have previously held sections should never be used for smears.

The fluid which is generally considered to be superior to all others for the killing and fixing of smears is Navashin's. The two portions composing this fluid are mixed together just before using. It may be necessary in some cases to vary the proportions of some of the ingredients, particularly the acetic acid; this can be judged only by trial.

The most satisfactory vessel in which to carry out the fixation of smears is the square Petri dish recently placed on the market (obtainable from E. H. Sargent and Company, 155 East Superior Street, Chicago, Ill.). Place slender glass rods, cut to the proper length, at opposite ends of the lower half of the dish to keep the slide off the bottom. The dish

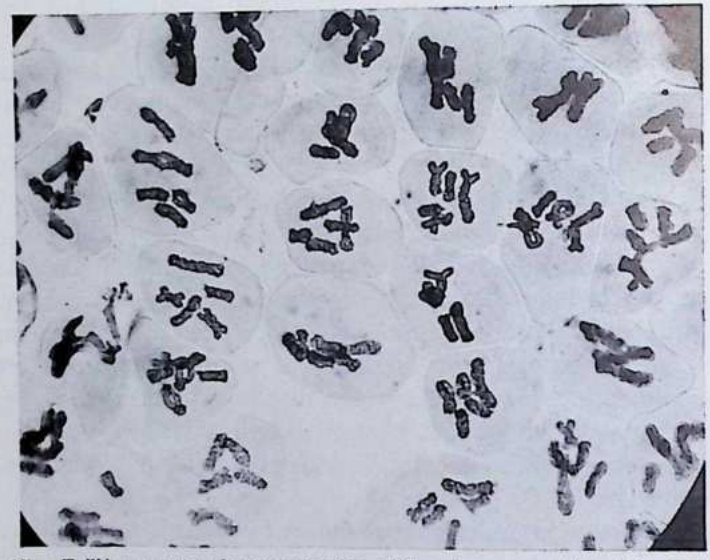


FIG. 17.—*Trillium ovatum:* low-power view of the microsporocytes at the first division; permanent iron-acetocarmin smear preparation. (*Smear and photomicrograph by Dr. H. E. Warmke.*)

will hold two slides placed one beside the other. Pour the killing solution into the dish just before making the smears; it should be more than enough to cover the rods (approximately 40 cc.).

Different technicians have methods of making smears adapted to their own needs. Most of them remove the anthers from the buds, cut off both ends, or cut each anther into short segments if it is over 2 mm. in length, and place near one end of the slide. Previously a mark should be made in the upper right corner of the slide with a grease pencil (or etched with a diamond) in order that one may know on which side the smear is made. With a clean scalpel, which should be honed flat and smooth, quickly and evenly crush and spread the anthers over the center of the slide so that the microsporocytes are in a single layer over the greater part of the slide. Immediately invert the slide and place

in the Petri dish in such a way that the entire smeared surface comes into instantaneous contact with the killing fluid. If the slide is brought into the fluid in any save a perfectly horizontal position, most, if not all, of the material will be washed off. The time elapsing from excising the anthers to placing the smear in the killing fluid should not exceed 4 seconds. As the capacity of the Petri dish is two slides, a second smear may now be made, using anthers from another bud if desired, and placed beside the first. Although the slides may be turned smear side up after about 10 minutes, it will be safer to leave them as they are for the duration of the time necessary for proper fixation, which with Navashin's fluid is about 4 hours.

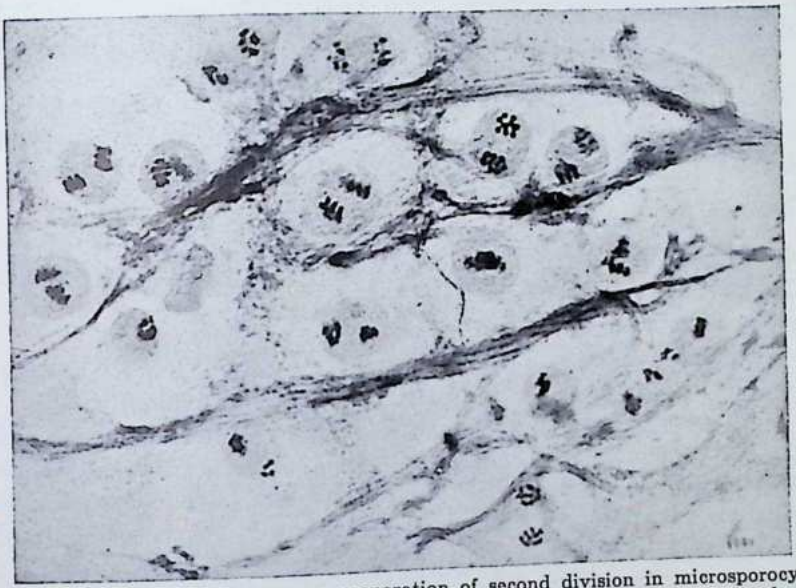


FIG. 18.—*Nicotiana alata:* smear preparation of second division in microsporocytes. The anthers contain a dense periplasmodium which absorbs some stain. Fixed with Navashin's fluid; stained by Johansen's methyl violet method.

Another method of smearing the anthers or pieces of anther is to place them in the center of a slide, to hold a second slide crosswise, and to exert just enough force firmly to extrude and to spread the microsporocytes. Immediately invert both slides in the dish containing the killing fluid.

Other killing fluids may, of course, be tried. The following has proved excellent for chromosome structure:

10% aqueous acetic acid	10.0 cc.
10% aqueous chromic acid	1.0 cc.
2% osmic acid in 2% chromic acid	7.5 cc.
Distilled water	41.5 cc.

To the mixture add approximately 1% saponin; the amount may have to be adjusted for different species. LaCour's 2BE fluid sometimes gives good fixation, but frequently leaves the cytoplasm in such a condition that it is too dense to permit the chromosomes to be clearly outlined by stains.

The fixation completed, turn the slide right side up, and with forceps remove anther fragments and other thick pieces of debris that might unduly elevate the coverslip when mounted. Then place in low staining dishes, and wash for about 15 minutes in gently running water. The slides should be examined under the microscope and any undesirable ones rejected. The smears are now ready to be stained. There are available a number of staining schedules, each adapted for particular needs and each giving a different picture of the chromosomes and their structure.

Johansen's Methyl Violet Method.—The principal attributes of this method are its brevity, ease of manipulation, and the certainty of obtaining sharply stained chromosomes from diaphase onward. It will ordinarily prove useless for prediaphase stages, except possibly in certain plants.

1. Transfer smears from water to a 1% aqueous solution of methyl violet 2B, preferably freshly made. This should should not have been boiled, otherwise precipitates will be left in the cells. The staining requires 15 minutes or less but may be increased if necessary.

2. Transfer slides to water, in which they may be left for as long as ½ hour. The slides should hereafter be handled individually.

3. Differentiate in 70 and 95% alcohol, to each of which is added 0.5% picric acid (if the crystals come in the moist form, add a little more to make up for the weight of the water). Hold the slide with broad forceps, and move back and forth gently in the vessel containing the alcohol. About 10 seconds in each alcohol is long enough. Do not use the series after they have become saturated with dye, nor should used solutions be allowed to stand unused for more than a few days, lest a greenish-yellow color be imparted to the cytoplasm.

4. Immerse the slide for 15 seconds or so in 95% alcohol containing about 4 drops ammonia to each 100 cc. alcohol.

5. Complete dehydration in absolute alcohol for about 10 to 12 seconds.

6. Complete differentiation in pure clove oil: this is the critical step in the procedure. Eight to fifteen seconds is generally long enough. Keep the slide moving back and forth to ensure even differentiation.

7. Wash in xylol containing a trace of absolute alcohol.

8. Leave the slides in pure xylol for about 2 hours, but not longer than 3 hours, before mounting: this will improve the sharpness of the

stain. The slides may be examined under the microscope and any unsatisfactory ones rejected before mounting in balsam.

In general, a counterstain is undesirable with smear preparations, and few if any cytologists use one. However, any of the usual cytoplasmic counterstains may be used if occasion demands.

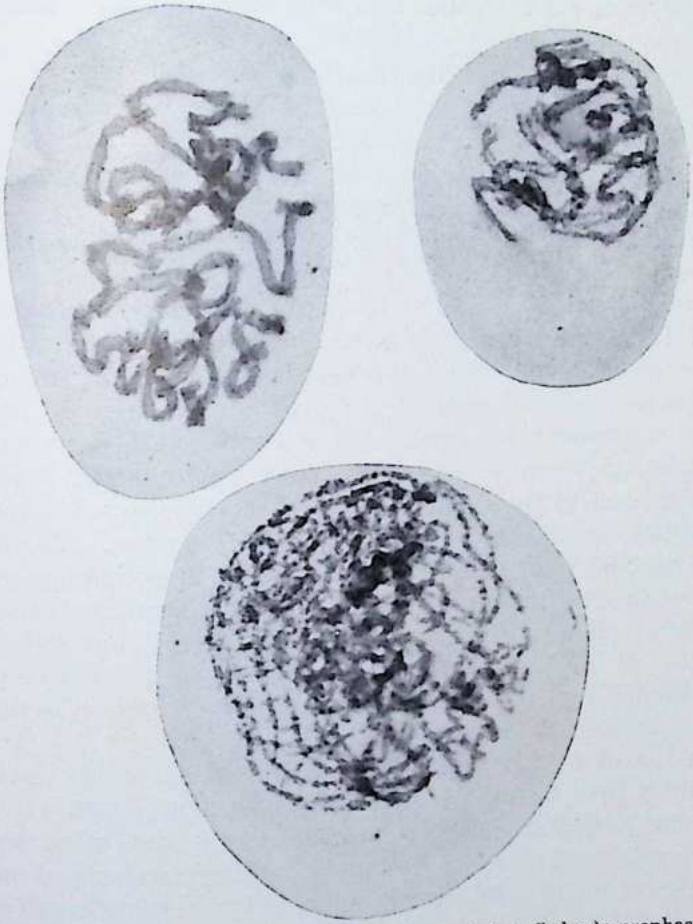


FIG. 19.—*Trillium ovatum* (above) and *T. chloropetalum* (below): prophase stages in meiosis. Permanent iron-acetocarmin smears. (*Preparations and photomicrographs by Dr. H. E. Warmke.*)

Newton's Gentian Violet-Iodine Method.—Newton originally added 1 g. each of potassium iodide and iodine to each 100 cc. of solution of each of the dehydrating alcohols, passed the slide through the series, washed thoroughly in absolute alcohol, cleared in xylol, and mounted in balsam. Any degree of depth of the stain could be obtained in this fashion. Present-day workers, however, have eliminated the chemicals from all

the dehydrating alcohols save either the 70 or the 85% one. The procedure is as follows:

1. Pass from water through 30 or 35% alcohol to the 70% containing 1 g. each of potassium iodide and iodine to each 100 cc. of solution. The slide should be kept immersed until no more color comes off, which will be in from a few seconds to a little over 1 minute. The smears should look quite black.

2. Remove the iodine by washing in 70% alcohol.

3. Pass quickly, depending on how much stain is coming out of the sections, through 85, 95%, and absolute alcohol.

4. Complete differentiation in clove oil. This should be rather brief, or if necessary may be omitted altogether.

5. Wash thoroughly in xylol, clear in pure xylol for 2 hours or longer, then mount in balsam.

Newton's method requires more experience than does the preceding one and, furthermore, demands greater accuracy in judgment in order to secure the optimum results. Most technicians use the gentian violet (crystal or methyl violet may also be used) at a strength of 1% (which is almost a saturated solution), while others use 0.75 down to 0.2%. If the stain appears to be too strong, it is a simpler matter to dilute it with water than to cut down the time. The time depends upon the material, but the optimum period is about 15 minutes. The method does not succeed with many plants and is said to be inferior to other procedures in the staining of the diaphase and earlier stages. The chromosomes often appear so transparent that this has called forth objections. It has been claimed that the transparency can be greatly reduced by judicious exposure of the completed preparation to sunlight. The stained slides last about 1 year, then begin to fade.

Sax's Variation of Newton's Method.—Make smears as usual, and fix preferably with Navashin's fluid for 1 to 2 hours (Sax 1931). Wash in 10 to 20% ethyl alcohol for 12 to 30 minutes, then stain with 1% aqueous crystal violet for 1 to 5 minutes. Rinse in water, and immerse in 30% alcohol for 15 to 20 seconds, followed by the same length of time in 50% alcohol. Iodize in 80% alcohol containing 1% iodine and 1% potassium iodide for 30 seconds. Destain and complete differentiation with absolute alcohol, clear with clove oil, pass through xylol, and mount in balsam.

LaCour's Method.—It has been found (LaCour 1935) that, if the anthers are smeared, immediately placed, smear side down, in a dish containing 3% aqueous cane sugar for a second or two, then laid flat over an open dish containing concentrated ammonium hydroxide or concentrated nitric acid for a few seconds, and next placed in the fixing fluid (preferably a Flemming medium solution plus osmic acid) for 2

hours, the spiral structure of the chromosomes is more clearly revealed. The main precaution in order to prevent loss of cells is always to keep the slip flat and to move it horizontally. After fixation, there is much less danger of the cells washing off. Wash, bleach if necessary, and stain as preferred.

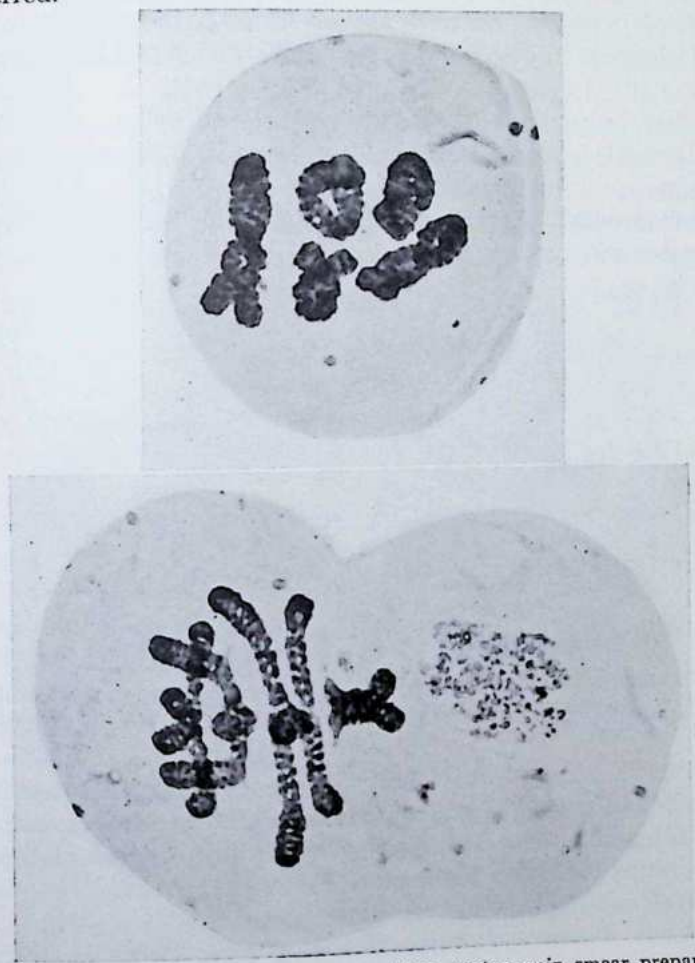


FIG. 20.—*Trillium chloropetalum:* permanent iron-acetocarmin smear preparations of microsporocytes; *A*, diaphase, showing chiasmata; *B*, early anaphase. (*Preparation and photomicrograph by Dr. H. E. Warmke.*)

Kauffmann's Iron-Hematoxylin Method.

1. Prepare smears, and fix in Navashin's or a chrom-osmo-acetic fluid.

2. Mordant in 2% aqueous ferric ammonium sulphate for 1 hour.

3. Wash carefully in running water for a few seconds and rinse in distilled water.

4. Stain for 1 hour or longer in 0.5% hematoxylin.

5. Rinse in water, then destain under observation in the same solution that was used for mordanting, which is discarded upon completion of the differentiation. When the stain seems satisfactory, transfer the slide to water.

6. Wash in running water for at least 1 hour to remove the iron alum.

7. Dehydrate by stages of not over 10%, with 2 or 3 minutes in each stage.

8. Clear in mixtures of absolute alcohol and xylol, of which about four intermediate stages suffice. Then leave in pure xylol for a short time before mounting in balsam.

Tuan's Modified Hematoxylin Method.—This is a method requiring considerable patience, but the preparations are usually of such surpassing

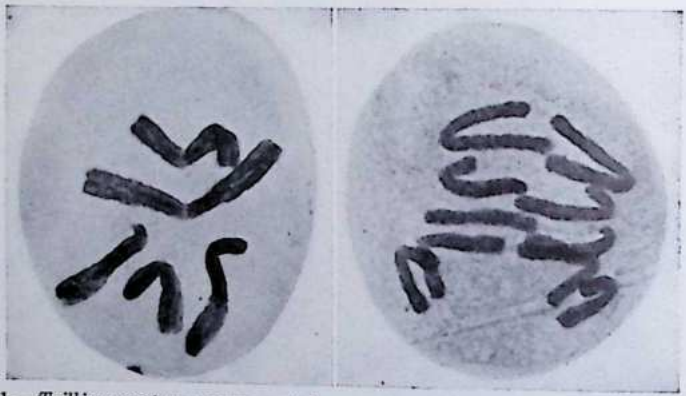


FIG. 21.—*Trillium ovatum:* permanent iron-acetocarmin smear preparations of mitoses in the young microspore; *A*, metaphase; *B*, anaphase. (*Preparation and photomicrograph by Dr. H. E. Warmke.*)

beauty that the extra effort is amply repaid. When properly carried out, it is possible to study the internal structure of metaphase chromosomes.

1. Kill and fix the smears in Navashin's fluid, or in a chrom-osmo-acetic mixture, for about 20 minutes.

2. Wash in running water for 20 minutes.

3. Bleach if necessary with diluted hydrogen peroxide, then wash again.

4. Mordant in 2% ferric ammonium sulphate for 20 minutes.

5. Wash in running water for 6 to 10 minutes, then rinse in distilled water.

6. Stain in 0.5% hematoxylin for 20 minutes.

7. Wash out excess stain with water.

8. Differentiate in a saturated aqueous solution of picric acid. The time varies according to the species and to the stage of development of the microsporocytes or microspores: 20 minutes for divisions in pollen

grains, 40 minutes for tetrad stages, and 100 to 120 minutes for dividing chromosomes. Examine occasionally under the microscope to note progress of the destaining. It must be carried considerably further than usual if one wishes to observe internal chromosomal details.

9. Wash in running water for 30 minutes, then proceed to the dehydration and mounting in balsam.

Capinpin's Brazilin Method.—Belling's original brazilin method lacked certain desirable qualities, one being permanence. It was better adapted for the Liliaceae than for dicotyledonous families, in the opinion of many critics. In the Onagraceae, and particularly for *Oenothera*, it was for years impossible to make really first-class smear preparations of the peculiar rings of chromosomes found at the diaphase of many species. A number of improvements over Belling's method have been devised (Capinpin 1930), and it is possible to secure results of unusual excellence.

1. Make smears as preferred, using Navashin's fluid for fixation.

2. Transfer to a mixture of 1 part of Solution A of Navashin's fluid (the chromic acid part) and 4 parts water. Turn slides right side up, and remove the thicker fragments and other debris. Leave for about 15 minutes in order to remove the formalin of the fixative.

3. Pass through 15, 30, and 50 to 70% alcohol, and allow to remain overnight.

4. Mordant overnight in a freshly prepared 1% solution of ferric ammonium sulphate in 70% alcohol.

5. Wash about 45 minutes in several changes of 70% alcohol.

6. Stain in a ripe 0.5% solution of brazilin in 70% alcohol. (The brazilin solution should be ripe a week after being prepared.) The time varies between 2 to 6 hours.

7. Wash briefly in 70% alcohol.

8. Differentiate in 1% ferric ammonium sulphate in 70% alcohol. This process takes from 5 to 10 minutes. When examined under the microscope, smears rightly stained show chromosomes brownish-black or black, cytoplasm pink or straw-colored, and cell wall colorless.

9. Wash in two changes of 70 and one of 95% alcohol, 10 minutes in each.

10. Dehydrate in absolute alcohol, and pass through the following mixtures:

Equal parts of absolute alcohol and cedar oil
Equal parts of xylol and thin cedar oil
9 parts xylol and 1 part cedar oil

11. Complete the clearing in two changes of xylol, then mount in balsam.

Backman's Method.—This procedure combines the stain with the fixing fluid (Backman 1935). It has not been thoroughly investigated but is being presented because of its possibilities.

1. Prepare smears as usual.
2. Fix for 3 to 5 minutes, depending upon the density of the cytoplasm, in the following mixture:

Bouin's fluid, using anthraquinone in place of the picric acid	100 cc.
Alizarin Red S	0.125 g.
Saponin	0.150 g.

3. Rinse for 2 to 5 minutes with tap water.
4. Dehydrate through a rather close alcohol series up to 95% for about 1 minute in each.
5. Differentiate by flooding the slide with 0.5% sulphuric acid in 95% alcohol saturated with picric acid until the chromosomes become clear; a few seconds generally suffice. A too prolonged treatment with the acid solution is inadvisable. If the differentiation is difficult, shorten the time in the fixative.
6. Wash thoroughly with 95% alcohol to remove the acids. A further neutralizing effect may be obtained by adding 0.5% potassium hydroxide to the alcohol.
7. Intensify the stain, if desired or if the preparation is required for immediate use, by washing for 30 seconds to 1 minute in 95% alcohol plus 4% by volume of cymene. (The originator does not specify what type of cymene should be employed.)
8. Complete dehydration with absolute alcohol.
9. Clear for 2 minutes in each of the following mixtures: 5 parts absolute alcohol and 3 parts xylol; 3 parts absolute alcohol and 5 parts xylol.
10. Wash for 5 minutes in each of two changes of xylol, then mount in balsam.

Belling's Iron-acetocarmin Method.—Really superb results have been secured by the use of this method. Much care is required to obtain successful preparations, and the method is not applicable to every organism. For example, beautiful preparations of the reduction divisions in the ovaries and testes of certain Orthoptera, or in anthers of *Triticum* or *Nicotiana* are easily made, but it is difficult to get even passable slides of meiosis in the microsporocytes of *Lilium* and impossible in *Oenothera* and related genera. The preparations, as a rule and at their best, do not remain in good condition for longer than a few months. Despite its disadvantages, the student will find the iron-acetocarmin method well worth trial and will feel more than repaid when good preparations are secured.

Belling's formulae for the preparation of iron-acetocarmin have varied somewhat. The one which the writer prefers is to add 90 cc.

glacial acetic acid to 110 cc. distilled water. Heat to boiling, remove from the flame, and immediately add 1 g. (roughly) certified carmin dye. Cool in the ice box, then decant. Add a few drops of an aqueous solution of ferric acetate until the color on standing is a dark wine red. Be careful not to add too much iron or the carmin will be precipitated. Keep in a well-stoppered bottle.

There are several methods of using iron-acetocarmin. The method to be used with a particular type of material depends upon the material itself. Four methods are outlined below.

1. Place a few small anthers, or small pieces of large anther, in a drop of acetocarmin on a slide. After a few minutes withdraw the fluid with absorbent paper, and replace with a fresh drop of dye. Press out the microsporocytes with a scalpel or by other means, and remove anther walls and debris. Put on a large coverslip, withdrawing any excess fluid with absorbent paper. Then seal as soon as possible by covering the edges of the coverslip with melted paraffin (of a low melting point) with a warmed metal instrument, say a flattened broad needle. Allow to stand for several days for the stain to penetrate thoroughly. Any particular microsporocyte may be flattened out, or the cytoplasm and chromosomes may even be forced from the cell, by careful pressure on the coverslip.

2. Anthers cut into small sections or very tiny pieces of testis or ovary are put into a test tube with at least fifty times their bulk of acetocarmin. After about five days, depending upon the species, preparations are made by putting a piece of the material on a slide in a drop of acetocarmin, squeezing out the microsporocytes or flattening or teasing the animal material, adding a coverslip, and sealing.

3. Smears of anthers or testis may be made on a coverslip in the usual way and the latter immediately placed upside down on a drop of acetocarmin on a slide.

4. If it is desired to stain the nuclei or chromosomes in microspores, add just enough crystals of chloral hydrate to a few drops of acetocarmin to clear the material. If too much of the chloral hydrate were added, plasmolysis will ensue. Leave in the stain for two days or so. Individual cells may be squeezed or partially crushed to give better views of their contents.

When viewing iron-acetocarmin preparations under the microscope, a green filter should be inserted between the microscope and the source of illumination. The bluish-red chromosomes will then appear nearly jet black.

McCallum's Iron-propionocarmin.—Propionic acid gives better fixation and staining than does acetic acid. The amount of propionic acid in the staining solution need not be so critical as with acetocarmin,

therefore the amount may be varied to give the optimum results for each type of material (G. A. McCallum, unpublished). Incidentally, the propionic acid solution dissolves more carmin dye and gives a more intense reaction, together with greater clarity of the cytoplasm.

Kill and fix the material in 1 part propionic acid and 2 parts absolute alcohol. Place the material in iron-propionocarmin (prepared in exactly the same manner as iron-acetocarmin, substituting the same volume of propionic acid for the acetic acid) for 2 to 3 minutes, then transfer to a slide in a drop of the staining solution, and flatten under a coverslip. If the material is difficult to flatten, heat the slide slightly. Float the coverslip off in 50% aqueous propionic acid, then if necessary destain in the same solution. Heat may be applied to accelerate the destaining. Next dehydrate through the following series of mixtures, allowing about five minutes in each:

¼ tertiary butyl alcohol, ½ propionic acid, ¼ water
½ tertiary butyl alcohol, ½ propionic acid
¾ tertiary butyl alcohol, ¼ propionic acid
9/10 tertiary butyl alcohol, 1/10 propionic acid
Pure tertiary butyl alcohol. (In the original method, tertiary butyl alcohol was employed, but it is probable that hygrobutol would work better.)

Mount in balsam dissolved in tertiary butyl alcohol or hygrobutol.

McClintock's Permanent Acetocarmin Method.—Place anthers directly in a bottle containing 1 part glacial acetic acid to 3 parts absolute alcohol. Material can be kept for 12 hours to several weeks (the stainability of the chromosomes is definitely improved by the fixative). For longer storage transfer directly to 70% alcohol.

Transfer anthers from either killing fluid or 70% alcohol to a small drop of acetocarmin. Carefully squeeze or tease out contents of anther. Remove all debris, then add coverslip. Heat the slide over an alcohol flame for about 1 second. This should be repeated four to five times until the cells are somewhat spread and flattened. The solution must not be allowed to boil. Examine slides at this juncture, and reject all undesired ones. Be careful not to shift the coverslip or the sporocytes will be loosened. Place slide in a Petri dish filled with 10% aqueous acetic acid. The coverslip will gradually rise somewhat from the slide, carrying some of the sporocytes with it. Push the coverslip gently to the edge of the slide so that it can be grasped with forceps, but wait until enough solution has run between the slide and coverslip to allow the latter to float freely. The sporocytes stick to both slide and coverslip; consequently both must be handled in the solutions that follow. Carefully transfer both to a jar containing equal parts of acetic acid and absolute alcohol. Then pass through the following mixtures, allowing a few minutes in each:

1 part acetic acid to 3 parts absolute alcohol
1 part acetic acid to 9 parts absolute alcohol
Equal parts of absolute alcohol and xylol

The slide and coverslip are recombined directly from the last solution in xylol-balsam. A pure xylol solution must not be used on fresh slides as it will cause a distortion of the sporocytes.

By one variation of the above method (Steere 1931), the anthers are smeared on clean slides, then immediately fixed and stained by placing the slides, smear side down, in a Petri dish full of steaming acetocarmin for from 1 to 10 minutes. The slides are then transferred rapidly through the following mixture: 2 parts glacial acetic acid plus 1 part absolute alcohol; 1 part acetic acid plus 2 parts absolute alcohol; 1 part acetic acid plus 9 parts absolute alcohol. Complete dehydration by immersion for a few minutes in absolute alcohol, then clear for 2 to 3 minutes in a mixture of equal parts of absolute alcohol and xylol, and mount in balsam from this solution, but work rapidly during the mounting in order to prevent absorption of moisture from the atmosphere.

Zirkle's Methods.—These methods have been designed to combine the acetocarmin staining mixture with inert substances which solidify on standing and seal the mounts, thus avoiding the difficulties usually attendant on transferring materials from the stain to a permanent mounting medium (Zirkle 1937).

1. The material is macerated on a slide in a drop of acetocarmin, then several drops of the following solution are added:

Acetocarmin	80 cc.
Karo corn sirup (dextrose)	10 cc.
Certo (pectin)	10 cc.

Next heat the preparation, then press down on the coverslip until the smears are flattened to the desired extent. Excess solution oozing from the coverslip need not be removed.

2. The following mixture combines in one operation fixing, staining, and mounting. It may be used as made up, or it may be diluted with acetocarmin in various proportions as required by the nature of the material. If diluted, more fluid should be allowed in order to compensate for the greater amount of water and acetic acid that evaporate.

Glacial acetic acid	50 cc.
Water	50 cc.
Glycerin	1 cc.
Gelatin (powdered)	10 g.
Dextrose	4 g.
Ferric chloride	0.05 g.
Carmin dye	To saturation

Dissolve the gelatin in the water, then add the other ingredients. Boil and filter the mixture, exactly as is done with plain acetocarmin. The medium becomes firm like balsam as the aqueous portions evaporate; it will not liquefy when heated, once it has solidified.

Warmke's Method.—The schedule affords permanent smears of root tips and gives exceptionally beautiful and useful preparations (Warmke 1935). It may also be used on microspores in order to study the first postmeiotic division.

1. Kill and fix tissues as directed in McClintock's method.

2. Remove from the killing fluid and place for 5 to 10 minutes in a mixture of equal portions of 95% alcohol and concentrated hydrochloric

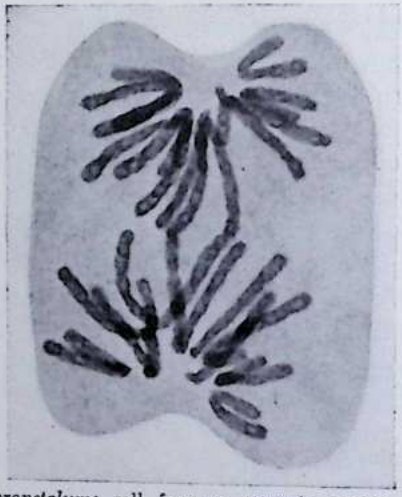


FIG. 22.—*Trillium chloropetalum:* cell from a root-tip smear prepared according to Warmke's method. (*Preparation and photomicrograph by Dr. H. E. Warmke.*)

acid. This serves to dissolve the pectic substances of the middle lamellae. The proportions may be varied considerably: 3 parts acid to 7 parts alcohol may be used, but the time must be prolonged.

3. Transfer to Carnoy's fluid (with chloroform) for 5 minutes or longer. The purpose is to harden the tissues again after the acid treatment, which more or less softens it.

4. Cut off a small piece (less than 0.5 mm. long) from a root tip or piece of anther, and place on a clean slide in a drop of acetocarmin. It will be difficult to flatten the cells sufficiently if it is attempted to use too large a piece of material. Press directly on the piece with a small flat scalpel: the cells will separate and float free in the stain. Place a coverslip over the drop of stain, and apply gentle pressure. The size of the drop of acetocarmin is governed by the dimensions of the coverslip. If too much stain is used, it will flow out from under the coverslip and

carry many of the cells with it. Small square coverslips will be found more satisfactory than large rectangles.

5. Heat the slide cautiously by passing three or four times through the flame of an alcohol lamp (take great care not to let the solution boil); this serves to clear the cytoplasm.

6. The preparation may be preserved temporarily by covering the edges of the coverslip with a mixture of equal parts of Parowax and gum mastic heated together. Such mounts will keep in good condition for about a week. Permanent mounts can be made as described under McClintock's method, beginning with the placing of the slide in 10% aqueous acetic acid.

Hillary's Method.—Fix the root tip with any suitable fluid, then wash out the fixing fluid thoroughly, and apply the Feulgen technique (Hillary 1938). Sufficient time should be allowed for the stain to penetrate thoroughly. Dehydrate through three changes of dioxan. Place a tip in a drop of balsam diluted with dioxan on a clean slide, and divide the tip into small longitudinal sections by means of sharp needles or a very small scalpel. Finally add more balsam if necessary, then apply a coverslip. Further pressure applied to the coverslip aids in spreading the cells.

This method may also be used on anthers that are difficult to smear before the microsporocytes have rounded up.

CHAPTER XIV

CYTOLOGICAL METHODS

Microtechnical methods employed in the field of cytology are the most critical of all, and before a person enters this domain, he should take the pains to become fairly well grounded in the basic principles and procedures of general botanical microtechnique. Only in this way is it possible to become sufficiently proficient to devise the special variations in schedules demanded by the differences in structure and chemical composition of the tissues of diverse plants. No two species react exactly alike to the identical technical schedule, and the beginning technician must learn how to adapt the composition of killing and fixing fluids and the staining procedures to the particular plants under investigation. It is impossible to interpret and to judge the final results accurately and adequately unless considerable preliminary experience has been gained. The common practice of attempting to learn microtechnique and to investigate a cytological research problem simultaneously cannot be too strongly condemned. Those who do this usually never become other than mediocre technicians and poor cytologists, since they are unable to master the finer points of microtechnique and consequently cannot appreciate the value, from the standpoint of interpretative cytology, of working with first-quality preparations.

Cytological technicians in the past have been too content to adopt pragmatism as their sole philosophy. The rationale of their methods has too often mattered little or not at all, provided the final result was according to their notions. As one critic succinctly put the matter: "As a result, textbooks of cytological and histological technique have tended to resemble the pharmacopoeas of the Middle Ages." A change in this viewpoint is a vital necessity if cytological technique is to progress.

A complete understanding of the behavior of the different substances entering into the composition of killing and fixing fluids, particularly with regard to their effect upon both resting and dividing chromatin, is the first and probably the most important point upon which the embryonic cytologist should become thoroughly grounded. Next in order of importance is the ability to manipulate stains and staining schedules to obtain the optimum optical contrast, bearing in mind the fact that cytologists never employ general staining, as is commonly used on purely anatomical materials, but always aim to procure the sharpest

possible staining of particular structures. The fact should incidentally be emphasized that not only do stains differ in their action upon different species, but even upon different stages in the same piece of material. Many cytologists use only one dye and concentrate on some one phase under specific investigation, such as the chromosomes at the metaphase stage in either mitosis or meiosis. The more critical cytologists employ more than one killing and fixing fluid and different staining methods in order to obtain confirmatory evidence through diversity of methods.

The beginning cytologist further needs to become proficient in the use of iron-acetocarmin or iron-propionocarmin, whether smears or crushed materials are used. The majority of the most competent technicians always make a preliminary study of their materials by means of temporary mounts stained by some carmin method.

Cytological methods, on the whole, are the most painstaking of all, but the final results, when successful, more than justify the trouble entailed in their preparation. John Belling, one of the most critical and competent of cytologists, thought nothing of spending weeks and even months preparing a single perfect preparation: every step was carefully considered in advance, and nothing was left to chance or guesswork. One would do well to emulate his brilliant example. Except for temporary carmin-stained preparations, a passable cytological slide cannot be made in a hurry, nor by lackadaisical methods.

It is not possible to write precise directions, step by step, for cytological methods. The best that can be done is to enumerate the methods which innumerable cytologists have found by sustained experience to afford the optimum results: using these methods as a guide, a person with sufficient preliminary training and experience will find himself able to adapt them to the peculiarities and idiosyncrasies of his particular materials.

Smears versus Sections.—The beginner who has followed the literature, especially that between 1925 and 1932, will doubtless have noted a sharp divergence of opinion between those cytologists who depend principally upon smears (or crushed and spread tissues) for their observations and those who favor sections. No one method, however, can possess any inherent superiority over another since none alone can be wholly sufficient by itself. Each method has its good points, as well as its disadvantages, consequently each should be considered as supplementary to the others. Each supplies evidence of a type not readily afforded by another; taken all together, each reveals a part of the whole. There are circumstances in which one method is more useful than another, but this does not necessarily rule out the applicability of the others. To cite a few instances: the pollinia of *Acacia* and the orchids cannot be smeared; the microsporocytes of the Onagraceae are easily smeared but cannot

always be well stained and show only a few metaphase stages at a time; the microsporocytes of the Liliaceae and related families are equally good smeared or sectioned but only smears will show the entire nucleus or chromosome garniture because of their large size; buds of many species are too small to be handled for smearing, and so on.

The rule to follow should be to make smears whenever satisfactory ones are permitted by the nature of the material and its staining capacity and always to make sections, checking the observations on the one against those on the other.

For smears, consult the chapter on Smear Methods (page 155). One should commence with temporary carmin-staining schedules, progressing to permanent carmin methods, thence to other permanent methods. For paraffin sections, the tertiary butyl alcohol method of dehydration is recommended. Celloidin methods are rarely satisfactory for cytological studies since it is so troublesome to arrange serial sections, but this difficulty can be partly circumvented by double-embedding.

Manipulation of Material.—Materials intended for cytological investigations must be more carefully handled than is generally recognized. Post-mortem changes must be completely avoided; the time from the removal from the plant to placing in the killing fluid should be as short a period as possible, and the plants should also be healthy and turgid, unless pathological material is being investigated (such as parasitic fungi, mosaic diseases, etc.). Wilted portions should never be fixed, otherwise the completed preparations will reveal excessive plasmolysis, clumping of chromosomes, cytomixis, and similar artifacts.

Root tips may be obtained from plants grown in nutrient solutions or in not too large pots. Aquatic plants or those grown in nutrient solutions may simply be lifted out of the water and the tips cut off behind the meristematic region (which is whiter and more opaque than elsewhere) by means of a small sharp scalpel or pinched off with fine-pointed forceps. If the plants are grown in pots, they should have been allowed to grow until the space between the soil and interior of the pot is beginning to become filled with roots. Invert the pot, holding the stem between the middle and index fingers of the left hand with the hand itself flat on the soil, knock the edge of the pot against something solid, and the mass of roots and soil generally comes out readily. With the whole thing still held in the hand, snip off the tips with fine forceps, and place immediately in the killing fluid. To be on the safe side, as many tips as possible should be removed from the potted plants. The tips from such a source rarely have particles of grit adhering, but care should be taken not to carry any over. Sometimes there may be no root tips visible; in such cases, the soil may be carefully washed away under a faucet, when it is generally possible to find tips near the crown. Plants dug out of gardens scarcely ever have tips in good condition.

Bulbs of *Allium cepa* may be grown in the dark in suitable containers, using a somewhat diluted Knop's or other nutrient solution. The tips will be long enough for removal in about three days. Bulbs of *Hyacinthus, Crocus, Fritillaria,* and similar genera are better grown in a cool, dark place in finely sifted soil (with no sand, if possible) or in shredded peat in pots or boxes, since they require from two weeks to a month to attain sufficient growth for removal of the root tips. Remove the bulbs from the container, wash quickly under a gently flowing faucet, then cut off the tips with a scalpel.

It is a simple matter to remove the buds from dicotyledonous plants. Anthers from one or two may be removed and smeared in iron-aceto-carmin to determine the stage of microsporogenesis, but ovules can only be collected blindly. If the anthers have a yellow or reddish color, the pollen grains have been formed; as a rule, meiosis has already taken place by this time in the megasporocytes. If the buds are large, either remove the anthers and ovaries individually, or reduce the entire buds to convenient small portions. With small buds the procedure depends on whether the ovaries are superior or inferior, and in the case of the latter whether both anther and ovary portion is to be removed as an entity or separately. If the ovaries are superior, separate from the plant by cutting between the base of the ovary and the apex of the pedicel, then cut off the overlapping sepals at the top in order to permit the killing and infiltrating reagents to enter more readily. In the large buds of most Asteraceae bisect each capitulum in the median vertical plane; it would be a good idea to do the same thing with other plants whenever the nature of the material permits it. In the case of flowers with inferior ovaries one may remove the whole thing if it is small enough, and simply remove the sepal tips. Otherwise separate the ovary and anther portions, and run up independently. If the flowers come in small dense bunches, it is the usual practice to treat the whole group as an entity, but care must be taken to insure complete penetration of the killing fluid by making a few incisions in places where they will do no damage. All superfluous tissue should always be removed.

The remarks in the preceding paragraph refer to buds that are readily visible externally. In many monocotyledons, particularly in the Liliaceae, the buds are concealed in bulbs or similar structures and must be dissected out. It is necessary to have some knowledge as to the time of year at which the various developmental processes are undergone. In *Trillium*, for example, meiosis in the microsporocytes occurs during September and early October; in *Hyacinthus* it may occur as early as August in some species or varieties, in October in others.

Plant groups other than the angiosperms, which have been considered above, may be treated as suggested in the special sections devoted to the pertinent groups.

Choice of Fixing Fluid.—If a proper fixing fluid, with the ultimate purpose for which the preparations are required always kept in mind, is not chosen at the beginning, little or nothing can be done afterward to remedy initial errors. Of course, there will be occasions when one will be forced to use materials which from necessity were fixed in the only available fluids (as on collecting trips to regions remote from a laboratory); in such instances it becomes a matter of adapting staining schedules to obtain the desired result. There is, however, no excuse, when adequate opportunities are at hand, for not using a carefully adjusted formula for the killing fluid. In determining the constituents of such a fluid and the proportions of the various ingredients, the factors which require to be taken into account include: (1) the nature of the material, (2) the size or bulk of the latter and its penetrability by fluids, and (3) the dye or stain conbination to be employed for staining. If a violet dye, for example, is to be used, the fluid should contain chromic acid; if a triple combination is to be used, the fluid should contain both chromic and osmic acids. If the bulk of the material is somewhat large, the fluid should either be somewhat stronger than usual or should contain ingredients of strong penetrating power. The same precautions should be observed if the material is heavily cutinized or suberized, since such tissues impede penetration of fluids. For cytological purposes aqueous fluids are always preferable to alcoholic mixtures, since alcohol is a poor preservative of dividing chromatin.

Statements in the literature to the effect that such-and-such a fluid "gives the best results," without specifying under what conditions such results were obtained, are meaningless. Conditions are not the same everywhere at all times. In England, for example, LaCour's fluids are popular and apparently afford excellent results, but on the Pacific Coast the fixation is atrocious, and staining is most difficult. Climatic conditions apparently have a definite bearing on fixation results and certainly have one on the subsequent staining. This statement refers more to terrestrial plants than to those growing in aquatic habitats, as the latter have a more nearly uniform milieu throughout the world when growing in the same type of water. It is both desirable and necessary to experiment with several fixatives and with variations in these fixatives in order to ascertain which provides exactly the desired results. One should not blindly accept some writer's statements, except insofar as these provide clues as to which formula might be most useful.

The majority of cytological technicians prefer killing fluids giving an acid fixation image. The more they concentrate on dividing chromatin, the more acid are the mixtures that they employ since their sole purpose is to observe the chromosomes during mitosis and meiosis. Fixing fluids which give basic images are employed only when cell contents and

inclusions are being studied; such fluids are useless for studies on dividing chromatin since they dissolve it more or less.

Navashin's fluid, or some variation thereof, is generally considered to be the most useful for cytological purposes. It is usually made up in two parts. Few workers change the proportions of the chemicals in the first part, but most vary the proportions of the formalin of the second part in accordance with the idiosyncrasies of their material. Many technicians, furthermore, especially when working with thick materials or when these are heavily suberized or cutinized or covered with dense hairs that prevent the material from sinking into the killing fluid, first immerse the material in Carnoy's fluid for from 5 to 10 minutes, then pour it off (some also give a quick rinsing with water) and replace with Navashin's fluid. The beginning cytologist cannot go far astray if he first tries Navashin's fluid, later making changes in the proportion of the formalin if this seems desirable or necessary.

For reasons which have been amplified elsewhere, the simultaneous use of potassium bichromate and chromic acid in a mixture is irrational. Tissues fixed in such mixtures are always overchromated and staining is difficult. It is usually necessary to resort to bleaching, which at times is dangerous since it may alter the fixation image.

If the morphology of the chromosomes is being studied, particular attention should be paid to the amount of chromic acid in the fixing fluid, since it has a direct effect upon accurate preservation of chromosomal structure. Fixatives somewhat weak in this acid will reveal constrictions that are not evident after fluids strong in chromic acid.

Infiltration and Embedding.—Standard methods of infiltration with paraffin are employed by most cytological technicians. It is very seldom that any difficulties are encountered. The tertiary butyl alcohol method is recommended since it produces the least variations in the fixation image and permits easy microtoming. The periods during which materials remain in the dehydrating series and in the paraffin oven should not be too prolonged.

Cytological materials are generally embedded differently than anatomical or other ordinary specimens. They are so embedded that the largest possible number of sections can be mounted on each slide (note Fig. 4*A*, *F*). For instance, root tips are embedded in bunches of from two to as many as a dozen, for microtoming transversely, and small buds are embedded in groups depending on their size. If the buds are of different sizes, one may either follow the less desirable method of grouping together buds of various sizes, particularly if the exact stage when meiosis occurs is not known and it is desired to ascertain this fact, or the buds may be all the same size. The group of buds should not exceed 15 mm. in any direction, but it does not matter whether they are arranged as

squares or rectangles. If they are to be cut longitudinally, they should lie flat on their sides, all preferably pointing in the same direction; if they are to be microtomed transversely, then the end which is to face the knife should be pointed down. Not all types of buds, however, can be oriented with reference to any particular plane, so they may simply be grouped together. Separate anthers, ovaries, etc., may be treated in the same fashion. All materials should be grouped together as closely as possible, since it is exasperating to have to move the slide across wide empty spaces when it is being examined under an immersion lens. Unless a special electrically heated embedding plate is used, do not try to arrange too large a quantity of material at one time lest the paraffin become too solid before the task is completed.

Microtoming.—Although most plant materials for general study are sectioned at from 10 to 12μ, cytological materials are not cut at any standard thicknesses. Materials fixed in fluids giving the basic fixation image are cut rather thin; some technicians consider that 2 and 3μ are none too thin, while others use sections as thick as 10μ. Mitochondria are usually studied in sections cut at between 6 and 8μ; the cytoplasm in sections anywhere from 2 to 10μ, depending upon the diameter of the cells in which it is being investigated. In other words, the smaller the mean diameter of the cells, the thinner the sections must be cut.

In sectioning root tips and buds for the study of the somatic and meiotic chromosomes, the material should be microtomed at about three-fourths of the average diameter of the cells. Microsporocytes and megasporocytes increase enormously in size at a very rapid rate at about the time meiosis is occurring in each, consequently sections should be somewhat thicker than one would judge the thickness to be before this stage is reached. Serious mistakes in the counting of chromosomes have been made when too thin sections have been employed. A commercial concern once advertised onion root tips allegedly sectioned at 1μ (which is extremely doubtful) and designed especially for chromosome studies. Such sections are very dangerous to use for that purpose since the chromosomes have been cut into innumerable portions scattered over as many as a dozen sections, and it is utterly impossible to match the portions, much less to count the chromosomes accurately. The optimum thickness for onion root tips, the more or less standard material upon which beginning cytological technicians work, when microtomed longitudinally is 11μ; when cut transversely it should be 13 to 14μ. The majority of root tips should be microtomed transversely. There are very few which are as satisfactory as the onion when cut longitudinally; among these may be mentioned *Vicia faba*, *Hyacinthus*, *Trillium*, *Lilium*, and *Zea mays*. It is problematical whether there is any species in which the chromosomes can be accurately counted in side views (*i.e.*, in longi-

tudinal section) of the spindle at either metaphase or anaphase of mitoses; in any event, cytologists are skeptical of counts made under such circumstances. These sections, however, are excellent for studying the structure of the chromosomes. Very small root tips (using the onion for comparison) with a comparatively short meristematic region should be sectioned transversely at about 10μ. In very large tips the diameter of the cells should first be ascertained, then the appropriate thickness may be calculated. In *Fritillaria meleagris*, for instance, the chromosomes are so large and numerous that sections 30 to 36μ thick are required. Such thick sections are better cut with safety razor blades held in a Craig-Wilson holder through which lukewarm water is allowed to run; this will cause them to form a ribbon, which they will not do on an ordinary knife. In *Crocus* the cells are larger than in the onion, but the chromosomes are mostly smaller and more numerous; hence sections should be cut transversely at 12 to 14μ.

The plane in which ovaries so small that it is impracticable to dissect out the ovules for individual treatment are sectioned depends entirely on the orientation of the ovules. It is invariably necessary to section the ovules in the longitudinal plane perpendicular to the raphe or funiculus; consequently the technician should become familiar with the arrangement of the ovules while the material is being prepared for fixation. In general, the ovules are arranged either horizontally or vertically, and thus the ovaries may be sectioned transversely in the first case and longitudinally in the latter instance. When they are in mixed positions, *i.e.*, radiating from a common center in all directions, one can only cut blindly and trust to luck to find ovules that were sectioned longitudinally. This obviously means that a larger quantity of material must be sectioned in order to afford a sufficient number of satisfactory sections. The megasporocyte differs greatly in size among different species, and there is little relation between the size of the ovule and the diameter of the megasporocyte. To contrast two genera: ovaries of *Lilium* and *Oenothera* of approximately the same size may be taken; those of *Lilium* are sectioned transversely at 24μ and those of *Oenothera* longitudinally at 12μ. The ovules of *Lilium* will be found to be rather large with few, large cells; those of *Oenothera* will be found to be small with numerous rather small cells. The megasporocyte of *Oenothera* averages less than one-fourth the size of that of *Lilium* and the chromosomes are even smaller.

One piece of material should first be cut, stained, and examined under the microscope. If the majority of the cells containing mitotic or meiotic figures show that all the chromosomes are contained in either uncut cells or in cells from which thin slices have been cut from either top or bottom or both, then the thickness is correct, and the remainder of the material may be sectioned at that thickness.

Staining Methods.—Cytological staining must, above all, be sharp, precise, and brilliant, with the clearest possible differentiation between structures. Sloppy staining is worse than useless. Many beginners in cytological technique overdo the staining and therefore should exercise rigid control over this aspect of their work. Cytological staining is at the same time the most simplified and the most difficult, yet if one takes the necessary pains it can be easy. The secret of success is not to attempt to do more than one thing at a time. To make this point clearer, it will be recalled that in the average morphological preparation it is the customary aim to attempt to stain as many structures as possible—cell walls (whether cellulose, lignified, cutinized or suberized), cytoplasm, nuclei, nucleoli, chromosomes, chloroplasts, sclereids, etc.; in cytological preparations, on the other hand, one should not attempt to stain anything besides the chromosomes, or the mitochondria, or the cytoplasm, as the case may be. If, for example, anything besides the chromosomes is stained, it should be looked upon as an accidental occurrence; if it does not obscure the chromosomes, it is of no moment, but if the chromosomes are rendered less visible, it is a serious matter that must immediately be remedied.

Most root tips take an adequate stain with iron hematoxylin for all stages of mitosis; others do not. Those taken from plants which naturally grow in alkaline soils are especially unsatisfactory with this stain, as are tips with very dense cytoplasm. Instances are known when certain chromosomes became completely destained long before the remainder were sufficiently differentiated, thus leading to inaccurate counts of the chromosomes. The tips of some species destain rapidly, but most of them destain somewhat slowly, and in many such cases great caution must be taken not to differentiate too far. A few cytologists destain the hematoxylin until only the outlines of the chromosomes remain visible, but this practice is hardly to be recommended. If the cytoplasm is very dense, one can only judge from experience as to when the differentiation should be stopped. Few cytological technicians use a counterstain on root tips in which the chromosomes are to be studied since it is liable to obscure their morphology. Sometimes, however, one may be required to reveal the otherwise invisible cell walls or to intensify the cytoplasmic background; orange G may be recommended for the purpose.

Practically all root tips take a sharp and brilliant cytological stain by one of the violet methods (page 89 *et seq.*). Most present-day cytologists prefer the violets, crystal or methyl, as they can be sharply differentiated and have a high specific affinity for dividing chromatin. The violets are not always so good for resting chromatin as is iron hematoxylin. There is considerable variation between species in the degree to which the violet is concentrated in the dividing chromatin and removed

from other structures. When the slide is removed from the differentiating fluid, the stain may seem to be still present in the sections generally, but if the slide is examined under the microscope while still wet, it will usually be observed that the stain is quite dilute in the cytoplasm and cell walls and sharp enough in the chromatin. In still other species every vestige of the violet is removed from the cytoplasm and other structures, and the dividing chromatin alone (if lignified tissues are present, they are usually also stained) stands out, brilliantly stained. It will therefore be seen that the macroscopic appearance of the sections is useless as a guide to the adequacy or nature of the staining, and it should not be depended upon. As a rule, the thicker the sections, the more generalized the stain appears to be, but macroscopic and microscopic examination are two very different things. Very few cytologists use counterstains after the violets, but if it appears desirable to add one, either erythrosin or orange G would be preferable.

Not all the violet dyes on the market are satisfactory for cytological staining. Some are, in fact, worthless for the purpose. Gentian violet, as explained in the chapter on stains, is not a single dye, but a mixture of crystal and methyl violets in different proportions. Crystal violet should be used whenever gentian violet is specified and is probably the most satisfactory for use by Newton's method. Either crystal violet or methyl violet 2B may be used in Johansen's method. Certified samples should always be used.

Sections thicker than 10μ cannot be satisfactorily stained with iron hematoxylin, but the violets give sharp staining at all thicknesses.

Other stains may be used on root tips, but none is of greater value than the violets or iron hematoxylin. Tips of *Allium cepa* are commonly stained with safranin and fast green or by the Cajal-Brozek method, and both are very useful in elementary classes; hence one might experiment with these two. If neither a violet nor iron hematoxylin stains adequately, resort may be had to a triple combination; Stockwell's modification is particularly useful, especially when the sections appear to be overchromated.

Buds for the study of meiosis are commonly stained with a violet, exactly as for root tips, followed by orange G. There is, however, far more difference in the case of buds with regard to the extent that they retain the violet stain. Sometimes it may be rather erratic, and in such cases mordanting of one type or another is indicated. This may consist in the application of 1% aqueous chromic acid for at least 1 hour, or of differential acidification previous to going into the staining solution. Iron hematoxylin is frequently very sharp on buds, and it is to be preferred for the prophase stages. The violets commonly do not stain the prophase stages at all.

Feulgen's reaction is of the utmost value in cytological investigations. The application of this chemical test appears to be rather complicated, but once it has been mastered, it is a simple procedure and should be adopted as a routine method.

The violet methods cannot be used on materials sectioned in celloidin, but iron hematoxylin is generally quite satisfactory. Apparently no one has yet applied the Feulgen reaction to celloidin sections.

A great many methods have been devised by animal cytologists for the staining of mitochondria, but the stains all fade within a short time and have never been any too successful on plant materials. Innumerable staining methods for other elements making up animal tissues have also been devised, but one need merely recall that animal and plant tissues are radically different both chemically and physically, consequently these methods are useless on plants.

Judgment of Results.—From the statements of many authors the conclusion is inevitable that the problem giving them the greatest concern is that of artifacts. The writer, on the other hand, is inclined to the opinion that the question has been considerably overemphasized. Modern technical methods, moreover, have gone far to remove the commonest sources of artifact production.

An artifact has been described as "an appearance that arises from treatment" (Darlington 1932). Consequently, when a living organism is subjected to chemical treatment, artifacts are so liable to be introduced that it could probably be stated categorically that no treated material is entirely free from such appearances. Artifacts, on the other hand, are rarely purely such. Those artifacts which are introduced extraneously and as such are "pure artifacts," include deposits from certain fixing fluids (*e.g.*, those containing mercuric chloride) and certain stains. The hematoxylins, especially when they have become aged and turn color, are notorious for leaving deposits when carelessly handled. All pure artifacts, fortunately, are promptly recognizable as such.

Since artifacts may be considered as always being present, the problem becomes simplified into one of determining the extent of their significance. The majority of artifacts are dependent upon the nature of the fixing fluid, but others have different causes. The appearance of cytoplasm following the use of a fixing fluid giving the basic fixation image is more nearly natural and normal; its appearance after a fluid giving an acid fixation image is always an artifact. To cite another cause of artifacts, if the chromosomes appear clumped following the use of Bouin's fluid, the blame rests with the dehydrating fluids. The significance of the artifact depends greatly on how far it was carried along to being what it is. The chromosomes may show only a slight rounding up after Bouin's fluid, in which case they may serve for counting purposes if they

can be clearly distinguished each from the others, but they are in no condition for any other purpose; if they are badly clumped, the preparation is worthless.

Appearances will frequently be encountered where it is necessary to determine whether an apparent artifact is natural or merely characteristic. If a presumable artifact appears after only one fixing fluid (generally also when the same staining method is used) and not after others, then it is a genuine, noncharacteristic artifact. If, on the contrary, it appears after a variety of fixing fluids and staining methods, it is a characteristic artifact. Again, if it can also be observed in the living material, it can scarcely be considered to be an artifact. From this discussion it becomes evident that the artifact question must be approached with caution, and it ill behooves a critic to call an appearance an artifact and thereafter ignore it. Its nature must be investigated; it may turn out to be a matter of greater significance than was at first realized.

Cytology texts should be consulted by those wishing to go further into the artifact question, since a discussion of the subject from the cytological standpoint is beyond the scope of the present text (*vide, e.g.*, Darlington 1932, Appendix I). Such texts should be considered in a critical vein since cytologists themselves are not yet in agreement on many points.

The final problem to be considered is that of cytomixis. It is a difficult one to answer. Most plant cytologists seem to consider the extrusion of chromatin during meiosis in microsporocytes to be the result of bad fixation or faulty technical treatment, but evidence has been presented to show that the extrusion of nuclear material regularly and constantly occurs in certain plants. It is also likely to be found in material collected during very warm periods of the daytime.

CHAPTER XV

MICROCHEMICAL METHODS

The term "microchemistry" connotes two distinct phases: purely chemical microchemistry and botanical microchemistry. We are not concerned with the first phase, but with the latter only. In botanical methods qualitative reactions alone are almost exclusively employed, whereas in chemistry quantitative methods are of greater importance. Those who wish to pursue the chemical aspects may consult the standard works (*e.g.*, Emich 1932, Haas and Hill 1928, Klein 1931–1933).

To many botanists microchemical tests are of significance only when correlated with either anatomical structures or, to a lesser extent, physiological appearances. These tests, unfortunately, are of a fugitive nature for the most part, and in but few cases is it possible to make permanent preparations demonstrating microchemical reactions.

No attempt has been made to present an exhaustive list of procedures or to include all substances for which tests have been devised.

Preparation of Sections.—Only strictly fresh material should be used, as a rule. Wilting of the tissues can sometimes produce changes in reactions, and in some cases plant material that has been kept indoors away from light and subjected to the influence of laboratory odors or reagents will give a very different reaction from that of material freshly collected outdoors.

Generally speaking, slightly thick sections are best for use in microchemical tests. Even those cut at around 50μ are not too thick. Very thin sections present too little tissue, especially if the substance for which the test is being made occurs in quite small amounts, and there is thus danger of obtaining a negative reaction. Such thin sections, moreover, are difficult to handle.

Sections can be cut with safety razor blades, razors, or sharp scalpels with thin blades. Clean the cutting implement thoroughly before each operation to avoid contaminating the sections. In most tests the sections are to be placed directly in the reagent, but if specific directions concerning the immediate treatment of the sections are not given, it is understood that they are to be placed in a few drops of pure distilled water.

Microchemical Reagents.—The majority of the reagents are simple solutions of various salts, dilutions of different acids, etc., and their

preparation is given in sufficient detail upon each occasion. Some general reagents in common use, however, are being described at this juncture and their ingredients and preparation described.

1. When the term "dilute" is used with reference to an acid, it usually means a solution in distilled water of not over 5% strength. Dilute solutions of salts may be of a strength not over 10% by weight.

2. *Iodine-Potassium Iodide.*—Under certain circumstances variations in the proportions of these two chemicals are required. When such are not specified, the general formula may be employed. Dissolve 1 g. potassium iodide in 100 cc. distilled water, then add 1 g. iodine flakes.

3. *Millon's Reagent.*—Dissolve 1 g. mercury in 9 cc. concentrated nitric acid of sp. gr. 1.52, and dilute the solution with an equal volume of water. The reagent does not keep long, but its usefulness may be prolonged by the addition of a few drops of potassium nitrite solution.

ALDEHYDES

Formaldehyde.—Sections of the fresh tissue to be tested are placed directly in a few drops of a 1% solution of diphenylamine in concentrated sulphuric acid on a slide. Heat slightly over a flame. A permanent green color, representing a condensation product of formaldehyde and diphenylamine, appears. With other aldehydes the green color shortly changes to red.

ALKALOIDS

The number of known alkaloids is now so large that tests for each of the individual alkaloids cannot be given here. Reference should be made to manuals or monographs on the group (*e.g.*, Henry 1924, Tunmann 1931). Alkaloids generally occur as colorless crystalline solids, but a few are liquids. As a rule, they are insoluble in water but will dissolve in neutral organic solvents. Most alkaloids do not occur free, but combined with some acid in the form of a salt.

Alkaloids, like most other substances having a nitrogen base, are precipitated by the salts of the heavy metals. The best of such reagents are 5% aqueous solutions of either gold or platinum chloride.

Parallel tests should be made on fresh sections and on sections which have been freed of their alkaloidal contents. To extract the alkaloids, put the sections in 5% tartaric acid in 95% ethyl alcohol for several days, then wash, and test. Iodine-potassium iodide solution gives a chocolate-brown precipitate with alkaloids; proteins give a yellow to brown color.

Innumerable color reactions for different alkaloids are described in monographs.

Occurrence.—Alkaloids are widely distributed. Saps of the Papaveraceae contain several different alkaloids. In the Berberidaceae,

berberin forms tufts of needles of berberin nitrate when sections are placed in 2% nitric acid.

Any species of *Nicotiana* may be used in tests for nicotine: with phosphotungstic acid, deep yellow colors becoming yellowish-green are produced; with saturated mercuric chloride the color is white; with platinum chloride (2%), yellowish-white; with iodine solution, brown-yellow.

AMINO ACIDS AND AMINES

In testing for most of the amino acids, it is best first to crystallize them out of absolute alcohol. Fresh sections are placed directly in a drop of the alcohol on a slide, and the alcohol allowed to evaporate. In addition to the crystals of various amino acids, crystals of potassium nitrate may appear. These crystals may be identified by their form, specific reactions, and optical characters. Add to the slide a saturated aqueous solution of asparagine (about 0.5 g. in 29 cc. of water). The water-insoluble needle crystals of tyrosine remain; water-soluble plates of leucine crystals dissolve; potassium nitrate crystals, which have one right angle, dissolve; asparagine crystals, which have a 51° acute angle and a 129° obtuse angle, are not readily soluble but increase in size. Transfer the sections again to absolute alcohol, add a coverslip, allow the alcohol to evaporate, then heat cautiously to 170°C. Leucine crystals sublime on the coverslip; potassium nitrate crystals are unchanged; asparagine crystals become transformed into foamy oil-like drops which are readily soluble in water.

Arginine, Histidine.—The addition of a few drops of a dilute solution of picrolonic acid to fresh sections produces a yellow crystalline precipitate.

Asparagine, Glutamine.—Quinone gives a red color to these two amino acids: first crystallize the acids out of absolute alcohol then add 1 drop quinone solution. The asparagine crystals take a red color along their boundaries, then dissolve slowly, and produce a red solution.

In order to distinguish between asparagine and glutamine, place the sections in absolute alcohol, and dry for several days in a desiccator. The glutamine crystals dissolve readily on the addition of water to the sections.

A further test for asparagine may be carried out as follows: place fresh sections in a drop of 7% aqueous cupric acetate on a slide. In about 15 minutes begin adding traces of alcohol until ultramarine-blue sphaerocrystals of copper asparagine appear.

Occurrence.—Asparagine is of very widespread occurrence in plants; it is easily demonstrated in etiolated seedlings of *Lupinus* and in tubers of *Dahlia*.

Glutathione.—Heat thin sections of fresh tissue in dilute acetic acid. Wash in a saturated solution of ammonium sulphate, then place in a watch glass containing about 5 cc. of this solution. Add 5 to 15 drops of a 5% aqueous solution of sodium nitroprusside. Agitate thoroughly for a short time, add about 1 cc. ammonium hydroxide, and at the same time watch the sections carefully. On the addition of the last reagent, a color between pale pink and magenta will flash in the cells, lasting usually for only a few seconds. The intensity and time of duration of the color reaction vary, presumably with the amount of glutathione present.

Leucine.—As crystallized out of absolute alcohol, crystals of leucine are in the form of thin plates arranged as rosettes, with angles of 70 and 110°. It commonly happens that leucine and tyrosine crystallize together as needles and planes arranged in sphaerite form. If the sections are covered with a coverslip and heated to 170°C., the leucine crystals sublime on the coverslip.

Occurrence.—Seedlings of most Papilionaceae, and those of *Cucumis* and *Chenopodium;* buds of *Aesculus;* tubers of *Solanum tuberosum* and *Dahlia.*

Tyrosine.—To sections crystallized out of absolute alcohol, add a drop of Millon's reagent. The tyrosine crystals are red at first but soon dissolve into a red solution.

Place fresh sections in absolute alcohol on a slide, then after the alcohol has evaporated, heat the sections in a few drops of a freshly prepared solution of 0.01 g. sodium molybdate in 1 cc. concentrated sulphuric acid. This dissolves the tyrosine crystals, giving a deep blue color which soon turns violet.

With a solution of 2 drops acetaldehyde plus 1 drop concentrated sulphuric acid, tyrosine gives a red color. When heated with a mixture of approximately equal parts of formalin and sulphuric acid, a green color is produced by tyrosine.

Occurrence.—Shoots and tubers of *Dahlia;* very young *Lupinus* seedlings.

CALLOSE

There are several color reactions distinguishing callose.

1. Permanent preparations of suitable small objects are easily made. Place the material for 30 minutes in a freshly prepared and not too strong aqueous solution of anilin blue. Transfer to a slide, add a small drop of levulose sirup (10 g. levulose to 8 cc. warm distilled water) and cover with a coverslip. After the sirup has evaporated slowly until it becomes thickened, ring the coverslip as for glycerin mounts. Callose is stained blue.

2. Rosolic acid (corallin) may be employed in place of anilin blue. Use a 1% solution in 4% aqueous sodium carbonate. A red color is imparted to callose membranes.

3. Place the sections in a drop of a 1:2500 solution of resorcin blue. After about 15 minutes, callose takes a brilliant blue stain. Transfer to a drop of glycerin or levulose sirup on a slide for examination.

Callose, whose exact chemical nature is not yet definitely known, may be distinguished from other membrane substances by its solubility characters: (1) callose is insoluble, cellulose and the hemicelluloses soluble, in copper oxide ammonia; (2) callose is soluble, cellulose and chitin insoluble, in glycerin heated to 280°C.; (3) callose swells but does not dissolve in the alkaline carbonates and in ammonia; pectic acid (or pectic substances changed to the acid form) dissolves.

Callose is also readily soluble in 1% aqueous potassium or sodium hydroxide and in calcium or stannous chloride solutions.

CARBOHYDRATES

Sugars.—Sucrose is the principal sugar stored in tissues, but other sugars are also present, notably fructose and glucose. It is necessary to ascertain whether such sugars are actually present before tests for sucrose are made. The Flückiger test may be employed for this purpose. In a drop of 15 to 20% aqueous sodium hydroxide on a slide dissolve a small quantity of copper tartrate, place the sections therein, and add a coverslip. Fructose immediately gives a yellowish-red precipitate of cuprous oxide. On gentle warming, glucose gives cuprous oxide crystals. On heating the sections for about 20 minutes, dextrin causes the formation of cuprous oxide crystals. No precipitate is given by sucrose. Upon the addition of 95% alcohol the fructose and glucose are dissolved, leaving the insoluble dextrin in the tissues.

The osazone test, when properly performed, is the most satisfactory test for sugars (Mangham 1911). Its only disadvantage lies in its comparative slowness. Make up the following solutions: (1) pulverize phenylhydrazine hydrochloride in a mortar, then dissolve 1 part of the powder in 10 parts glycerin, filter, and store in a brown bottle; (2) make a solution of 1 part sodium acetate in 10 parts glycerin, and likewise filter and store in a brown bottle. On the slide mix 1 drop of the phenylhydrazine solution and 2 drops of the sodium acetate mixture, then place the sections in the reagent, and add a coverslip. Avoid using too much of the reagent. Keep at a temperature of about 20°C. In from 7 to 12 hours the formation of osazone crystals indicates the presence of fructose; in two days glucose gives identical osazones. The difference in the rate of crystallization makes it possible to distinguish between fructose and glucose by the forms and general appearance of the crystals.

The appearance of yellow droplets in the cells indicates the beginning of the reaction. In the case of glucose these droplets crystallize slowly and produce dense, deep yellow to orange sphaeroclusters of needle crystals. With fructose the clusters of crystals are less dense and clear yellow in color. These distinctions are obtained only if there is more than 1% of the sugars present.

Maltose gives an osazone, which is soluble in 75 parts of boiling water and can be crystallized from this solvent in rosettes of plates or broad needles resembling sword blades. In the sections the osazone rosettes are dense, lemon-yellow in color, and broader and larger than those obtained with fructose and glucose.

Another procedure is to heat the slide carefully on a water bath for 15 minutes. The fructosazones are formed during the heating. Set the slide aside to cool; the glucosazones form in about 30 minutes after cooling.

Methylphenylhydrazine gives osazones with fructose and sorbose only. Since the sorbose osazone is soluble, the appearance of osazone crystals indicates the presence of fructose alone. Prepare the reagent by putting 1 part methylphenylhydrazine and 10 parts glycerin in a brown bottle, add acetic acid until the pH is about 4.7, and shake the bottle at intervals for several hours until the mixture becomes homogeneous. Employ the reagent with sodium acetate as described for phenylhydrazine hydrochloride above. The crystals appear in about 24 hours in the cold or in 15 minutes upon heating the slide on a water bath.

Sucrose can be inverted by dilute acids, but since the acids would also hydrolyze any glucosides and hemicelluloses that might be present in the tissues, this test is scarcely to be recommended. Instead, inversion by means of the enzyme invertase is preferable. Invertin, a water-soluble powder containing invertase, should first be prepared: mix enough water with a cake of yeast to make a paste, keep at 40°C. for 12 hours, then press to extract the enzyme, filter, and precipitate with 95% alcohol. The resulting white powder is invertin. (1) Put sections of the fresh material in a drop of invertin solution on a slide, and keep at 40°C. for 3 hours or for a longer period if the tissues are somewhat impenetrable, then test for glucose. (2) Put more sections in 5% citric acid on a slide, heat for 10 minutes, then test for glucose. If much glucose is found to accompany the sucrose, test for sucrose as follows: perform the Flückiger test (page 186) for glucose and fructose; wash the sections with 5% tartaric acid; add warm magnesium chloride solution to dissolve the cuprous oxide precipitate; wash again with the tartaric acid, then test for glucose. The test should react negatively; if so, invert the sucrose, and test.

Amylodextrin.—Amylodextrin is intermediate between maltose and starch and is commonly present in solution in storage organs where starch is being hydrolyzed. It gives a red color with iodine-potassium iodide solution.

Inulin.—Inulin occurs as sphaerocrystals in roots and tubers. Put small pieces of fresh tissue in 70% ethyl alcohol for two to four days, then cut off smaller portions, place on a slide in alcohol, add a coverslip, and examine. To bring out the concentric layers of the crystals, add a drop of chloral hydrate solution (5 parts hydrate to 2 parts water) to the sections.

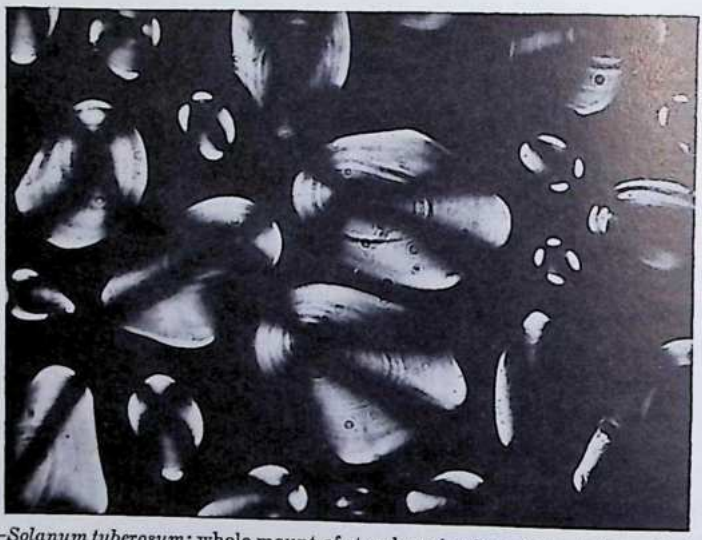


FIG. 23.—*Solanum tuberosum:* whole mount of starch grains from living tuber, photographed by polarized light. Unfixed and unstained.

To identify the inulin crystals, locate some in the sections under the microscope, and add a drop of a 15% solution of thymol in alcohol and a drop of concentrated sulphuric acid. The crystals become carmine-red immediately and soon pass into solution.

Inulin is readily hydrolyzed to fructose. Consequently, to show typical crystals in the cells, a nonhydrolyzing killing fluid must be used when it is desired to make permanent sections by the paraffin method.

Occurrence.—Tubers of *Dahlia* (in autumn); in *Taraxacum, Helianthus tuberosus, Allium, Galanthus, Leucojum,* etc.

Starch.—The familiar iodine test is still the most satisfactory one, even if other substances also give a blue color reaction with iodine. Use a weak solution (0.3 g. iodine, 1.5 g. potassium iodide, 100 cc. water). The blue color disappears on heating but returns on cooling. Small starch grains may be better observed if the chlorophyll is removed from

the sections with alcohol before applying the iodine. The grains will appear black. Heat the slide, then place under the microscope, and watch for the return of the color.

Different types of starch may be distinguished by their behavior under polarized light (note the appearance of potato starch in Fig. 23).

Glycogen.—Glycogen occurs in animal tissues and in certain fungi, such as *Saccharomyces cerevisiae*, in Myxothallophyta and in the Cyanophyta. With iodine solution, glycogen gives colors which vary according to the amount present. If a definite iodine solution (0.1 g. iodine, 0.3 g. potassium iodide, 45 cc. water) is used, a fair determination of the relative amount of glycogen present can be made. A brown, orange, or yellow color indicates traces of glycogen, while considerable amounts give a wine-red color. Glycogen is soluble in water and will soon disappear if the tissues are left in the reagent.

Glycogen may be coagulated and then stained with any basic coal-tar dye (*e.g.*, safranin, methylene blue, or Bismarck brown). Put the material in strong alcohol for about 15 minutes, then transfer directly to a 10% aqueous solution of tannin. After 10 minutes transfer the material directly to 1% aqueous potassium bichromate for a few minutes, then to 10% aqueous potassium bichromate for 10 minutes. This procedure renders the glycogen insoluble in water.

CELLULOSE

There are two kinds of cellulose, *viz.*, true celluloses and hemicelluloses, that are readily distinguished by the fact that the true celluloses are not hydrolyzed by acids, whereas the hemicelluloses are hydrolyzed by both 3% and concentrated (sp. gr. 1.19) hydrochloric acid and by 3% sulphuric acid. (Hydrochloric acid of a specific gravity other than the one noted dissolves cellulose immediately.)

Cellulose is hydrolyzed by 70 to 75% sulphuric acid into a colloid substance, hydrocellulose, which forms a blue adsorption compound with iodine solution. Place sections in a drop of iodine solution (0.3 g. iodine, 1.5 g. potassium iodide, 100 cc. water) on a slide, cover, and observe the localization of the blue color reaction. Allow a drop of 75% sulphuric acid to diffuse in from one side of the coverslip, and watch the swelling of the cellulose membranes. A few other plant substances also give a blue color reaction, consequently the localization of any blue color which appears before the addition of the acid should be noted.

Hemicelluloses are discussed below.

CHITIN

In an open beaker heat 100 cc. of a saturated aqueous solution of potassium hydroxide to boiling. Put the sections in the boiling solution,

cover the beaker, and continue the boiling for 20 to 30 minutes. Remove the sections, and wash in 90% alcohol. The chitin has been transformed into chitosan. Test for the latter with a weak iodine solution (0.2 g. iodine, 2 g. potassium iodide to 100 cc. water): a red-violet color demonstrates the presence of chitin.

Occurrence.—Principally in the higher fungi, but also in a few of the lower; however, there is some question whether the substance actually is chitin.

CUTIN, SUBERIN

A saturated solution of Sudan IV in 70% alcohol imparts a red color to all fatty substances. Leave the fresh sections in the stain for 20 minutes, wash with 50% alcohol to remove excess stain and transfer to a drop of glycerin for observation. Fats may be distinguished from the other colored substances by the fact that they exist as drops within the cells. Fuelgen's reaction also distinguishes cutin, suberin, and lignified elements (Margolena 1932).

To differentiate suberized and cutinized cell walls from cellulose membranes, concentrated sulphuric acid or copper oxide-ammonia may be used; these fluids dissolve the cellulose.

All membrane substances except cutin, suberin, and chitin dissolve quickly in the cold in 50% chromic acid; however, if the acid is heated, it will dissolve all membranes after a time.

The fact that suberin contains phellic acid and cutin does not provides the method of differentiating between the two substances. After several hours' maceration of the sections in concentrated potassium hydroxide, suberin and also lignin become yellow. Upon slight warming, the layers of suberin swell, and the yellow color becomes darker. Raise the temperature to boiling: granular masses of potassium phellonate, which become larger as the heating is continued, appear. Let cool, wash thoroughly with water, and add a drop of chlorzinc-iodide. The potassium phellonate acquires a reddish-violet color.

ENZYMES

There are but few microchemical localization tests for enzymes, and these are not always either satisfactory or conclusive. Fairly good tests for proteases, oxidases, peroxidases, and catalase can, however, be obtained.

Oxidases.—These enzymes are probably universally distributed in plants. Place sections in a drop of 1% benzidine in 60% alcohol. The reaction will appear in about 15 minutes. If the tissue is acid, oxidase-containing cells turn blue; if the sections are alkaline, the color is brown.

A second test for oxidases is to place the sections in a drop of a 10% solution of guaiaconic acid in strong alcohol. On oxidation there appears a blue to blue-black precipitate, insoluble in water, but soluble in chloroform, ether, and benzol. The presence of tannins or weak acids retards the reaction.

Peroxidases.—The two reagents described as tests for oxidases are oxidized by peroxidases only if hydrogen peroxide is added.

Ursol tartrate may be used as a reagent. Prepare it by mixing a saturated alcoholic solution of ursol with a saturated aqueous solution of tartaric acid; filter to remove the precipitate, wash it with alcohol, and recrystallize from ether. On a slide put 1 drop of this reagent, add 1 drop of hydrogen peroxide, and place the sections in the mixture. The immediate appearance of a green color, which changes to blue and finally to gray, indicates the presence of peroxidases.

Catalase.—The prompt evolution of gas bubbles when hydrogen peroxide is added to sections in a drop of 1% gum arabic or gelatin solution demonstrates that catalase releases oxygen from the peroxide.

FATS

True fats are always readily distinguished by their affinity for the specific dyes, Sudan III and IV. The majority of lipoids do not take up these dyes, nor are they absorbed by vacuoles. To distinguish fat globules from vacuoles, neutral red dye, which is taken in by the latter but not by the former, may be used.

The stain solution is prepared by dissolving 0.5 g. of the dye in 100 cc. of 70% alcohol. Leave the sections in the stain for 20 minutes, wash carefully but quickly with 50% alcohol, and transfer to glycerin for observation.

GLUCOSIDES

Amygdalin.—Tests for this substance are based upon the production of hydrocyanic acid during its decomposition.

Guignard's test is one of the easiest. Immerse the sections in 1% aqueous picric acid for 30 minutes, wash with water, and place in 1 drop of 10% aqueous sodium carbonate on a slide. A red color appears if hydrocyanic acid is released.

If the test is made quickly, the Berlin blue reaction is fairly good. Place the sections in a potassium hydroxide solution composed of 20 parts of 20% aqueous potassium hydroxide and 80 parts 90% alcohol, for a few minutes. In a small watch glass mix equal parts of a 2.5% aqueous ferrous sulphate solution and 20% aqueous ferric chloride solution, and heat to boiling. Transfer the sections to this mixture; after 5 to 10 minutes, transfer the sections to a slide holding a drop of 20% hydro-

chloric acid. A deep blue precipitate indicates the presence of hydrocyanic acid.

A test that requires more skill in manipulation and interpretation is the mercurous nitrate reaction. Place the sections on a slide in a few drops of 3% aqueous mercurous nitrate for 1 to 2 minutes, wash in distilled water, and place in glycerin for observation. The performance must be carried out rapidly, and it would be well to moisten scalpel and object with the reagent. If the sections are heated, other substances will give the reaction.

Occurrence.—Seeds of *Amygdalus, Pyrus, Crataegus,* and related genera; leaves of *Prunus laurocerasus.*

Anthocyanin.—The color of most red and blue flowers is due to anthocyanin. If petals of such a flower are held in ammonia vapor for a minute, the color changes to green. Anthocyanin is red in acid solutions, blue violet when there are traces of alkali, and green in alkaline solutions.

Put the petals or portions of such in a drop of water under a coverslip on a slide, and crush out the colored liquid. Some anthocyanins form liquid globules on evaporation; others produce crystals. Or the petals may be placed in a small drop of glacial acetic acid or 10% hydrochloric acid and the acid (which extracts the anthocyanin) evaporated slowly in a moist chamber. The anthocyanin forms various sorts of red crystals.

Arbutin.—Place sections in 10% nitric acid. A dark orange color immediately appears in cells containing arbutin, but it soon changes to yellow and slowly disappears.

Arbutin on hydrolysis is transformed into glucose and hydroquinone. Heat the sections, placed dry or in a drop of water on a slide; the arbutin sublimes in crystals. The latter become red-brown upon the addition of ammonia, or pale green with ferric chloride solution.

Occurrence.—Ericaceae and Pyrolaceae.

Saponin.—Sections placed directly in 1 drop concentrated sulphuric acid on a slide exhibit a characteristic sequence of color reactions, beginning immediately with yellow, changing to red within 30 minutes, and finally becoming violet, or blue-green in a few instances. This test is merely an indication of the presence of some type of saponin. To determine the localization of the saponin, put the sections in a saturated solution of barium hydroxide for about 24 hours. A practically insoluble, colorless compound is formed by the barium and saponin. Wash the sections in a weak aqueous solution of calcium chloride, then place in 10% aqueous potassium bichromate. The compound first formed is broken down, and the barium unites with the chromium to form barium chromate, yellow in color. Tannin-containing cells become brownish-red during the reaction.

Occurrence.—Roots of *Saponaria, Laothoe* (*Chlorogalum*), etc.

Tannin.—The word tannin does not denote a single substance but is a generic name covering a whole group of substances having certain characteristics in common. They are mostly colloidal substances and occur either in solution in the cell sap or not infrequently in distinct vacuoles. The amount of tannin present varies with the state of growth, time of year, physiological condition, and other factors; but in some organs, such as root tips of *Pinus*, the variations are within rather narrow limits (McNair 1930).

Tissues, such as root tips, suspected of containing tannin localized in vacuoles or circumscribed cell areas, may be fixed in an aqueous solution containing 3 to 5% formalin and 10% ferrous sulphate for 24 to 48 hours, washed, dehydrated, sectioned, the paraffin removed, and balsam and a coverslip applied. The iron compound both fixes and stains the tannin.

If sections of fresh tissue are placed in 10% aqueous ferric chloride plus a little sodium carbonate, a blue-green color is given by tannins.

Tannins are readily oxidized. In many types of prepared slides the presence of dark red or reddish-brown, more or less isolated cells indicates the occurrence of phlobaphene, which results from the oxidation of tannins.

Occurrence.—Tannin is rather commonly present. All parts of *Quercus* spp., the Crassulaceae, and the Coniferophyta readily react for its presence.

HEMICELLULOSES

Hemicelluloses are divided into two groups: (*A*) those entering into the constitution of permanent cell walls, and (*B*) those occurring in storage organs and which are consumed during growth. The distinction is rather weak, since the same substance can occur in both groups. No really distinctive localization reactions have been reported for the hemicelluloses.

A. Skeleton Hemicelluloses Occur Chiefly in Woody Cell Walls and in Seed Coats.

Galactase.—There is no really satisfactory test for this substance.

Araban, Xylan.—The phloroglucin-hydrochloric acid test may be carried out. Place sections in a drop of 1% phloroglucin solution (0.1 g. phloroglucin, 10 cc. 95% alcohol) on a slide, add 1 drop hydrochloric acid, and observe. Lignin gives an immediate violet-red color. Heat cautiously for about 10 minutes to hydrolyze the xylan to the pentose xylose, which takes a cherry-red color.

Methyl Pentoses.—Place the sections in 1 or 2 drops acetone on a slide, add 1 drop concentrated hydrochloric acid, and warm gently for 15 minutes. The appearance of a red or violet color indicates the presence

of methyl pentosans (rhamnose, fucose, quinovose, etc.). The methyl pentoses occur associated with pentoses as cell-wall constituents. The pentoses also give a violet color by the above reaction, but this color fades within 1 hour.

B. Storage or Reserve Hemicelluloses Are Found Principally in the Cell Walls of Endosperm and in Very Young Bast Fibers.

Amyloid.—This occurs mostly in fatty seeds. It gives a blue color with iodine solution: by adding 2% hydrochloric acid and warming, the intensity of the reaction is increased.

Mannan.—Mannan is of somewhat widespread occurrence. Hydrolyze rather thin sections in a small watch glass in 2% hydrochloric acid for 30 minutes or slightly longer. Transfer the sections to a slide, neutralize with a few drops of 2% ammonia, then test for mannose as described for the osazone reaction for glucose. Colorless plate-like crystals of mannose hydrazone should appear within a short time.

Galactan.—This reserve sugar usually occurs accompanied by other sugars. If mannose or amyloid is found to be present, galactan is presumably also present.

LIGNIN

Sections are placed on a slide in a large drop of a solution of 0.1 g. phloroglucin in 10 cc. of 95% alcohol and covered with a coverslip. Allow part of the solution to evaporate, then let a little 25% hydrochloric acid diffuse in at the edge of the coverslip. The appearance of a red-violet color indicates the presence of lignin.

To a 0.001% aqueous solution of methyl red, add just enough alkali to render it yellow. A permanent color resembling that imparted by phloroglucin is acquired by lignified cell walls in either fresh or paraffin sections.

To test for the lignin oxide present in the walls of young bast fibers: cover the sections on a slide with 1% neutral aqueous potassium permanganate for 15 to 20 minutes (the Maule test). Manganese dioxide is deposited on the woody tissues. Wash thoroughly, and place in 2% hydrochloric acid (sp. gr. 1.06). The acid reacts with the manganese to produce chlorine which in turn produces a chlorination. After the dioxide has been dissolved, wash the sections thoroughly with distilled water. Add a few drops of either ammonium hydroxide or sodium bicarbonate solution. A deep red color develops in the lignified elements of deciduous plants, an indefinite brown in those of coniferous trees. This test is said to afford a means of distinguishing between deciduous and coniferous (except *Ginkgo*) woods.

Solubility tests may be used to distinguish lignified membranes from other membrane substances:

1. Suberin, cutin, and chitin are insoluble in 50% chromic acid; lignin and all other membrane substances are dissolved.

2. Lignin is not soluble, cellulose and the hemicelluloses are soluble, in copper oxide-ammonia.

Most oxidizing agents, such as hydrogen peroxide, potassium chlorate, nitric acid, etc., also dissolve lignin from membranes.

LIPOIDS

Lecithin.—The same dyes, Sudan III and IV, for which fats have a marked affinity, are also taken up by lecithin. It is therefore necessary first to rid the sections of fats by placing them in a vial of acetone for about 12 hours. The sections may then be stained with Sudan IV or with 1% aqueous osmic acid. With the latter reagent, the lecithin acquires a light brown color.

Phytosterol.—Thick sections of tissue are placed in concentrated sulphuric acid: they first show a red color, then the phytosterol dissolves into a foamy mass whose particles turn olive green in 3 or 4 hours and finally become colorless. Or the tissues may first be placed in chloroform and the sulphuric acid then added: the phytosterol is first rose-red and finally brown. A second reaction is to add a trace of iodine-potassium iodide solution after the sections have been placed in concentrated sulphuric acid. The color at first is red, then after the addition of the iodine solution it changes to violet, next to blue, and finally to yellowish-red or brown. A third method is to place the sections in strong trichloracetic acid (9 parts acid to 1 part water), heating slightly or adding a little hydrochloric acid: the resulting color is violet or reddish-purple.

MINERAL SUBSTANCES

Calcium.—The most sensitive test is the formation of calcium oxalate. Place the sections on a slide in an excess of 2% aqueous oxalic acid. Leave uncovered. After about 30 minutes, withdraw some of the acid, add a coverslip, and very carefully place 1 drop alcohol at one edge of the coverslip. Very small but readily recognizable crystals of calcium oxalate will indicate the presence of calcium.

When iron occurs in plants, it is often accompanied by calcium. Fixing fluids containing acids might dissolve calcium compounds; formalin should also be avoided. Calcium is stained by hematoxylin and has an affinity for silver and other metals. The latter fact permits the use of a simple method involving the reduction of silver nitrate. Bring fresh sections from distilled water into a weak solution of silver nitrate in distilled water, and leave for 5 minutes. Wash with distilled

water, then transfer to a weak solution of a photographic developer such as pyrogallol, where the sections may remain until the calcium-containing portions become blackened. Then wash out with absolute alcohol, and pass through xylol into balsam. Keep such slides in the dark.

Calcium Oxalate.—Small pieces of tissue or sections are treated with a saturated aqueous solution of cupric acetate. The crystals of calcium oxalate, if present, dissolve, and the oxalic acid diffuses into the intercellular spaces where cupric oxalate crystals are formed. To test for the dissolved oxalate, a solution of ferric sulphate (made by adding 5 g. ferric sulphate and 20 cc. acetic acid to 80 cc. water) may be used, resulting in the formation of yellow crystals of ferrous sulphate.

Magnesium.—If the cells are presumed to contain both magnesium and a phosphate, the addition of a little concentrated ammonia to the sections in 1 drop of water on a slide will cause the appearance of crystals of ammonium-magnesium phosphate. Or if phosphates are absent, one may be added in the form of sodium phosphate. Prepare the reagent by adding a few drops of free ammonia to a saturated aqueous solution of ammonium chloride, then sufficient sodium phosphate to make a 0.1% solution. Put the fresh sections in 1 drop of this reagent, and warm the slide a little. In about 10 minutes add a coverslip, and examine.

Occurrence.—Magnesium is found in the meristematic tissues of most plants and in latex (*Ficus, Euphorbia*) and resinous secretions (*Pinus*).

Potassium.—Sections of tissue, which should be neutral or slightly acid, may be placed directly in a 10% aqueous (some workers prefer an alcoholic) solution of platinum chloride: yellow octahedron crystals of potassium chloroplatinate appear. If ammonium is present, similar crystals of ammonium chloroplatinate will be formed.

A second indicator of the presence of potassium is by the formation of crystals of potassium-cobalt nitrite. The reagent is prepared by dissolving 20 g. cobalt nitrate and 35 g. sodium nitrite in 65 cc. water plus 10 cc. glacial acetic acid. A precipitate of fine yellow crystals appears if potassium is present.

Occurrence.—Potassium is found in the cytoplasm of most plants; it is absent in nuclei and chloroplasts. Favorable objects are the stem apices and storage tissues of *Daucus carota, Solanum tuberosum,* and *Beta vulgaris.*

Sodium.—Place the sections in a saturated aqueous solution of uranium acetate on a slide, add a small drop of hydrochloric acid, then place the slide, without a coverslip, in a desiccator so that the fluids may evaporate slowly. In two to eight hours pale yellow tetrahedral and rhomboidal crystals of sodium-uranium acetate appear. If magnesium should be present, this will be indicated by the formation of large rhomboidal crystals of uranium-magnesium-sodium acetate.

Occurrence.—Sodium is abundant in marine and strand plants and also in many cultivated plants. Among the latter are *Solanum tuberosum*, *Spinacia oleracea*, *Brassica oleracea* and *B. rapa*.

Ammonium.—The least complicated test is to observe the formation of crystals of ammonium-magnesium phosphate. The sections may be placed in a solution of sodium phosphate and magnesium chloride slightly alkalinized with sodium hydroxide.

Occurrence.—Stems of *Helianthus tuberosus;* bulb scales of *Allium cepa;* leaves of *Tradescantia.*

Iodine.—Tests for iodine are indirect, and all are based upon the ability of iodine to color starch grains blue. Iodine occurs in the Phaeophyta, particularly in *Laminaria*. Place fresh sections in a small watch glass with about 2 cc. of a 1% aqueous suspension of potato starch flour, together with 1 to 3 drops of a 20% aqueous solution of potassium nitrite and 1 to 3 drops of 5% hydrochloric acid. The starch becomes colored blue if iodine is present.

Iron.—In manipulating tissues to be tested for iron, take extreme care to avoid contacts with iron or steel instruments. If a scalpel or knife is used for cutting sections, clean it very thoroughly; use glass needles for handling the sections after they have been cut.

Have the sections in water; immerse for 5 minutes in 1% aqueous hydrochloric acid, to each 25 cc. of which is added 3 drops of a freshly prepared concentrated aqueous solution of potassium ferrocyanide (some workers advise using 8 to 10 drops to each 25 cc. of a 2% solution of the acid, for 30 minutes to 1 hour). Wash out the acid with distilled water, and stain the nuclei with alum carmin. Wash out excess dye, mount, and examine. The sections can be dehydrated and mounted in balsam if desired. Any iron present should be stained a deep blue, the nuclei red.

Or immerse the sections in a slightly old (yellowish) solution of ammonium sulphate for 5 to 30 minutes or until the sections become dark green. Wash out quickly, then transfer to glycerin to which a little ammonium sulphate has been added, and examine. If satisfactory, wash out with absolute alcohol, pass through xylol, and mount in balsam. Carmin may be used as a counterstain.

Occurrence.—The capsules of the iron bacteria are saturated with iron. This element is also present in many lichens and in the seeds of *Sinapis alba.*

Manganese.—Oxalic acid combines with manganese to form manganous oxalate crystals. If a prompt reaction does not take place, let the sections dry, then touch a speck of potassium oxalate to them. Immediate crystal formation should take place.

Occurrence.—Manganese is especially abundant in the cortex and wood of the Coniferales and in the epidermis of aquatic plants.

Silicon.—Place the sections on a slide, then, without adding any liquid, cover them with a crystal of phenol, and heat until a red color appears wherever silicon occurs. If it is desired to preserve the sections, clear with clove oil, and mount in balsam.

Silicon crystals frequently stain with methylene blue and crystal violet.

Occurrence.—Silicon is especially abundant in diatoms, in the Equisetums, Poaceae, and Cyperaceae; and occurs in the form of crystals in the epidermal cells of the Orchidaceae and Palmaceae.

Nitrates.—Cover the sections with a coverslip, and allow diphenylamine (0.1 g. diphenylamine in 10 cc. 75% sulphuric acid) to run in from one side of the coverslip. A deep blue color indicates the presence of nitrates; as the sulphuric acid begins to disintegrate the tissues, the color changes to a yellowish-brown.

Occurrence.—Plants growing in waste places and around rubbish heaps generally are rich in nitrates. Such are to be found among the following genera: *Chenopodium, Urtica, Mercurialis, Solanum, Helianthus. Zea mays* and *Cucurbita pepo* are also favorable plants.

Phosphates.—If both magnesium and phosphate are present, the addition of ammonia causes the formation of crystals of ammonium-magnesium phosphate. If magnesium is presumably absent, treat the sections with 1 drop of the following reagent: to 15 cc. water add 25 cc. of a saturated aqueous solution of magnesium sulphate and 2 cc. of a saturated solution of ammonium chloride. Ammonium-magnesium phosphate crystals should form.

Another test is to place the sections on a slide in a drop of a solution of 1 g. ammonium molybdate in 12 cc. nitric acid. The presence of phosphates is indicated by the appearance of small yellow black-bordered drops, which turn into sphaerocrystals, then into cubes and octahedrons.

Occurrence.—Parenchyma of leaves, the most favorable being those of *Aesculus, Allium cepa,* and *Ranunculus.*

Sulphur and Sulphates.—Sulphur occurs in plants principally in the bound form but is found free in some of the lower plants.

Any of the sulphur bacteria (such as *Beggiatoa,* or *Oscillatoria* among the Cyanophyta) may be mounted in glycerin and the grains of sulphur are usually visible. This type of sulphur occurs free. Free sulphur is insoluble in weak acetic acid, weak hydrochloric acid, 1% chromic acid, saturated aqueous solutions of picric acid, concentrated sulphuric acid, glycerin, nitric acid, and potassium hydroxide; it is soluble in absolute alcohol, chloroform, and partially so in carbon bisulphide.

Organically bound sulphur occurs in meristematic tissues. Place sections in 10% potassium hydroxide, then add 1 or 2 drops of a fresh

10% aqueous solution of sodium nitroprusside; a red color reveals the presence of sulphur.

Sections to be tested for sulphates may be placed in a 1% solution of benzidine chloride in 3% hydrochloric acid. Small colorless needles or glistening scale-like crystals of benzidine sulphate may appear.

Oil- or fat-containing tissues, such as those of cereal seeds, should first be treated with ether to remove the fats. After the sections have dried, add 1 drop of 10% hydrochloric acid, then 1 drop of 10% barium chloride. A granular precipitate of barium sulphate is brought into being, but it may be somewhat difficult to observe.

Carbonates.—Add a saturated solution of strontium acetate to the sections on a slide, then place the latter in a moist chamber for half a day or longer, since the reaction proceeds slowly.

Chlorides.—Put several sections, cut with a scrupulously clean scalpel, in a drop of 5% aqueous silver nitrate on a slide. Examine microscopically without covering; the precipitate of silver chloride, which seems to be white to the unaided eye, appears black under the microscope. Transfer some of the sections, by means of glass needles, to another slide carrying a drop of 1.5% aqueous nitric acid, add a coverslip, and examine. The acid clears the sections sufficiently to permit localization of the reactions. To the remaining sections in the nitrate solution, add ammonia slowly until the precipitate becomes dissolved. Set aside: in about 1 hour the precipitate will reappear in the crystalline form. The crystals acquire a deep violet color on reduction.

The silver chloride crystals are stained brilliantly with methylene blue, Bismarck brown, and eosin.

Thallium sulphate or thallium acetate (1 g. in 15 cc. water and 2 cc. glycerin) will develop gray or black crystals of thallium chloride in sections.

Occurrence.—Roots of *Daucus carota* and *Beta; Solanum;* stems of *Primula obconica.*

ORGANIC ACIDS

Formic Acid.—Sections may be placed in a few drops of mercuric chloride solution (1 part of a concentrated solution diluted with 5 parts water), heated on a water bath for 1 hour, and washed with water acidified with 1 drop of hydrochloric acid. Then transfer the sections to 1 drop of 1% potassium hydroxide on a slide. Cells containing formic acid become blackened.

Occurrence.—Leaves of *Abies* or *Sempervivum;* cell sap of *Urtica.*

Oxalic Acid.—Treat sections with a solution of 1 part ferrous phosphate in 8 parts of phosphoric acid; an intense yellow color indicates the presence of oxalic acid. If a considerable quantity of the acid is present,

crystals of ferrous oxalate are formed. If calcium oxalate crystals are also present, first dissolve them in dilute hydrochloric acid, then add the ferrous salt.

With manganous nitrate, crystals of manganous oxalate in the form of six-pointed stars are formed.

Occurrence.—Oxalis, Begonia, Mesembryanthemum, Rheum, Rumex, Salsola, and *Salicornia.*

Citric Acid.—Sections presumed to contain citric acid may be neutralized with sodium hydroxide and heated in a 5% aqueous calcium chloride solution. A crystalline precipitate of calcium citrate is produced. This precipitate is soluble in acetic acid, which distinguishes it from calcium oxalate; it is insoluble in water, thus distinguishing it from the oxalate and tartrate of calcium.

Calcium citrate crystals can frequently be found in small pieces of tissue that have been preserved for a long time in alcohol or glycerin.

Malic Acid.—Sublimation of malic acid into maleic acid and maleic acid anhydride is claimed to be the best reaction for the acid. This must be carried out at a very high temperature in order to avoid carbonization of the tissues. The crystals will appear in about 24 hours after sublimation.

If there is much of the acid present, it will crystallize out when the sections are dried; or if put into 70% alcohol, the crystals appear on the surface.

Tartaric Acid.—Sections of tissue treated with a 4% aqueous solution of any ferrous salt and a few drops of hydrogen peroxide, or 10% potassium permanganate with the addition of an excess of sodium hydroxide solution, after a few minutes give a violet color which is produced only by tartaric acid.

Large crystals of calcium tartrate, which recrystallize from hot solutions, are produced when the sections are heated in 20% calcium acetate or 10% calcium chloride.

PECTIC SUBSTANCES

Ruthenium red is the classical dye indicator for pectic substances. It is a very expensive dye, and its solutions are quite unstable. Make up only a small quantity of solution as needed: to two or three tiny crystals of the dye in a watch glass add distilled water drop by drop until the solution is a clear reddish-pink in color. Leave the sections in the ruthenium red solution for 30 minutes, then wash thoroughly, and mount in glycerin on a slide. All pectic substances acquire a red color.

A 1:10,000 solution of methylene blue likewise stains all pectic substances, giving a violet color. This color reaction is not so precise as that given by ruthenium red, since other membrane substances are

also stained. For example, cellulose is usually stained blue, lignin and some of the hemicelluloses green.

The general procedure of testing sections for pectic substances is as follows:

1. Stain with ruthenium red (or methylene blue); note localization of pectic substances. The pectine becomes dissolved in the water of the reagent.

2. Either heat carefully in 2% aqueous hydrochloric acid for 20 minutes, or leave in a solution of 1 part hydrochloric acid to 4 parts alcohol for 12 hours. Pectose is transformed into pectine, which dissolves, or into insoluble pectic acid. (The conditions which determine whether pectose is to be changed into pectine or pectic acid are not understood.) Calcium pectate is also broken down, resulting in the formation of calcium chloride and pectic acid.

3. Wash thoroughly with water. Of all the pectic substances originally present, pectic acid is the only one left. Stain the sections again, and note localization of reaction.

4. Wash in 2% ammonia. This dissolves the pectic acid.

5. After 30 minutes wash in water, and stain once more. There should be no reaction for pectic substances.

PIGMENTS

Carotin.—Place fresh pieces of young green leaves in a solution of 20 g. potassium hydroxide in 80 cc. 95% alcohol in a closed vessel kept in the dark until the chlorophyll is extracted. Remove the sections, wash for about 12 hours in numerous changes of distilled water, then mount in glycerin on a slide. Orange-red crystals of carotin and yellow crystals of xanthophyll will appear in a day or two.

If older leaves are used, the carotin probably will not crystallize, because of the heavier oil content in such leaves; the carotin dissolves in the oil and appears as yellow droplets.

Carotin and xanthophyll both dissolve and become blue when treated with concentrated sulphuric acid.

Crystals of carotin are soluble in ether, chloroform, xylol, and benzol but are insoluble in water and dilute alkalies and acids.

Chlorophyll.—Put the sections in 1 drop of ether on a slide, and add a little of a mixture of 20 g. potassium hydroxide in 80 cc. of strong methyl alcohol. The chlorophyll immediately turns brown, then after a while changes back to green.

Or place the sections in a very small drop of 25% hydrochloric or glacial acetic acid on a slide. Large yellowish-green drops ooze out of the chloroplasts within a few minutes and in about ½ hour long curved brown crystals of chlorophyllan appear in the cells. Heat the preparation

slowly to about 90°C., whereupon the chlorophyllan is recrystallized into clusters of straight needles. These crystals are soluble in ether or chloroform.

Xanthophyll.—Place fresh sections in 1 drop of chloroform on a slide. In a few minutes add 1 drop of petroleum ether. The insoluble xanthophyll crystallizes out, while the carotin, which is soluble in ether, remains uncrystallized.

PROTEINS

Proteins.—Most of the tests recommended for the detection of proteins are unsatisfactory because other substances simultaneously react. With such reactions confirmatory tests should also be made.

A saturated aqueous solution of picric acid is an excellent precipitating agent for proteins, staining them an intense yellow. Allow the reagent to react for 24 hours, then mount the sections in glycerin for examination.

Extremely dilute solutions of eosin, reacting for about 10 minutes, stain proteins red.

A fair localization reaction is given by potassium ferrocyanide. Make up the reagent by adding 1 g. of the cyanide to 20 cc. water and 100 cc. glacial acetic acid (sp. gr. 1.063). Leave the sections in this solution for 1 hour, during which time the proteins will be fixed, then wash briefly with 60% alcohol, and add a few drops of aqueous ferric chloride. A blue color results.

Aleurone.—Aleurone grains are made up of protein crystals and globules in a protein matrix. Saturate absolute alcohol with picric acid and nigrosin dye. Place the sections on a slide in a few drops of this reagent, and observe under the microscope. When the ground substance of the grains appears blue, stop the reaction by adding absolute alcohol. The globules should be colorless and the crystals yellowish-green. The sections can be cleared with clove oil and mounted in balsam if permanent mounts are desired. In place of the nigrosin, eosin may be employed. With this dye the ground substance becomes dark red, the globules slightly tinted red, and the crystals yellow.

Occurrence.—Seeds of the Poaceae.

SECRETIONS

Ethereal Oils.—The ethereal oils resemble the fatty oils in that they stain with the usual fat stains, are blackened by osmic acid, and are dissolved by fat solvents. The ethereal oils are odorous and volatile, while the fatty oils are not. If the sections are placed on a slide and covered and if either glacial acetic acid or chloral hydrate solution is run under the cover, the ethereal oils are easily dissolved, but the fats gener-

ally remain unaffected. Sections, uncovered, may be placed on a slide and heated over a water bath for about 10 minutes: the ethereal oils evaporate, and the fatty oils remain.

Resins.—Resins, found in secretory passages and heartwood, are divided into seven groups (McNair 1930) which are of only theoretical interest to the technician.

Thin fresh sections may be placed in a watch glass, covered with 7% aqueous cupric acetate, and allowed to stand for from one to six days until a greenish resin-copper precipitate appears. It would be desirable first to remove ethereal oils from the sections by distillation. Tests for fatty oils should also be performed.

Aromatic Acids.—The aromatic acids, which occur in secretions, resins, etc., can scarcely be identified by localization reactions. They are detected mostly by the appearance of the crystals resulting from sublimation. Benzoic acid sublimes out as feathery clusters of crystals, soluble in sodium hydroxide. Cinnamic acid gives thin plate crystals. Ferulic acid crystals are in the form of feathery rosettes and brush-like clusters at the ends of narrow plates.

Gums.—Gums, as they occur in plants, are mixtures of various substances, consequently there are no microchemical tests for gums as such but only for the constituent substances.

Four distinct classes of gums are recognized (McNair 1930): (1) those containing arabin or arabic acid, (2) those consisting of mixtures of arabin and cerasin (cerasic acid), (3) those containing bassorin, and (4) those containing mixtures of cerasin and bassorin.

Mucilages.—In some plants, such as the marine algae, mucilage is very abundant, and in many others it is of constant occurrence. No satisfactory tests are on record, probably because mucilages include a number of chemically distinct substances, but the technician when working with fresh tissues can generally recognize mucilage by its slimy character.

Occurrence.—Most of the larger Phaeophyta; seeds of *Linum* and *Cydonia*.

WAXES

Microchemical tests for waxes are the same as those cited for fats. Waxes are soluble in all ordinary fat solvents, but somewhat less so than the fats.

Most waxes are to be found on the epidermis or in the cuticles of leaves and stems. Place sections on a dry slide, cover with a coverslip, and run in ether from one side of the cover. Allow the ether to evaporate slowly. Needles, plates, and aggregates of crystals will appear after the ether has evaporated.

CHAPTER XVI

SOURCES OF MATERIALS

Many persons have on occasion felt dissuaded from attempting the preparation of microscopic slides of certain plant materials because of the presumed difficulty or impossibility of obtaining what might be wanted. This difficulty is really more apparent than actual, except generally in the case of tropical plants not in cultivation or of those which are of quite rare occurrence. There are at present several satisfactory sources for plant materials, equally available to all interested, and the cost is usually far less than one would have to stand if the material were personally collected.

Practically everything in the way of plant life is potential and legitimate game for the botanical technician. The whole problem is simply one of knowing where, when, and how to look for things, if one must depend primarily on one's own efforts. One should become familiar with local floras, special manuals on the various groups, and other sources which give detailed information on the occurrence, life histories, and other facts regarding the special plants in which one might be interested. Make the acquaintance of local florists and nurserymen, as well as of both amateur and professional botanists of the vicinity. Establishments conducted by elderly persons are a more likely source of interesting plant material than are those run by younger persons primarily concerned in quick financial returns from commercial crops. However, an acquaintance with the latter would be profitable to those interested in plant pathology. All these people will usually be much interested in what one is doing; one should be patient and cordial in explaining matters, as it frequently happens that the most unexpected favors are bestowed. It makes literally no difference where one might be located: plant materials of one sort or another are available everywhere.

The modern biological supply concerns, conducted as most of them are by trained specialists, are veritable lifesavers for the technician. It has long been the writer's opinion that too little is generally known of the services that certain of them are equipped to render. There are, of course, a number that are in business merely to make money rather than primarily to serve biologists, but one quickly learns of their unreliability from the character of the goods which they furnish. Most of the ethical concerns, unfortunately for botanists, are directed by persons trained

primarily as zoologists, who therefore specialize in animal materials. A zoologist who can turn out superbly injected cats or dogfish simply does not know a thing about preserving the Chlorophyta without plasmolysis. For the most part, such concerns deal simply in "preserved materials," which are not always suitable for slide-making purposes.

There are two concerns which deal exclusively in plant materials and both are staffed by competent botanists; these are also the only ones which offer both fixed and embedded materials to technicians who wish to make their own slides but are unable personally to collect the desired material. Neither definitely offers such materials in their catalogues, mostly because of the difficulty of keeping adequate stocks always on hand, but specific requests will receive careful consideration, and every possible effort will be made to supply the desired materials. These concerns are:

California Botanical Materials Company, 787 Melville Avenue, Palo Alto, Calif. (Offers the wider variety of all types; the only source for Pacific Coast algal material.)

Dr. Geo. H. Conant, Ripon, Wis. (Best source for the commoner laboratory types, also for all fungi.)

In addition, there are a few other concerns which, because they are in excellent collecting regions, can supply certain materials in better quality than can be obtained elsewhere:

Oregon Biological Supply Company, 4514 S. E. Eighteenth Avenue, Portland, Ore. (Excellent for *Mnium*, *Polytrichum*, and a number of other Bryophyta; also for *Selaginella*.)

Carolina Biological Supply Company, Elon College, N. C. (Fine source for *Volvox*, *Spirogyra*, *Euglena*, *Nitella*, and other aquatic organisms.)

Supply Department, Marine Biological Laboratory, Woods Hole, Mass. (For certain east coast algae. Living *Fucus* can be ordered shipped by air mail during cold weather, and it usually arrives in good condition for immediate fixation. Preservation excellent on some materials, extremely poor on others. The author has been unable to obtain unlisted species, and special fixation methods will not be used.)

American Type Culture Collection, George Washington University, Washington, D. C. (Carries cultures of a great variety of bacteria and fungi, including forms like *Aspergillus*, *Rhizopus* (both plus and minus strains), *Mucor*, *Actinomyces*, *Fusarium*, *Zygorhynchus*, and innumerable yeasts. A highly recommended source.)

GLASSWARE, APPARATUS, AND GENERAL SUPPLIES

There are numerous concerns scattered over the country which carry supplies of a general nature, such as staining dishes, dyes, slide boxes, miscellaneous glassware, chemicals, etc. Some are also the agents for other concerns which specialize in the manufacture of dyes, reagents, and similar products. Inspection of their catalogues, obtainable upon request, will reveal the extent and specific nature of their stocks. The following, selected for their location in different regions, large stock listings, and known reliability, may be recommended:

Braun Corporation, 2260 East Fifteenth Street, Los Angeles, Calif.
Braun-Knecht-Heimann-Co., 1400 Sixteenth Street, San Francisco, Calif.
Central Scientific Company, 1700 Irving Park Boulevard, Chicago, Ill. (Pacific Coast representative: Redman Scientific Company, 585 Howard Street, San Francisco, Calif.)
Chicago Apparatus Company, 1735 North Ashland Avenue, Chicago, Ill.
Standard Scientific Supply Corp., 34 West Fourth Street, New York, N. Y.
A. H. Thomas Company, West Washington Square, Philadelphia, Pa.
W. M. Welch Manufacturing Company, 1515 Sedgwick Street, Chicago, Ill.
Will Corporation, Rochester, N. Y.

Slides and Coverslips

Most general supply dealers list slides and coverslips, but the following supply superior grades of both items:

A. H. Thomas Company, West Washington Square, Philadelphia, Pa.
Hellige, Inc., 3702 Northern Boulevard, Long Island City, N. Y.
Redman Scientific Company, 585 Howard Street, San Francisco, Calif.

Microtomes

Instruments of American manufacture are preferable:

Spencer Lens Company, Buffalo, N. Y.
Bausch and Lomb Optical Company, Rochester, N. Y.

Microscopes

Students in collegiate institutions should already be provided with suitable microscopes. If one desires to possess his own microscope, American instruments will be found to be as satisfactory as, if not superior to, those of foreign make. They may be purchased from local optical dealers or from the optical firms listed above under Microtomes.

Chemicals and Reagents

All ordinary chemicals may be purchased from the general supply firms. Well-known brands should always be specified, and care should be taken to see that the materials come in the original containers; this is the only preventive against adulterations or substitution and deterioration. The following list includes the manufacturers or sources of special chemicals and reagents:

Eastman Kodak Company, Chemical Sales Division, Rochester, N. Y. (Special organic chemicals.)
Merck and Company, Rahway, N. J. (Pacific Coast distributor: Griffin Chemical Company, 1000 Sixteenth Street, San Francisco, Calif.) (For all reagent chemicals.)
General Chemical Company, 40 Rector Street, New York, N. Y. (Baker and Adamson Reagent Chemicals.) (Pacific Coast distributor: Redman Scientific Company, 585 Howard Street, San Francisco, Calif.) (Organic and inorganic chemicals; xylol.)

J. T. Baker Chemical Company, Phillipsburg, N. J. (Pacific Coast distributors: Braun Corporation, Los Angeles, Calif.; Braun-Knecht-Heimann-Co., San Francisco, Calif.) (General and reagent chemicals.)

Carbide and Carbon Chemicals Corporation, 30 East Forty-second Street, New York, N. Y. (Pacific Coast distributor: Griffin Chemical Company, 1000 Sixteenth Street, San Francisco, Calif.) (Synthetic organic chemicals; methyl cellosolve, dioxan, synthetic ethyl alcohol, etc.)

Eimer and Amend, Third Avenue between Eighteenth and Nineteenth Streets, New York, N. Y. (Essential oils, waxes, special chemicals.)

California Botanical Materials Company, 787 Melville Avenue, Palo Alto, Calif. (For special chemicals for botanical microtechnique: tertiary butyl alcohol, hygrobutol, etc.)

Glyco Products Company, Inc., 148 Lafayette Street, New York, N. Y. (Synthetic chemicals, waxes, resins, and special solvents.)

Baker and Company, Inc., Newark, N. J. (Osmic and chromic acids.)

Dyes and Stains

National Anilin and Dye Company, 40 Rector Street, New York, N. Y. (Stocks are carried by most general dealers.)

Hartman-Leddon Company, 6010 Haverford Avenue, Philadelphia, Pa.

MacAndrews and Forbes Company, 200 Fifth Avenue, New York, N. Y. (Hematoxylin, hematein, and brazilin dyes.)

Akatos, Inc., 55 Van Dam Street, New York, N. Y. (For the true Grübler dyes.)

Pfaltz and Bauer, Inc., 300 Pearl Street, New York, N. Y. (For Grübler-Hollborn dyes and stains.)

Embedding Media

Parowax may be purchased at any large grocery store. The brand manufactured by the Standard Oil Company of Indiana is far superior to others in that it does not crystallize readily.

All supply concerns list paraffin in their catalogues, but such paraffins can be almost anything. It would be advisable to make one's own rubber-paraffin embedding medium, as described on page 22, or to purchase a similar brand, such as Parlax.

Most technicians prefer the Schering-Kahlbaum brand of celloidin. This is of German manufacture but is obtainable through most dealers or from Akatos, Inc., 55 Van Dam Street, New York, N.Y. The product called "colloidon" in some catalogues is merely a solution of celloidin and is intended for medical purposes, not for embedding. There are a number of types of celloidin on the market, going under such names as Parloidin, but the author is not prepared to express an opinion on their suitability for microtechnique purposes.

Mounting Media

Canada balsam of satisfactory quality is difficult to obtain. Most of the balsam on the market has been highly diluted with xylol or other

solvents, and many samples show the dark brown color caused by excessive heating to accelerate solution. One may, of course, purchase the dry balsam and dissolve it; but the pure, neutral, nearly colorless, thick solution, filtered through paper, is best of all. Such a balsam, which can be diluted with the proper solvents required by special techniques (*e.g.*, hygrobutol, dioxan), may be obtained from the following:

Redman Scientific Company, 585 Howard Street, San Francisco, Calif.
Coleman and Bell Company, Norwood, Ohio.
California Botanical Materials Company, 787 Melville Avenue, Palo Alto, Calif.

Diaphane and Euparal are essentially similar products. The first, which is American in origin, may be obtained from the Will Corporation, Rochester, New York.

The special synthetic resins used for mounting diatoms are quite difficult to secure and are expensive. A high-quality Hyrax may be purchased from the Braun-Knecht-Heimann-Co., 1400 Sixteenth Street, San Francisco, Calif.

SECTION II
SPECIAL METHODS FOR THE VARIOUS PHYLA

CHAPTER XVII

SCHIZOPHYTA

SCHIZOMYCETES

Bacteria, which are to be found everywhere under the most extreme of conditions, constitute a very low or primitive type of plant life, despite the high specialization of some kinds. They are extremely minute, simple, unicellular organisms. A microscope equipped with an immersion lens is usually required for their examination.

The professional bacteriologist has evolved the study of the bacteria into a separate science—one so involved and with such a special terminology that the average botanist feels hopelessly bewildered at every contact with the subject. Botanists are interested in bacteria primarily for their own sake, not in phagocytosis, hypersensitiveness, or anaphylaxis. The teaching of bacteriology is commonly conditioned by medical school requirements, hence the subject is usually viewed with distaste by the young botanist, who in most colleges is compelled to study the bacteria on his own initiative. The methods of most bacteriologists, moreover, are more complicated or specialized than the simple needs of the botanist require. An attempt is therefore being made in the present chapter to cover the technical aspects of bacteriology as simply and adequately as possible.

Morphology.—Bacteria of known dimensions range in size from $0.5 \times 0.2\mu$ up to 50 to 60 $\times$ 4 to 5μ. Some of the pathogenic forms are so small that they are invisible microscopically.

Three basic forms are recognized: the coccus (spherical), bacillus (rod-like), and spirillum (spiral or segment of a spiral). For a given species the shape is fairly constant. During multiplication bacteria exhibit more or less of a tendency to remain attached.

Single coccus forms are nearly spherical, but during multiplication the form may become elongated or lanceolate. Cells which divide in one plane only and remain attached occur as pairs, known as "diplococci," or in shorter or longer chains, designated as "streptococci." Those which divide in two directions, one at right angles to the other, form groups of four known as "tetrads." Division in three directions to form cubes produces what are called "sarcinae." Finally, those which divide in any direction to produce irregular bunches are described as "staphylococci."

Bacilli characteristically are cylinders with a straight axis, of uniform thickness and flat ends, but there are numerous exceptions. Some types,

used. If agar is added, dissolve it first by placing the container in a boiling water bath. Have standard Pyrex test tubes, scrupulously cleaned, ready. Fill each tube with about 5 cc. of medium, then plug with a generous wad of ordinary nonabsorbent cotton, leaving just enough exposed for the plug to be pulled out without difficulty. Place the tubes in the autoclave and sterilize for the specified time at the required pressure (this is usually cited as 15 pounds pressure for 20 or 30 minutes). After autoclaving, wait for the pressure to go down completely before removing the tubes. Before the agar has a chance to solidify, place the tubes on their sides, with the plugged end slightly raised. Take care that the medium covers the bottom end of the tube, otherwise the slant might collapse of its own weight after cooling. The bacterium to be cultivated is streaked over the slanting surface of the solidified medium.

For transferring cultures, a platinum needle or small loop, attached in a glass or special aluminum holder, is employed. Between every transfer the wire must be sterilized by heating in a gas flame until white hot. While making transfers, the tubes must not be kept open any longer than necessary, otherwise contamination might occur. Hold the tube by the base in the left hand. The right hand, also holding the platinum wire, is used for removing the cotton plugs, the plug of one tube being grasped by the little finger and that of the second tube between the third and fourth fingers. After withdrawing the plugs, pass the mouths of the tubes three or four times through a flame, then transfer the growth, and replace the plugs. Draw the needle or loop over the growth in the culture tube, then in the new tube commence at the bottom and streak the needle diagonally back and forth until the upper limits of the agar are reached. Sterilize the platinum immediately after using and before placing it down. Be extremely careful to avoid splattering.

Most bacterial cultures grow best at either of two standard temperatures, 22 or 37°C. For general purposes the first temperature is that of the average room, but if a more uniform and exact temperature is indicated, an incubator must be employed.

In place of test tubes Petri dishes may be used as culture vessels. An ordinary Petri dish requires about 10 cc. of medium. It is more desirable to fill test tubes with that amount of medium in each, then to plug, and to sterilize. The Petri dishes are cleaned and sterilized. When one wishes to make a culture, a tube of medium is melted in hot water and poured into the Petri dish: the cover is opened just enough to allow the mouth of the tube to be inserted. As soon as the agar has solidified, the transfer of culture may be made. As with slants, it should be streaked diagonally across the surface.

Bacteria are either aerobic or anaerobic, but the average botanist will scarcely meet with bacteria that can grow only in an atmosphere freed of oxygen.

Preparation of Smears.—The slides or coverslips on which bacteria are to be smeared must be chemically clean. In the center of either, place a small drop of distilled water. With the platinum needle or loop take up a very small amount of the culture, place in the drop, and stir the two together thoroughly but not violently, then spread the drop out somewhat. It is best to place the slides in a warm protected place for 24 hours to dry, but if in a rush, dry the drop by passing four or five times through a clean gas flame.

Or the smears, partially dried but still moist, may be fixed in any standard fixative or over the fumes of osmic acid.

Dry, fixed, unstained bacterial smears keep indefinitely in a cool place. If mounted in balsam or other mounting media, stained slides tend gradually to fade.

Fixation of Tissues Containing Bacteria.—Plant tissues infected with bacterial diseases, such as *Aplanobacter insidiosum* in the roots of *Medicago sativa*, should be cut into small portions not over 3 mm. square and 2 mm. thick. Fluids containing mercuric chloride are said to be better than others, but it appears that very little research has been done on this phase of the problem. It seems probable, in any event, that any fluid which gives satisfactory preservation of the host tissues will likewise preserve the bacteria, but dehydration should be carried out with tertiary butyl alcohol as other fluids are suspected of damaging the bacteria.

Animal tissues should be fixed in Zenker's fluid:

Potassium bichromate	2.5 g.
Sodium sulphate	1.0 g.
Distilled water	100 cc.
Mercuric chloride	5.0 g.
Glacial acetic acid	5 cc.

Dissolve the bichromate and sublimate in the water with the aid of heat. Add the acetic acid just before the fluid is to be used. Wash the fluid out thoroughly with running water, pass through three changes of pure dioxan over a 24-hour period, transfer to a mixture of equal parts of dioxan and paraffin oil, then into melted parafin, and embed after several changes of paraffin have been made.

Stains.—The staining process, so far as bacteria are concerned, depends upon the solvent condition of the dye. Stains dissolved in absolute alcohol do not stain well, if at all. Absolute alcohol, furthermore, does not decolorize bacteria, while diluted alcohol is an active decolorizer. The more completely a dye is dissolved, the weaker its staining power becomes. The addition of an alkali to a stain renders the solvent action less complete and intensifies the staining power.

The staining process is further dependent upon the nature of the bacterium. Some forms stain easily, others do not. Spores and flagella, as has already been mentioned, are difficult to stain. Many species

possess highly selective staining properties, and this fact is of practical importance with regard to certain staining schedules. Gram's stain is an example, but the differentiation of bacteria into two classes—those which react to Gram's stain and those which do not—is unreliable and impractical because external circumstances often modify the reaction. Previous treatment of the bacteria with acids or alkalies frequently inhibits the reaction. There is, however, no difficulty in finding a suitable stain for all the different species.

Methyl and crystal violet are the most intense stains which may be used on bacteria; they usually overstain and must therefore be differentiated. Methylene blue, thionin, basic fuchsin, and safranin give better differentiation since they do not easily overstain. Bismarck brown and eosin stain weakly and are commonly used as counterstains.

Mitochondria, in general, are also stained by bacteriological methods (see page 101 for a staining method to differentiate between the two).

General Staining Methods.—Bacterial smears are placed directly into the stain after first having been thoroughly dried. Smears fixed in killing fluids are washed carefully and then transferred to the stain. Paraffin sections must, of course, first be deparaffined and brought down to the appropriate alcohol or to water.

Practically all the coal-tar dyes are first made up as saturated alcoholic solutions. Stock solutions are prepared by filling a bottle about one-fourth full with the dry dye, then nearly filling the bottle with 95% alcohol. The bottle is then tightly corked, well shaken, and set aside for 24 hours. If at the end of this time all the dye has dissolved, more should be added, the bottle shaken again, and set aside for another 24 hours. The process should be repeated until some undissolved dye remains at the bottom of the bottle.

Methyl (or Crystal) Violet.—The simplest staining method is as follows:

1. Stain 2 to 5 minutes in crystal or methyl violet (1 part saturated alcoholic solution to 10 parts water).
2. Rinse quickly in water.
3. Differentiate by dipping into 95% alcohol until most of the color is gone.
4. Dry as much as possible by blotting with filter paper, then pass through a flame or put on the slide warming table to complete the drying.
5. The preparation may be examined without mounting, or balsam and a coverslip may be added.

Anilin Water-Methyl Violet.—An anilin water solution of either crystal or methyl violet is considered to give a better stain. First prepare the anilin water by shaking up 4 cc. anilin oil with 90 cc. distilled water and filtering the mixture through a wet filter. Add 10 cc. of the

saturated alcoholic solution of either violet to 100 cc. anilin water, and set aside for 24 hours to allow the precipitate which soon forms to settle. The stain solution keeps for a week or ten days. Staining occurs within 5 minutes; differentiate as described for the preceding method.

Loeffler's Methylene Blue.—This is one of the most widely used stains. Mix 30 cc. of a saturated alcoholic solution of methylene blue with 100 cc. of 0.05% potassium hydroxide solution. Smears are stained in 2 to 5 minutes; if the solution is heated, the stain is intensified. Sections are stained for 15 minutes to several hours; decolorized with weak acetic acid (1:1000) until a faint blue color remains; counterstained with 1% aqueous eosin, dehydrated with absolute alcohol for 20 seconds, cleared, and mounted.

Ziehl-Neelson's Carbol-Fuchsin.—Add 10 cc. of a saturated alcoholic solution of pararosanilin (basic fuchsin) to 100 cc. of 5% aqueous carbolic acid. This is a good spore stain and is used as described below under Spore Stains. The solution keeps well.

Carbol-Methyl Violet.—One part of a saturated alcoholic solution of methyl (or crystal) violet is added to 10 parts of a 2 to 5% aqueous solution of carbolic acid.

Carbol-Methylene Blue.—Add 15 cc. of a saturated alcoholic solution of methylene blue to 100 cc. of 5% aqueous carbolic acid.

Carbol-Thionin.—This is made up of 10 cc. of a saturated alcoholic solution of thionin and 100 cc. of 1% aqueous carbolic acid.

Gram's Method.—First stain the sections or smears with anilin water-crystal violet. From the stain transfer to Gram's iodine solution (1 g. iodine and 2 g. potassium iodide in 300 cc. distilled water) for 1 to 2 minutes. Rinse in 95% alcohol until the violet color is no longer apparent to the naked eye. If the slides are insufficiently decolorized by the alcohol, treat again in the iodine solution. If permanent preparations are wanted, rinse in absolute alcohol, pass through xylol, and mount in balsam. Otherwise wash smears with water, and dry. With smears the method works satisfactorily on young, actively growing cultures. Some bacteria do not stain by the method: such are described as being Gram-negative, in contrast to the Gram-positive types, which retain the stain.

The above schedule is the classical one used by bacteriologists. A version which will probably be found more satisfactory by botanists is as follows.

The anilin water staining solution should be made up with methyl violet 6B, or the most highly methylated violet obtainable. Stain the slides for 5 to 30 minutes, the length of time depending upon the species and whether the slide holds smears or sections; this time will need to be determined by trial. Rinse in anilin water for 30 seconds (the slides can remain in this solution for some time if necessary), then transfer

to the iodine solution for 1 to 2 minutes. Sections become brown-black in color. Wash in 95% alcohol for 30 seconds, then wash in 95% alcohol plus 10% hydrochloric acid for 10 seconds, followed by washing in absolute alcohol, at which juncture no more color should come out of the sections. Complete clearing in cedar oil (do not use clove oil, as it will extract the remaining stain), proceed to xylol, and mount in balsam. The bacteria should be blue or blue-black, tissues nearly or quite colorless. A weak solution of safranin can be used to stain the nuclei, with orange G for the cytoplasm. Before one attempts to stain bacteria by this schedule, it should be ascertained if the bacterium is Gram-positive.

The schedule recommended by the Society of American Bacteriologists (Hucker modification) is as follows. Prepare two separate basic dye solutions: (1) Dissolve 4 g. crystal violet (85% dye content) in 20 cc. 95% ethyl alcohol; (2) dissolve 0.8 g. ammonium oxalate in 80 cc. distilled water. Mix the two, and stain smears for 1 minute in the mixture. Wash in water. Immerse in Lugol's iodine solution (1 g. iodine, 2 g. potassium iodide, and 300 cc. distilled water) for 1 minute. Wash in water, and blot dry. Decolorize in 95% alcohol for 30 seconds with gentle agitation. Cover with safranin counterstain (10 cc. of a 2.5% solution of safranin in 95% alcohol to 100 cc. distilled water) for 10 seconds. Wash, blot, and dry.

There is available a method for the differential staining of Gram-positive and Gram-negative bacteria in sections of tissue (Brown and Brenn 1931). Stain for about 5 minutes in Harris' hematoxylin (filter just before using), wash with acid alcohol (3% hydrochloric acid in 95% alcohol) until the sections are a light purplish-pink, alkalinize the color to blue with 1% ammonia in water, and finally wash thoroughly with water. Stain for 2 minutes in the following freshly prepared solution: mix together 5 drops of 5% aqueous sodium bicarbonate to which has been added 0.5% phenol and 0.75 cc. of 1% aqueous crystal or methyl violet. Wash off excess stain with water and treat with Gram's iodine solution for 1 minute. Wash again with water, then blot the sections carefully to remove all excess water possible, but do not allow them to dry. Put the slides in acetone for a few minutes, remove, hold level with the sections up, and cover with 0.1% aqueous picric acid until the sections become yellowish-pink. The last step is the critical one in the schedule; the slides should be held above a white plate or other white surface. Stop the action by draining off the acid and plunging the slide into a jar of acetone. Pass through a mixture of equal parts of acetone and xylol, then pass through pure xylol, and mount in balsam. The nuclei of the tissue are dark reddish-brown, the cytoplasm yellowish, Gram-positive bacteria deep violet to almost black, Gram-negative bacteria bright red.

Fuelgen's reaction may also be used on bacteria.

Staining Methods for Spores.—Smears should be made on coverslips. Fix by passing many times through a flame.

Put the fixed smear in 5% aqueous chromic acid for 5 seconds to 10 minutes (if the spores are not stained at the end of the schedule, lengthen the time in the chromic solution). Wash in water. Stain with Ziehl-Neelson's carbol-fuchsin or anilin water-fuchsin (use pararosanilin in place of methyl violet as described for anilin water-methyl violet above) for 1 minute, heating the solution until it steams but does not boil. Destain 5 seconds in 5% aqueous sulphuric acid. Wash in water. Counterstain with methylene blue, wash with water, dry in the air, and finally mount in balsam. The spores should be red, the bacteria blue.

Some workers contend that the use of an acid for differentiation is undesirable because it damages the organisms and tends to decolorize the capsules of the spores (Löte 1931). To avoid acids, use may be made of the fact that some dyes differentiate other stains. Stain smears for 1 to 2 minutes with carbol-fuchsin, rinse with water, dry, and differentiate with 1 to 1.5% methylene blue. The time required differs according to the species: some need only 10 minutes, others as long as 1 hour. Spores, also certain bacteria, are red, other bodies bluish-purple, blue, or gray. Bismarck brown (0.5 to 0.25%) may be substituted for methylene blue.

The following method gives excellent results, with sharp differentiation (Schaeffer and Fulton 1933). Smears are fixed by passing through a flame three times. Flood the smear with 5% aqueous malachite green, and heat to steaming several times within 30 seconds. Wash in running water for about 30 seconds. Cover with 0.5% aqueous safranin for 30 seconds. Wash, dry, and mount in balsam if desired. The spores are stained green, cells red.

Staining Methods for Capsules.—Smears to be stained for capsules should be made from cultures not over 24 hours old. Dry the smears in the air, without using heat. Perhaps the simplest method is the following (Churchman and Emelianoff 1932): flood the smear with 10 drops of Wright's stain (best purchased made up for use), and leave until the stain has nearly, but not completely, evaporated. This takes 3 to 4 minutes, and the original blue changes to a pinkish color. Next wash off as rapidly as possible with a buffer solution of pH 6.4 to 6.5, and dry without blotting. If the buffer wash is used before the stain has evaporated sufficiently, the capsule will not be stained. If preferred, the smears may be left in the stain overnight. The smears are removed and the stain allowed to evaporate as before, dipped in the buffer solution and dried. Occasionally there might be a precipitation of the stain to cause granular deposits.

The Hiss capsule stain also gives excellent results and may be employed if the preceding method presents difficulties. Prepare the smears on a very clean coverslip, dry in the air, and fix by passing through a flame. Flood the coverslip with methyl violet solution (5 cc. saturated aqueous solution to 95 cc. distilled water), and steam for a few seconds over a hot flame. Wash off the stain with 20% aqueous copper sulphate. Do not wash with water at any time. Blot dry with filter paper (do not dry in the air or the copper salt will be precipitated out). The capsule is a faint blue halo around a dark purple cell.

Staining Methods for Flagella.—Make a thin smear by transferring a very tiny amount from a culture not over 24 hours old, to 1 drop of water on a slide. Let dry in the air. Cover with a mordant composed of 3 parts 5% aqueous tannic acid and 1 part 10% aqueous ferric chloride for 4 minutes (after Bailey 1930). Pour off the mordant, and cover for 9 minutes with the following freshly prepared solution: 7 drops of the mordant mixture with 1 drop Ziehl-Neelson's carbol-fuchsin, with the successive admixture of 1 drop concentrated hydrochloric acid and 1 drop concentrated formaldehyde. Wash in running water. Flood with Ziehl-Neelson's carbol-fuchsin, and steam gently for 3 minutes. Wash in running water, blot, and dry.

There is a second method which affords a slightly better differential staining (Leifson 1930). Mix the following ingredients in the order cited:

Ammonium (or potassium) sulphate, saturated aqueous solution	20 cc.
Tannic acid, 20% aqueous solution	10 cc.
Distilled water	10 cc.
95% ethyl alcohol	15 cc.
Pararosanilin, saturated aqueous solution	3 cc.

Crystal violet may be substituted for the pararosanilin at the rate of 1.5 cc. instead of 3 cc. of the saturated solution; or methylene blue by using 5 cc. and reducing the additional 15 cc. of ethyl alcohol to 10 cc. The stain solution keeps for about a week. Stain for 10 minutes, first warming the solution to not over 38°C. Wash in water, and dry. To counterstain: if pararosanilin has been used, 0.1% aqueous methylene blue plus 1% borax reacts nicely; or if either crystal violet or methylene blue has been used to stain the flagella, carbol-fuchsin diluted 1:10 counterstains excellently.

Silver staining may also be employed (Safford and Fleisher 1931). Make smears as usual. Cover with a fixative composed of 100 cc. of one-fourth saturated aqueous solution of picric acid, 5 g. tannic acid and 7.5 g. ferrous sulphate. A few minutes suffices for the mordanting. Wash with tap water, dry, and cover with the silver stain (to 25 cc. of 2% aque-

ous silver nitrate add dilute ammonia until the precipitate which forms is redissolved, then add more of the silver nitrate until a faint turbidity results—clear solutions are useless), heat to steaming, and allow to react for 1 to 2 minutes. Wash in tap water, then dry.

Staining Methods for Metachromatic Granules.—Prepare smears as usual. Flood with Gram's iodine for 1 minute, rinse in tap water, stain with Loeffler's methylene blue for 20 to 30 seconds, rinse, stain with 1% aqueous safranin for 10 to 15 seconds, rinse, dry, and finally mount in balsam. Polar bodies are dark bluish-black, while the rest of the bacterium is red (Kemp 1931).

Soil Microflora.—The microscopic flora of the soil includes algae and various fungi and bacteria. Formerly it was considered necessary to dig a trench in the field soil for inserting slides, but a simpler method is now in vogue (Conn 1933). Merely fill a jelly tumbler with the soil whose flora is to be examined, insert two or more cleaned slides, cover and set aside for 5 days. Remove one of the slides at the end of the incubation period, and replace by another. Do the same with a second slide on the seventh day, and repeat again after the lapse of another 5 days and 7 days for the replaced slides, respectively.

For the miscellaneous collection of organisms that will probably be found on the slides, only a general stain serves satisfactorily. Rose bengal is perhaps the most useful. It is prepared in the following proportions: rose bengal, 1 g.; calcium chloride, 0.01 g.; 5% aqueous phenol, 100 cc. The slides are preferably laid on a flat surface over a boiling water bath, the stain solution is poured upon them and allowed to remain for 1 minute, care being taken that the slides do not become dry; but if desired, the slides may be stained in beakers of the steaming stain solution.

Determination of Motility.—When examined under the microscope in the actively living condition, many bacteria exhibit motility in fluids. Some species have a rapid movement; others move so slowly that it is difficult to distinguish between actual movement and pedesis ("Brownian movement"). Again, not all bacteria which possess flagella exhibit spontaneous movement under all conditions, since external circumstances such as heat, light, electricity, and chemicals may influence the movements.

To test for motility, make hanging drop cultures from very young cultures (not over 4 hours old) preferably grown in neutral bouillon. A hanging drop culture is made by placing a glass ring, 2 to 5 mm. deep, on a slide; take a circular coverslip of greater diameter than that of the ring, put a large drop of the culture in the center of the coverslip, carefully reverse, and place over the ring so that the drop remains suspended in the well. To prevent excessive evaporation, both ends of the glass ring may be coated with a thin layer of vaseline or petrolatum.

Special Methods.—It is not possible here to present detailed methods for all bacteria, consequently manuals which describe all that is known about each form should be consulted (*e.g.*, Bergey 1939). Other manuals should be consulted for culture media and cultural methods (Levine and Schoenlein 1930). However, methods are cited below for some of the commoner and more interesting bacteria.

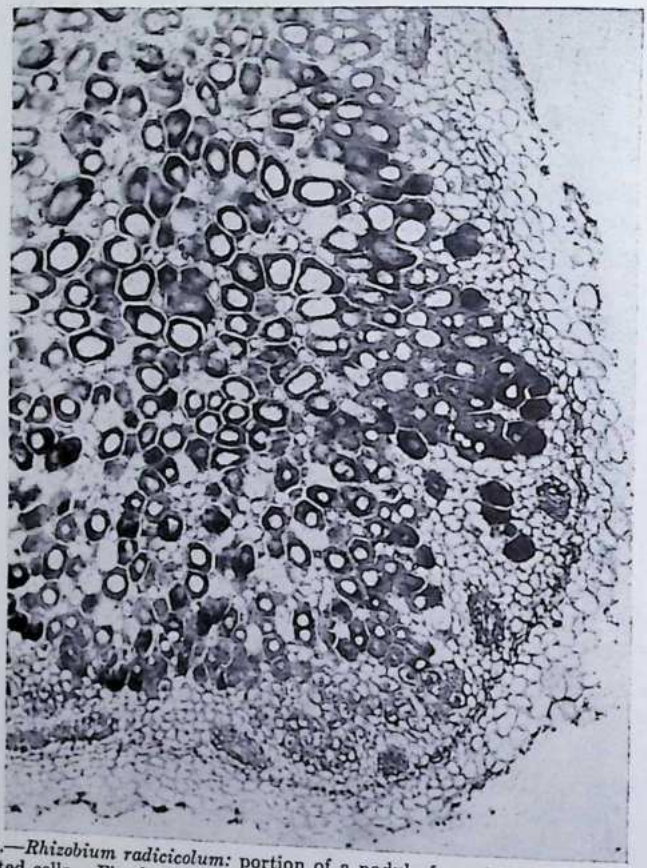


FIG. 24.—*Rhizobium radicicolum:* portion of a nodule from *Melilotus indica* with bacterial-infected cells. Fixed with chrom-osmo-acetic; differentially acidified with HCl and stained with methyl violet.

Rhizobium includes the forms which are capable of fixing free nitrogen when growing in nodules on the roots of legumes. Some writers call the association parasitism, others designate it as symbiontism, and still others claim it to be a fortituous association. There are two species generally recognized: *R. leguminosum* consists of rods occurring singly and in Y-shaped formations; *R. radicicolum* (Fig. 24) occurs singly and in pairs, often swollen at one end or near the middle. The bacterial cells always stain unevenly, perhaps because they secrete a mucilaginous

substance. They are actively motile by means of a polar flagellum, but some types are peritrichous (Stern and Sarles 1938). Nodules from the roots of almost any of the Fabaceae or Papilionaceae, or less desirably from the Mimosaceae, may be fixed in a solution made up of 4 cc. of 2% aqueous osmic acid and 6 cc. of 1% aqueous chromic acid, and the specimens may be embedded in paraffin. Sections should be cut not thicker than 4μ in the lengthwise plane of the nodule. Methyl violet, carbolfuchsin, or Gram's method may be employed for staining.

If slides of the cause of gonorrhea, *Neisseria gonorrheae*, are desired, take clean slides to a friendly physician, and ask him to make thin

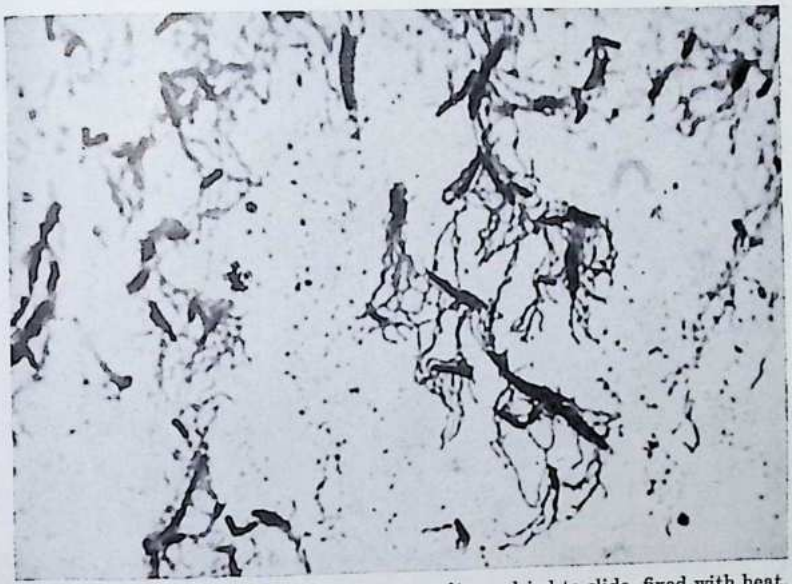


FIG. 25.—*Eberthella typhi:* smear from a young culture, dried to slide, fixed with heat, and stained by Leifson's method (using crystal violet) for the flagellae.

smears of urethral discharge from a three- or four-day preferably male case. Fix by exposure to sunlight for 10 minutes. The Pappenheim-Saathof stain for phagocytes containing gonococci is specific and strikingly beautiful. Stain smears in the following solution (use specially certified dyes) for 2 minutes, then wash, dry, and mount in balsam:

Methyl green	0.15 g.
Pyronin	0.05 g.
96% alcohol	5.00 cc.
Glycerin	20.00 cc.
2% phenol in distilled water	100.0 cc.

The genera *Erwinia* and *Phytomonas* contain practically all bacteria pathogenic to other plants (Elliott 1930). On artificial media, growth

is usually whitish and often slimy. *Erwinia* includes motile rod-shaped forms with peritrichous flagella, while the motile forms in *Phytomonas* have polar flagella. To show the bacterium in tissues of the host plant, remove small pieces of the infected tissue, kill and fix preferably in a strong chrom-osmo-acetic fluid, dehydrate and embed in paraffin, section at not over 5μ, and stain with carbol-fuchsin or methyl violet, counterstaining with erythrosin.

Eberthella typhi, the cause of typhoid fever, is one of the best forms in which to demonstrate peritrichous flagella (Fig. 25). Smears should be obtained from a professional bacteriologist.

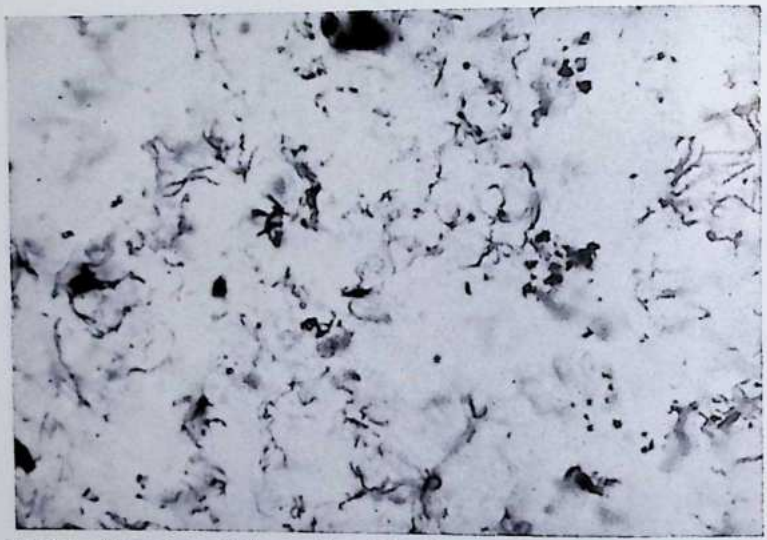


FIG. 26.—*Treponema pallidum:* section of fetal lung showing the spirochaetes in abundance. Fixed with 10% formalin; stained by the Warthin-Starry silver impregnation method. (*From a preparation by Miss Enid Larson.*)

There are five genera of so-called "iron bacteria." Most of them cannot be grown on artificial media. They are typically aquatic, algal-like filamentous bacteria which may develop conidia but never endospores. The sheath surrounding them is impregnated with iron. It may be necessary to remove the iron encrustations with dilute acid in order to reveal and to stain the cells more clearly.

Rotting marine algae are an excellent source for the so-called "sulphur bacteria," *Thiothrix* and *Beggiatoa*. They are filamentous forms which show an oscillating motion similar to that of *Oscillatoria*.

One will have to go to a clinical pathologist for material of *Treponema pallidum*, the cause of syphilis (Fig. 26). Despite the prevalence of the disease, it is actually quite difficult to secure suitable infected material; fetal lung or liver is superior to other tissues. The tissue, cut into very

tiny portions, should be fixed for several days in 10% aqueous neutral formalin, then washed thoroughly with water, and embedded in paraffin. Sections should be cut at 5 or 6μ. Clinical technicians and medical pathologists commonly use the Dieterle silver-impregnation method (Dieterle 1927), but the Warthin-Starry method (Farrier and Warthin 1930) will give results more to the liking of botanists.

ACTINOMYCETES

The Actinomycetes constitute a group apparently intermediate between bacteria and molds. They contain innumerable soil organisms and a few forms pathogenic to man. They consist of extremely delicate branching threads greatly resembling the mycelium of molds. The ends of the filaments break up into spores.

The peculiar characteristic of the soil Actinomycetes is their conspicuous musty odor. When these organisms are abundant in a given soil, they give to that soil its characteristic odor. When grown in the laboratory, the Actinomycetes produce pigments which are frequently very beautiful and serve for purposes of identification and classification.

The Actinomycetes are better known to medical bacteriologists and soil scientists than to botanists.

Cultivation.—The Actinomycetes cannot tolerate an acid medium, consequently the acid media recommended for yeasts and molds cannot be used. Most of them will grow on the same media used for bacteria, but it is too difficult to keep down the growth of contaminating bacteria on such media. Most Actinomycetes thrive on very weak media which will scarcely support bacterial growth (Heinrici 1930).

For general isolation of the saprophytic forms soil-extract agar or Czapek's agar may be used. The method of growing cultures on slides as described for the Myxothallophyta may be used for some forms, provided bacterial growth can be kept down sufficiently. Coat the slides with Czapek's agar.

Pure cultures of an unusually large number of Actinomycetes may be secured from the American Type Culture Collection.

Preparation of Slides.—For general morphological studies, Actinomycetes may be treated like yeasts (page 334) or similar fungi, but the very small size of the former requires more careful methods. An oil lens must ordinarily be used for examining them.

Permanent preparations of the spore-bearing bodies and the spores are not difficult to make (Drechsler 1919). Smear a thin film of Mayer's adhesive over a coverslip. Carefully drop the coverslip, smeared side down, on the surface of an actively sporulating colony, and then gently lift it off again. The spore-bearing filaments will adhere to the coverslip and will be broken off from the culture but will retain their normal

arrangement. The coverslip with the adhering organisms may now be placed in a dish of fixative and subsequently carried through the staining, dehydrating, and mounting processes. Any of the basic coal-tar dyes may be used.

Two staining methods adapted for sections are as follows.

Israel's Method.—1. Stain the sections in a concentrated aqueous solution of orcein, to which has been added a little acetic acid, for several hours.

2. Wash with water, then dehydrate rapidly.

3. Wash in absolute alcohol for several hours.

4. Wash in xylol, then mount in balsam.

Actinomycetes are blue, clubs a light red.

Bostroem's Method.—1. Stain in a 1% solution of crystal violet in anilin water for 10 to 15 minutes.

2. Transfer directly to picrocarmin (add a strong solution of carmin in ammonia to a saturated aqueous solution of picric acid until a precipitate is formed; add a trace of phenol to prevent mold growth, then allow to stand exposed to the air until the solution has evaporated down to a fourth of its original volume; filter, and allow the filtrate to evaporate to dryness; make a saturated solution of the resulting crystalline substance in distilled water for staining) for 5 to 10 minutes.

3. Wash with water, then wash in absolute alcohol until the sections appear reddish-yellow.

4. Clear in cedar oil or oil of origanum, and mount in balsam.

The Actinomycetes are dark blue, clubs red, and nuclei reddish-yellow.

CHAPTER XVIII

CHLOROPHYTA

CHLOROPHYCEAE

The green algae from the earliest days of microtechnique have been favorite subjects with botanical technicians. *Spirogyra*, for example, has universally been worked upon perhaps more than any other plant. The reason is that every reaction can be critically observed, and the results, when different procedures are followed, can be readily compared. Killing and fixing, dehydrating, staining, and even infiltration with paraffin or celloidin may be carried out under direct observation. These algae serve as superb practice objects for the technician; they are to the botanist what the guinea pig or rabbit is to the animal experimenter. They are superior to all other experimental types in that what is happening is always plainly visible. This is quite apart from their own inherent interest.

Occurrence.—Chlorophyta occur everywhere—in pools, lakes, streams, along rocky shores of the oceans, on damp soils, in ordinary soils, in salt lakes and salterns, in aerial habitats, and as symbionts or parasites. Many genera have a specific preference for particular habitats and are to be found nowhere else.

Collection of Material.—Most of the Chlorophyta are easy to collect, even if the collector has only the haziest idea just what genus or species it is that is being collected. Simply place the material, if taken from water, in a bottle of convenient size. The bottle should not be filled more than a quarter of its capacity with any alga, the remainder of the bottle is to be filled with the water in which the plants are growing. Lack of air causes algae to begin rather prompt deterioration. Remove the cork or cap from the bottle as soon as the return to the laboratory has been made.

Material collected on damp earth can be put in a pasteboard box or in one of the cellophane wrappers from a pack of cigarettes for transportation to the laboratory. Do not let such algae dry out.

Examine the immediate neighborhood of aquatic plants for flocculent masses of algae. Take up some of the plants carefully, hold over the opening of a wide-mouthed bottle and by running the fingers down the length of the plant squeeze out the organisms which grow more or less attached to the plants. Of course, one will scarcely get a pure collection, but many exceedingly interesting species are to be obtained in this fashion.

If one finds especially desirable forms, cultures can usually be started from them.

As soon as the return to the laboratory has been made, the collections should be examined microscopically. Discard all undesired material, and preserve the remainder if cultures are not to be made. Watch particularly for the motile forms while these are still in the living, active condition. It will probably be impossible to make slides of such organisms, when very few in number and together with other and larger forms, as they are very easily lost. It would be more practicable to grow a large quantity of such species in more or less pure ("unialgal") culture and to make permanent mounts from such a culture. If only a few are available, they can be mounted directly into glycerin or glycerin jelly and might retain their color for some time.

Cultivation.—The unicellular and smaller colonial algae are not, as a rule, difficult to cultivate and to obtain in practically pure culture. Various methods following the well known plating procedure as used in bacteriology have been proposed.

In preparing media on which to cultivate the Chlorophyta, the important aspect to keep in mind is that the algae are extremely sensitive to slight traces of metals. Pyrex glass-distilled water should always be used, and containers which have previously held solutions of metallic salts, mineral acids, or formalin should never be used.

If the alga is one that has motile spores, the following method is excellent. If the form is sufficiently abundant, platings may be made on a 1% agar medium prepared with 0.2% Knop's solution. The alga will grow rather slowly; it may be three weeks or longer before the colonies can be observed amongst the numerous bacterial colonies which are rather certain to appear in the meantime. After a plate showing a good growth has been secured, allow it to dry slightly for several days to a week, then transfer small colonies, as aseptically as possible, to vials of sterile distilled water. Zoospores should be liberated promptly. These will generally swim to the surface of the water and some can be scooped up with a platinum loop and spread in a drop of nutrient solution over the surface of sterile nutrient agar in a Petri dish. In this manner pure cultures are readily obtained and may be transferred repeatedly to both solid and liquid media. Hanging drop cultures and permanent preparations can be made of almost any stage in the life history. To embed material from an agar culture, cut out small rectangular blocks of the agar bearing the culture. The algae will adhere to the substrate throughout the dehydration process (the agar is easily embedded and sectioned). Sections should be cut at from 2 to 5μ. To distinguish between the nucleus and pyrenoids, use a triple combination; for other purposes use iron hematoxylin.

Present-day algologists consider soil-extract media to be far superior to those composed solely of mineral salts for the cultivation of the green algae. To prepare the stock solution (Bold 1936), autoclave 500 g. of good field or garden soil in a flask with 1 liter of glass-distilled water, at 15 pounds pressure for 2 hours. Cool, decant, and filter several times until the filtrate is clear. The filtrate is designated as the "stock soil solution." It may be diluted in various proportions with distilled water and to each 100 cc. of dilution should be added 1 cc. of 5% aqueous potassium nitrate. The following dilutions are the most useful (quantities are in cubic centimeters):

Solution number	1	2	3	4
Distilled water	94	84	74	64
Stock soil solution	5	15	25	35
5% aqueous potassium nitrate	1	1	1	1

The above dilutions may be solidified by the addition of 1, 2, or 3 g. agar to each 100 cc. of solution. Since the agar may contain impurities (unless a specially prepared granulated bacteriological agar is used), it is wiser to take the precaution to place the agar in a cheesecloth bag, to wash for 24 hours under running water, and then to soak in several changes of distilled water.

A large number of purely chemical solutions have been proposed. The following are the most useful of those which have given excellent results in the hands of experienced algologists.

Modified Chodat-Grintzesco Medium (Fred and Peterson 1925):

Calcium nitrate	1.0 g.
Dibasic potassium phosphate	0.2 g.
Magnesium sulphate	0.2 g.
Potassium chloride	0.1 g.
Ferric sulphate	Trace
Distilled water	1 liter
Agar, washed and rinsed	10.0 g.

Autoclave. The reaction should be about 7.3. Pour a large number of plates of high dilution of the alga to be cultivated. Invert the plates, place under a bell jar in a north window or under artificial illumination (see below), and allow to incubate for several weeks. From well-isolated colonies pick cultures, and streak on the surface of agar slants. To determine whether contamination is present, make transfers from the slopes which show a profuse growth into glucose broth and glucose yeast water. To secure the maximum growth of algae in a short time, add glucose or sucrose to the Chodat-Grintzesco medium. The carbohy-

drates are very beneficial, and the sugars are entirely assimilated in about 60 days. Large amounts of alga growth can be obtained by adding 75 cc. of the medium, without the agar, to tall 16-ounce flat-shaped bottles; keep in the greenhouse, flat side down and plugged with cotton.

Modified Detmer's Solution (Bold 1936):

2% monobasic potassium phosphate	72.5 cc.
2% dibasic potassium phosphate	21.0 cc.
2% calcium nitrate	140.0 cc.
2% magnesium sulphate	87.5 cc.
2% potassium chloride	87.5 cc.
0.1% ferric sulphate	19.5 cc.
Water	6572.0 cc.

Dissolve each of the chemicals separately in a large portion of the water, then mix together, adding the calcium nitrate last.

Modified Knop's Solution (Bold 1936):

Calcium nitrate	2.0 g.
Monobasic potassium phosphate	0.5 g.
Potassium nitrate	0.5 g.
Ferric chloride (1% aqueous)	1 drop
Water	350.0 cc.

Dissolve the chemicals separately in portions of the water, then mix together. The solution is considered to be a 1% Knop solution. For the Volvocales, dilute to 0.05%.

Modified Benecke's Solution (Bold 1936):

Ammonium nitrate	0.02%
Calcium chloride	0.01%
Dibasic potassium phosphate	0.01%
Magnesium sulphate	0.01%
Ferric chloride (1% solution)	1 drop per liter

An intense but cold source of illumination is needed for the successful cultivation of the Chlorophyta. The temperature should never exceed 22°C.; some species require a considerably cooler temperature. Most people simply keep cultures in a window with a northern exposure, but uniformly satisfactory results are rarely attained in this manner. A source of artificial, controlled illumination is superior. The best type is undoubtedly the new "cold light" apparatus recently devised by the General Electric Company, but an equally satisfactory setup can be arranged by utilizing apparatus found in practically every laboratory. As depicted in the diagram (Fig. 27), the items consist of a bell jar with a hole at the top for the insertion of a rubber stopper, a suitable tripod, a 500-watt electric lamp connected to an electric outlet in a suitable man-

ner, glass tubing, and rubber tubing for connecting the apparatus to a water faucet. The diagram illustrates how the whole is put together. As originally devised, the lamp was placed inside a large beaker placed inside the bell jar, but experience showed that it was too difficult to keep the beaker in position. Most 500-watt lamps have long necks, which makes it possible to immerse the lamp in the water if care is taken not to allow the metal screw end to get under water. The rate of flow of water should be regulated so that no heat radiates below the bell jar. The culture dishes may be arranged on glass shelves placed around the bell jar, as well as directly below the latter, and should be illuminated for about 10 to 12 hours daily.

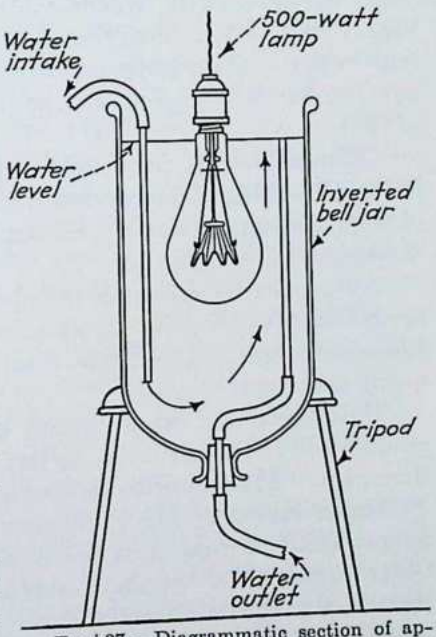


FIG. 27.—Diagrammatic section of apparatus for furnishing artificial light for algal cultures. (*Based upon Bold* 1936.)

More detailed directions for cultivating certain species, especially where concerned with the inducing of reproductive phases, will be given under the species concerned.

Herbarium Specimens.—The older botanists made a regular practice of drying Chlorophyta on paper for preservation as herbarium specimens, but not many follow it nowadays. Thick cellophane or sheet mica is often substituted for the paper.

Preservation.—All Chlorophyta can be permanently preserved, after fixation and washing, in 3% formalin, to which should be added 6% glycerin in order that the material may not become completely dry in case the water evaporates. Formalin-aceto-alcohol will also preserve material almost indefinitely.

For preservation in natural colors, use copper-lactophenol:

Phenol, c.p.	20 g.
Lactic acid, sp. gr. 1.21	40 g.
Glycerin, sp. gr. 1.25	20 cc.
Distilled water	0.2 g.
Cupric chloride	0.2 g.
Cupric acetate	

Fixation.—Weak chrom-acetic fluids are by far the best for the majority of Chlorophyta, although with some species other fluids are strongly

indicated. Some technicians claim that the addition of osmic acid is necessary for the most precise results, but in the writer's experience this claim has never been substantiated.

The proportions of chromic and acetic acids to be used vary according to the species, and even to different lots of the same species. One may take 1 g. chromic acid and 1 cc. glacial acetic acid to 100 cc. water as the standard proportions. If fixation appears to be inadequate after these proportions, reduce the amount of chromic acid, and increase the amount of acetic acid. Thus 0.7 g. chromic acid and 5 cc. acetic acid to 160 cc. water may give better results. Fix for 10 to 24 hours, and wash thoroughly with water. Some algae, such as certain of the Chlorococcales, may be fixed satisfactorily for immediate transformation into permanent mounts, but they cannot well be preserved unmounted for any length of time.

Chlorophyta are frequently well fixed with formalin-aceto-alcohol in the proportion of 5 cc. formalin and 5 cc. glacial acetic acid to 100 cc. of 50% alcohol. Wash in a change of 50% alcohol after 24 hours, and proceed to the staining.

For marine Chlorophyta the above fluids (except for the formalin-aceto-alcohol) may be used, but substitute sea water for distilled water. Wash out with sea water, then transfer from sea to tap water for subsequent treatment.

The flagella and other delicate structures in the small motile forms are difficult to fix and preserve, but any sort of attempt may be better than none. Try mixtures of iodine and formalin as suggested for *Volvox*.

Whole Mounts.—The procedures outlined in the chapter on Whole-mount Methods were designed primarily with the requirements of the Chlorophyta under constant consideration, consequently reference to that chapter should be made.

To prepare whole mounts of filamentous forms in nearly natural color, add 6 parts formalin to 1 part of the copper-lacto-phenol mixture described above, and fix the alga in this mixture. Then mount in glycerin to which has been added 10% copper-lactophenol.

Staining.—As a rule, iron hematoxylin, with a suitable counterstain, is the stain par excellence for the Chlorophyta. Since the cell walls are composed mainly of cellulose, a dye having an affinity for this substance should be employed if it is desired that the cell walls stand out prominently. Delafield's or Harris' hematoxylin can be combined with iron hematoxylin (for the nuclei and pyrenoids) and fast green or safranin (for the chloroplasts and cytoplasm, respectively) for this purpose. Picro-indigocarmin gives a naturalistic green color to many of the colonial species. Mayer's carmalum (made up with pure carminic acid) may also be used for the same species.

Pyrenoids stain red or reddish after triple combinations; the starch grains surrounding the pyrenoids should stain violet.

Volvocales

Members of the order are flagellated in the vegetative condition and may be either solitary or colonial. Typically, the cell is enclosed by a definite wall, with a cellulose layer next the protoplast. The colonial species are commonly surrounded by a gelatinous matrix.

Polyblepharidaceae.—In this primitive family the protoplast is not surrounded by a cellulose wall but is fairly rigid in the peripheral portion.

Dunaliella, invariably present in salterns (thus giving them their characteristic red color), is probably the only form which the technician might encounter. It may be mounted directly in glycerin. If found in a concentrated salt solution, put a drop of the brine containing the organism on a slide, fix for 1 or 2 minutes by suspending the drop over the mouth of a bottle containing osmic acid, then let dry, stain in hematoxylin, and mount in diaphane. Too much salt may cause trouble in the foregoing method, consequently a better plan would be to kill the organisms in formol-iodine and to centrifuge to concentrate the very small cells, whereupon they may be washed, placed in distilled water, and subsequently treated for whole mounts.

Chlamydomonadaceae.—*Chlamydomonas*, naturally, is the genus of greatest significance in the family. It is most likely to be found in pools rich in ammonium compounds. Once one collects this genus, a culture should be made; if a small amount is placed over clean white sand in a Petri dish or similar receptacle and kept just moist, it will remain in good condition for a long time. If zygotes should be formed, the culture may be allowed to dry out and can be started into growth again by simply adding sterile water. If grown on Detmer's solution solidified with agar, the colonies remain in a "*Palmella*" condition and divide actively; if transferred to distilled water or to 0.05% Knop's solution, they will return rapidly to the motile condition. Formol-iodine fixatives (see below under *Volvox*) are preferable. Iron hematoxylin or a carmin stain is satisfactory. To reveal the flagella clearly, special staining is required; fast green might be tried.

Phacotaceae.—Treat like *Chlamydomonas*.

Volvocaceae.—The most interesting and intensively studied of the Volvocales belong in this family. The cells are arranged into coenobic disks or hollow spheres. The genera may all be treated alike in the manner described below in considerable detail for *Volvox*. The latter genus has been selected simply because it is more likely to be found and recognized than are the other genera.

Getting *Volvox* in a satisfactory condition in nature is mostly a matter of chance. The best places are deep ponds, small lakes or large quiet ditches, whose waters are wholly or principally rain water. The plants are more likely to be found along the edges, but the location varies according to the species: some grow among grasses and aquatic plants along the margins, others float in the middle of ponds together with copepods and other plankton organisms, while still other species prefer the bottoms. If the colonies are present in small numbers, they may be collected in quantity by means of a plankton net of fine bolting silk: draw it slowly through the water in a likely spot, and if *Volvox* is present, the bright green colonies are easily recognized. Let nearly all the water filter out, then transfer the colonies to a large bottle or battery jar by means of a giant pipette. The writer once collected more than a quart of pure *Volvox* in less than half an hour by this method.

Volvox, as well as *Gonium*, *Pandorina*, and *Eudorina*, can be cultivated without much difficulty. Luxuriant growth has been obtained on Nos. 2, 3, and 4 soil solutions (page 229) and in 0.05% Benecke's and 0.05% Knop's solutions (Bold 1936). Cultures may be grown on 1.5% Detmer's agar, on which palmelloid colonies are produced, and may be kept in this condition for some time. The following solution has also been recommended:

Potassium nitrate	0.25 g.
Magnesium sulphate	0.25 g.
Calcium nitrate	1.0 g.
Monobasic potassium phosphate	0.25 g.
Potassium carbonate	0.345 g.
Ferric sulphate	0.0125 g.
Distilled water	1 liter

The pH of the above solution should be about 7.6 at 18 to 20°C. When ready to use, take 1 part of the solution and 9 parts distilled water, and pour 100 cc. into flasks of 200-cc. capacity. The flasks should be of alkali-free glass; if the freedom of the glass from soluble alkalies is questionable, wash with strong sulphuric acid, and rinse out the acid thoroughly with distilled water. Be sure that the colonies forming the basis of the culture are reasonably free from other algae, and use only a very few colonies for starting each culture. Grow under cool artificial illumination.

Volvox does not keep in preserving solutions without gradually undergoing collapse. Consequently, when one obtains an excellent culture, whether in nature or by cultivation, it would be well to make it into slides at once.

In handling *Volvox* through the killing and subsequent procedures, it is important to prevent the colonies from becoming clumped together.

This can be done by keeping all fluids slightly acidified (with acetic acid if any particular solution is not already acid). The most satisfactory killing fluid is composed of the following:

Potassium iodide	2 g.
Iodine	1 g.
Formalin	24 cc.
Glacial acetic acid	4 cc.
Water	400 cc.

Dissolve the iodine and iodide in the water first, then add the other ingredients. The colonies may be concentrated, if necessary, by filtering out the water through coarse filter paper or bolting silk. The colonies should be left in the killing solution for about 48 hours, preferably longer. Protoplasmic connections, flagella, and internal details of reproduction should all be perfectly preserved. Wash out with water slightly acidified.

Picro-indigocarmin (0.25 g. indigocarmin in 100 cc. of a saturated aqueous solution of picric acid) is an exceedingly beautiful stain for *Volvox*, provided it can be kept from becoming extracted during the dehydration. Leave the *Volvox* in the stain for a week or much longer, as there is very little chance of overstaining (Fig. 28). Mayer's carmalum is also excellent, as is Lynch's precipitated carmin, but neither is too precise. The carmin dyes also require a long period to take effect, and dehydration should be by an extremely gradual process. With some species, but not all, iron hematoxylin can be used, but the mordant must be used in very dilute concentration as it tends both to collapse and to clump the colonies. About 1 drop of 2% ferric ammonium sulphate in 250 to 300 cc. of acidified water may be tried. Use for about 24 hours, wash with acidified water, then apply the staining solution in high dilution. Differentiate in a warm saturated aqueous solution of picric acid, and counterstain with highly diluted fast green.

The problem of getting *Volvox* through any dehydrating process is exceedingly difficult. Stages must be extremely gradual, and all diffusion currents must be avoided. The glycerin evaporation process, with washing in 95% alcohol, is excellent. The gradual drop method may be employed with hygrobutol, or dioxan in a very close series of stages sometimes works beautifully.

Volvox is readily embedded and sectioned. Use the iodine-formalin fixing fluid, wash out quickly, and dehydrate by a very gradual series. Almost any desired series may be used. The material is kept in thin shell vials during all the various processes (the vials in which photographic developers come are useful); the colonies will sink soon after each change of fluid, and the liquids may be withdrawn with a pipette. Leave in the paraffin oven for only a short time, and make changes of paraffin by means of a pipette warmed to the temperature of the paraffin.

The paraffin may be solidified in the vial and the glass broken after cooling, or the material may be poured into a small paper tray to give a layer of material 2 to 3 mm. thick. One will have to sacrifice some material in trimming down the block for microtoming. Cut sections

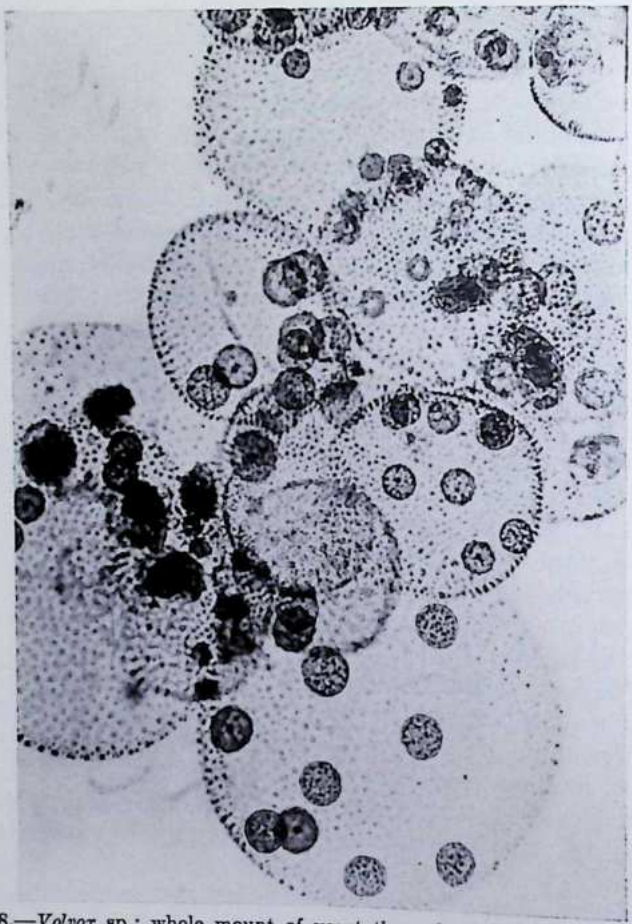


FIG. 28.—*Volvox* sp.: whole mount of vegetative colonies. The colonies commonly become more or less clumped together. Fixed in special formol-iodine fluid; stained with picro-indigocarmin. Dehydrated with hygrobutol and infiltrated with balsam.

at about 4μ. Stain preferably in iron hematoxylin with orange G for counterstain.

Spondylomoraceae, Sphaerellaceae.—Treat members of these families exactly like *Chlamydomonas*.

TETRASPORALES

This order is made up principally of genera which may only occasionally be encountered. Whether unicellular or united to one another in

colonies, the cells are surrounded by gelatinous sheaths. These sheaths are either distinct or confluent.

Cultivation may be in one of the soil solutions, the optimum dilution needing to be determined by experiment.

The smaller forms may either be dried down on the slide directly after first killing over the fumes of osmic acid; or they may be killed and dehydrated as far as 95% alcohol, when a drop of the material may be placed on a slide smeared with Mayer's adhesive and then stained with dyes dissolved in 95% alcohol or clove oil. If the species forms colonies of a sufficiently large size to be easily handled, they may be killed, dehydrated very gently, embedded in paraffin, and sectioned. Most dehydrating fluids are apt to cause excessive hardening of the gelatinous matrix.

Ulotrichales

The genera in the order possess simple or branched filaments which make unusually good preparations.

Ulotrichaceae.—Filaments of all genera are unbranched. *Ulothrix* is the commonest genus, but it is not so readily found in most regions as are others. It occurs as light green masses in quiet or running cold water, attached to stones. Reproductive phases occur only after midnight and are completed early the next morning; vegetative phases only are found after about 9 A.M.

Kill and fix in chrom-acetic. Wash, stain with iron hematoxylin and follow the hygrobutol method, counterstaining with fast green. Filaments of *Ulothrix* commonly have the annoying peculiarity of clumping together during the later stages of the dehydration, or at the beginning of the infiltration, process. However, if the bunched filaments are cut into portions not over 5 mm. in length, placed in a drop of balsam on a slide, and then gently pressed with a needle spatula, they will become separated. Care should therefore be taken not to place too much material on each slide.

Ulothrix is easily embedded and sectioned. A large quantity of filaments showing the formation of zoospores and gametes may be wrapped in lens paper, carefully tied at the ends with fine thread, and the whole run up into paraffin. Microtome at 3 to 6μ, and stain with iron hematoxylin and fast green.

Cultivation of vegetative filaments may be in undiluted Detmer's solution or in No. 2 soil solution. Zygotes of *Ulothrix* may be germinated on an agar medium:

Distilled water	200 cc.
0.05% aqueous potassium nitrate	100 cc.
Dried earth or loam	30 g.
Agar	To 1%

Autoclave, then pour into Petri dishes or similar containers. Keep the cultures at 10°C. under artificial light.

Ulothrix is perhaps one of the most desirable forms for demonstrating the differences between zoospores and gametes. There is an alternation of generations in the genus (Grosse 1931).

Microsporaceae.—*Microspora*, to be expected in early spring in pools and ditches, has a peculiar wall structure. Treat it as described for *Ulothrix*.

Cylindrocapsaceae.—Treat as for *Ulothrix*.

Chaetophoraceae.—*Stigeoclonium* and *Draparnaldia* are found in clear, cool, running water, but the first genus sometimes occurs in quiet ponds or lakes. In such situations it is frequently found producing akinetes and is one of the best Chlorophyta in which to demonstrate this method of reproduction. Kill with 1% chrom-acetic, stain with iron hematoxylin (paying particular attention to the differentiation of the akinetes) and fast green, and follow the hygrobutol method. *Stigeoclonium* usually breaks apart more or less, but *Draparnaldia* sometimes forms such large plants that they must be reduced to smaller portions for mounting.

Genera such as *Aphanochaete*, *Thamniochaete*, and *Chaetopeltis*, which grow upon other filamentous algae, should be worked up together with the host since it is quite impossible to separate the two.

Protococcaceae.—*Protococcus* (*Pleurococcus*) is the only genus occurring in the United States, and should be available almost everywhere. There is, however, great confusion as to the identification of the Protococcaceae and one should therefore be cautious before concluding that any unicellular aerial green alga is a *Protococcus*. *Protococcus* is easily isolated and cultivated on Beijerinck's ammonium nitrate agar:

Distilled water	1000 cc.
Ammonium nitrate	0.5 g.
Monobasic potassium phosphate	0.2 g.
Magnesium sulphate	0.2 g.
Calcium chloride	0.1 g.

After the chemicals have been dissolved separately in portions of the water and then mixed together, add 20 g. agar, boil until the latter is dissolved, then autoclave. Pour into Petri dishes or similar containers. To inoculate, mix a little of a coating of the alga from tree trunks (preferably from *Tilia*), and pour over the plates. Pour or pipette off any excess water. The colonies will appear after about three weeks.

Staining of *Protococcus* is not easy. Many technicians stain with fast green alone, others with a carmin stain (which obviously gives them an unnatural color) or with picro-indigocarmin. A really satisfactory stain has not been described. When iron hematoxylin is used, it is

extracted from the very small nuclei before the cytoplasm is sufficiently differentiated.

Coleochaetaceae.—*Coleochaete* is such a small plant that the use of a high-power microscope is required to detect it. The largest colonies or thalli probably will not be found to exceed 5 mm. in diameter. The thalli grow on the leaves, culms, or stems of various water plants, particularly *Sagittaria*, *Isoetes*, *Elodea*, or *Typha*, and occur from just below the surface of the water to about 6 inches below (Wesley 1928) and on the sides of the host that receive the most light.

It is inadvisable to attempt to separate the plants entirely from the host. Small portions of the host bearing the thalli may be cut away; if the plants grow on *Elodea* leaves, whole mounts of the latter are excellent. Kill and fix with 1% chrom-acetic for 1 hour, wash thoroughly, then stain with either safranin and fast green (the latter in aqueous rather than alcoholic solution) or Harris' hematoxylin and fast green, and follow the hygrobutol method. Beechwood creosote or dioxan might be tried, but the material will be so hardened after xylol or a similar fluid that it cannot be mounted. Except for details of zoospore formation, sections are of little service. Material which is to be embedded should be fixed in a strong chrom-acetic fluid, dehydrated by a very gradual series, embedded in bunches, microtomed not thicker than 8μ, and stained with iron hematoxylin and fast green.

If zoospores are wanted, arrange slides on the bottom of a large, shallow dish, fill the latter partially with tap water, place some mature thalli of *Coleochaete* in the water, and set the whole in the sun. The zoospores will attach themselves firmly to the slides, and the latter may be carried through all the killing, fixing, and staining processes as if they carried sections. For staining, try acid fuchsin.

Trentepohliaceae.—*Trentepohlia* is a strictly aerial genus. Filaments may be scraped off the bark on which they grow and mounted directly in glycerin jelly without any special treatment. The color is retained for many years.

Ulvales

Ulvaceae.—Most of the species are marine in habit, but some grow in brackish, a few in fresh, waters.

Ulva, a marine genus, is very common along both coasts and is easily recognized and collected. Fix portions of the thallus, including some from the marginal region, in 1% chrom-acetic in sea water, dehydrate, and embed in paraffin. *Ulva* tends to become excessively hardened during the clearing and infiltration if either xylol or chloroform was employed, consequently, tertiary butyl alcohol, which has little or no hardening effect, should be used. Since sections cut perpendicular to the flat

surface are required if details of zoospore and gamete formation are desired, pile several portions of thalli on top of one another to a depth of 2 mm. or so while the embedding is being done, and press together gently with a blunt instrument if they do not lie perfectly flat. In this manner a long series of sections can be cut with a minimum of effort. For reproductive details sections should not be over 3μ; for general structure 10μ is thin enough. Stain in iron hematoxylin with orange G, or a triple combination might be substituted.

Even if the thallus of *Ulva* is only two layers of cells in thickness, whole mounts are usually unsatisfactory. The presence of considerable iron in the cell walls inhibits proper staining.

Monostroma, which favors brackish waters, forms a sheet-like thallus one cell in thickness. Use a weak chrom-acetic, but make the fixation period very brief. The gelatinous consistency of the thallus causes it to become readily dissociated. By fixing the entire plant rather than small portions of the whole, one has a greater chance of avoiding disaster. Embed as described for *Ulva*. Material intended for whole mounts should be dehydrated with hygrobutol, or with glycerin, which may in turn be washed out with 95% alcohol. Picro-indigocarmin is the preferred stain.

Enteromorpha has a hollow tubular thallus one cell thick and favors more or less saline habitats. It may be treated like *Monostroma*, but the thallus would have to be cut open and spread apart for mounting.

Schizogoniales

Thalli of the Schizogoniales resemble those of the Ulvales. Reproduction is by akinetes and aplanospores. It is scarcely to be expected that any technicians save those specializing on the algae will come into contact with members of the order. Nothing has been described in the literature about their treatment, but *Prasiola*, which is to be found in the Rocky Mountains and the Sierra Nevada at isolated stations and at a few marine localities on both coasts, may be dealt with exactly as if it were like *Ulva* (Fig. 29). For whole mounts, stain with picro-indigocarmin.

Cladophorales

The multinucleate cells of the simple or branched filaments have numerous chloroplasts. Some species are marine, others are fresh-water.

Cladophoraceae.—The thick stratified cell walls are composed of three layers: an outermost zone consisting chiefly of chitin, a middle zone rich in pectic substances, and an inner cellulose layer. The absence of an external pectic layer explains why Cladophoraceae are usually so heavily encrusted with diatoms and other epiphytic algae.

Cladophora is one of the commonest of marine and fresh-water Chlorophyta in the United States. It will be found more expedient to select species, from either fresh or saline waters, that have short branching filaments not so densely interwoven as to make it difficult or impossible to untangle them when they have finally been brought into balsam for whole mounts. Such forms, also, are more likely to be found with reproductive stages.

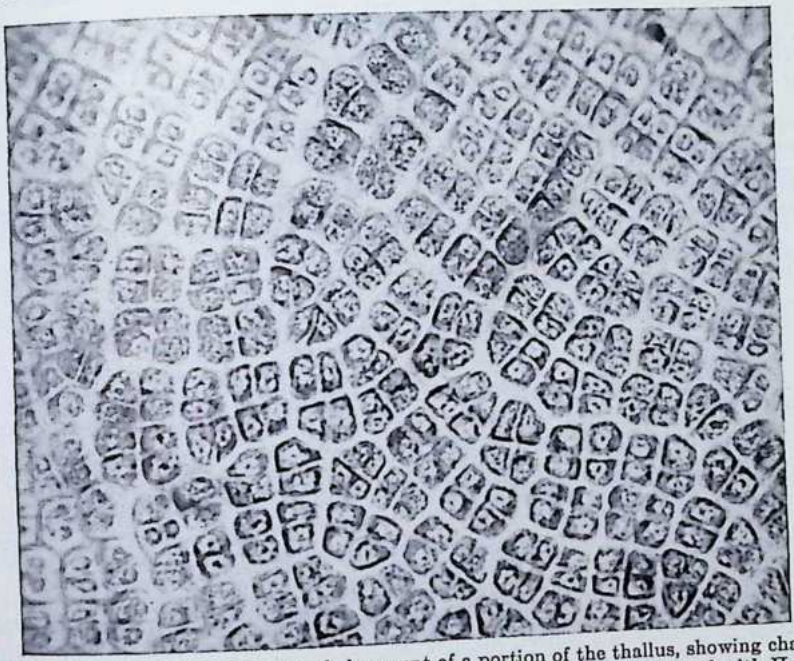


FIG. 29.—*Prasiola nevadensis:* whole mount of a portion of the thallus, showing characteristic arrangement of the cells. Fixed with formalin-aceto-alcohol; stained with Harris' hematoxylin and fast green.

Kill and fix with a medium chrom-acetic fluid, stain with iron hematoxylin and fast green, and run into balsam preferably by the hygrobutol method.

Rhizoclonium, Chaetomorpha, and other common genera of the order may be treated in the same manner as *Cladophora.* Some care should be exercised not to destain the iron hematoxylin too far or to overstain with the fast green.

Sphaeropleaceae.—*Sphaeroplea* is of particular interest because of its method of oögamous sexual reproduction. It develops to maturity and disappears within a month; consequently it is not often collected. It is most likely to be found in early or late spring. Fix with chrom-acetic, stain with iron hematoxylin, but take care not to overdo the counterstaining with either fast green or orange G. Make transfers from one fluid

to another very gradual as the unusually large cells of the filaments collapse readily.

Oedogoniales

Members of the order are particularly noted for the unique method of cell division; manuals should be consulted for a description of the process (G. M. Smith 1933). Very critical staining is required to bring out the details of the process, and one should employ material killed after midnight. The presence in certain species of dwarf male filaments bearing antheridia makes the group especially interesting.

All the genera, *Oedogonium*, *Bulbochaete*, and *Oedocladium*, may be treated alike technically. Species of all genera may be easily grown in soil solution No. 3 (see also Mainx 1931). If the material collected is sterile, it can sometimes be induced to produce antheridia and oögonia by artificial means. Place not too many of the filaments in a large container of any nutrient solution, preferably Knop's, highly diluted. After several days transfer to distilled water, and observe from time to time. Do not place in direct sunlight.

Fix in a chrom-acetic fluid, with the following proportions: 1 g. chromic acid, 2 cc. glacial acetic acid, and 100 cc. water. The formol-iodine fluid recommended for *Volvox*, or even formalin-aceto-alcohol, may be tried. Iron hematoxylin is the best stain, but the oögonia must be very carefully destained to the proper degree. For details of the methods of cell division and reproduction paraffin sections are deemed necessary by most workers.

Material may be preserved in a 2% aqueous solution of potassium acetate to which are added sufficient cupric acetate to color the mixture and a little phenol to prevent mold growth.

Zygnematales

Members of the order are common, readily distinguished, and easily manipulated algae. The cell wall is two-layered, consisting of an external pectic layer and an internal cellulose layer. The pectic external layer becomes so mucilaginous in the filamentous species that they feel slippery to the touch. In the Desmidiaceae there is a third, intermediate layer composed of cellulose impregnated with pectic substances and ferric salts. Special staining technique is required to differentiate between the cell layers and their constituent substances (Lütkemüller 1902).

There are no marine Zygnematales; the species are all confined to fresh-water ponds, swamps, creeks, rivers, or ditches.

Zygnemataceae.—The most intensively studied of all algae, *Spirogyra*, belongs in this family. The genus is cosmopolitan and should be

readily available to every student commencing microtechnique. Where *Spirogyra* is too rare, related genera should be easily found in place of that genus. In many localities *Zygnema*, as for instance in southern California, is more common than *Spirogyra*. As a last resort, well-fixed material is obtainable from the botanical supply concerns.

It is not an easy matter under average circumstances to obtain *Spirogyra* and other filamentous Zygnematales in the conjugating condition. This requires periodic examination of specimens from any given locality. There is a strong seasonal periodicity in the occurrence of conjugation, which varies according to the species. Most species conjugate in the spring, but there are some which do so in summer and others in the autumn. Contrary to the claims made by a few writers (*e.g.*, Chamberlain 1932), conjugation cannot be induced at will (G. M. Smith 1933); the writer, despite repeated attempts, has never been able to get any species to conjugate by artificial inducements. It is probably impossible to duplicate under artificial conditions the exact combination of circumstances which induces conjugation in nature. It is also difficult to keep Zygnematales in culture vegetatively and is scarcely worth attempting.

Methods for preparing whole mounts of *Spirogyra* are given in considerable detail in the chapter on Whole-mount Methods, to which reference should be made. These schedules are applicable to all the other genera. The combination of Delafield's or Harris' hematoxylin with iron hematoxylin and fast green should particularly yield preparations of surpassing beauty.

All the genera are easily embedded in paraffin, but such preparations are of value only in critical cytological investigations.

Mesotaeniaceae.—Treat like members of the following family. There is, however, no impregnation of the cell walls with iron compounds.

Desmidiaceae.—The unicellular Zygnematales, the desmids, are among the most beautiful of all unicellular organisms because of their astonishing diversity in cell structure and especially in ornamentation. Unfortunately for the technician, who usually does not like to see "something of everything" in his preparations, too many of the desmids occur in mixtures of several species or even genera. Sometimes, however, the patient collector may have the good fortune to find some particular form in unialgal culture in nature. Under such circumstances the most should be made of these opportunities. Most desmids occur in somewhat acid waters, or where the pH ranges between 5 and 6. This fact gives a clue to the method of treatment for these organisms. Follow the suggestions given for *Volvox*. Use a centrifuge to get the algae to settle between changes of fluids. Iron hematoxylin is the best of all nuclear stains, especially for the larger species, and any desired counterstain may be used when one seems advisable.

It has also been stated that excellent fixation may be obtained with 2 to 3% formalin plus a few drops of acetic acid. Dehydration is by a 5% series of steps of ethyl alcohol to 95% alcohol, allowing 15 minutes in each percentage. Staining is for 12 to 48 hours in 1% light green in 95% alcohol; wash in clean 95%, then complete dehydration with hygrobutol, and infiltrate with balsam.

Chlorococcales

The Chlorococcales are mostly unicellular or colonial organisms, of the greatest diversity in form. Because of the small size of most of the species, they are difficult to handle during the dehydrating and mounting. However, if one is fortunate enough to obtain any species in sufficient abundance, the loss of a considerable proportion of the collection is not such an exasperating matter.

The proper staining of Chlorococcales is a problem which has scarcely been attacked. Different lots of the same species react differently to the same staining procedure. The writer once had the experience of obtaining a beautiful iron hematoxylin stain on one lot of *Scenedesmus* but failed completely with material collected from the same locality ten days later. Others report experiences of the same sort. The difficulty apparently resides in differences in the pH of the water in which the organisms were growing and which had a direct effect upon the affinity of the cells for dyes.

For fixation of bulk material use a weak chrom-acetic (with 1 or 2 drops osmic acid solution to each 100 cc. fixative, if desired), or 5 cc. formalin and 5 cc. glacial acetic acid in 100 cc. distilled water.

A method which has been found to be entirely adequate for all but the most exacting cytological investigations is to smear a drop of Haupt's adhesive over a chemically clean slide, add 1 drop of the suspension containing the alga, then invert the drop over the mouth of a bottle containing any osmic acid solution for about 10 seconds. Next put the slide in a slide box, drop up, and when the box has been filled but before the drops dry out entirely, place the box, together with a watch glass full of formalin, in the paraffin oven. The fumes of the formalin will fix the gelatin of the adhesive and cause the organisms to stick to the slides. Remove the slides from the oven as soon as the film has dried completely.

Iron hematoxylin staining may be attempted. When successful, the results are of surpassing beauty. Counterstaining may be with fast green. For ordinary purposes, such as class use, a variation of Flemming's triple combination usually gives an excellent though exceedingly variable stain. When this combination is used on *Scenedesmus*, there is no consistency in the affinity of the different structures for the three dyes involved. First immerse the slides in 10% alcohol, for overnight or

longer, to extract the chlorophyll. Place in 1% safranin in 50% alcohol for 12 hours to 4 days; the time varies with each collection of material, but there is little danger of overstaining. Wash in running water for 5 minutes, then place the slides in 1% aqueous crystal violet for two days. There is more danger in overstaining with the violet than with safranin. Rinse off excess stain with water, then place in a saturated aqueous solution of orange G for not longer than 5 minutes. Rinse quickly with water, then with 95% and absolute alcohol for a few seconds in each. Differentiate with clove oil; the time is variable, but one should leave the slides in the oil until the nuclei are clearly visible when the preparation is examined under the microscope. Wash in two changes of xylol, and mount in balsam.

These algae may, of course, be mounted entire in glycerin without any special treatment, but they will retain their color for only a short time.

Only the more prominent genera are being mentioned in the following discussion.

Chlorococcaceae.—*Trebouxia* is the alga most commonly present in lichens, though not the only unicellular one to be found in these plants. Sections of the lichen thallus (*Sticta* or *Usnea* is to be recommended) should readily reveal the alga. When staining such material for the algal member alone, disregard the fungal member of the association as it is impossible to stain both equally well in most lichens. Iron hematoxylin or a quadruple schedule might be followed.

Protosiphonaceae.—*Protosiphon* is usually found associated with *Botrydium*, and the two are difficult to distinguish in the field. In the laboratory the iodine reaction for starch may be applied: *Protosiphon* reacts for starch, *Botrydium* does not. It is usually too difficult to separate the plants completely from the mud in which they grow. Cultures of *Protosiphon* are easy to secure (Bold 1936). Remove some of the plants from the mud with a microneedle, and place in the darkened portion of a unilaterally illuminated Petri dish containing sterile distilled water. Gametes which are strongly positively phototaxic are liberated in 5 or 6 hours. Some of these may be taken up in a loop and streaked over the surface of sterile solidified Detmer's agar in Petri dishes. The germlings will require about two weeks under artificial illumination to reach a sufficient size. Gamete formation and conjugation may again be induced as just described. Kill in a weak chrom-acetic fluid, and run into balsam by a very gradual method, as plasmolysis easily ensues if sudden changes are made.

Hydrodictyaceae.—*Pediastrum* is frequently encountered in plankton collections from pools or large ditches, but it is rarely found in abundance at most localities where it occurs. Specimens may be isolated and grown in No. 2, 3, or 4 soil solutions (Bold 1936). This fascinating little alga

may be treated as described for *Scenedesmus* (page 244), or if sufficiently abundant to be handled in bulk, fix with a weak chrom-acetic or with formalin-aceto-alcohol, stain with iron hematoxylin, safranin, or picro-indigocarmin, and run up by the hygrobutol method.

Hydrodictyon is widespread and can sometimes be found in enormous quantities in quiet waters. The nets will generally be in the vegetative condition, but one might be fortunate enough to encounter young nets within the mother cells of the old nets. Under artificial illumination, growth in soil solutions Nos. 3 and 4 has been luxuriant. A single young

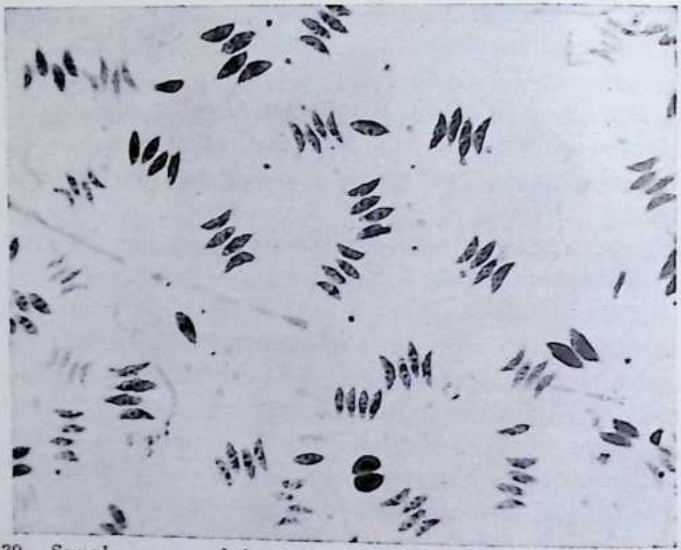


FIG. 30.—*Scenedesmus* sp.: whole mount from a pure culture. Fixed with osmic acid vapor and dried to the slide; stained with safranin, methyl violet, and orange G. Note the differences in stain retention by the various colonies.

net or a small portion of an older net should be rinsed through several changes of sterile soil solutions 1 or 2 before being placed in No. 3 or 4 in a large culture vessel (Bold 1936). If vigorously growing nets are transferred to fresh nutrient solution, zoospore formation will be stimulated (Mainx 1931*b*). Various fixatives have been recommended for *Hydrodictyon*. All of them may cause plasmolysis under circumstances for which there is no plausible explanation. Formalin-aceto-alcohol may be used on species which feel rather brittle when handled; for the others a weak chrom-acetic (say, 0.5 g. chromic acid and 5 cc. acetic acid to 100 cc. water) may be satisfactory. Many of the older technicians found that Juel's fluid gave excellent fixation (96 cc. 50% alcohol, 2 g. zinc chloride, 2 cc. glacial acetic acid). Iron hematoxylin with fast green is a good stain combination, but take care not to overstain with the green. Run up by a very gradual method into dilute balsam. In mounting, use scissors freely to cut the nets into suitable portions.

Oöcystaceae.—Free-living species may be treated as described for *Scenedesmus*. Species of *Chlorella* are found in *Hydra*, *Stentor*, and *Paramecium*. Texts on zoological technique should be consulted for methods of preparing slides of these animals.

Scenedesmaceae.—General methods of dealing with the species included in this family have been described above. *Scenedesmus* is an ubiquitous alga and exceptionally easy to culture (Fig. 30).

Siphonales

The genera are mostly marine and confined to warmer waters, but a few are predominantly fresh-water, and three are parasitic.

Bryopsidaceae.—*Bryopsis* prefers warmer seas but at least one species extends into icy waters on both the Atlantic and Pacific Coasts. This genus usually fruits in spring or early summer and occurs at, or below, low-tide level. The species are rarely found in abundance. Most of the available forms are sufficiently small for whole mounts. Kill and fix in a weak chrom-acetic in sea water, transfer to distilled water, stain with iron hematoxylin with a suitable counterstain, and run into balsam by an extremely gradual schedule. The plants, as usually found, are sterile, but if brought into the laboratory and kept in cold sea water, the gametes will appear in three or four days.

Codiaceae.—The family is a peculiar and interesting one. The plant body consists of branched coenocytic filamentous cells twisted together to form spongy thalli of more or less specific form. A few species are calcified.

Codium was not easy to get into paraffin by the older methods and usually cracked to pieces during the microtoming. Sections are of dubious value, except for the reproductive stages. For such studies kill in a strong chrom-acetic and get into paraffin by the method described for the Phaeophyta (page 268). Microtome at not over 10μ, preferably transversely, and stain in iron hematoxylin with either erythrosin or orange G as counterstain. Whole mounts of the individual cells with their gametangia attached at one side are of considerable service. Cut out portions of the thallus under water, and place in 6% hydrochloric acid until, on gentle stirring, the mass becomes dissociated fragments. Wash by decantation or by pipetting off the water after the mass of cells settles down, then place in a solution of 10% formalin and 3% glacial acetic acid in sea water to complete fixation. Wash out this mixture thoroughly, stain in iron hematoxylin or Harris' hematoxylin, counterstain lightly with fast green, and proceed to the hygrobutol method.

Halimeda is a tropical genus and may be found in Florida. The plants are calcified but are easily sectioned after fixation in a fluid con-

taining at least 5% acetic acid. Microtome perpendicular to one flat surface, and stain with iron hematoxylin and fast green.

Derbesiaceae.—*Derbesia* somewhat resembles *Bryopsis* in habit, also prefers warmer seas and has about the same geographical range. Treat like *Bryopsis*.

Vaucheriaceae.—*Vaucheria* is the most conspicuous fresh-water representative of the order since it is to be found almost everywhere. One may find it on pots in the greenhouse, on damp soil, in quiet fresh waters, in brackish and saline waters. It usually forms felt-like masses, easily distinguishable by their glistening appearance when examined in sunlight. Sterile material is worthless and uninteresting; only material showing at least antheridia and oögonia is worth preserving. *Vaucheria* is reputed to be difficult to kill and fix satisfactorily, and this reputation is often found to be sustained. Formalin-propiono-alcohol gives good results with most species; the weak and medium chrom-acetic mixtures too often cause considerable plasmolysis and, as a rule, are not to be recommended. The amount of formalin in the formalin-aceto-alcohol should be increased to 10 cc. with the usual 5 cc. acetic acid to 100 cc. of 50% alcohol. After this fluid very satisfactory results have been obtained with even a short balsam infiltration schedule, since *Vaucheria*, if properly fixed, is not so prone to become plasmolyzed during the later processes as are many other filamentous Chlorophyta. Iron hematoxylin is by far the best basic stain, but great care must be taken to differentiate the oögonia properly, as they commonly tend to remain overstained. Use fast green for counterstain.

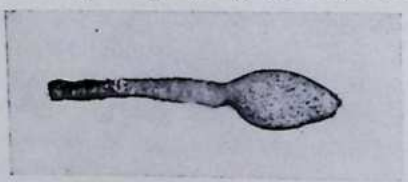


FIG. 31.—*Vaucheria terrestris:* whole mount of a germinating aplanospore. Fixed with formalin-aceto-alcohol; stained with iron hematoxylin and fast green.

If the only available material is sterile, it can sometimes but not often be induced to produce the reproductive structures. The following methods have been suggested. If placed in a 4% aqueous solution of sucrose, aplanospores may be obtained in a few days and then germinated in a nutrient solution (Fig. 31). In the same solution, if placed in bright sunlight, antheridia and oögonia may also appear. To induce the formation of zoospores, place the material in Knop's solution diluted to 0.2% strength for a week or longer, then place in tap water, and put in a cool dark place. Signs of zoospore development may be seen within two days. If such signs are not forthcoming, strengthen the nutrient solution, place the culture in the sunlight for several days, and then put back in the dark. Production of zoospores under such conditions may continue for a fortnight. In place of Knop's diluted solution, one might try a 2% sucrose solution.

For habit studies preserve the material in 3% formalin.

SIPHONOCLADIALES

The genera are all subtropical or tropical marine plants.

Valoniaceae.—*Valonia* is common in Florida. Portions of the thallus may be cut out and fixed in Bouin's fluid, which appears to be as good as any (Schechner-Fries 1934). Rather thin sections (at about 4μ) should be cut, and these may be stained with iron hematoxylin.

Dasycladaceae.—Most of the species are calcareous, which explains why exceedingly little about the technical manipulation of these forms is available. However, since the lime-encrusted Rhodophyta have been shown to offer practically no difficulty, there is no reason why the similar forms in the Chlorophyta should not be equally amenable to treatment. Species of *Acetabularia* and *Neomeris* from Florida are less than 10 mm. in height and should be easy enough to decalcify and mount entire. Other species of much larger size are also to be found in Florida.

CHAROPHYCEAE

The Charophyceae are placed by most recent writers with the Chlorophyceae (G. M. Smith 1938). *Chara* and *Nitella* are the principal genera; both are aquatics inhabiting ponds and lagoons and to a lesser extent ditches. *Chara* generally grows in warmer waters; the most luxuriant growth the writer has ever seen occurred in small pools on the floor of Death Valley.

Chara is not mentioned in most botanical texts, but it nevertheless is a most interesting and instructive plant and incidentally is one in which the fixation and staining will try the skill of the technician. This is because of its fragile structure, the calcium deposits, and its tendency to become exceedingly brittle if proper care in the dehydration is not taken. It is as difficult as with the aquatic Angiosperms to stain sections sharply.

Cultivation.—*Chara* is easily grown in a large aquarium or in a jar of at least 2-gallon capacity. Only a few plants should be put in the container, and they should be rooted in a layer of pond mud and sand about 1 inch deep.

Germinating stages can be secured by the culture method only. It has been claimed (Chamberlain 1932), but not substantiated by the experience of others, that old plants of *Chara* can be collected from a dried-up pond, stored for at least a month, and the zygotes removed and germinated in a shallow container containing tap water. Filamentous growths are supposed to appear shortly and lateral buds give rise to the typical *Chara* plant. These germlings, if available, are easily mounted entire by the general methods for the Chlorophyta.

Whole Mounts.—There are two general methods of preparing whole mounts of the sexual organs and thallus of the Charophyceae. In one the customary killing, fixing, and staining are carried out as desired; in

the other the plants are killed and fixed so as to retain the natural colors of the different organs.

In preparing mounts by the first general method, kill and fix in a strong chrom-acetic fluid or in formalin-aceto-alcohol. Wash out the killing fluid, which should have dissolved most of the encrustations, and stain by any desired *in toto* combination. Mayer's carmalum, Harris' hematoxylin with fast green, methyl green acidified with acetic acid, or even safranin and fast green, may be tried. It will scarcely be possible to obtain excellent internal staining of older antheridia or oögonia, but the development of the younger sex organs may be easily demonstrated. The apical cell will be too hidden by the enveloping leaves to be seen in whole mounts, although there may be species of *Nitella* in which this would be possible. Older antheridia may be run up entire, then crushed, teased apart carefully in a drop of balsam, and a coverslip added; thus the filaments, shield, manubrium, and capitula may be observed.

Permanent mounts of the sexual organs and thallus, in which all the natural colors are retained, are most instructive and apparently not too difficult to prepare (Woods 1929).

1. Soak the desired portions in cool tap water for two days to remove part of the encrusting lime.

2. Remove air by pumping or by immersion in cool boiled tap water.

3. Fix in the following solution for 4 hours:

Water	200 cc.
Glacial acetic acid	8 cc.
Cupric acetate, c.p.	1 g.

(Cupric sulphate may be used in place of the acetate.) The volume of solution should be fifty times that of the material. Agitate occasionally to dislodge the bubbles of carbon dioxide.

4. Pour off the fixing fluid, and add to it just sufficient concentrated ammonia to change the blue color to a decidedly purplish tint. Pour back over the material, and let it remain for about 1 hour or until the thallus has turned a markedly blue-green color.

5. Wash in running tap water for 10 minutes, then soak in several changes of distilled water for 5 minutes. The thallus should now be a bluish-green, the antheridia orange, and the oögonia brown and green. Transfer to 5% glycerin in distilled water. If plasmolysis occurs here, omit the washing in tap water, and go directly to the 5% glycerin.

6. This step must be carefully followed if the orange and brown pigments are to be preserved. Place portions of the material on chemically clean slides, add sufficient of the 5% glycerin, then cover with No. 2 coverslips, and begin artificial evaporation at once. In warm and dry regions this may be done outdoors. Otherwise place the slides on an

asbestos board, and adjust in front of an electric heater in such a manner that the water evaporates steadily, but not suddenly, from under the coverslips. Add 5 or 10% glycerin as needed, but use solutions of higher concentrations toward the end of the process, which should not require more than 10 to 12 hours. Examine occasionally to avoid plasmolysis or swelling. A little practice is needed to perform this step properly.

7. When the solution has reached the consistency of pure glycerin, remove the coverslips, transfer the material to new chemically clean slides, and mount in glycerin jelly. Put aside for a week to harden, then seal carefully with balsam.

Fixation.—All calcified species require a fluid containing acetic acid in order that the calcium encrustations may be dissolved. For the apical cell and younger stages of sexual development, a medium to strong chrom-acetic fluid is satisfactory. It would also be useful to fix some of the material in a fluid giving a basic fixation image to reveal the vacuolar system more clearly. Since the nuclei in the internodal regions increase in number amitotically, special attention should be devoted to the fixation and subsequent staining if it is desired that this feature be emphasized. Formalin-aceto-alcohol has been found to fix the older reproductive organs satisfactorily, but it is useless for the apical cells.

The apical portions of the branches are easily removed; it is not necessary to dissect away the side "leaves" or smaller branches since it is not difficult to orient the pieces so that longitudinal sections through the apical cell are secured. The side branches bearing the developing sex organs should be cut off separately, and as the organs are approaching maturity, they should be removed individually or in groups of two or three. Orientation otherwise will be difficult since the internodes have a tendency to curl during dehydration. Embed the branches on one side, and section parallel in this plane at 10μ for the younger reproductive organs and at 12μ for the older stages. Apical cells should be cut at 8μ.

Staining.—The quality of the staining is largely dependent upon the fixation, and with some species it is rather difficult to obtain a sharp differentiation. Frequently everything else must be sacrificed for some particular structure, such as the antheridia. A triple combination is good for the apical cell and for anatomical details. For the earlier stages in the development of the sex organs iron hematoxylin is satisfactory, but it is usually rather unsatisfactory for the later stages. For these, safranin and fast green have given sharp staining. In staining the developing antheridia, care must be taken not to overstain, and all other structures should be ignored. One might attempt Feulgen's reaction. The developing oögonium is so gorged with foodstuffs that it is not easy to give it a satisfactory stain. During the metamorphosis of the antherozoids, the blepharoplast may be revealed with iron hematoxylin.

CHAPTER XIII

EUGLENOPHYTA

The euglenoid flagellates may not be considered as belonging among plants, but as they contain pigments identical with those in the Chlorophyceae, one might as well be somewhat arbitrary with classificatory ideas and treat them as plants (G. M. Smith 1933, 1938).

Euglenaceae

Occurrence.—These organisms are most commonly found in small pools rich in organic matter; the pigmented species are far more abundant than the colorless types. Forms which are not free-floating occur on algae, plant debris, or upon small crustaceans. One genus, *Euglenamorpha*, is endozoic in habit, occurring in the intestinal tract of tadpoles of *Rana*.

Structure.—All free-swimming species possess a periplast, which may be flexible or rigid. In two genera the protoplast is surrounded by a lorica with an opening at the anterior end through which the flagella project. The lorica, composed of a firm gelatinous substance, is without any trace of cellulose but usually is heavily impregnated with iron compounds. Paramylum, an insoluble carbohydrate with a chemical formula similar to that of starch, is the chief product of photosynthesis; it does not react to the ordinary chemical tests for starch. Cysts with thick walls are common in many genera.

Cultivation and Manipulation.—Since only one genus, *Euglena*, is of common occurrence and has been cultivated artificially with its technical handling worked out, the discussion of the various genera will be limited to this group. Most of the free floating species can be treated similarly so far as fixation and staining are concerned. *Euglenamorpha* must be treated as noted.

Euglena occurs in pools or ponds rich in organic matter, forming a greenish scum. Other organisms will probably be found in company with it, but a pure culture of *Euglena* may be obtained by placing a small amount of the scum in a large covered dish containing 1 liter of pure tap water and 1 g. of boiled rice. In a few days there should be an abundance of *Euglena*. Material can be prepared with this culture as a

A great many methods have been proposed for cultivating *Euglena* (see also Mainx 1927). A choice may therefore be made between the following:

1. Although not all species will grow equally well on this particular medium, perhaps the most useful one is split pea infusion, prepared by

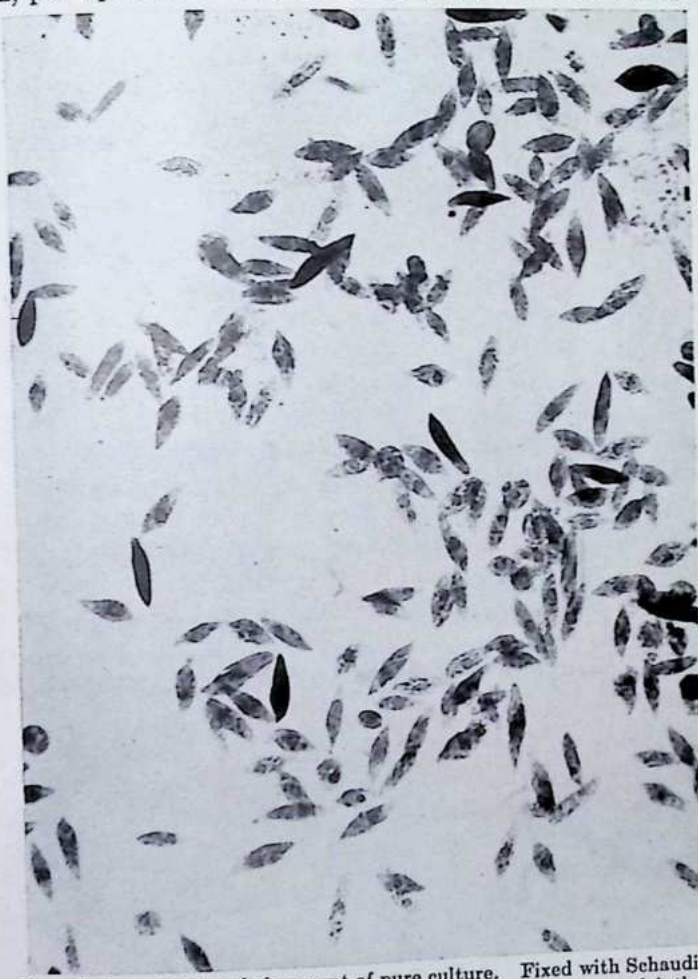


FIG. 32.—*Euglena viridis:* whole mount of pure culture. Fixed with Schaudinn's fluid; stained with Lynch's precipitated carmin and indulin. The flagella are faintly visible in some specimens. (*From a preparation by Miss Enid A. Larson.*)

boiling 40 split peas in 1 liter of tap water (Baker 1926). Add enough citric acid to prevent excessive growth of bacteria.

2. An autoclaved mixture containing 0.1% yeast extract and 2 g. sodium acetate per liter of distilled water has given excellent results.

3. Quince-seed jelly may be used for keeping cultures in an inactive condition: boil 20 g. dry quince seeds for 30 minutes in 1.5 liters distilled

water, then pass the thick exudate, together with the water, through a fine wire (80-mesh) sieve. Increase the volume to 2.5 liters with distilled water, and place in a stoppered bottle, where it will keep for many months. Put some of this medium in a test tube or small flask, and inoculate. Such cultures will last almost indefinitely. Molds are likely to put in an appearance; they may be prevented by rendering the medium slightly alkaline. Place the cultures in moderate light, and look for the *Euglena* on the side of the vessel toward the light. If a split pea infusion be inoculated with material from a quince-seed jelly culture, abundant active forms will be produced within a few days.

4. Small cubes of coagulated egg albumen added to sterilized tap water constitute a good medium.

5. Soil dilutions No. 2 or 3 as recommended for the Chlorophyceae are reported to support luxuriant growth (Bold 1936).

The most satisfactory killing fluid appears to be Schaudinn's heated to 56°C. For best results when studying nuclear details, the organisms should be killed late in the evening; the largest number of mitoses are to be found at 10 P.M. Material should be stained *in toto*. For staining, a long iron hematoxylin method or Lynch's precipitated carmin (Fig. 32) is preferable. A counterstain is required to reveal the motor apparatus; eosin or Bordeaux red, or even light green, may be used. If eosin or Bordeaux red is selected, which seems recommendable, apply it *before* using the ferric mordant in hematoxylin staining. Other stains that may be employed include Harris' hematoxylin, safranin and fast green, or a triple combination.

Get the *Euglena* into dilute balsam by the hygrobutol or beechwood creosote method.

CHAPTER XX

PYRROPHYTA

DINOPHYCEAE

The Dinophyceae are mostly marine planktonic organisms restricted to the warmer parts of the ocean. The fresh-water species, with a strictly algal organization, are not known in the United States. Several species occur as parasites in protozoa and metazoa. The majority of the species are unicellular (G. M. Smith 1933, 1938).

A few species have naked protoplasts, but the majority have cellulose walls which usually consist of a definite number of articulated plates. The unicellular motile forms, as well as the zoospores of immobile forms, have a distinctive structure. There is always a transverse or a spiral groove completely or partially encircling the cell. All motile forms have two flagella inserted in the groove. The general color of the organisms is yellow-brown, but others may be blue-green. The principal food reserve of the fresh-water species, as a rule, is starch, that of the marine species is an oil.

Some investigators claim that the Dinophyceae are best studied in the living condition, but this appears to have come about because they paid more attention to the plates on the external walls than to internal details. For permanent preparations, fix with 1% chrom-acetic, stain with iron hematoxylin, Harris' hematoxylin, Mayer's carmalum, or safranin, and counterstain with a transparent dye such as erythrosin or orange G. Dehydrate rather slowly with hygrobutol, and infiltrate with very highly dilute balsam.

CHAPTER XXI

CHRYSOPHYTA

With the exception of the diatoms, members of the Chrysophyta are rarely collected in the United States, simply because so few searches are made for them. Most of them are plankton organisms existing below the surface of the water, but others are to be found in pure waters of pools and ditches. The diatoms, on the other hand, are to be found in all kinds of waters and are decidedly cosmopolitan.

All Chrysophyta have cell walls that are usually composed of two overlapping halves and are frequently more or less silicified; there is an absence of starch formation, the reserve foodstuffs consisting of oils and leucosin (Pascher 1924, 1937; G. M. Smith 1938).

Most of the Chrysophyta degenerate very rapidly after removal from the habitat, consequently fixation should be effected as promptly as possible.

XANTHOPHYCEAE (HETEROKONTAE)

The Xanthophyceae are so rarely collected and so little known to the average American botanist that an extended discussion appears to be unnecessary. Two genera, however, are common and may be encountered. *Tribonema*, a filamentous genus, may be dealt with as if it were one of the filamentous Chlorophyta. *Botrydium* is the only siphonaceous genus in the class and may, when conditions are favorable, form an extensive growth. A portion of the damp substrate may be dug up and the organisms carefully washed out under running water. Kill and fix in a medium chrom-acetic fluid, stain with iron hematoxylin, and bring up to 85% ethyl alcohol extremely gradually over a period of a week or longer. Counterstain with fast green in 95% alcohol plus a little methyl cellosolve. Then wash with a change of 95% alcohol, and add hygrobutol at the rate of 1 drop per ½ hour or longer; days or even two weeks should be allowed for the dehydration process. Infiltrate just as gradually with highly dilute balsam. Mount in depression slides.

CHRYSOPHYCEAE

Chrysophyceae are as rare and as seldom collected as are the Xanthophyceae.

Dinobryon is said to be widespread in standing fresh waters, and occasionally to occur in abundance. Most illustrations depict merely

the more or less silicified lorica. There is no record of dependable methods for fixing and staining material.

Hydrurus is occasionally very abundant in the cold waters of montane streams in mid- or late spring. The plants have a feather-like appearance but are tough in consistency. As the name of the sole species, *H. foetidus*, indicates, it generally has a disagreeable odor. Because of the yellowish-brown color, it may easily be ignored on the assumption that it is merely a mass of disintegrating green algae. Kill and fix with formalin-aceto-alcohol or a 1% chrom-acetic fluid; stain with iron hematoxylin and fast green. If dehydration and infiltration with balsam are attempted, it should be done very slowly because of the danger of collapsing the gelatinous portion of the thallus. Direct mounting in Karo would probably be better.

BACILLARIOPHYCEAE

Diatoms are almost ubiquitous organisms wherever there are aquatic or moist situations exposed to sunlight (Boyer 1927*a*, 1927*b*). Some genera prefer fresh waters and others marine habitats; again certain species are strictly planktonic, and others grow attached to rocks, to other algae, or to aquatic Angiosperms. Most species prefer cooler waters or at least are more abundant during the spring and autumn, but that others can survive in hot waters is evidenced by their abundant occurrence in Death Valley streams.

Collection and Preservation.—Diatoms may for convenience be divided into two groups: fossil and living.

Among the living diatoms most collectors will find the fresh-water forms more easily obtainable, although under average conditions the marine species can be secured in greater abundance and in purer cultures. At most times of year, but particularly under any given set of circumstances, certain species will be found to predominate over others.

In fresh- and brackish-water situations, diatoms may be free floating on or below the surface. One method of securing such species is to obtain a jar of at least 1-gallon capacity, with not too wide a mouth, whose sides are painted black or covered with black paper, and to fill with the water in which the diatoms are living. Place in bright sunlight; within a day or so the living diatoms will congregate on the top and around the edge, from which they can be removed by means of a giant pipette. Mud from the bottoms of ponds, lakes, shallow bays, and sluggish streams may be treated in the same manner.

Diatoms grow very abundantly in the form of slimy masses on rocks and on other algae and aquatic Angiosperms that have been standing in the water for some time. Such slimy masses are easily scraped off, but if the host is old and decaying, it is a better plan to place the entire mass

in the collecting bottle and later to get rid of the host tissues by chemical maceration and dissolution.

Marine plankton forms are most easily collected by towing a plankton net, made of fine bolting silk, through the water. In order to collect a sufficiently large volume of material, a large net should be towed behind a motorboat. The net should float near the surface. About ½ hour's towing will be required. Many marine species possess long hair-like processes (as in *Chaetoceros*); consequently the materials should not be handled roughly at any stage of treatment. Marine species growing attached to various large algae frequently occur as "unialgal cultures," either as single specimens in large numbers or grouped in fasciae by the millions. Those species which develop fasciae exhibit marked preferences as to their hosts and also in many cases produce the fasciae in such definite macroscopic forms that they are commonly mistaken for members of the Phaeophyta.

All living diatoms are easily fixed and stained if it is desired that internal structural details be revealed. A weak chrom-acetic fluid will give the best fixation; it should be based on sea water in the case of marine species. The standard stains may be employed (iron hematoxylin is recommended), but differentiation will be somewhat difficult because of the siliceous envelopes, and some unevenness must be expected. Dehydrate by the hygrobutol or dioxan method; infiltrate with highly dilute balsam, and evaporate the solvent very gradually.

Plants of *Polysiphonia*, *Vaucheria*, *Cladophora*, and *Chara* made into whole mounts, according to methods applicable to those genera, frequently have excellently fixed and stained diatoms attached to their thalli.

"Diatomaceous earth" consists mainly of fossil diatoms, which may be either fresh-water or marine in origin, and is found in various parts of the world, as in Ireland, New Zealand, Nevada, and particularly in California. If samples cannot be obtained by personal collection, it is a simple matter to purchase a small quantity from certain of the supply concerns or from diatom specialists.

Preparation of Diatoms for Taxonomic Study.—Most diatomologists are not interested in internal structure, their attention being primarily concentrated on the surface sculpturing of the cell walls. Their methods of preservation are, therefore, crude in the extreme, since the siliceous walls are usually not damaged by ordinary reagents. Most of them simply add from 5 to 10% formalin to the water to act as a preservative. The formalin should be added as soon after collection as possible because degeneration changes set in promptly.

To determine some genera and most species accurately, everything except the cell walls must be removed: diatomologists call this the "clean-

ing" of diatoms. The mass containing the diatoms is placed in a large test tube or small beaker, and a small quantity of hydrochloric acid is added. The acid serves a twofold purpose. If an effervescence is noted, it indicates the presence of chalk, limestone, or other calcium compounds, and these substances must be entirely removed by the addition of more acid until all effervescence has ceased. The other purpose of the acid is to loosen those which adhere to other objects, consequently the diatom material should be left in the acid for a day or two. Shake the contents well, add considerable water, and strain into a small beaker through coarse muslin (to remove the larger pieces of debris, portions of other plants, sand, etc.). If calcium compounds appear to be present, the material must be washed thoroughly with water, otherwise the next step in treatment will leave irremovable precipitates of calcium sulphate. For making rapid washings, a centrifuge may be employed. The tubes should have round bottoms (like those of test tubes) rather than the narrowly attenuated ones with which most centrifuges are equipped. The centrifuge should be revolved slowly; since the diatoms will settle quickly, not many turnings will be needed. If no calcium compounds appear to be present, two thorough washings suffice. Pour off most of the water, add a small quantity of sulphuric acid, and warm gently over a flame. The acid will char or dissolve most of the organic matter present. Next add a small crystal of potassium bichromate. Considerable chemical activity is usually generated, and chromic acid is liberated. The liquid turns greenish. If the mass is not completely freed of debris and the diatoms well cleaned, wash with water, and repeat the sulphuric acid-bichromate process. When finally clean, the diatoms are well washed with water. If the diatoms are to be mounted as strews in balsam, simply wash with two or three changes of hygrobutol or dioxan, and infiltrate with balsam. Mounting of individual specimens may be carried out as directed below.

The manipulation of diatomaceous earths depends on whether the mass is composed almost exclusively of empty shells or mixed with more or less debris. The former condition rarely occurs, but in such cases the material requires no further cleaning. Most samples of diatomaceous earth must be subjected to a laborious cleaning process before the diatoms will be in a condition suitable for critical study (Shropshire 1931).

A 1-inch cube of the earth is broken up into portions about the size of peas. These are placed in a small Erlenmeyer flask together with three times the bulk of sodium acetate and enough water to moisten the entire mass thoroughly. The flask is boiled in a water bath for 15 minutes, then set aside to cool quite undisturbed. When cold, a small crystal of sodium acetate is dropped in; the forces of crystallization thus set up serve to break up the masses of earth. Warm water is added in excess and the mass brought to a boil. The flocculent material is poured into a beaker,

then the boiling and crystallizing process is repeated with the unbroken part until all the material is broken up. The sodium acetate must now be removed by repeated washings. Water is added to the material, and the container is allowed to stand undisturbed until the diatoms have settled; the water is then cautiously decanted, and more clean water is added. The process should be repeated several times, until it is certain that all traces of the acetate have been removed. If only small quantities are handled at a time and proper care is taken, a centrifuge may be employed to hasten the washing process. The washing completed, all possible water is poured off, and concentrated sulphuric acid is added in an amount equal to about three times the bulk of the material. The container is placed in a sand bath, brought to a boil, and the boiling permitted to continue for at least 15 minutes. The sand bath should be placed under a hood or out in the open air because of the dangerous fumes evolved during the next operation. Remove the source of heat. Add carefully drop by drop (by means of a pipette) a concentrated aqueous solution of potassium permanganate until the solution becomes bleached. The mass of material is then cautiously poured into a large flask containing at least 500 cc. of cold water. The material is next washed thoroughly with several changes of clean water until all acid is removed (the centrifuge may again be utilized). The material is then poured into a 500-cc. beaker, sufficient water and a teaspoon of any good soap powder are added, and the solution is boiled for about 20 minutes. The material is thereupon again washed thoroughly. Most diatomaceous earths have been completely cleaned after this laborious treatment, but if small particles of dirt still adhere to the diatoms, the soap treatment may be repeated. If microscopic examination shows the material not to be clean enough for mounting, the only recourse is to repeat the entire process, beginning with the sodium acetate. If the material is quite clean, it is ready for mounting.

Mounting of Diatoms.—For most practical purposes, mounting in balsam is as good as in any other medium. The larger diatoms have their charateristic markings revealed clearly enough, but for the very small species, which require examination under an immersion lens, mounting must be in Hyrax, Styrax, or some other synthetic resin with a high index of refraction. It should be borne in mind that the diatoms are entirely without color (they cannot be stained after having been cleaned); consequently a mounting medium with a different refractive index is required. It is, however, not an easy matter to obtain satisfactory samples of synthetic mounting media, and the aid of a specialist on the group may have to be enlisted.

The elaborate groupings of diatoms which give the "cranks" on the subject so much delight are of no particular scientific value; sometimes

even the alleged aesthetic value is questionable. However, if one wants to go to the trouble of designing a rosette or what not with different diatoms, he will gain the skill and fine touch necessary for the preparation of type slides of diatoms for taxonomic purposes.

The simplest method of preparing strew diatom slides, especially where quantities of such are required, is to drain off all possible water from the cleaned material, to wash with three changes of hygrobutol, and to transfer to diluted balsam. Instead of evaporating the solvent until the balsam has become sufficiently thickened, as is ordinarily done, mounting should be begun as soon as the diatoms have settled down. Place a small drop of thin balsam on the slide, pick up a tiny amount of diatom material with a needle spatula, and place in the drop of balsam. Pass a cleaned 15 or 18 mm. No. 1 circular coverslip through an alcohol flame, then place on the drop of balsam. Accurate judgment is needed both in estimating the size of the drop of balsam, which should be as small as possible yet enough to spread to the periphery of the coverslip, and in picking up just enough of the diatom material so that it becomes spread as evenly as possible throughout the balsam without excessive overlapping of individual diatoms.

Some diatomists merely place a drop of the watery solution of diatoms on a coverslip, let it dry, then heat on a copper plate and, while still warm, reverse over a drop of mounting medium on a slide. By this method too many diatoms tend to collect at the periphery of the drop of water; the difficulty can be remedied by smearing the slip with a thin film of Mayer's adhesive. The diatoms must be thoroughly dried before being mounted. Air is frequently trapped within them, but most of it can be caused to disappear if the slide is heated.

In making designs or special-type slides, the desired diatoms are first picked out of the general mass by means of fine pipettes (perform this operation under a binocular microscope with suitable magnifications) and set aside to dry. Diatoms are more easily removed from alcoholic than from aqueous solutions. Small circular coverslips are smeared with a very thin film of acetic gelatin (liquid gelatin thinned with an equal volume of 50% acetic acid) and this allowed almost to dry. Many mounters prefer to make their adhesive by adding 2 parts clove oil to 3 parts acetone-soluble celloidin, which is spread as thinly as possible on the chemically clean coverslip. The diatoms are picked up by means of a bristle (a cat's whisker will do) and arranged as desired under the microscope. Place the completed coverslip on a warming plate until thoroughly dry. A drop of Hyrax (or similar medium) is placed on the coverslip and the latter inverted and gently pressed down on a warmed slide. If the Hyrax is too thick, it may be thinned with benzene.

CHAPTER XXII

PHAEOPHYTA

PHAEOPHYCEAE (MELANOPHYCEAE)

In North America the brown algae are exclusively marine plants. Elsewhere only three fresh-water species are known. The brown algae are far more complex in both structure and reproductive processes than are the Chlorophyta or the Cyanophyta, but less so in both respects than the Rhodophyta.

In size the thallus of the Phaeophyta varies from simple or slightly branched filaments of a single row of cells and simple membranaceous forms, varying from a single layer to several layers of cells in thickness, up to solid plant bodies of various forms. Many of the smaller forms are epiphytic upon or endophytic within other algae. An alternation of generations occurs in most if not all of the Phaeophyta outside of the Fucales. The two generations are either similar or dissimilar in size and vegetative structure. The sporophyte may be smaller or larger than the gametophyte. In some genera the gametophyte is an annual and the sporophyte a perennial, but in other genera both generations are annuals and in still other genera both are perennials.

Occurrence.—Phaeophyta are predominantly cold-water plants, but there are a few which prefer warm seas. Some, such as *Pelvetiopsis* and an occasional *Fucus*, are found at the high-tide line, but the majority prefer the middle littoral and sublittoral zones. In a few particularly favorable locations species normally occurring at deeper levels may be collected without much difficulty during extremely low tides. Under such conditions on the Pacific Coast it is easy to obtain small plants of the giant kelps, *Macrocystis* and *Nereocystis*.

There is not so great a variation in the colors of the various Phaeophyta as exists among the Rhodophyta. Some are a light yellowish-brown, others appear darker because of a greater thickness of the tissues, while many crustose species become so dark brown as to appear black when more or less dried out during low tides. There are a few Rhodophyta which might be mistaken by the inexperienced collector for Phaeophyta, but such can readily be distinguished if the chemical tests noted under the former phylum are applied.

There is an excellent manual for the Phaeophyta of the north Atlantic Coast (Taylor 1937) and another for the Pacific Coast species (Setchell and Gardner 1925).

Collecting.—Nearly all Phaeophyta which are not crustose, endophytic, or epiphytic grow firmly attached to rocks and frequently where they enjoy the full force of the surf. It is generally difficult to get them loose; at times one may have to work quickly between waves, when extreme caution is necessary. The upper part of the plants may be cut off with a large sharp knife, but one usually wants to get the entire plant. The easiest method of prying the plants loose is to use an old chisel with a blade about 1 inch wide or a stout geologist's pick, and to cut against the rocky substrate. The rocks are usually in a disintegrating condition, and it is easier and quicker to chip pieces of rock loose than to chop through the mass of stout hapteres.

Although some collecting can be done at all times, the best specimens are always obtainable during the lowest tides. At such periods the larger forms, which grow below mean low-tide levels, can be secured. A tide table for the locality should be available; it may be found on the maritime or "waterfront" pages of local seaport newspapers, or a tide table for the entire year may be obtained, usually gratis, at sporting goods stores that cater to fishermen. Tide levels and times differ enormously for localities a few miles apart; consequently the tables of mean differences and times, which accompany the tide tables, should be consulted and the proper allowances made.

Masses of algae cast ashore should, as a rule, be ignored, since they are either too dry or in too advanced a stage of decomposition to be worth examining. At certain parts of every rocky beach, determined by a combination of currents and winds, masses of floating algae, cut adrift from deeper regions, occur. One may cautiously wade out in such areas and select the better, fully intact and nondiscolored specimens. Material from such sources is fully satisfactory for slide-making purposes, but one runs a certain danger of criticism if locality herbarium specimens are made therefrom. If the material is dried, it should be used merely for purposes of identification and the label should always bear the notation "cast ashore."

In clambering over rocks in search of material, one should proceed with great caution, as it is very easy to slip and receive bruises or abrasions. If one slips against jagged rocks, barnacles or mussels, open cuts on legs readily result, and when the salt water gets into the wounds, they can become very painful. Concentrate on collecting algae and forget about surf bathing or getting a tan or other extraneous matters—except for keeping an eye open for waves and the incoming tide. Wear stout hip boots even if the water is warm; these are mostly for protection against slips and consequent abrasions. If the rocks are densely covered with algae, place one foot down firmly, twisting it sidewise to get a good hold, before lifting the other foot, and do not attempt to take too long

steps or to jump from one projecting rock to another. In wading through pools, walk very slowly, with short steps and the foot not lifted too high, and in a direction so as to avoid reflections from the surface. Search under and around large masses of algae for smaller forms. Examine shady banks and overhanging ledges; the plants in such situations are apt to be different from those growing on the sunny sides. In removing plants, grasp the entire mass firmly as close to the base as possible and pull up with a twisting motion, or use the chisel or pick to assist in loosening them.

The plants, as removed, may be placed in a galvanized or enamelware bucket of about 2-gallon capacity. Do not put any water in the bucket, but if the algae show signs of drying out, simply immerse momentarily in cold sea water, and replace. If the algae are placed in water, most of the spores, gametes, oögonia, etc., may be liberated. Do not cut the smaller epiphytic species from their hosts since it is frequently necessary to know the identity of the host when classifying the epiphytes. If cultures are to be started, put only one species in a bucket; otherwise contamination may result. In a general collection there is little danger of spoiling if the different sorts are mixed, but *Polysiphonia*, *Pterosiphonia*, and *Desmarestia* (particularly *D. herbacea*) should never be permitted to come into contact with other algae.

As soon as the return to the laboratory has been made, sort out the material. The species which cannot withstand much drying should be worked up first. If an immediate identification is not possible, place some of the material in a tank of running sea water for later study. If any material appears to have suffered from drying, place in cold sea water for a short time to restore turgidity.

Preservation of Material. 1. *In Liquid.*—In general, all Phaeophyta may be preserved in 8 to 10% formalin in sea water, plus the addition of enough borax to render the solution distinctly alkaline. Unfortunately, this solution corrodes all except all-glass receptacles. After remaining in the formalin solution in sea water for about a week, this fluid may be washed out with two or three graduated mixtures of sea water and tap water and finally stored in 3 to 5% formalin in tap water plus sufficient borax to prevent the fluid from becoming acid and about 5% glycerin.

2. *Rough Drying.*—Old newspapers may be spread in some place where the air is as dry as possible and not in full sun. The material may then be spread apart on the newspapers, then turned over from time to time so that the drying may be more uniform and molding is avoided. The specimens should be taken up once in a while and shaken gently so that the branches will not adhere but will dry separately. If the atmosphere is damp or if the specimens are unusually large, it may be necessary

to utilize gentle artificial heat in order to avoid rotting or molding. The specimens might be placed in a warm room or near a radiator, stove, or other source of heat. On the other hand, the specimens must not be dried too rapidly, or they will become too brittle. When the specimens have become fairly dry, but before they have become too brittle to handle without damage, they should be rolled into a ball or roll, tied with twine, and allowed to continue drying. They may finally be wrapped up in newspapers or placed in suitable boxes.

The plants should not have been washed in other than sea water; the salt left in them during the drying process serves to keep them flexible. After drying, even if they have remained in that condition for years, they may be soaked out in either fresh or salt water and studied as if they were freshly collected specimens.

To prepare dried specimens for mounting as herbarium specimens, soak them in sea water until they are restored to approximately the original condition, then transfer to a mixture of equal parts of water, ethyl alcohol, and glycerin plus 10% phenol until thoroughly penetrated, then they may be dried on paper. Or they may be dried without placing on paper and will retain their flexibility indefinitely if stored in tin or airtight boxes.

3. *Herbarium Specimens.*—Practically all forms can be dried on paper. The more slimy forms, such as *Fucus*, *Pelvetia*, and *Hesperophycus*, will shrink badly by the time they are thoroughly dry. Most small specimens will adhere readily to the paper but species belonging in the Fucales, Dictyotales, and Laminariales will need to be glued to the paper with waterproof glue after they have dried completely. Most of the Laminariales are too large for the ordinary herbarium sheet; one must either search for small specimens, cut the specimens into suitable lengths, or mount selected portions of mature plants.

To prepare herbarium specimens, obtain a flat tray of a size to receive easily a standard herbarium sheet. Of course, one does not have to use the regulation-size herbarium mounting paper: almost any convenient size of paper that will not shrink badly from wetting may be used. *Ectocarpus* and similar small forms may, for example, be mounted on plain standard 3 × 5-inch index cards. Students have mounted specimens on thick 8½ × 11-inch notebook paper and incorporated the finished mounts in their notebooks. Fill the tray somewhat less than half full with sea water, slip the sheet of paper in and float the specimen above the paper. Hold the stipe or thicker end of the specimen at one end of the sheet and partially raise this end out of the water. With a small brush or needle arrange the rest of the specimen as the paper is gradually lifted out of the water. Drain off excess water, lay the paper on a drying blotter, and cover the specimen with a piece or two of cheesecloth

or part of an old linen bed sheet. Place a couple of driers over the cloth, followed by a piece of corrugated board. The next specimen may now be added. If the specimens are being mounted on small pieces of paper, several such pieces may be arranged on a single drier. After 2 hours remove all driers, and replace with dry ones; do not try to remove the cloths. If the atmosphere is too damp for drying to take place, some form of gentle artificial heat must be arranged. The driers should be replaced at least twice a day with dry ones until the specimens are thoroughly dry. Between changes, keep the mass under moderate pressure.

Cultivation.—Methods have never been devised for growing to maturity other than the smaller filamentous species; the larger species with massive mature structures have been grown to the older germling stage only. Cultivation methods have, in fact, been directed mainly toward obtaining the gametophytes in genera in which these structures are microscopic in size and cannot be found in nature.

No matter what the means of reproduction, cultural methods and solutions are essentially identical. The following solution has given excellent results (Schreiber 1931):

Sodium nitrate	0.1 g.
Dibasic sodium phosphate	0.02 g.
Distilled water	50.0 cc.
Sea water	To 1 liter

Autoclave at 15 pounds pressure for about 20 minutes. The solution may be solidified with 1½% agar if it is desired to raise the germlings on such a medium. If the cultures are to be carried for longer than three months and slides are not to be made of stages younger than well-developed sporelings, it is preferable to use a solid substrate. Cultures have been carried along for 3 to 5 years on such media.

If zoospores are to be germinated, obtain a small battery jar about 4½ inches in diameter and 3 inches deep. Place two clean slides at opposite sides on the bottom, then lay two more slides across the ends of these slides and continue stacking slides until the pile is about 1½ or 2 inches deep. Pour in the culture solution, using more than enough to cover the stack. If gametophytes with sex organs are to be raised, use a similar jar of wider diameter, and arrange the slides in one layer on the bottom and stacked in one row around the periphery, then fill with nutrient solution. It may be more satisfactory to use large coverslips (24 × 50 mm., No. 2) in place of slides for gametophyte growth.

Place small pieces of fruiting material in the culture dishes. Portions of the laminae of the Laminariales bearing sori, or receptacles of the Fucales, should have been collected some time previously and kept in the refrigerator for several hours or overnight, then rinsed thoroughly

but quickly in many changes of sterile sea water before being placed in the culture. The aim of this procedure is to get rid of any foreign spores and diatoms that might be present, but it is nevertheless very difficult to keep down diatom growth in cultures. Most of the spores will have been discharged by the next morning, whereupon the pieces of fruiting material should then be removed. The spores of most species will have germinated by the second or third day; growth at first is usually rapid so that within a week there is a brown layer over the bottom and sides of the culture vessel and on the slides placed therein. Keep the cultures in a cool location in moderate light.

Slides may be removed at any desired stage of growth of the germlings and plunged into 1% chrom-acetic in sea water. Fix for about 2 hours. Wash thoroughly in sea water, then transfer gradually to distilled water. Staining of both germlings and gametophytes may be with iron hematoxylin; but if details are obscured, treat for 1 hour with cold 1/N hydrochloric acid, rinse with distilled water, and apply Harris' hematoxylin. After either hematoxylin, counterstaining may be with orange G. Before proceeding with the staining, determine on which side of the slide the best or desired plants occur, mark this side so that the growths will not be accidentally wiped off, then clean the other side of the slide and the edges.

Two methods are available for dehydrating and mounting the slides. The germlings and gametophytes are easily plasmolyzed by too violent changes of fluids.

1. Place the slides in a large, shallow flat-bottomed dish containing an ample amount of 7% glycerin to which a trace of formaldehyde has been added (G. J. Hollenberg, unpublished). The reason for the formaldehyde is that fungal growths occasionally appear in the glycerin. Place the container in a warm place, protected from dust, for the glycerin to concentrate. Remove the glycerin with 95% and absolute alcohol, as in the glycerin method (p. 119), and follow with a graded series of absolute alcohol and xylol mixtures, allowing 30 minutes in each, until the material is in pure xylol. Return the slides again to a large flat-bottomed dish containing a little xylol. This time place the slides with the attached gametophytes on the lower side, and support each end of the slides by means of glass rods or thin slides. Carefully add an ample quantity of 3% balsam in xylol, and allow the solvent to evaporate in a warm dust-free place. Just before the balsam becomes of a mounting consistency, remove a slide, quickly wipe the balsam from the back and other exposed portions of the slide with a cloth moistened with xylol, then add a coverslip.

2. A faster and equally good procedure is to dehydrate with hygrobutol after the staining has been completed. Place the slides in a suitable

container in water or in alcohol, if they have already been partially dehydrated, and add small quantities of hygrobutol at a time. Do not add too much hygrobutol at a time, and allow at least 30 minutes between additions. Pour off some of the mixture from time to time as the proportion of hygrobutol increases. Finally give two changes of pure hygrobutol, and mount in thin balsam. However, if plasmolysis results upon mounting, which is not likely since the hygrobutol will harden the specimens somewhat, place the slides in 5% balsam in hygrobutol, then evaporate the solvent, and mount as described in the first procedure outlined above.

Whole Mounts.—Filamentous forms such as *Pylaiella*, *Ectocarpus*, *Streblonema*, and *Sphacelaria*, frond tips of *Desmarestia*, *Myriogloia* and similar forms, gametophytes of the Laminariales if loose rather than attached to slides, etc., are perfectly fixed in 10% formalin in sea water. If a chrom-acetic fixation image is desired, use the standard formula given below, but for the more delicate forms, dilute it considerably with sea water.

Whether fixed with formalin or chrom-acetic, wash out the fixative thoroughly with sea water, and transfer the material through (1) a mixture of 75 parts sea water and 25 parts distilled water, (2) equal portions of sea and distilled water, (3) 25 parts sea water and 75 parts distilled water, allowing the material to remain in each mixture for at least 1 hour. The transfer to plain water may be completed by washing in several changes of distilled water, whereupon the material is ready for staining. Iron hematoxylin may be recommended for most species (but not for those with thick thalli), with a counterstain of orange G. Differentiation of the hematoxylin is more satisfactory with a 1% solution of ferric chloride. Or one may use Harris' hematoxylin, especially for reproductive phases, particularly when a sharp, transparent stain is required for revealing internal details of reproductive phases. For example, whole mounts of the thalli of *Myriogloia* with all stages in the development of the zoosporangia are very satisfactory after this stain. A counterstain should be used on filamentous species, but it is usually better not to employ one on thick thalli. Dehydrate with hygrobutol, and transfer to highly dilute balsam. Plenty of material of the filamentous species should be mounted on each slide as stages in the development of the reproductive bodies are usually none too abundant.

Fixation and Embedding.—With the exception of species belonging among the Fucaceae, the Phaeophyta are exceptionally easy subjects technically, this statement embracing all forms from those that are tiny filaments up to the giant kelps of the Pacific Coast. The Fucaceae are very troublesome to manipulate because of the excessive slime content of the thalli.

Two standard fixing fluids are applicable to the majority of the Phaeophyta and will be found to meet practically all needs. The simplest is a 10% solution of formalin in sea water, in which the material should be allowed to remain for at least several days before being subjected to further treatment. The second fluid is a 1% chrom-acetic in the following proportions:

Sea water (filtered)	100 cc.
Chromic acid	1 g.
Glacial acetic acid	1 cc.
Saponin	0.5 g.

If a quantity of the stock solution is to be made up, add the saponin only as needed. Material should be allowed to remain just sufficiently long to become properly fixed; wash out thoroughly with copious quantities of sea water. For many softer forms and for those which are delicately filamentous, the fluid should be diluted up to one-half its volume with sea water. Such forms are sufficiently fixed in about 4 hours, but portions of the thick fruiting laminae of the Laminariales need to remain in the fluid for about 24 hours. The transfer from sea water to a paraffin solvent is readily effected by a graduated series of fluids, as noted in the following table (the volumes are in cubic centimeters):

Sea water	90	80	65	50	35	20
Distilled water	5	10	20	30	35	40
95% ethyl alcohol	5	10	15	20	30	40

From the last mixture proceed to the 50% solution of the tertiary butyl alcohol method. Since the tissues are readily penetrated by the fluids, changes may be a little more rapid than usual: 1 hour is enough. Only sufficient fluid to cover the material completely is required; it should then be discarded and not used over again. Dioxan has proved to be very unsatisfactory for the Phaeophyta, as it is nearly impossible to remove it completely during infiltration. Xylol and similar fluids will harden the tissues excessively. The time in the paraffin oven needs to be as short as possible since the heat of the oven has a deleterious effect upon the tissues. The paraffin penetrates rapidly; consequently the time in the oven may be half that ordinarily required for vascular plants.

Microtoming.—All phaeophytean tissues are quite easily sectioned. Very few are so hard that they cause trouble; such tissues might better be embedded in celloidin. Among these are the hard stipes of *Cystoseira* and old stipes of the larger Laminariales. Sections of embedded material as thin as 2 and 3μ are readily cut, provided a paraffin of extremely fine consistency (such as Parlax) is employed for the embedding.

Staining.—The Phaeophyta, because of the nature of their cell-wall structure, are not easy to stain adequately and sharply by ordinary procedures. The cell walls are composed principally of cellulose, and the gelatinous portion is a pectic compound know as "algin." The proportion of the two substances varies greatly both according to the species and to the tissues. The walls and cytoplasm as a rule both stain deeply and quickly and are very hard to differentiate if overstained. Care must also be taken not to allow the tissues to remain too long in killing fluids containing chromic acid since the tissues are readily overchromated and bleaching is useless. Overchromated tissues are notoriously difficult to retain on the slides, and this is particularly true of the brown algae. Except for sections of blades with zoosporangia, it is always advisable to avoid going below 70% alcohol during the staining process, but a momentary rinsing in water to remove excess stain usually does not cause the sections to float off.

Combinations of stains commonly used on stems, leaves, roots, etc., of the vascular plants are entirely useless on the Phaeophyta. There is, for example, no xylem in these plants; consequently the use of safranin would serve no purpose. Other combinations are therefore necessary.

A combination that gives excellent results and rather good differentiation on anatomical material is Bismarck brown and fast green. Deparaffin the slides and bring down to 70% ethyl alcohol. Place in a 1% solution of Bismarck brown in 70% alcohol for 20 minutes (if left in the stain for longer than 30 minutes, it becomes impossible to extract the stain). Remove slides individually and wash for 5 to 10 seconds in each of two jars of 95% alcohol. (After the first jar becomes too saturated with excess stain, discard this alcohol, replace with the alcohol from the second jar, refilling the latter with fresh alcohol.) Remove the slide from the alcohol, quickly wipe the underside dry with a clean cloth, then apply the fast green from a dropping bottle (the dye should be a methyl cellosolve-clove oil-alcohol solution), and allow to remain for about 5 to 8 seconds. Differentiate for a few seconds in a mixture of 2 parts clove oil and 1 part each of absolute alcohol and xylol, pass through a xylol wash, thence into pure xylol, and mount in balsam. In sections of the stipe of *Macrocystis* the sieve tubes and plates take the green and all other structures the brown in shades varying from pale straw to dark brown.

Zoosporangia are best stained with iron hematoxylin and orange G. Mordant for 20 minutes, and leave in the hematoxylin for not longer than 2 hours; it is very easy to overdo the staining. If it appears to be impossible to obtain a clear differentiation with the hematoxylin, advantage may be taken of the affinity of zoospores for acid fuchsin. The zoosporangia of *Postelsia*, which do not take a good hematoxylin stain,

are clearly stained by the fuchsin. Use a 1% solution of acid fuchsin in 70% alcohol and stain for not longer than 20 minutes. Rinse the slide by plunging two or three times in tap water, then pass through two jars of 95% alcohol (strong alcohol has little effect on the stain, hence the necessity for rinsing in water). Then counterstain with fast green as described in the preceding paragraph.

Antheridia and oögonia, microspores and macrospores, and similar reproductive bodies should be stained with iron hematoxylin, although the male elements occasionally take a better stain with Harris' hematoxylin. If a counterstain is desired, use orange G.

Taxonomy.—The classification of the Phaeophyta is now based upon the life cycle (Kylin 1933, G. M. Smith 1938, Taylor 1937), and this scheme has been adhered to in the following discussion.

ISOGENERATAE

Two similar generations alternate; they are identical in vegetative structure.

Ectocarpales

A large number of families were formerly included in the order, but as the life histories of the various species became better known, they were transferred to other orders. This has incidentally made possible a greater uniformity in technical procedures.

Ectocarpaceae.—The species in the family are all filamentous forms, commonly epiphytic on other algae. If large enough and sufficiently abundant, remove from the host or other attachment. If too small, work up both host and epiphyte together, and scrape the latter off with needles when ready to mount; or the two may be brought into paraffin and sectioned together. For whole mounts fixation is superior in a weak chrom-acetic fluid. *Ascocyclus, Streblonema* and some species of *Ectocarpus* bearing sporangia on the prostrate filaments are better sectioned than mounted entire. Iron hematoxylin with a light counterstain of orange G is the most pleasing stain combination, but care should be taken not to leave plurilocular sporangia overstained. Iron hematoxylin, for some reason, does not always stain some species sharply; in such cases, resort to differential acidification, and stain with Harris' hematoxylin and orange G. This combination is especially good for critical cytological investigations (Fig. 33).

In *Pylaiella* the sporangia are mostly catenate, usually intercalary in position. Some species have a strong tendency to become brittle during dehydration for whole mounts; consequently the process should be rather gradual.

Ralfsiaceae.—The flat, crustaceous thalli may be scraped off the rocks on which they grow, all the debris possible washed away, and the specimens embedded and sectioned.

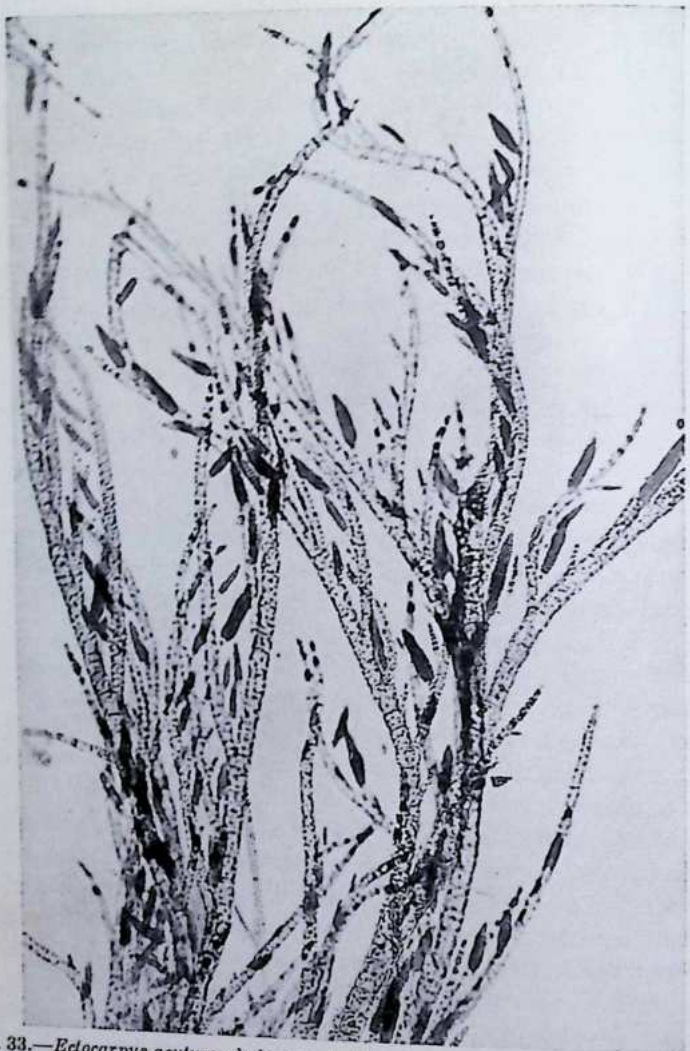


FIG. 33.—*Ectocarpus acutus:* whole mount of portion of mature plant with gametangia. Fixed in 10 % formalin in sea water, ionized with HCl, stained with Harris' hematoxylin and orange G, dehydrated with hygrobutol and mounted in balsam.

Heterochordariaceae.—*Heterochordaria* is one of the commonest brown algae on the Pacific Coast. The plants are too thick for whole mounts but are easily embedded and sectioned. Microtome transverse sections of the ramuli for the gametangia at not over 6μ and stain sharply with iron hematoxylin and orange G.

Sphacelariales

The family Sphacelariaceae is the only one, members of which are likely to be found in the United States. The plants occur rather rarely.

Most members of the order are filamentous plants ranging from 4 mm. to 5 cm. in height, all species being characterized by prominent apical cells. The plants become polysiphonous in the older portions. In some species of *Sphacelaria* lateral branches are converted into propagulae. Permanent whole mounts are best made by the hygrobutol method, after staining with iron hematoxylin. The counterstain should be applied in very dilute solution since the tissues have an unusually strong affinity for acid stains. In *Sphacelaria* and *Stypocaulon*, the latter occurring only on the north Atlantic Coast, mitoses are conspicuous. At midnight or shortly thereafter, fix portions of the tips about 6 mm. long in 1% chrom-acetic, embed, and section longitudinally, perpendicular to the flat surface, at 3μ. Stain critically with iron hematoxylin, and counterstain very lightly with orange G.

Cutleriales

Cutleria is said to occur in Florida; otherwise the order is not represented in the United States. (For technical methods, consult Yamanouchi 1909, 1912.)

Dictyotales

The Dictyotales differ from the other orders in the presence of aplanospores and in a heterogamous method of reproduction. Members of the one family, Dictyotaceae, prefer the warmer southern waters, and representatives occur on both coasts.

In *Dictyota* growth is by means of an apical cell; in the other genera it is by division of marginal cells. The apical end of the thallus of *Dictyota* should be sectioned parallel to the flat surface, the thalli of the other genera should be cut perpendicular to the flat surface. Sections should be cut at about 12μ; staining is superior with iron hematoxylin with or without a counterstain of orange G or erythrosin. Many people make whole mounts of the apices of the thalli.

The aplanospores, oögonia, and antheridia are arranged differently in the various genera; a knowledge of their location or the nature of their distribution is therefore necessary when fruiting material is desired. The antheridia and oögonia, however, are usually in dense sori and always project beyond the surface. The sporangia are usually scattered, but in *Neurocarpus*, for example, they are arranged in sori along either side of the midrib. The oögonia and antheridia may be on the same frond or plant or on different individuals and disposed in some definite arrange-

ment or scattered. In many species one is more likely to find aplanospores than antheridia or oögonia. In order to obtain the best fixation of the critical stages in the development of the antheridium and oögonium of the forms with thick thalli, a chrom-osmo-acetic fluid is preferable. The following formula has proved to be satisfactory with *Dictyota*, *Padina*, and *Zonaria:*

1% chromic acid in sea water	10 cc.
2% osmic acid in 2% chromic acid	0.05 cc.
Glacial acetic acid	0.5 cc.

The centrosomes and radiations should show up clearly after staining with iron hematoxylin and no counterstain.

In *Zonaria*, which is common on the southern California coast, an astonishingly large number of apical cells are readily obtained, and developmental stages are easy to follow out. The following formula has been used:

Chromic acid	1 g.
Glacial acetic acid	3 cc.
1% aqueous osmic acid	1 cc.
Sea water	96 cc.

HETEROGENERATAE

Two dissimilar generations alternate. The sporophytic is usually macroscopic, the gametophytic microscopic.

Haplostichineae

Growth is trichothallic, the thallus being composed of one or more filaments and their branches.

Chordariales

Practically all material of the Chordariales that one collects is the sporophytic generation; the gametophytes are known only from cultural studies. The cultural methods cited in the introductory paragraphs to the Phaeophyta may be followed to obtain the gametophytic phases.

Chordariaceae.—*Chordaria*, the more prominent one of the several genera, grows farther south on the Atlantic Coast than on the Pacific. Sections of the cylindrical fronds should readily reveal the zoosporangia.

Coliodesmaceae.—*Coliodesme* is common on the Pacific Coast. It usually grows on large specimens of *Cystoseira* or *Cystophyllum*. Portions of fronds of various developmental stages may be cut out, embedded, and sectioned in the vertical plane to show the unilocular zoosporangia.

Elachisteaceae.—The species are pulvinate, microscopic in size, and epiphytic on various larger algae. Work up portions of the host bearing

the epiphytes by the hygrobutol method, finally scraping off the filaments carefully, and mount in balsam.

Myrionemataceae.—The thallus consists of a prostrate disk, composed of radiating filaments more or less closely united and not more than two cells in thickness, and erect filaments, which may be free or united in a common jelly. All the genera grow upon other algae or on marine Angiosperms; the pneumatocysts of *Macrocystis* are particularly favored by species of *Compsonema*. Cut out portions of the host bearing the epiphyte, embed, and section in the transverse plane of the host tissues (Fig. 34).

Leathesiaceae.—Most of the species grow on rocks, a few on other plants. The thallus is thick, carnose, and composed of filaments held

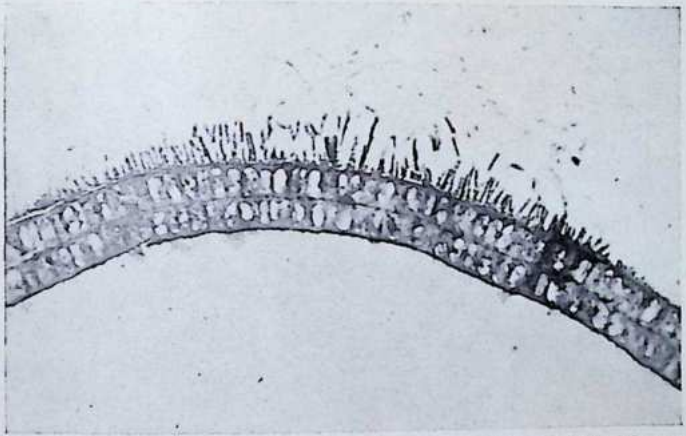


FIG. 34.—*Myrionema strangulans:* section of a young colony on *Ulva lobata*, with two young zoosporangia in the center. Fixed with chrom-acetic in sea water; stained with iron hematoxylin and orange G.

together in a thick jelly. Cut portions of the thallus into small pieces, embed, and section.

SPOROCHNALES

Sporochnaceae.—Two species of *Sporochnus* occur rarely on the Atlantic Coast from South Carolina southward. Whole mounts may be made of portions of the branches with terminal filaments; otherwise sections are indicated.

DESMARESTIALES

Myriogloiaceae.—Sections of embedded portions of the filaments should show the unilocular zoosporangia. Prepare whole mounts of the tips of the branches to show their interesting structure; stain with Harris' hematoxylin (Fig. 35).

Desmarestiaceae.—Members of the family occur on both coasts, *Desmarestia* being the most widespread genus, and certain of the species have the characteristic peculiarity of turning a verdigris green on drying and of bleaching other algae when coming into contact with them. Decomposition commences almost as soon as the plants are taken from the ocean. For this reason, when preserving material of the genus, the plants should be placed in a quiet pool, and the desired tissues removed and immediately immersed in the killing fluid. Portions of the stipe

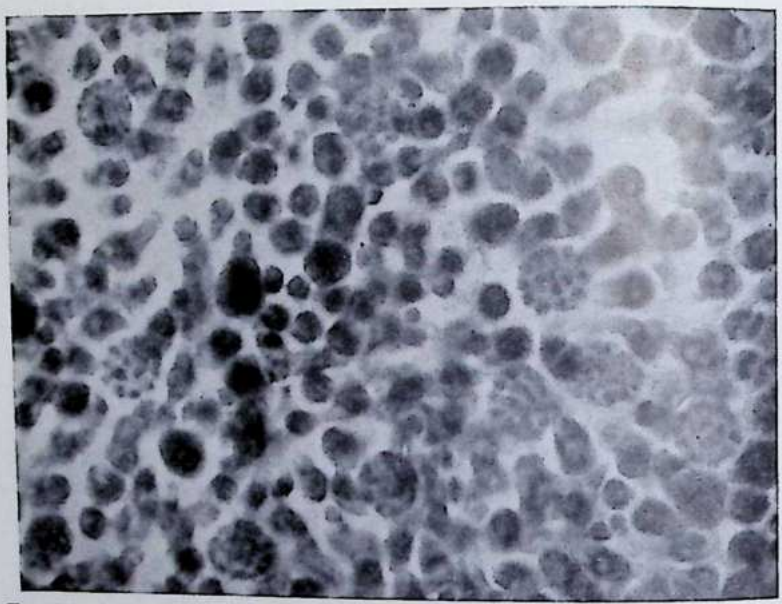


FIG. 35.—*Myriogloia andersonii:* portion of a whole mount of thallus with radiating filaments and zoosporangia. Fixed with 1 % chrom-acetic in sea water; stained with Harris' hematoxylin, dehydrated with hygrobutol and infiltrated with balsam.

and frond are valuable for morphological study; whole mounts of the apices of the fronds to demonstrate the trichothallic apical growth are easily made. The zoosporangia are very difficult to locate, and one may have to examine a large number of fronds of fully mature plants before finding them; they probably appear only in the autumn.

Polystichineae

Growth not trichothallic; thallus parenchymatous.

PUNCTARIALES

Stilophoraceae.—A single representative, *Stilophora rhizoides*, occurs from North Carolina to Massachusetts and fruits at the end of the summer. Whole mounts of the apices are possible, but sections should be cut for the reproductive structures.

Asperococcaceae.—The fronds are sometimes ligulate, but the more common species are saccate. *Soranthera* is a widespread Pacific Coast epiphyte (Fig. 36), occurring generally on *Rhodomela*. Plants in all stages of development are usually to be found on a single host plant; consequently it is not difficult to prepare sections showing all stages in the growth of the sori and zoosporangia. Section the thallus transversely

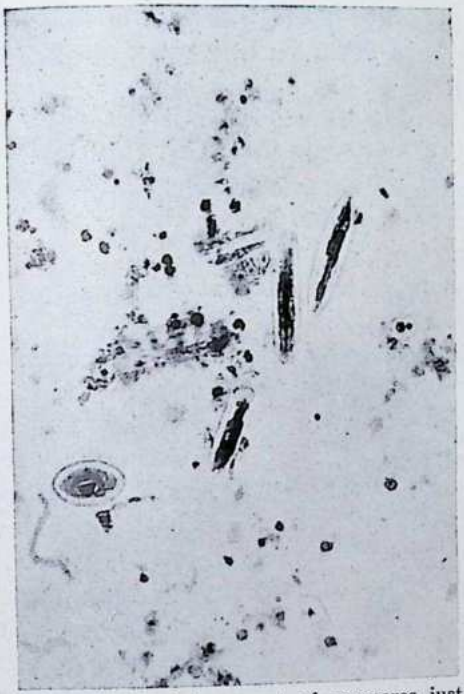


FIG. 36.—*Soranthera ulvoidea:* whole mount of zoospores just before germination. Diatoms are also present. Fixed with 10 % formalin in sea water; stained with iron hematoxylin.

at about 7μ. *Asperococcus*, equally common on the Atlantic Coast, is also excellent and may be dealt with similarly.

Punctariaceae.—The simplest forms in the family are monosiphonous; the more complex species are either filamentous and solid, saccate, or membranous. The zoosporangia and gametangia form definite sori. Whole mounts of portions of the thallus are easily prepared, but as they are useless for detailed studies, sections are advisable.

Scytosiphonaceae.—*Scytosiphon lomentaria*, the best-known species, occurs on both coasts and is easily found. It usually produces gametangia in the autumn. Cut the long frond into pieces about 5 mm. in length, embed, and section transversely at about 8μ. The frond is likely to be very densely covered with diatoms and other small organisms.

Laminariales

The order includes the giant kelps, largest of all known marine plants. The plants, as ordinarily seen, represent the sporophytic generation: the gametophyte is microscopic and in most of the genera is very much reduced. The fronds are usually differentiated into three regions: (1) a holdfast, varying from discoidal in some species to clusters of simple or branched hapteres in others; (2) a stipe, cylindrical or more or less flattened and simple to dichotomously or irregularly branched, and (3) one or more flattened blades. The zoosporangia may be borne either on the typical blades or on specialized sporophylls and are generally grouped into sori of considerable extent. Growth takes place in most species in a meristematic tissue intercalated between the blade and stipe; in *Nereocystis* growth occurs throughout the entire plant.

There is no necessity for dealing with the families or genera separately since the same technique serves for the entire group with respect to the various structures.

Holdfast.—Cut out small portions of the main part; or in the case of branching hapteres, remove pieces of both the older parts and the apical end. Embed, and cut both longitudinal and transverse sections at 10μ. There is little or no tissue differentiation. A single stain suffices: a 1% solution of Bismarck brown in 70% alcohol or a 0.2% solution of fast green in 95% alcohol will stain deeply enough in a few minutes. Wash quickly in 95% alcohol, clear, and mount.

Stipe.—In some species the stipe may attain a diameter of more than 15 cm., thus becoming so large that portions must be cut out for easier manipulation. It is easy to cut such material in the fresh condition with a sliding microtome, exactly as in the case of stems. However, it will be better to embed since the cells are so small that rather thin sections are indicated. The solid portions of the hollow stipes of *Nereocystis* and *Postelsia* average 1.2 cm. in thickness, but it is not in the least difficult to embed and section these parts. The stipes of the other genera are decidedly variable in their internal structure.

Mucilage ducts are present in some, absent in others. When present, there may be some difficulty in fixation; the remedy is to increase the percentage of acetic acid in the fixative. As the sections are retained on the slide only with great care, it is advisable to cover them with a thin film of celloidin before removing the paraffin with carbol-xylol.

The stipe of *Macrocystis* is peculiarly interesting because of the presence of large sieve tubes and sieve plates. Embed, and cut transverse sections at 14μ and longitudinal sections at 12μ. On account of the more or less accentuated twisting of the stipe, the longitudinal sections will rarely show entire sieve tubes and on the whole are usually disappointing. Stain with Bismarck brown and fast green.

The stipes of *Pterygophora* and those of some species of *Alaria* exhibit concentric rings. In a few species the stipe becomes too cartilaginous to be sectioned readily in paraffin. Resort might be had to celloidin. The stipes of the algae cannot be soaked in water in order to soften them previous to microtoming, as is frequently done with woody stems, because they will promptly swell up and disintegrate.

Blade and Sporophylls.—Despite their rather thick and tough, leathery character the blades and sporophylls of all members of the order present no difficulty whatever.

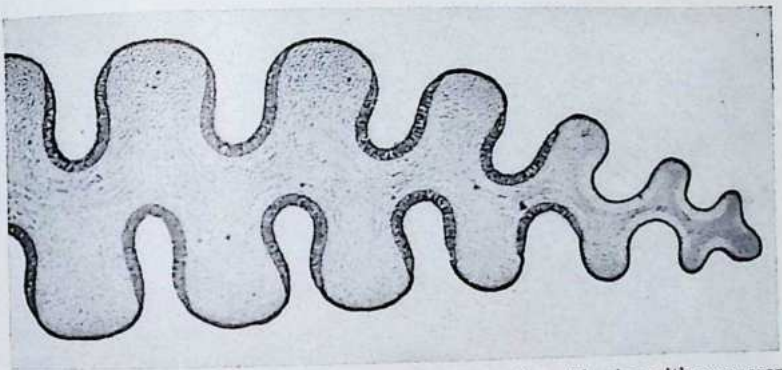


FIG. 37.—*Postelsia palmaeformis:* Portion of cross section of lamina with zoosporangia in the depressions. Fixed with 1 % chrom-acetic in sea water; stained with acid fuchsin and fast green.

When borne on the blade, the areas containing the sori appear a darker brown against the light; but in some forms such as *Postelsia* the presence of zoosporangia can be determined only by microscopic examination of small pieces of the blade. In the Alariaceae and in *Lessoniopsis* of the Laminariaceae the zoosporangia are borne not on the blade but on specialized sporophylls. The latter appear as a rule at certain seasons only and a careful watch must be kept for their appearance. Cut out small portions of the blade or sporophyll, taking care to obtain a series of stages from the youngest to the oldest, and embed in paraffin (Fig. 37). In *Laminaria* especially, the tissues tend to become very hard, but nevertheless they are surprisingly easy to microtome.

To show the origin and development of the zoosporangia and paraphyses, the sections should not be thicker than 3μ. Staining is preferably with iron hematoxylin alone. On thicker sections, for general morphology, acid fuchsin and fast green, or Bismarck brown and fast green, should be employed.

Gametophytes.—These are obtainable only by special culture methods, which can be readily carried out only at a properly equipped marine station. Methods are described in the introductory paragraphs to the Phaeophyta.

Cyclosporeae

A diploid generation only is present.

Fucales

The order occurs on both coasts of North America. It contains two families: Fucaceae, in which the fronds are flattened in two ranks in one

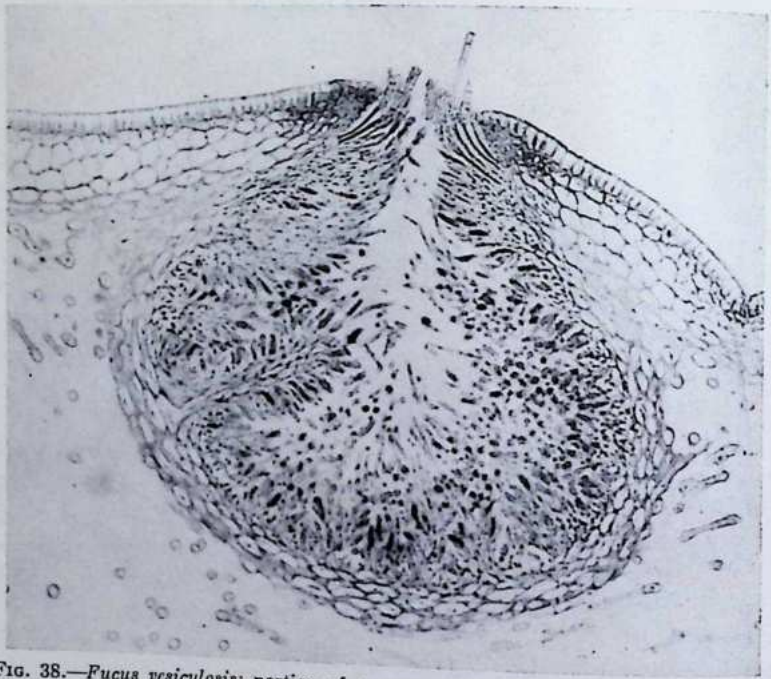


Fig. 38.—*Fucus vesiculosis:* portion of a cross section of a receptacle with a microsporangial conceptacle. Fixed with chrom-acetic in sea water; stained with iron hematoxylin and orange G.

plane without differentiation into axial and lateral branches, and Sargassaceae, in which the branches arise on all sides of the main axis.

Fucaceae.—*Fucus* is unquestionably the most widely studied of all Phaeophyta but is exasperatingly troublesome to the technician because of the chemical nature of the mucilage. The pieces of frond are usually left hard and "dry" by the usual fixation and dehydration methods; the macrosporangia are too often completely disrupted.

Fixation may be in the following fluid:

Chromic acid	1 g.
Glacial acetic acid	3 cc.
Glycerin	10 cc.
Sea water	90 cc.

Wash with 10% glycerin in sea water, then place in 10% glycerin, and set aside for the water to evaporate. Wash out the concentrated glycerin with a mixture of equal parts of 95% alcohol and tertiary butyl alcohol, then with a short series of mixtures of these two alcohols in which the butyl alcohol is gradually increased. Finally give two changes of pure tertiary butyl alcohol, and infiltrate with paraffin. Or one might, after washing out the glycerin thoroughly with the mixture of equal parts of

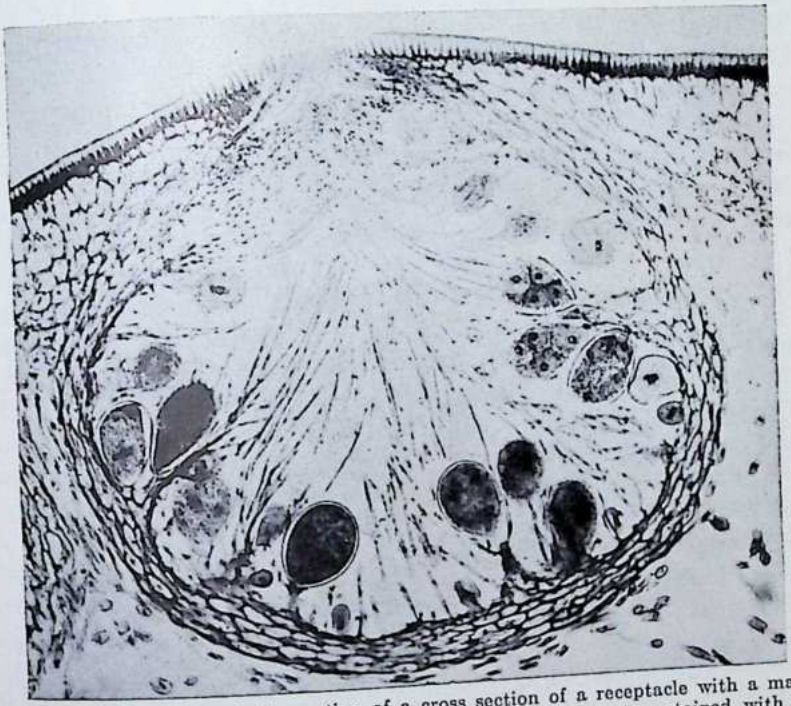


FIG. 39.—*Fucus vesiculosis:* portion of a cross section of a receptacle with a macrosporangial conceptacle. Fixed with 1% chrom-acetic in sea water; stained with iron hematoxylin and orange G.

the two alcohols, go to a mixture of equal parts of absolute alcohol and tertiary butyl alcohol (giving at least three changes to make certain that all glycerin has been removed), thence to one of equal parts of absolute alcohol, tertiary butyl alcohol and ether, and infiltrate with celloidin.

For the younger stages of micro- and macrospore development, the very tips of the fronds should be selected for fixation. On the Atlantic Coast, *F. vesiculosis*, a dioecious species, predominates. On the Pacific Coast, *F. furcatus* is a widespread monoecious species. Botanists on the east coast prefer the first-named species, whereas those on the west coast use the monoecious species in the classroom. From the technical standpoint, *F. furcatus* is the more difficult of the two. For the first

8 to 10 mm. of the tips, the sections should be microtomed in the longitudinal plane, perpendicular to one flat face, at 10μ. Older portions should be cut transversely at about the same thickness. Stain with iron hematoxylin and orange G (Figs. 38, 39).

For demonstrating the apical cell of the Fucaceae with its five cutting faces (four lateral and one posterior) and for the origin and development of the conceptacles, nothing is better than *Pelvetia fastigiata*. The conceptacles originate quite close to the apical cell (many tips will have two adjoining apical cells); the one-celled to several-celled stages are usually found in preparations showing the apical cell or cut for this structure. Remove the anterior 2 mm. of the apices of vigorously growing young fronds, taken preferably from plants which show definite indications of being in the fruiting condition. Fix with 1% chrom-acetic, and embed by the standard tertiary butyl alcohol method. Section the tips transversely, parallel to, and perpendicular to the flat surface in order that the apical cell may be observed in different views. This method ought also to show several stages in the development of the conceptacles; as these are scattered irregularly over the thallus, they can be obtained only in a hit-or-miss fashion. Sections should be at 8μ, and staining is precise with iron hematoxylin and orange G.

If one is located in the Middle West or far from the coast, that need be no deterrent to working with living material of *Fucus* or of many other Phaeophyta. During cold weather material will travel safely from either coast.

Sargassaceae.—Probably all the genera within the confines of the United States possess fronds provided with vesicles, which constitute an easy character for distinguishing the genera. In *Sargassum* the vesicles occur singly; in the other genera they are seriate. *Sargassum* for the most part prefers the warmer seas, but at least one species ranges as far north on the Atlantic Coast as New England. *Cystoseira* is to be found farther north on the Pacific side than on the Atlantic (Florida). Standard methods are applicable to these two genera. In *Cystoseira* the conceptacles are formed in the more or less metamorphosed terminal branchlets; in *Sargassum* they are borne in specialized axillary branches, which are slender and more or less forked. *Cystoseira* is scarcely less favorable than *Pelvetia* for illustrating the origin and development of the conceptacles. In *Sargassum* it is known that the development of the oögonia in general is periodic and simultaneous. Desired stages can be found only at certain hours on definite days. Meiosis can be found in the oögonium in the daytime as well as at night.

In most of the genera, but particularly in *Cystoseira*, the macrospores contain numerous large chromatophores which take a deep stain with iron hematoxylin and are difficult to differentiate clearly.

CHAPTER XXIII

CYANOPHYTA

MYXOPHYCEAE (CYANOPHYCEAE)

The Myxophyceae are of widespread occurrence (Geitler 1930–1932, G. M. Smith 1933) and one cannot fail sooner or later to come into contact with them. There are only three things about them to annoy the technician: (1) to get any particular free-living species, with certain exceptions, in a sufficiently pure condition; (2) to get enough material of such forms; and (3) the primitivity of the nuclear organization, which frequently makes exact stain differentiation difficult. If these do not present too great annoyances to the technician, the group will be found to be decidedly interesting, and preparations of any type will be most valuable acquisitions to one's collection of microscope slides.

Before starting to work up any species, it should be carefully studied in the living condition. Generic and specific determinations are, as a rule, best made on living material, supplemented where necessary by simple chemical tests.

A few Cyanophyta are unicellular, but most of them form filamentous or other types of colonies. In filamentous colonies, a single row of cells is called a "trichome"; the trichome together with the gelatinous sheath is called a "filament." Some filaments may contain more than one trichome.

The cell wall is composed of cellulose or hemicellulose. Earlier reports that the wall is chitinous have been disproved by recent investigations. The gelatinous sheath is composed mostly of pectic compounds.

A definite nucleus or nucleolus is absent, but the central body, which takes up most chromatin stains, contains nucleic acid and therefore may be considered as being nuclear in nature. The reserve foodstuffs are mostly glycoproteins but include other proteins and also oils.

Occurrence.—Cyanophyta are to be found everywhere in an astonishing diversity of habitats, but they are principally fresh-water organisms. Pure growths, however, are rarely found; these are usually species of either *Anabaena*, *Nostoc*, or *Oscillatoria*. The habitats of the more prominent forms will be described in the discussions under the orders.

Cultivation.—Many Cyanophyta are easy to culture. Knop's, Benecke's, or Detmer's solutions, diluted to about 0.2/N strength, are excellent culture media. Other fluids, such as artificial sea water, Wettstein's fluid (Wettstein 1921), or the following may be used:

Tap water	100 cc.
Ammonium nitrate	0.02 g.
Dibasic potassium phosphate	0.05 g.

To obtain soil Myxophyceae, inoculate a medium consisting of 100 cc. water and 0.02 g. dibasic potassium phosphate (in a 3-liter flask) with 1 or 2 g. garden earth. Place in partial sunlight at 16 to 20°C. Or make the following solution and inoculate in the same manner:

Water	1 liter
Neutral potassium phosphate	0.2 g.
Magnesium sulphate	0.2 g.
Potassium sulphate	0.2 g.
Calcium carbonate	0.1 g.
Ferric chloride	Trace

Preservation of Specimens.—Herbarium specimens are easily prepared. Simply spread and dry the specimens on pieces of mica or thick cellophane. They may be wetted when it is desired to examine them microscopically, and then allowed to dry again. The process may be repeated indefinitely. Habit material may be preserved in 3 or 4% formalin.

Fixation.—Fixation, in general, is easily accomplished. The simplest fluid is a 3 to 5% formalin solution, in which the algae should remain for a week or longer (Haupt 1923). Bouin's fluid is said to work well. The material may be dehydrated by any desired method and embedded in paraffin. Sections should be cut at 2μ for detailed cytological investigations.

Mitochondrial methods are wholly unsatisfactory (Guilliermond 1926).

Staining.—Staining may be for one of three purposes: (1) general, (2) for identification of various cell contents, or (3) to outline the gelatinous structure. It is rarely possible to accomplish all three simultaneously.

General staining, in turn, may be vital or on killed material. For vital staining very dilute aqueous solutions of either neutral red, cresyl blue, or toluidin blue are most useful. Preserved or microtomed material will be more or less stained by almost any of the coal-tar dyes; methylene blue is often used.

Volutin may be identified by placing the material in 0.1% aqueous methylene blue and, after the stain has reacted, adding a little 1% sulphuric acid; a deep blue or black color results.

Plasmodesma, if present, may be demonstrated with a mixture of 6 cc. of a concentrated alcoholic solution of basic fuchsin and 100 cc. of 3% aqueous phenol. Place some of the filaments on a slide in a large drop of the solution, and heat gently over a flame. The protoplast will

shrink and stain an intense red, but the plasmodesma will be clearly revealed.

For internal details iron hematoxylin surpasses all other stains in the quality of the results obtained. Counterstaining may be with erythrosin, fast green, or anilin blue. It would also be productive of valuable results to try Feulgen's reaction. Picro-indigocarmin gives a superb and occasionally quite life-like stain to many species.

The gelatinous envelopes may be demonstrated by using moderate concentrations of the counterstains mentioned above, or of crystal violet, ruthenium red, toluidin blue, or methylene blue.

Whole Mounts.—All the Cyanophyta, with the exception of those which form huge gelatinous colonies (such as *Aphanothece*, *Aphanocapsa*, and *Coelosphaerium*) and those which invade other plants, are easily mounted entire. Stain as desired, dehydrate by a gradual hygrobutol method or glycerin concentration procedure, and infiltrate with very highly diluted balsam. A very short schedule, satisfactory for all but critical studies, consists simply of placing a drop of formalin-fixed material to dry on a clean slide and proceeding directly to the staining, mounting in balsam from xylol. Of course, one can mount directly in glycerin or glycerin jelly without staining.

Chroococcales

Chroococcaceae.—*Gloeocapsa* and *Gloeothece* may be treated as dried mounts, as they will stick readily to the slip, whether or not Mayer's adhesive is used. *Merismopedia* is frequently found; the flat colonies are easily stained and mounted. Stain some colonies with iron hematoxylin, others with an alcoholic carmin stain or with picro-indigocarmin, and mount some of the different colorations under the same cover.

Aphanocapsa, *Aphanothece*, *Microcystis*, and *Coelosphaerium* are difficult subjects; quite apart from this fact, many algologists claim that these genera are not worth mounting. The writer has found *Microcystis ichthyloblobe* to be comparatively easy to manipulate; stain with Mayer's carmalum. The others can probably be mounted in glycerin only, as all attempts to get them into balsam or paraffin have met with failure because of the complete collapse of the gelatinous matrix. Dissect the larger colonies into fragments before proceeding with the staining or dehydration.

Chamaesiphonales

Dermocarpaceae.—*Dermocarpa*, a marine genus, is common on both the Atlantic and Pacific coasts. On the west coast *Rhodomela* is almost certain to be liberally encrusted with *D. fucicola* (Fig. 40). Embed not too old portions of the host, cut transversely at 8 to 10μ, and stain with

iron hematoxylin and erythrosin. Reproduction is by means of endospores, whose development is easily followed out, since a series of stages are usually found in each preparation. (Some writers describe the endospores as gonidia, produced in an enlarged cell which functions as a gonidangium.) *Ceramium* is frequently found covered with *Dermocarpa* on the older filaments; host and epiphyte may be mounted together as whole mounts. On the Atlantic Coast, *D. prasina* is abundant on different algae and may be treated like the west coast species.

FIG. 40.—*Dermocarpa fucicola:* section of two colonies on *Rhodomela larix*, showing the formation of endospores. Fixed with 1 % chrom-acetic in sea water for 10 minutes; stained with iron hematoxylin and erythrosin.

Xenococcus grows, usually in profusion, on other marine algae, which are small enough to permit the epiphyte and its host to be worked up together and mounted entire. If the staining is critical enough, such preparations are just as good as sections.

HORMOGONALES

Oscillatoriaceae.—The best-known genus is *Oscillatoria*, although there are others which are perhaps rather more interesting. *Spirulina*, *Arthrospira*, *Lyngbya*, *Phormidium*, and *Microcoleus*, all of which have fresh-water, brackish-water, and marine species, are of the greatest value both for technique and for instructional purposes.

These forms may all be treated technically according to the general methods. The cells of the larger species, especially in *Oscillatoria*, are rather liable to collapse if too violent changes of fluids are made. Those growing in warm salt water should also be handled cautiously; it would be well to warm the killing fluid to the temperature of the water in which they are growing, and let it cool slowly. It would be interesting

to compare the fresh-water and marine species when prepared by identical procedures.

Nostocaceae.—*Anabaena* and *Nostoc* are cosmopolitan forms and are readily obtained in abundance and generally also in a pure condition. *Nostoc*, which sometimes is found in enormous gelatinous balls in the Rocky Mountain region and in British Columbia, seems to prefer cooler and more protected habitats than does *Anabaena*. The latter is usually

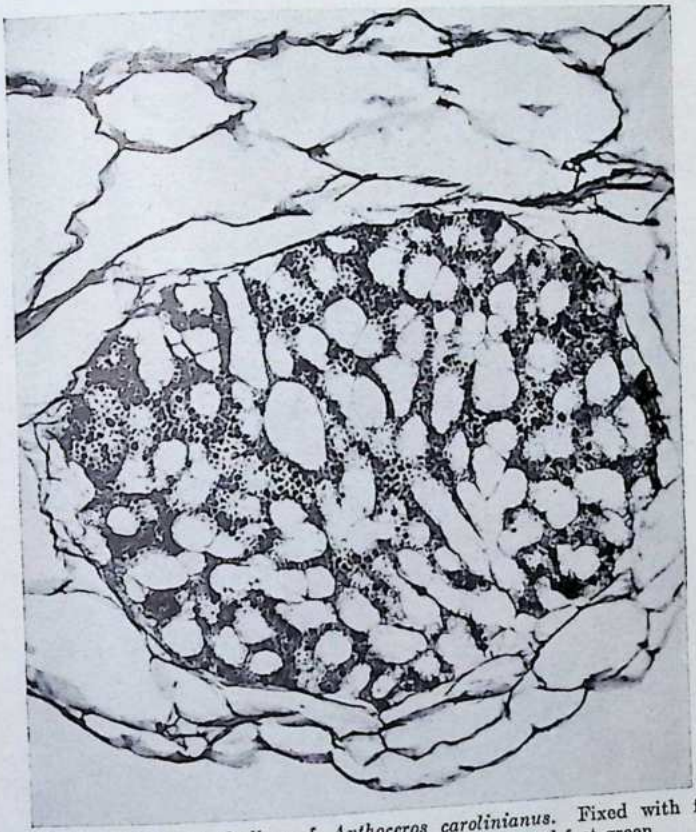


FIG. 41.—*Nostoc* colony in thallus of *Anthoceros carolinianus*. Fixed with formalin-propiono-alcohol; stained with iron hematoxylin and fast green.

a free-living aquatic alga, but *Nostoc* is sometimes found on damp soil. Two species of *Anabaena* are parasitic. *A. azollae* infests *Azolla;* material of *Azolla* fixed and stained as described for that genus will afford excellent preparations of the alga. Section the younger and vigorously growing branches in the vertical-longitudinal plane at 11μ; the alga will be found at even the very apex of the host and is easily identified if staining is in safranin and fast green. *A. cycadeae* inhabits the cortical tissues of the roots of various species of *Cycas*. Section portions of the embedded roots transversely at 10μ. The parasite is not easy to locate in most

roots. For whole mounts of free-living species, stain with iron hematoxylin or picro-indigocarmin. *Nostoc* may be terrestrial, aquatic, or parasitic, and occurs in the form of gelatinous nodules of various sizes, some becoming as large as 50 cm. across. The species found free floating or attached to various substrata in swiftly flowing streams are more

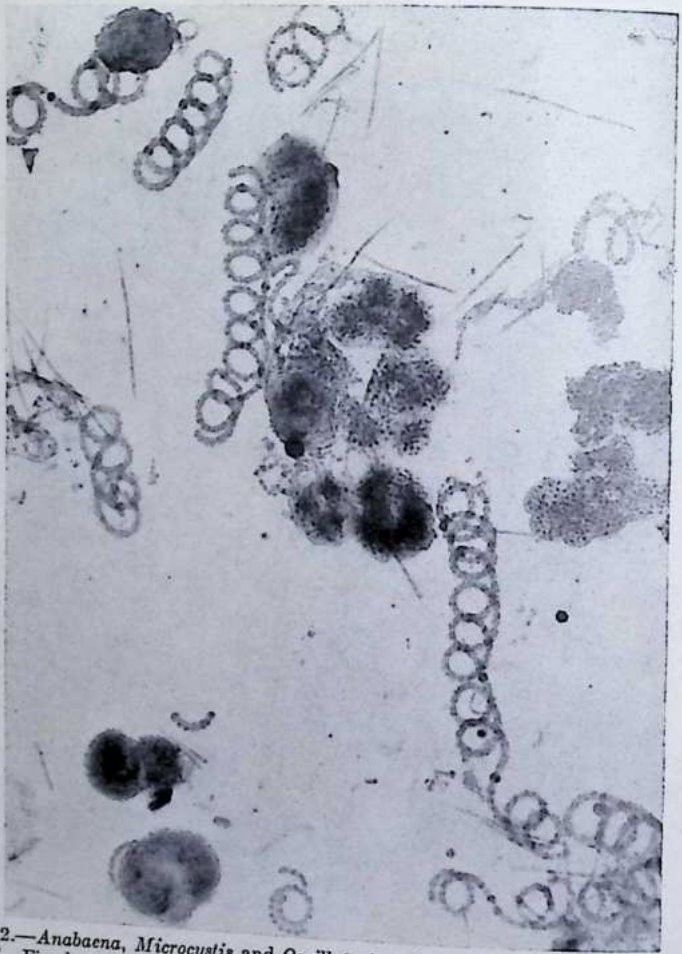


FIG. 42.—*Anabaena, Microcystis* and *Oscillatoria:* whole mount of the so-called "Wasserblüte." Fixed with formalin-aceto-alcohol; stained with iron hematoxylin and fast green.

easily manipulated technically than those growing on damp ground. Kill and fix in weak chrom-acetic, 6% formalin, or in formalin-aceto-alcohol. Small colonies may be run up entire and the colony crushed just before the coverslip is applied. Larger colonies are very easily embedded and sectioned for detailed investigations. Cut at any thickness up to 8μ. Stain critically in iron hematoxylin. A counterstain of acid fuchsin (1% in 70% alcohol) will differentiate the sheaths enclosing

the individual trichomes from the common gelatinous matrix; anilin blue is also excellent for the purpose and is perhaps less gaudy. *Anthoceros* and its allies are parasitized by *Nostoc*, which can be easily recognized in sections of the host thallus (Fig. 41).

Akinetes are most easily found in *Cylindrospermum:* they are developed next to the heterocysts at one end of each trichome and are sometimes found in a catenate series. Treat like free-living species of *Anabaena*.

Scytonemataceae.—Members of this family are easily recognized by their false branching. This character is shared by the Rivulariaceae, but in this family the trichomes are conspicuously attenuated and possess terminal hairs. Most of the genera are cosmopolitan in distribution. In *Scytonema* the filaments grow over damp soil or on dripping cliffs and are usually so interwoven as to form a felt-like mass of considerable extent. *Tolypothrix* and the remaining genera are aquatic.

All species may be treated alike. The hygrobutol method has been the most successful one. The basic stain is preferably iron hematoxylin. A counterstain is necessary to reveal the sheaths and the nature of the false branching. For this purpose orange G, fast green, acid fuchsin, or anilin blue may be employed. These algae are easily embedded and sectioned, but whole mounts are ordinarily adequate.

Stigonemataceae.—Genera belonging to the family are characterized by true branching. Heterocysts are always present, but akinetes are of rare occurrence. *Stigonema* is most likely to be collected. Treat as in the preceding family.

Rivulariaceae.—Cyanophyta which have trichomes conspicuously attenuated either from base to apex or from the center toward both extremities belong in this family. Each trichome usually terminates in a hair. There are both fresh-water and marine species, the latter predominating. Submerged species usually grow on the stems of other plants, within the gelatinous envelopes of other algae, or on rocks or wood.

Rivularia and *Gloeotrichia* are perhaps the best known of the genera, but others, such as *Calothrix* and *Dichothrix*, are quite as interesting and useful. The nodule-forming species may be treated like similar species of *Nostoc*, other species by the general methods for the phylum. The soft nodules of *Gloeotrichia* are very apt to become dissociated in the killing fluid. A drop containing dissociated filaments may be spread on a slide smeared with a thin layer of adhesive and fixed in 95% alcohol for a few minutes. Stain for 24 hours or longer in safranin, extract the stain with acidulated water until only the internal details remain clear, then counterstain with fast green to bring out the sheaths; or iron hematoxylin with any suitable counterstain may be used. Complete dehydration and mount in balsam.

Colonies of *Rivularia* may be fixed with 1% chrom-acetic or with formalin-aceto-alcohol and the fluid washed out; then the trichomes may be critically stained with iron hematoxylin, dehydrated to 85% alcohol, and counterstained with fast green, and dehydration may be completed with hygrobutol, and infiltrated with balsam; finally a single colony may be crushed on a slide and the coverslip applied.

CHAPTER XXIV

RHODOPHYTA

RHODOPHYCEAE

In structure and particularly in methods of reproduction the Rhodophyta are the most diverse and complicated of all algae. From the technical standpoint very little indeed has previously been done on the phylum as a whole.

In size the Rhodophyta generally are smaller than most of the Phaeophyta, but larger than the Chlorophyta. The designation "red algae" is somewhat of a misnomer, for some species appear to be greenish (*Iridaea, Halosaccion*) and others have a brownish color (*Nemalion, Gracilaria, Ceramium*). It has been claimed that sterile Rhodophyta may be distinguished from Phaeophyta by the slower rate at which they turn green when dipped in water heated to a temperature between 60 and 70°C. Some known member of the Phaeophyta, such as *Fucus* or *Laminaria*, should be used as a guide in making this test. A simpler method may be found in the fact that when the larger Rhodophyta are placed in the standard chrom-acetic fixing fluid, they lose all color very rapidly, whereas most Phaeophyta are scarcely affected.

Occurrence.—Although the Rhodophyta are predominantly marine plants, there is a considerable number of fresh-water species (Skuja 1938). These prefer shallow running waters and belong mostly to the primitive groups. *Batrachospermum*, so commonly employed in elementary botany courses as a representative of the red algae, is the most widespread genus as well as the one with the largest number of species. Very few of the fresh-water species are red in color; they are mostly bluish, green, grayish, or brownish.

The Rhodophyta prefer warmer waters, but a few representatives may be found in northern latitudes. In the United States the greatest range in forms is to be found in Florida and along the Pacific Coast from Puget Sound southward. Like the brown algae, the red algae grow mostly on rocks or where the shores are rocky. On sandy beaches few, if any, red algae can be found, except on pilings, stone jetties or similar structures. The coralline types are practically the only red algae occurring in tide pools, which are otherwise well filled with representatives of other algae and aquatic Angiosperms. The Rhodophyta prefer the middle and lower littoral regions: the more deeply the plants grow, the

brighter red their color becomes. Those growing near the upper tide line become more brownish (*Agardhiella*), darker (*Chondrus* and some species of *Gigartina*), olivaceous (*Gastroclonium, Lomentaria*), or even blackish (*Endocladia, Rhodomela*). One should therefore beware of being deceived by color differences when collecting Rhodophyta.

There is no satisfactory manual available for the Pacific Coast Rhodophyta, outside of one regional manual (Kylin 1925), but an excellent manual for the Atlantic Coast species was recently published (Taylor 1937). A comprehensive review of the anatomical features of the phylum (Kylin 1937; see the bibliography for references to the literature) has recently been published.

Collection and Preservation.—These procedures are identically as described for the Phaeophyta (p. 263). In making herbarium specimens, most Rhodophyta will adhere firmly to the paper and need no gluing or taping. They dry quickly but are susceptible to mold infection.

Fixation.—The two standard fixing fluids described as being satisfactory for the Phaeophyta serve equally well for most Rhodophyta; *viz.*, 8 to 10% formalin (neutral) in sea water, and a 1% chrom-acetic fluid. Certain groups, however, notably the Bangiales, Ceramiales, and Gigartinales, present exceptional difficulties, and special methods will occasionally be cited.

It must be emphasized in the strongest possible terms that Rhodophyta should not be left in any of the various chrom-acetic mixtures any longer than it takes to fix them properly. In some cases this is a matter of seconds. The filamentous forms and some thalloid genera, such as *Porphyra*, will break up quickly, and others will literally become dissolved into a gelatinous mass if left in the killing fluid for even 1 minute too long. Such Rhodophyta are good illustrations of the fact that killing and fixing are two separate processes, even if they are usually obtained with the same fluid; all Rhodophyta are promptly killed, but few ever become sufficiently fixed for further treatment. As a safe rule, it may be stated that fixation is completed in about half again the length of time that it takes for the natural color to disappear. For example, if the color disappears in 2 minutes, fixation (or what passes for fixation) is completed 1 minute later, hence the washing out of the fixative should be begun immediately. In neutral 10% formalin in sea water, material may be left for several months without appreciable harm, provided it is kept out of the light.

Embedding.—This may be carried out exactly as described for the Phaeophyta. Difficulty is likely to be encountered with many species. Even if the material appears to have been killed satisfactorily, there will be many occasions when it will be discovered that fixation actually was not affected; the material either becomes excessively hardened or shrinks

completely after it has been carried beyond 80% alcohol. The Rhodophyta have a tendency to become brittle if left too long in the paraffin oven, consequently the time devoted to this process should be as brief as possible. In any event, infiltration takes place very quickly with practically all species.

Whole Mounts.—The best fixing medium is an 8 to 10% solution of formalin in sea water, but as it is necessary to get rid of the natural pigments before staining can be effected in certain genera, a 1% chrom-acetic should be used on these. Even the fronds of large forms like *Ceramium* and *Ptilota* are perfectly preserved by the formalin solution and are easily stained and mounted entire. Fresh-water forms may be fixed in the standard chrom-acetic or formalin-aceto-alcohol fluids commonly used on the fresh-water Chlorophyta.

On no account should iron hematoxylin be used on whole-mount material of marine Rhodophyta: it is practically a certainty that the material will become completely dissociated when the mordant is poured on or when the stain solution is applied after washing out the mordant. The reason is that the acid of the mordant dissolves the gelatinous matrix and allows the cells to become separated. By far the most satisfactory results, as well as exceedingly beautiful preparations, may be secured with Harris' hematoxylin and an erythrosin counterstain.

Species that have a loose filamentous structure held together in a gelatinous matrix are adapted for the making of temporary mounts which are exceedingly serviceable for investigating the structure and development of complicated carpogonial branch systems. Place the fresh material in 6% hydrochloric acid in sea water, and let remain overnight or at least for several hours, whereupon it will be found to have become more or less dissociated. Put a small portion on a slide, cover with a No. 2 coverslip, and crush and spread gently. The filaments will become separated, and the entire reproductive structure with all its ramifications may be located and studied. This is impossible in the case of even very thick paraffin sections. If it would be possible to do so without too much handling of the material, the dissociated mass might be stained, dehydrated with hygrobutol, and mounted in balsam.

Bangioideae

Bangiales

The majority of the species are marine, but a few fresh-water species are found in each of the families.

Erythrotrichiaceae.—Most of the genera are marine, and when growing epiphytically are inseparable from the host. *Erythrotrichia* is common on both coasts and is an instructive form because of the formation

of monospores by oblique walls in vegetative cells. Fix in 8% formalin in sea water, stain carefully with Harris' hematoxylin, counterstain with erythrosin, and run up by a gradual hygrobutol method (Fig. 43). Disregard fixation and staining of the host, which is usually in a bad condition.

Bangiaceae.—*Bangia* is fairly common on both coasts. Fixation is difficult, whether for whole mounts or embedding, because of excessive

FIG. 43.—*Erythrotrichia carnea* epiphytic on old filaments of *Cladophora:* this preparation well illustrates the utter impossibility of separating host and epiphyte and why they must be run up and mounted together. Fixed with 1 % chrom-acetic in sea water; stained with Harris' hematoxylin and erythrosin.

plasmolysis. As the thick gelatinous envelope will prevent the cells from breaking apart, iron hematoxylin may be used on material intended for whole mounts. The standard chrom-acetic fluid diluted in half with sea water sometimes gives good results on material to be embedded. A group of thalli should be embedded and sectioned together transversely at about 5μ.

Porphyra is very common and abundant on both coasts. It is a difficult alga to fix adequately, but in this respect it is not so difficult as *Bangia*. The thallus in most species is too thick for clear staining for whole mounts. Picro-indigocarmin is the only stain which, in the writer's experience, does not hopelessly overstain. The reproductive regions may usually be recognized as differently colored areas along the margins of

the thallus. The fluid which has given the best fixation for embedding and sectioning is composed of:

Saturated aqueous picric acid	100 cc.
Chromic acid	1 g.
Hydrochloric acid, c.p	6 cc.

While observing the color changes, watch the vegetative areas of small portions of the thallus, not the already discolored reproductive regions, and stop fixation as soon as all color has disappeared. Microtome trans-

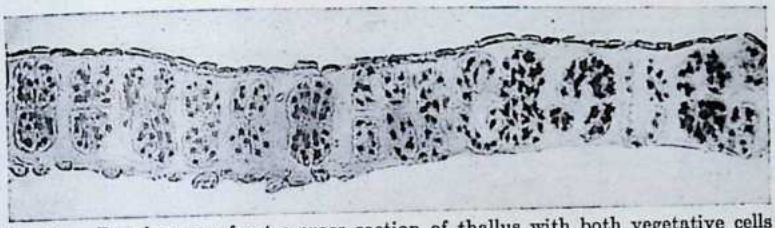


FIG. 44.—*Porphyra perforata:* cross section of thallus with both vegetative cells and reproductive regions. Fixed with 1 % chrom-acetic in sea water; stained with iron hematoxylin and fast green.

versely at about 7μ; staining is generally very good in iron hematoxylin (Fig. 44).

Florideae

The great diversity in thallus structure and methods of reproduction lead to technical difficulties of one sort or another. Even species in the same genus will not react equally well to an identical method of treatment. *Laurencia spectabilis* and *L. virgata* afford an example of this sort; the latter is far more amenable to treatment than the former, although the two are scarcely different structurally.

The following discussions will not go into any great detail, except in the case of such common laboratory forms as *Polysiphonia*. This, however, should emphatically not deter one from trying the admittedly difficult Florideae, for these plants, when properly worked up, constitute some of the most beautiful and instructive preparations imaginable.

NEMALIONALES

The order includes a few fresh-water and innumerable marine species. It differs from the other Florideae in that a tetrasporic generation is absent. The general impression seems to be that *Nemalion* is the only representative of the order worth studying. Such is not the case: *Nemalion* is rather unsatisfactory if one wishes to secure an abundance of all stages in the development of the carpogonia, gonimoblasts, and carpospores. *Cumagloia*, which unfortunately is restricted to the Pacific Coast, is a more satisfactory genus.

Chantransiaceae.—*Acrochaetium*, a filamentous marine alga, which is far more abundant on the Atlantic than on the Pacific Coast, is especially desirable for illustrating reproduction by means of monospores. The plants are said to be not always in a good condition for study. Spermatia and carpogonia occur in some species. Pieces of the host bearing the epiphyte may be fixed for about 10 minutes in 1% chrom-acetic, stained with Harris' hematoxylin and erythrosin, and then dehydrated by a gradual hygrobutol method; the epiphyte may be carefully scraped off with needles and mounted alone. Some species are endophytic and others endozoic; sections apparently can be made without difficulty.

Batrachospermaceae.—One of the commonest fresh-water Rhodophyta is *Batrachospermum*, found in cool small streams, ponds, and pools. It is easily recognized by the whorls of lateral branches on the conspicuous central axis. Most species are a pronounced bluish or olive-green in color when fresh and growing in the light; when found in shady places or deep water, the color changes from reddish to purple. Adult fruiting plants may be found only during the spring season. Fix in formalin-aceto-alcohol or in a weak chrom-acetic, stain in iron or Harris' hematoxylin, counterstain with erythrosin and make whole mounts. When ready to mount, cut the thalli into suitable small portions.

Lemaneaceae.—Small plants of *Lemanea*, which grows on rocks in turbulent fresh waters, are readily mounted whole, but longitudinal and transverse sections of the maturing thallus are required if stages in the development of spermatia and carpogonia are desired. Sections should be cut at 4μ for spermatia and 7μ for carpogonia. In staining sections, iron hematoxylin is preferable, but it is not always possible to get the spermatia and carpogonia differentiated to an optimum point for both on the same slide. The carpogonia do not retain the stain well, whereas a prolonged treatment is required by the spermatia. Counterstain with erythrosin.

Helminthocladiaceae.—The more interesting species are grouped in this family. A given species may be either monoecious, dioecious or rarely both. The spermatia are borne on the ends of short, and often densely crowded, branches. The carpogonia originate on short carpogonial branches; the terminal cell elongates into a trichogyne. The fertilized egg develops into gonimoblasts whose terminal cells produce carpospores.

Cumagloia andersonii, as previously noted, is one of the best species to use (Fig. 45). *Helminthora*, which does not occur in the United States, and *Liagora*, to be expected in Florida and perhaps farther north, are the next best, but *Nemalion* can, of course, be used if none of the other genera is available. *Nemalion*, which grows in scattered colonies on rocks in the upper or middle littoral zone and is not common, is readily recognized

in the field by the very slippery feeling of its dull brownish-red, terete thalli. In *Liagora* some species are slimy, but in others the gelatinous matrix is more or less calcified; if a fluid containing acetic acid is used on the calcified forms, no trouble should be encountered.

In order to obtain the youngest stages in the development of both spermatia and carpogonia, the very tips of the thalli must be used. The younger plants are better. The terminal 1.5 or 2 mm. of the thallus should be placed in one vial containing the killing fluid, the next 2 mm. in

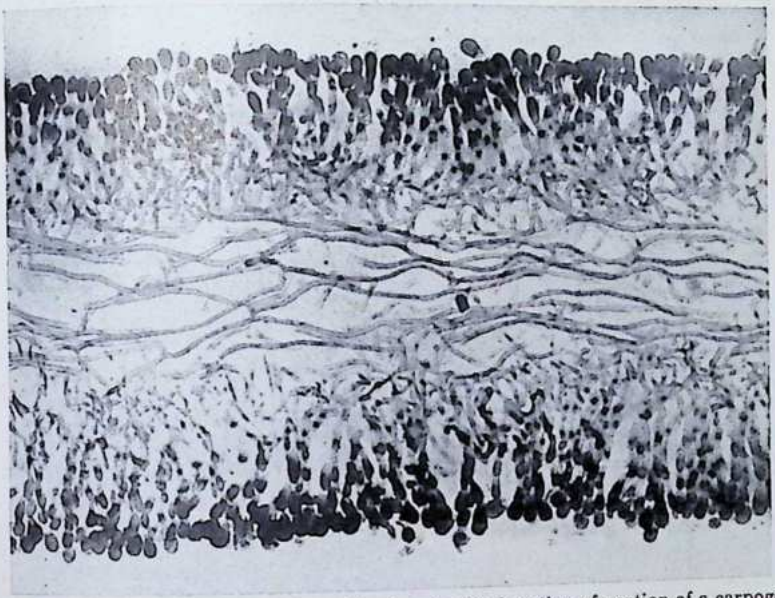


FIG. 45.—*Cumagloia andersonii:* median longitudinal section of portion of a carpogonial plant, showing the essentially filamentous structure of the thallus. Fixed with chrom-acetic in sea water; microtomed at 16μ; stained with iron hematoxylin and erythrosin.

a second vial, and the third 2 mm. in still another vial; portions of the thallus over 5 or 6 mm. from the tip will ordinarily show only mature structures. The standard chrom-acetic fluid may be used, or a few drops of a 1% aqueous solution of osmic acid may be added if desired, as well as 1 g. of urea or saponin.

To show the filamentous structure of the thallus, longitudinal sections of the apex should be microtomed at about 18μ, transverse sections a few microns thinner. For the development of the spermatia, longitudinal sections are best, cut at 8μ. To show the characteristic downward growth of the carposporophyte in *Nemalion*, which is rarely diagrammed accurately in botanical texts, longitudinal sections may be cut at 12μ. For studies in greater detail of either the spermatia or carpospores, the sections should be much thinner; 5μ is about right. Such thin sections

naturally do not show the general topography of the thallus as well as do the thicker sections.

Staining is exceedingly difficult with some genera, but iron hematoxylin may be tried. The destaining must be rather carefully controlled

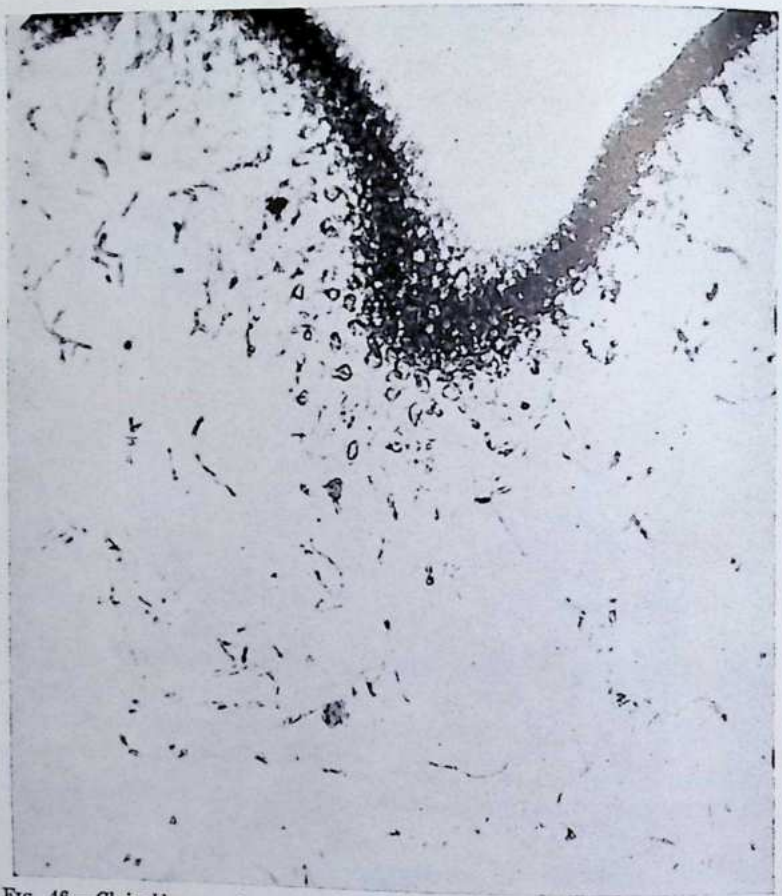


FIG. 46.—*Gloiophloea confusa:* longitudinal section of thallus with three carpogonia (one above the other, slightly to the left of the center). The very small size of the carpogonia and their burial within the thallus indicate the careful search that must be made to locate them. Fixed for 10 minutes in 1 % chrom-acetic in sea water; stained with iron hematoxylin and fast green.

as the sex organs lose the stain long before the vegetative portions are properly differentiated. Erythrosin differentiates the carpogonia and spermatia unusually well, but the staining period with this dye should be twice as long as when used on other plants.

It has lately been found that Johansen's quadruple stain affords clear and sharp results not obtainable with other stain combinations; the material should have been fixed in a chrom-acetic fluid as formalin-preserved material does not take a good stain.

All the genera in the family lend themselves to the preparation of whole mounts, but only fresh material should be used for this purpose. Preserved material becomes too acid to take and retain stains. The thalli of *Nemalion* and *Cumagloia* are so slippery that they can hardly be kept in glass containers; small strainers, obtainable at any five- and ten-cent store, should be used to hold them. Pieces of thallus about 2 inches in length should be fixed in 1% chrom-acetic in sea water to remove the pigment; wash thoroughly, transfer gradually to distilled water, then

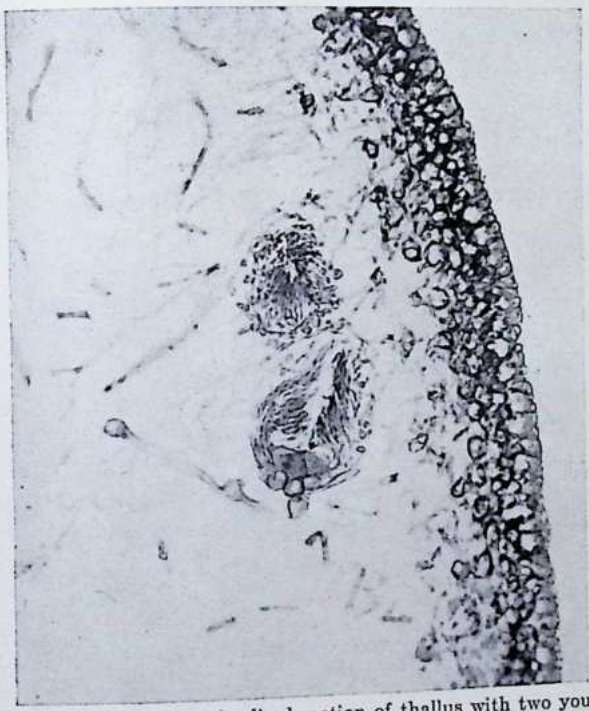


FIG. 47.—*Gloiophloea confusa:* longitudinal section of thallus with two young carposporophytes, the lower one being in median section. Fixation and staining as in Fig. 46.

pass through 10, 20, and 30% ethyl alcohol. Stain with Harris' hematoxylin, wash, and differentiate. Dehydrate somewhat slowly by a gradual hygrobutol method, and infiltrate with balsam. When ready to mount, cut the thallus into portions about 1 cm. in length with scissors. It will be necessary to apply considerable pressure on the coverslip to spread the thallus sufficiently; it would be better to crush the piece of thallus with the flat side of a scalpel before adding the coverslip.

Chaetangiaceae.—*Scinaia, Gloiophloea,* and *Whidbeyella* are found on the Pacific Coast, *Galaxaura* on the south Atlantic. All are equally interesting, but a little difficult to manipulate. The first three are soft plants, but this is a deceptive quality, as they occasionally become so

cartilaginous after passing through the higher alcohols that they can be neither embedded nor sectioned. *Gloiophloea* is exceptionally fine for showing the development of the gonimoblasts from the carpogonium (Figs. 46, 47). *Galaxaura* is more or less calcareous, but the hints given as to the treatment of *Liagora* also apply here.

The apical tips of the plants should be collected if one is studying the reproductive phases, as suggested under the Helminthocladiaceae. The cells composing filaments, antheridia, and carpogonia are so small that rather thin sections, at least 7 to 8μ, are indicated. For general topography the sections may be cut a little thicker. Staining is very beautiful with iron hematoxylin, which is comparatively easy to differentiate in this family.

Gelidiales

The order differs from the haplobiont Nemalionales in being diplobiont; the carposporophyte develops directly from the carpogonium. The plants are mostly slender or wiry and rather tough.

Gelidiaceae.—*Gelidium* is a widespread genus, and some species are of considerable economic importance as the source of agar. *G. cartilagineum* is common on the Pacific Coast, *G. crinale* on the Atlantic, and other species are to be found on both coasts. The species first mentioned is excellent for illustrating the development of the carpogonium peculiar to the family, but the trouble is to get the material into paraffin without its shrinking or becoming too cartilaginous. Satisfactory methods have not yet been devised. The standard chrom-acetic fluid succeeds only with the apices of the fronds or with the very youngest plants. Section the material in the longitudinal plane.

Cryptonemiales

Dumontiaceae.—In this family the carpogonial branches and auxiliary cell branches are distinctly separated from each other. Several genera are available for demonstrating this condition. *Cryptosiphonia woodii* is supposed to be one of the best, but the technique for this plant has not yet been sufficiently refined; the material is very apt to shrink completely during dehydration. Fresh or well-preserved material may be treated with hydrochloric acid and the complicated carpogonial filament and the carposporophyte observed in their entirety.

Squamariaceae.—The anatomy of *Peyssonnelia* is rather interesting; the genus occurs from Florida to Maine. There are several other Atlantic Coast genera. The usual methods should prove satisfactory.

Grateloupiaceae.—In this family the carpogonia and auxiliary cells are formed on inner branches of the cortical filaments; the gonimoblast arises from a large basal stalk cell. On both coasts *Grateloupia* and

Halymenia are the genera most likely to be found, and on the west coast *Prionitis* is very favorable material. It is not at all difficult to secure series of stages by following the general methods.

Corallinaceae.—The coralline red algae superficially form a decidedly forbidding group of plants because of the heavy incrustation of calcium salts on their fronds. There is little real basis for the claim that the group is technically impossible since preparations of even the larger and more heavily encrusted species are easily made. One need only make certain that the lime has been completely removed during fixation. The compact nature of the fronds permits exposure of the pieces of material to the softening action of acids for long periods.

The larger crustaceous forms, which grow on rocks, stones, or woodwork, should be carefully scraped off in small portions. The small crustose forms, which usually grow on other algae, are best removed together with part of the host. For example, leaves of *Phyllospadix* or *Zostera* or the main axis of *Laurencia* encrusted with *Epilithon* or *Melobesia* may be cut into short portions, dropped into the killing fluid, embedded, and sectioned together. It is of no avail to attempt to separate them. All the erect-branched species should be cut into convenient short lengths. It is better to cut across the middle of an internode (or segment) of the larger species than to break the joints apart at a node. The nodes are not calcified.

All the smaller species included in *Melobesia* and *Epilithon* are perfectly fixed and sufficiently softened within 10 minutes or slightly longer in the standard chrom-acetic fluid. In *Epilithon* (which is placed in *Lithothamnion* by some authors and in *Melobesia* by others), particularly, a fine series of developmental stages of all the reproductive phases are easily obtained. The larger forms may be placed in the following solution until soft (a month or longer may sometimes be required):

Distilled water	100 cc.
Sodium chloride	1 g.
Formalin	10 cc.
Glacial acetic acid	8 cc.
95% alcohol	50 cc.

Dissolve the salt in the distilled water first, and add the alcohol last, pouring it in slowly while stirring the mixture; there should not be a white turbidity. Wash out with 50% alcohol, and begin the dehydration process with the 50% step of the tertiary butyl alcohol method. The material usually microtomes with surprising ease. Staining is excellent with either iron or Harris' hematoxylin and erythrosin.

In genera with such broad joints as those of *Amphiroa* there may be some difficulty in locating the sex organs, although tetrasporangia are easily located.

Gloiosiphoniaceae.—One species of *Gloiosiphonia* occurs on the Atlantic Coast, two on the Pacific. Whole mounts of portions of the plant bearing tetrasporangia are possible, but since the material responds nicely to treatment, sections should be made.

Callymeniaceae.—The two most important genera are *Callophyllis*, with several species on the Pacific Coast, and *Callymenia*, with one species on the west and two on the Atlantic coasts. Plants of the former are easily collected at very low tides, but *Callymenia* grows in such deep water that only floating or dredged plants can be picked up.

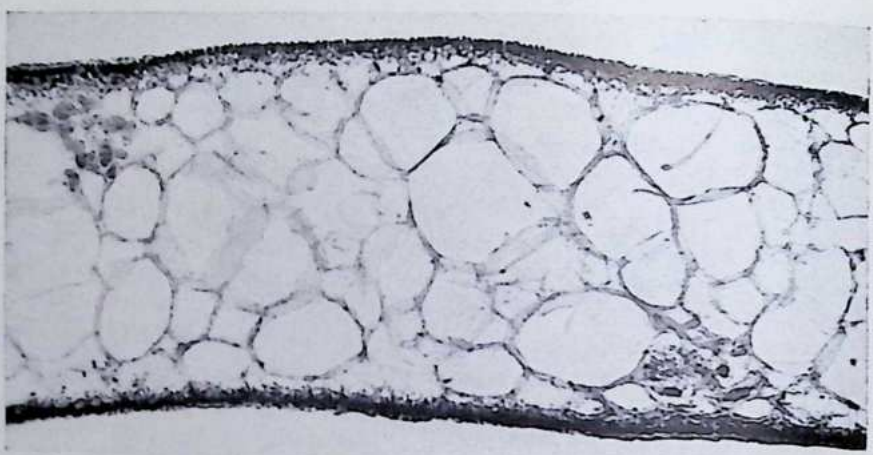


FIG. 48.—*Callophyllis furcata:* longitudinal section of thallus perpendicular to flat surface, with two young carposporophytes. Fixed with 1% chrom-acetic in sea water; stained with iron hematoxylin and erythrosin.

Callophyllis is probably the easiest of all Rhodophyta with thin, flat fronds with which to work. Fixation with chrom-acetic is perfect; there is no shrinkage or hardening during the embedding process, staining is precise with iron hematoxylin, and it is a comparatively simple matter to secure a complete series of stages from the two-celled procarp to mature cystocarps (Fig. 48). Cut off the tips of the plants about 6 mm. back from the apex, and section these in a vertical longitudinal direction at 10μ. The remainder of the thallus may be cut up into portions about 4 mm. long and microtomed transversely. Most of the plants collected will be cystocarpic; finding the spermatia and tetrasporangia is a matter of sectioning and examining material from a large number of plants. For this reason only material from a given plant should be placed in each vial.

GIGARTINALES

For a discussion of this order, one cannot do better than to study Kylin's papers (Kylin 1928, 1932) and to follow the bibliographies listed

therein. A large number of the genera mentioned in these papers are to be found on either coast of the United States (see also Taylor 1937).

Practically all of the species are perfectly fixed with the standard chrom-acetic fluid, but the species in the Gigartinaceae are exceedingly troublesome. The writer has never obtained really good fixation of either *Chondrus* or *Iridaea* after innumerable trials; success was had with two or three species of *Gigartina* but not at all with others. Species with the cells closely packed together are easier to manipulate than those with a rather loose cellular organization. Firmness to the touch does not always connote solidity of structure; for instance, thalli of species of *Gigartina* which have a rigid feeling when pressed with the fingers nevertheless become reduced to formless masses either during fixation or washing. Since the gelatinous substance permeating the large intercellular spaces is of a carbohydrate nature, there is no known method of precipitating or coagulating it, as can be done with proteinaceous substances. Sometimes treatment with hydrochloric acid, either during or immediately after fixation, prevents excessive dissociation. Very young portions of the thallus are generally more solid than the older parts; since these bear the spermatangia and carpogonia, good fixation can usually be obtained. Small portions of the thallus cortex with mature cystocarps can be removed and fixed separately.

Agardhiella and *Gracilaria* are particularly to be recommended; they are easy to fix, to section, and to stain with iron hematoxylin. If care is taken to obtain the youngest fruiting tips, a fine series of stages in the development of the carpogonial branches and carpospores is readily secured.

In the following discussion, those families which have no easily collected representatives on either the Atlantic or Pacific Coast are omitted.

Solieriaceae.—*Agardhiella tenera* is found all along the Atlantic Coast and *A. coulteri* on the Pacific side. They are exceptionally easy algae to work with. *Sarcodiotheca*, occurring in Washington, is likewise easy. The plants are heterothallic, large, bushy, with cylindrical, tapering branches that have a peculiarly fleshy, firm texture. The tetrasporangia and cystocarps are scattered, the spermatia are formed in patches on young branches. For the origin of all stages, remove 2 to 3 mm. lengths of the tips of the branches, and microtome longitudinally at 7 to 11μ. For developing gonimoblasts and tetraspores, use the next few millimeters of the tips, but section transversely at 12μ. The mature cystocarps are easily recognized as darker red spots in the thalli; cut out short portions bearing such spots, and microtome them transversely at 10μ. Stain all stages with iron hematoxylin and fast green.

Rhodophyllidaceae.—Two genera occur on the north Atlantic Coast. *Rhodophyllis* has flat, compact membranous thalli which appear to be

unlikely to give difficulty during fixation and dehydration. Fix short portions of the apices for the younger developmental stages; otherwise treat as in the preceding family.

Plocamiaceae.—Two species of *Plocamium* are to be found on the Pacific Coast. The thalli are richly branched, somewhat flattened, and bear the reproductive bodies on the small side branches. Remove a group of these branches, embed, and microtome in the horizontal longitudinal plane at 10μ. Mount as serial sections, since neither spermatangia nor carpogonia are abundant. Stain with iron hematoxylin, but avoid overdoing the counterstain.

Gracilariaceae.—On the Pacific Coast *Gracilaria sjostedtii* is very abundant in late spring, and its manipulation gives absolutely no trouble, although spermatangia and carpogonia are difficult to find. The developing and maturing cystocarps are recognizable immediately as "warts" scattered along the long, cylindrical thallus; section these both longitudinally and transversely, mounting only sections going through the pore. There are two species on the Atlantic Coast; one is cylindrical and the other flattened. All these species are found in shallow, quiet, and warm bays or coves.

Gigartinaceae.—Four genera which superficially do not appear to be closely related are placed in the family: *Chondrus*, *Iridaea* (*Iridophycus*), *Rhodoglossum*, and *Gigartina*. The exceedingly variable "Irish moss," *Chondrus crispus*, occurs on both coasts. Spermatia and carpospores are produced during the summer and tetraspores during the autumn. The plants become extremely brittle during dehydration, but if the process is made very gradual, efforts to get young tips into paraffin may be successful. Section these tips in the vertical longitudinal plane at 10μ.

Only one very small species of *Gigartina* grows on the north Atlantic Coast, whereas the genus has about 10 species on the Pacific side, some of them being among the largest of all Rhodophyta. The taxonomy of these species is still chaotic despite recent attempts to straighten it out. The cystocarps of *Gigartina* are borne in special short branches which densely cover the thallus in most species. As all stages in development save the very youngest are readily recognized externally, a series of stages is easy to collect. *G. canaliculata*, a slender plant which feels soft when handled in the fresh condition, is really cartilaginous and becomes so hardened and desiccated during dehydration that it cannot be microtomed. If paraffin blocks of this and other species of the Gigartinales are placed in water, the portions promptly "blow out." In fact the sections can hardly be placed in water to straighten them out, as they invariably twist themselves out of the paraffin. Celloidin embedding is not much better. *G. binghamiae* has proved to be the species most amen-

able to treatment, but it must be said that a satisfactory fixing fluid and dehydration method for the entire family has yet to be devised.

Iridaea is one of the commonest red algae during the summer and autumn along the Pacific Coast. All reproductive phases are buried within the thallus. A long series of killing fluids and various dehydration methods were utilized in an attempt to manipulate this genus, but no conclusive results were obtained. The most satisfactory fixation was obtained with the following:

1/N hydrochloric acid in sea water	90 cc.
Formalin	6 cc.
Glacial acetic acid	5 cc.
Chromic acid	1 g.

Stenogramme interrupta, which may be found at, or below, the lowest tide levels along the central California coast, is the only species in the family with which the writer has had consistently good fixation and staining results. Remove 4 mm. of the apices of the thalli, then fix separately the next, the third, and the fourth 4-mm. portions. The apices should be microtomed in the vertical longitudinal plane, the others transversely, at not over 8μ. In the apical portions the origin and development of the procarps or spermatia will be found; young stages of tetraspore formation will be found in similar portions from tetrasporangial plants.

Ceramiales

Included in the order are innumerable forms which are exceedingly beautiful when observed under the microscope, whether living or stained and mounted. For the most part these species are filamentous, but some are polysiphonous, others frondrose, while still others are more or less saccate and solitary or colonial. Few technique difficulties are likely to be encountered with any of the species.

Ceramiaceae.—Material of the species belonging to this family is abundant and easily collected. The difficulty is to obtain the sexual reproductive phases, as the bulk of the material usually collected tends to show only tetraspores, if fruiting at all.

All the species are readily transformed into permanent mounts. As the family is strictly marine, fixation of material intended for whole mounts is excellent in 10% formalin in sea water, or the usual 1% chrom-acetic fluid may be employed. The change from sea water to distilled water should be more gradual than usual as there is sometimes a tendency for the tetraspores to collapse either during this change or during the dehydration process. Staining is very good in Harris' hematoxylin with counterstaining in erythrosin.

Material intended for embedding may be fixed in the standard chrom-acetic solution, which should be diluted with an equal portion of sea water. If division figures are desired, the material must be transferred directly from the ocean into the killing fluid, and the only time when mitoses are likely to be caught is around midnight. The time of fixation

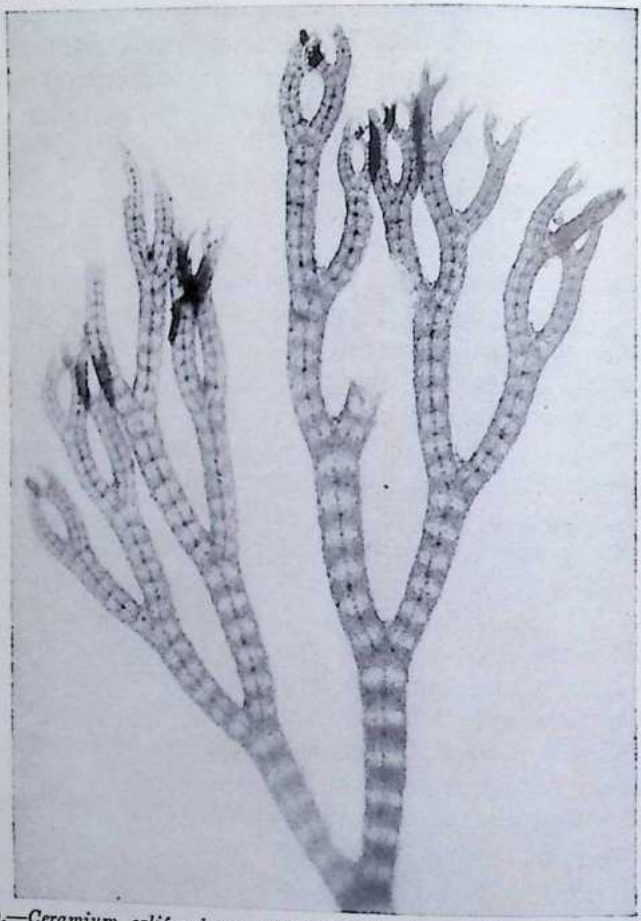


FIG. 49.—*Ceramium californicum:* whole mount of a portion of a vegetative plant. The banded appearance characteristic of the genus, and the protoplasmic connections between adjoining cells, are clearly shown. Fixed in 1 % chrom-acetic in sea water for 4 minutes; stained with Harris' hematoxylin and erythrosin.

is extremely short, even with the highly diluted fluids, hence its progress must be carefully watched. The material should remain in the paraffin oven for the briefest possible period. It would save much time and work later on if the pieces of material were embedded in bunches. Staining seems to be best with iron hematoxylin and erythrosin.

Antithamnion is one of the most widespread of the various genera. It grows on the piles of wharves and docks, on the bottoms of small boats,

and on the laminae and stipes of the larger Laminariales. The plants generally contain tetraspores; plants with gonimoblasts and spermatia are not often encountered, but when found a fine series of developmental stages is often present. Whole mounts are preferable; critical attention needs to be paid to the staining.

Callithamnion and *Ceramium* are also common, but it is sometimes difficult to collect them, especially the latter, when in fruit. *Ceramium*, however, has such an interesting thallus structure that even sterile material is well worth mounting entire (Fig. 49). Compared to the other genera, *Ptilota* may appear to have too thick a frond for whole mounts to be made, but such is not the case since a transparent stain is readily obtained with Harris' hematoxylin and erythrosin. *Griffithsia* is easily mounted whole, but as the reproductive regions may be obscured if the pieces of frond are not carefully examined before the coverslip is applied, sections may be more useful. Fix in diluted 1% chrom-acetic, section at 3 to 5μ, and stain with iron hematoxylin.

Spermothamnion is known from the Atlantic Coast, but it is said to be difficult to find in the fruiting condition, and spermatangia have not been found.

Delesseriaceae.—Most of the species in this family possess membranous fronds, and all are easily fixed and microtomed. On the Pacific Coast one may select *Nitophyllum*, which attains a considerable size and is easily collected. There are a number of other genera, species of which may be found on either or both coasts, which are so closely related to, or otherwise so resemble, *Nitophyllum* that they may be treated similarly. These include: *Erythroglossum*, *Polyneura*, *Heteronema*, *Delesseria*, *Cryptopleura*, *Membranoptera*, and others.

Remove the very tips of the fronds from the plants, and microtome these longitudinally both perpendicular and parallel to the flat surface at about 10μ. Stain with iron hematoxylin and counterstain cautiously to avoid overstaining. The procarps are rather difficult to locate, not being any too numerous. Older portions of the fronds may be sectioned transversely.

Rhodomelaceae.—Some of the commonest and most interesting of all red algae are included in the Rhodomelaceae. It contains *Polysiphonia*, which has doubtless been studied more extensively than any other red alga. This perhaps is more because of its general availability than for any other reasons, since there are other genera easier to handle and of simpler structure but with the same methods of forming the reproductive bodies. *Pterosiphonia* is an example.

Despite their wide distribution many of the Rhodomelaceae are difficult to collect at most localities, either for lack of abundance or of a healthy and vigorously growing or fruiting condition. The various

species grow on rocks, in sandy areas between protecting rocks (*Polysiphonia* especially), or on piles or other larger algae. One will find oneself compelled to study the habitat preferences of the different genera rather carefully and to collect material frequently over a long period in order to secure all the stages in reproduction. If not sterile, one too frequently finds only tetrasporangia in most of the material collected. Some species have been found to complete development and disappear within 40 to 60 days.

A word of caution concerning *Polysiphonia* and related genera is in order: the plants rapidly begin to putrefy after being removed from the water and evolve a characteristic odor resembling that of hydrogen sulphide; consequently they should be placed in a 6% formalin solution in sea water while being collected and later transferred to some other fixing fluid.

All the polysiphonous forms may be treated alike. This statement includes the following genera: *Polysiphonia*, *Pterosiphonia*, *Heterosiphonia*, *Herposiphonia*, and *Lophosiphonia*, most of which are Pacific Coast genera. For whole mounts 10% formalin in sea water is satisfactory; for embedding and sectioning, the regular chrom-acetic or the following fluid may be used:

1% chromic acid in sea water	25 cc.
1% glacial acetic acid	10 cc.
Sea water (filtered)	65 cc.

Fix 5 to 40 minutes, depending upon the "toughness" of the species, and wash out thoroughly with sea water. Cut longitudinally at 5 to 10μ and stain with iron hematoxylin or a triple combination. If the species is one that shows a tendency to shoot to pieces when placed in a chrom-acetic fluid, the following alcoholic formula will overcome the trouble:

70% ethyl alcohol	100 cc.
Formalin	6 cc.

Wash with 50% ethyl alcohol, and avoid going into water.

With material intended for whole mounts it is necessary to get rid of the natural pigments before a satisfactory stain can be obtained. The pigments inhibit staining. The process of extracting them is simple: wash out the formalin killing fluid, place the material in 1% chromic acid in sea water, watch the process of color extraction (which should take place rapidly if the material is exposed to sunlight), and stop as soon as completed by washing with sea water. Harris' hematoxylin gives a superb differentiation to material intended for whole mounts; counterstain with erythrosin. Dehydration is preferably with hygrobutol.

The nonpolysiphonous forms, which for the most part possess thalli too thick for whole mounts, present no difficulties in the way of fixation

and staining. Portions of the older thalli of the polysiphonous forms may also be sectioned for anatomical details.

Rhodomela larix is very common on rocks in the middle littoral zone on the Pacific Coast, and there are three species along the Atlantic. Embed portions of the thallus, and make both transverse and longitudinal sections.

Ricardia saccata is an unusually good type in which to demonstrate the origin of the procarps and the development of the cystocarps (Fig. 50). The plants form brownish grape-like clusters on *Rhodomela larix*. Small specimens may be fixed entire, but portions should be cut out of the older inflated sacs. Microtome transversely at 6 to 8μ.

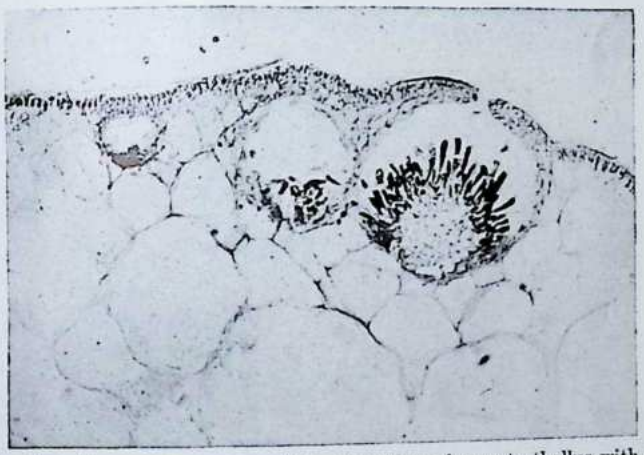


FIG. 50.—*Ricardia saccata:* cross section of portion of saccate thallus with cystocarps containing, at left, a young carpogonium and, at right, carpospores. Fixed with chrom-acetic in sea water; stained with iron hematoxylin and erythrosin.

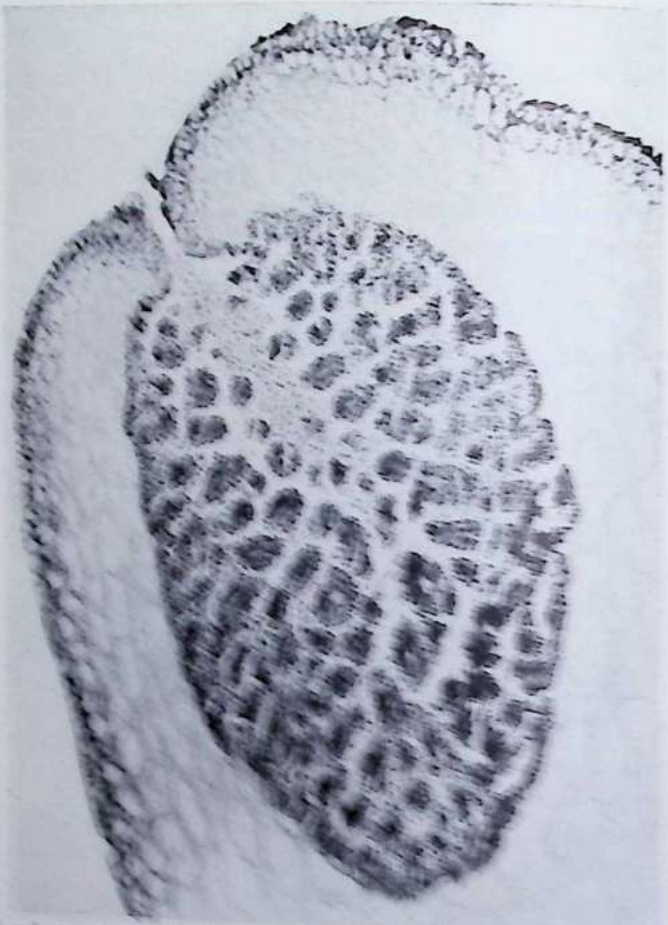
Laurencia includes two or three species on the Pacific Coast and several in Florida. *L. virgata* is an unusually good form with which to work, as it is easily embedded and all developmental stages can be followed out (Figs. 51, 52, 53).

RHODYMENIALES

There are far more tropical and subtropical representatives of the order than there are species occurring in the United States, and there also are more on the Pacific than on the Atlantic Coast. The order is made up of plants somewhat diverse in habit, but all give little or no difficulty from this standpoint, with the exception of *Halosaccion*.

Rhodymeniaceae.—*Halosaccion* forms obovate, hollow plants which are difficult to section and stain and are not worth the trouble that they entail.

Rhodymenia has one species which extends from New Jersey into the Arctic regions and is a deep-water perennial; there are several species on the Pacific Coast. *Chrysmenia* occurs in Florida and California. General methods are applicable to both genera.



filaments or gonimoblasts. Somewhat older portions of the branches may be selected and sectioned transversely for the carpospores. Tetrasporangial plants are less abundant than the cystocarpic; they may show

FIG. 52.—*Laurencia virgata:* longitudinal section of apex of frond with very young carposporophyte. Fixed in chrom-acetic in sea water; stained with iron hematoxylin and fast green.

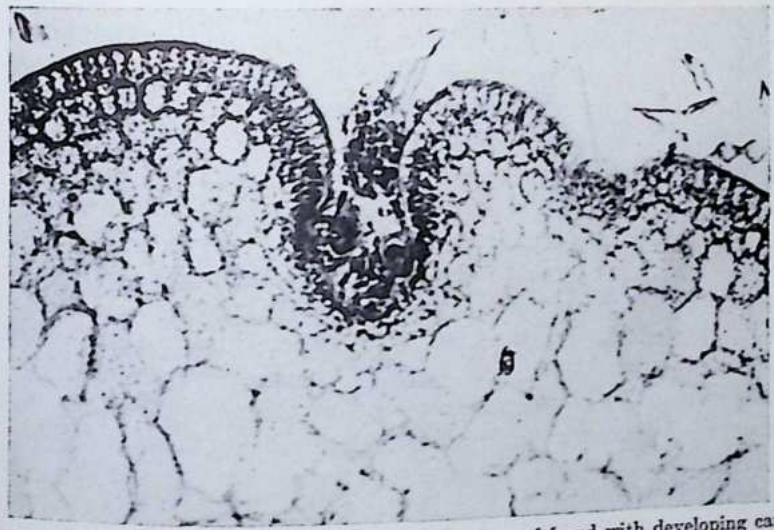


FIG. 53.—*Laurencia virgata:* longitudinal section of apex of frond with developing carposporophyte. Fixation and staining as in Fig. 52.

either tetrasporangia with four tetraspores or parasporangia with 15 to 20 paraspores.

Two species of *Lomentaria* occur on the Atlantic side and may be treated like *Gastroclonium*. One species of *Champia* also occurs on the East coast and is so small that whole mounts may be prepared.

CHAPTER XXV

MYXOTHALLOPHYTA

MYXOMYCETES

The Myxothallophyta are the simplest of the plants commonly known as "fungi" (MacBride and Martin 1934). They are characterized by an assimilative phase which consists of a naked, multinucleate, and mobile mass of protoplasm known as the "plasmodium"; and a reproductive phase which in most cases consists of a membranous spore case. The latter frequently contains, in addition to the spores, a capillitium of netted or free threads and sometimes bears, inside or on the outside, calcareous deposits.

Occurrence.—Myxomycetes occur in all except the coldest regions, thriving best in damp habitats. Most of them are very small and the free-living forms are exceedingly fragile structures. Some of the latter can be found in warm, moist locations at almost any time of year. The amoeboid and plasmodial stages occur in the upper layers of soil and in decaying surface vegetation. The beautifully colored saprophytic forms are easily collected. *Stemonitis*, the most widespread and readily recognized form, grows on rotting wood, and its sporophore is dark brown in color. Other forms are found on beams in damp cellars and on other fungi, such as agarics, boletes, and *Polyporus*.

Collection and Preservation.—These delicate organisms must obviously be handled with great care.

If the organisms are not to be killed and fixed in the field, they may be placed in boxes for transportation to the laboratory. An ordinary pasteboard box with a piece of cork linoleum covering the bottom serves satisfactorily. The pieces of wood or other substrate carrying the organisms may be pinned to the cork to hold them in place. Damage to the organisms causes either cessation of development or abnormal growth of the fruiting bodies, and the greatest care, particularly, must be taken not to let them become dried. It is usually impossible to bring immature fruit to normal maturity in the laboratory; if the myxomycete is in the immature fruiting condition, leave it where it is, watch carefully until it matures, then collect, and place immediately in the killing fluid. For herbarium purposes only mature fruiting material should be collected.

Most botanists simply let the organisms dry on the substrate and later glue or sew them to the bottom of small boxes, one species to a container. The boxes in which slides come are ideal for the purpose. Entire colonies

may be preserved in a mixture of 5 cc. formalin and 10 cc. glycerin in 100 cc. distilled water, but there is always the danger that colors other than brown or black will disappear in time.

Fixation.—A weak chrom-acetic fluid is good. About 1 g. chromic acid and 3 cc. glacial acetic acid to 100 cc. water have also proved to be satisfactory. Allen's modification of Bouin's fluid has been strongly recommended (Howard 1931*a*, *b*). Formalin-aceto-alcohol has worked very well, but it is probably not to be recommended for the finest details. For dehydrating, avoid xylol and similar fluids which tend to harden the tissues so excessively that they crack during microtoming. Infiltration is not difficult, but avoid leaving overlong in the paraffin oven.

Free-living forms growing on rotten wood (*e.g.*, *Trichia*) should not be scraped off the wood, as damage inevitably results. Cut out suitable portions of the wood bearing the plasmodia and sporangia, and work up as if they were one. Rotten wood presents no difficulties whatever in embedding and microtoming.

Sections need not necessarily be very thin, save for cytological details. For general morphology, 10μ is not too thick; half that thickness is thin enough for most purposes.

Simple Whole Mounts.—Put fresh fruiting material on a slide, wet with alcohol, and then, if necessary, with 2 to 3% potassium hydroxide to restore plumpness. The hydroxide may be mixed with a little glycerin if the material is required for prolonged examination. Permanent preparations may be made by placing the material on a circular coverslip in a drop of Amann's medium:

Phenol	20 g.
Lactic acid	20 g.
Glycerin	40 cc.
Distilled water	20 cc.

Cover with another coverslip of slightly smaller diameter, set aside until the medium has hardened sufficiently, and then the whole may be inverted over a drop of balsam on a slide.

Whole mounts of the forms which are naturally dark colored are readily made. Kill in 95% alcohol for a few hours, and wash out with a change of 95% alcohol. Transfer to a deep watch glass and give two or three changes of hygrobutol, then add balsam diluted with hygrobutol. Evaporate to a mountable consistency. To show up the capillitium, however, it is necessary to get rid of most of the spores. Place the myxomycete in a warm place for ½ hour, then, by tapping smartly against the substrate, displace the spores, and when enough have been removed, proceed to the killing.

Sections.—All forms are very easily embedded and sectioned. Do not leave the material in the oven too long since heat tends to harden it.

Staining.—Most forms which are naturally dark colored can be mounted entire without staining, as has been described above. *Stemonitis* is an example of the types which make beautiful whole mounts without staining.

For the plasmodium iron hematoxylin is the best stain. Resting and germinating spores, killed and fixed, acquire a sharp and precise stain with crystal violet (Cotner 1930). Acid fuchsin is said to be an excellent stain for the plasmodial strands, and rose bengal is recommended for soil forms.

Spores may be readily germinated in aqueous dilutions of the vital dyes (Howard 1931). Use dilutions of about 1:20,000. Place a large drop of the dye solution on a slide, sow the spores in the drop, then place the whole in a humid chamber. The myxomycete will go through its normal course of development, unless this is checked by contaminating bacteria or protozoa, and abundant plasmodia may develop in about 72 hours after sowing the spores. To study the vacuoles, their size, distribution, and movement, use neutral red. The cytoplasm takes an intense stain with methylene blue. Both the cytoplasm and mitochondria take up Janus green B. These methods have been tried only with the Myxogastres.

Cultivation.—Spores of most forms will germinate as soon as they are thoroughly ripened. Vitality of spores probably does not extend over three years. Germination takes place in from 20 minutes to three or four days, depending upon the species and the age of the spores. For the details of spore germination, reference should be made to published papers (Howard 1931).

Spores may be germinated in autoclaved tap water. Young plasmodia can be obtained in this fashion, and portions may be transferred to solid media for further developmental stages. An acidified oatmeal agar is probably the best medium. Put 30 g. rolled oats or oatmeal, 15 g. agar, and 1 liter water in a double boiler, and cook for 15 minutes. Transfer the gruel to a flask, and autoclave for 15 minutes at 15 pounds pressure. When sufficiently cool, pour into Petri dishes, flasks, or other suitable containers. The medium should be definitely acid in reaction in order to prevent excessive growth of bacteria. The temperature at which the plasmodium grows best is slightly above room temperature (about 20 to 26°C.).

Mannite agar forms a favorite substratum for the cultivation of plasmodia. The formula is as follows:

Mannite	15 g.
Dibasic potassium phosphate	0.2 g.
Magnesium sulphate	0.2 g.
Sodium chloride	0.2 g.
Calcium sulphate	0.1 g.
Agar	15 g.
Distilled water	1 liter

Autoclave, and pour into Petri dishes. This medium is unfavorable for the growth of bacteria and most other fungi.

A great many other methods have also been proposed (*e.g.*, Camp 1936).

The above methods yield an abundance of plasmodia but not in a condition for them to be of direct use for slide-making purposes. It will be necessary to grow the plasmodium directly upon the slides; this, fortunately can be readily accomplished. Obtain a shallow wooden box about 2 inches deep and a pane of glass large enough to cover it. Place

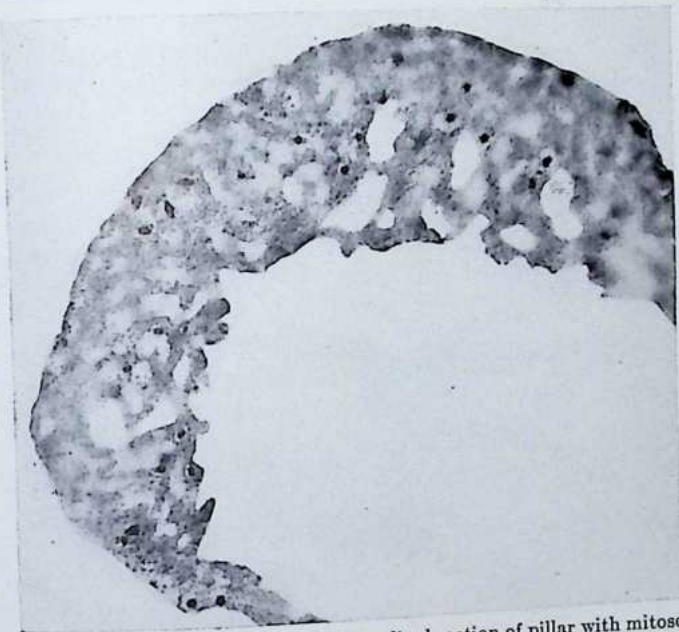


FIG. 54.—*Ceratiomyxa porioides:* vertical longitudinal section of pillar with mitoses. Fixed with alcoholic Bouin's; stained with iron hematoxylin and orange G.

two or three layers of blotting paper or drying felt on the bottom and moisten thoroughly with sterile water. On another piece of stiff absorbent paper, cut so that it can be easily placed in and removed from the box, arrange chemically clean slides side by side in rows. With a sterilized wide, thin, and soft-bristled paintbrush, cover the slides with a thin film of the melted oatmeal agar medium described in the preceding paragraph. Try to make the film as even as possible over the slides and uniformly thick; as the plasmodium will grow rapidly, the film should not be over 0.1 mm. thick. Place the paper with the slides in the box, and cover until the agar solidifies. The dry paper will absorb sufficient water from the lower dampened layers. Next, taking all possible precautions to avoid bacterial and mold contaminations, remove tiny portions of vigorously growing plasmodium from a culture by means of a platinum

loop, and place one in the center of each slide. Replace the cover, and set the giant moist chamber in a warm place to incubate. In only a few hours the slides may be covered with plasmodia. When growth has reached the desired stage, remove the slides, and plunge into jars of fixative (Allen's Bouin has been recommended) for several hours. Remove from the fixative, wash, scrape off excess or unwanted growth, then proceed to the staining. If the film of agar was thin and perfectly flat, no difficulty in mounting in balsam should be experienced.

FIG. 55.—*Ceratiomyxa porioides:* section of pillar with early spore formation. Fixed with alcoholic Bouin's; stained with iron hematoxylin.

The agar may be omitted and the plasmodium grown directly on the slips, but growth may not be quite so regular and abundant.

By exposing the plasmodia gradually to the air, sclerotization will take place. After desiccation has been completed, the sclerotia may be stored for at least a year. Reactivation of the sclerotium and development of new plasmodia may be brought about by wetting the material (but not submerging in water). An abundance of oxygen appears to be necessary for regrowth.

Exosporeae

The division contains a single genus, *Ceratiomyxa*, which in turn is probably monospecific. It is actually an extremely common myxomycete

but is not often collected. It occurs on decaying wood. The plasmodium is colorless when very young, but as it grows older, it turns white or occasionally has a pink or bluish tinge. The plasmodium grows within the wood; when ready to fruit, it breaks through at the surface and produces a stalk-line fructification composed of membranous, columnar, simple or branching sporophores on whose surface stalked spores are borne.

Ceratiomyxa is an exceedingly fragile plant: the slightest shaking, high temperatures, or sudden drying will ruin the culture. It is better to fix the material in the field rather than attempt to bring specimens to the laboratory for the purpose. The spores, however, may be germinated in the laboratory, using small culture vessels for the purpose, and the entire culture may be killed by cautiously adding the killing fluid. The most satisfactory reagent is a variation of Bouin's fluid, made by using a saturated solution of picric acid in 70% alcohol in place of the usual saturated aqueous solution (Gilbert 1935). Embed material as usual and microtome at 5μ. Staining should be with iron hematoxylin for all stages (Figs. 54, 55).

Myxogastres

The forms whose beauty is so captivating belong among the Myxogastres, which includes upward of 400 species. The general methods for the Myxothallophyta apply.

Acrasieae

The Acrasieae resemble the other myxomycetes only superficially. They pass the whole of their vegetative period as naked amoeboids; neither swarm spores nor a true plasmodium occur. In their fruiting phase they merely become loosely aggregated together.

The Acrasieae occur in the soil, in decaying wood, and on dung or other organic matter. *Polysphondylium* and *Dictyostelium* have frequently figured in morphogenesis investigations (Harper 1926, 1929; Raper and Thom 1932). Growth appears to be most luxuriant on mannite agar to which sterile shredded rat dung has been added just before the agar solidifies. The medium should be adjusted to a pH of 6.0. Permanent preparations may be made according to the general methods cited above.

CHAPTER XXVI

MYCOPHYTA (EUMYCETAE)

The Mycophyta include the vast group of saprophytic or parasitic plants known as "fungi." They differ from practically all other plants in that they are unable to carry on photosynthesis.

The carbohydrate reserve is glycogen, never starch. In the cell wall of primitive species cellulose predominates, but in the more advanced species so-called "chitin" is commonly found. Pectose and callose also occur in fungal cell walls.

Cultivation.—Methods of cultivating the fungi are so numerous that it is impossible in the space available to cite even the general methods. A compilation of culture methods for all the fungi has apparently never been made. Most fungi grow well on all ordinary bacteriological media (Levine and Schoenlein 1930), but flourish better if a sugar is added. Decoctions of horse dung, potatoes, beans, prunes, etc., may be solidified with agar and used as culture media. Or 1% Difco peptone plus 5% crude dextrose solidified with 1.5% agar is excellent for nearly all types. Sabouraud's medium is extensively employed for the culture of fungi:

Maltose	20 g.
Peptone	10 g.
Agar	15 g.
Glycerin	5 cc.
Distilled water	1 liter

Dissolve the substances, adjust the pH to 5.5, filter, tube for slants or talls, and sterilize at 15 pounds pressure for 15 minutes.

Still another medium is the Dox and Thom modification of Czapek's solution agar, which is the standard medium on which to grow *Aspergillus* (Thom and Church 1926) in controlled cultures; it is also suitable for fungi in general, Actinomycetes, and *Penicillium*, but not for *Mucor:*

Sucrose	30.0 g.
Sodium nitrate	2.0 g.
Dibasic potassium phosphate	1.0 g.
Magnesium sulphate	0.5 g.
Ferric chloride	0.5 g.
Ferrous sulphate	0.01 g.
Distilled water	1 liter
Agar	15 g.

One difficulty in cultivating molds is to prevent or keep down the growth of bacteria. Most of the proposed methods are unnecessarily complicated, requiring special apparatus, difficult techniques, or time-consuming procedures. A simple mechanical method (Raper 1937) is one of the easiest. Fuse three small glass beads not over 0.5 mm. deep to one end of a Van Tieghem ring (merely a piece of glass tubing about 1.5 cm. in diameter and 6 to 7 mm. in length), and place the ring in a Petri dish with the beaded end on the bottom. Pour sufficient nutrient agar into the Petri dish until about three-fourths of the depth of the ring is covered. After the agar has solidified, place a bit of the inoculum on the agar within the ring. The mycelium will grow down within the ring and out into the surrounding agar between the beads, thence to the surface outside the exposed top of the ring. The contaminating bacteria will not be carried along by the mycelium, consequently small portions of the latter may be removed and transferred to fresh media and bacteria-free cultures obtained.

It has been recommended that citric acid be added: the addition of about 5% of the acid should be sufficient. Tartaric acid also retards the growth of bacteria. Make a solution containing 5% dextrose and 5% tartaric acid. After autoclaving, this mixture may be kept for some time. When a culture medium is needed, melt a deep tube (about 10 cc.) of ordinary nutrient agar (preferably containing meat extract and peptone), and add to it 1 cc. of the dextrose-acid solution. This gives a final concentration of about 5% dextrose and 0.5% tartaric acid, with a pH of about 3.8.

When fungi are grown on an agar medium and both fungus and substrate are to be fixed and sectioned, the addition of a little lampblack to the agar just before sterilizing has been recommended, as making it easier to locate the sections of the fungus. The lampblack has not been found to have any effect upon the development of any fungus grown on media to which it has been added.

Further cultural directions will be outlined for many of the species described in detail below.

Fixation.—All the fleshy and parasitic fungi are excellently fixed with formalin-propiono-alcohol. A weak or medium chrom-acetic or a weak chrom-osmo-acetic also serve well. About 1% saponin or 0.2% ethyl or methyl acetate is advantageously added to all aqueous fluids in order to lessen the surface tension. Gelatinous forms are apparently better fixed in aqueous than in alcoholic fluids, since the latter may sometimes cause such a contraction of the gelatinous substance that the hyphae are distorted. Care should be taken not to overdo the fixation.

For mitochondria in fungi, any fixing fluid giving a basic fixation image may be employed.

Many plant pathologists claim that it is impossible to obtain adequate preservation of both fungus and host in the case of parasitic species, and there appears to be considerable ground for their complaint. The trouble apparently has been due principally to the older dehydration methods.

General Staining.—Most fungi are easily stained with all the various hematoxylin schedules. Iron hematoxylin is particularly favored as a cytological stain. The coal-tar dyes as a rule stain well but are frequently difficult to differentiate satisfactorily, particularly in the spores.

Differential Staining of Fungus and Host.—Quadruple staining combinations are the most satisfactory on parasitic species since the mycelium is well brought out in the host tissues. Many methods have, in fact, been proposed for distinguishing mycelium in woody tissues, but all of them have the disadvantage of requiring much experience to obtain a really first-class differentiation (see Stoughton 1930, Vaughan 1914).

Advantage may be taken of the oxidizing or reducing powers of the mycelium: silver nitrate in weak solution stains mycelium brown or orange and the wood a light brown.

The following is a rapid method of detecting mycelium of wood-rotting fungi:

1. Boil infected blocks (about 1-cm. cubes) for 30 minutes or longer, and soak for several hours to a few days in equal parts of glycerin and 70% alcohol, then cut freehand sections in a sliding microtome.

2. Flood sections with Bismarck brown (2% solution in 70% alcohol). Time varies according to the material, but 1 or 2 minutes usually suffices.

3. Drain excess stain, and wash with distilled water.

4. Flood sections with a saturated aqueous solution of methyl violet for 2 minutes (or use a diluted stain and increase the time).

5. Wash out excess stain with distilled water. Mount a specimen section in water, and examine microscopically. If the brown is too faint, begin the staining process again with step 2; if the violet is not strong enough, return to the violet stain. Wash again thoroughly before proceeding to the following step.

6. Flood sections with 50% dioxan, pour off immediately, and replace with 70% dioxan. After 10 seconds replace the 70% dioxan with pure dioxan. Give two more changes of pure dioxan, then mount the sections in balsam. The sections should never be placed directly in pure dioxan as the latter will precipitate dye all over the sections. Hyphae are stained deep violet; cell walls of the wood are yellow to brown. The bordered pits and medullary rays of conifers are usually stained violet.

The following is an excellent method for paraffin sections of infected tissues (Mangenot 1929): bring slides down to water, and stain for 1 hour in a solution of 0.1 g. thionin and 100 cc. of a 3% aqueous solution of phenol. Rinse in water, counterstain in 0.3% light green in 95% alcohol

until the sections appear green; wash with water, which will remove all the green color except from the xylem, pass through 95% alcohol and absolute alcohol, and again counterstain in a mixture of orange G and erythrosin (1 part saturated orange G in absolute alcohol to 2 parts saturated erythrosin in clove oil). Clear in xylol, and mount in balsam. Cutin and spore walls are yellow, cellulose yellowish-pink, cytoplasm pink, lignified elements green, middle lamellae reddish, hyphae and spores purple.

Another excellent method for sections (Ikata 1932) consists in staining them 5 to 10 minutes in 1% methyl violet in 50% ethyl alcohol, washing briefly in 50% alcohol, and then staining for 30 to 60 minutes in 1% eosin in 50% alcohol. Differentiate in a mixture of 2 parts turpentine, 1 part cedar oil, and 2 parts phenol crystals. Next wash in xylol, then mount. The cell walls of the host are stained violet, the chloroplasts and granules red, and the hyphae an intense red.

PHYCOMYCETAE

Members of the Mycophyta in which the asexual spores are produced in indefinite numbers within a sporangium are included in the Phycomycetae. No single distinctive type of spore is produced. In most species the zygote resulting from gametic union produces a thick wall and a resting period precedes germination. The mycelium is generally nonseptate but may occasionally be transversely septate. Some genera do not produce a mycelium. There is, in any event, no production of a macroscopic plant body.

All the Phycomycetae are either saprophytic or parasitic and many genera are aquatic in habitat.

Plasmodiophorales

The two more prominent genera are *Plasmodiophora*, in which the spores at maturity lie free in the host cell, and *Spongospora*, in which the spores are united in a solid sponge-like ball at maturity.

The single species of *Spongospora*, *S. subterranea*, is the cause of powdery scab of potatoes. Accounts of the life history are contradictory. Portions of infected tubers may be fixed in a medium chrom-acetic fluid. For the myxamoeba stages thin sections of very recently affected tubers are needed. For nuclear details stain in iron hematoxylin and fast green; for general morphology any triple combination may be used.

Plasmodiophora brassicae occurs in the roots of *Brassica oleracea* and related species, causing readily apparent malformations (Fig. 56). For the myxamoeba, roots of seedlings should be used if there is reason to suspect that they are infected. Roots that have begun to swell will show the later stages; spores will be found in badly swollen roots. Formalin-

aceto-alcohol has given excellent fixation of all stages when followed by tertiary butyl alcohol dehydration. Navashin's fluid or a medium chromosmo-acetic fluid might also be tried. The stains recommended for *Spongospora* may be applied.

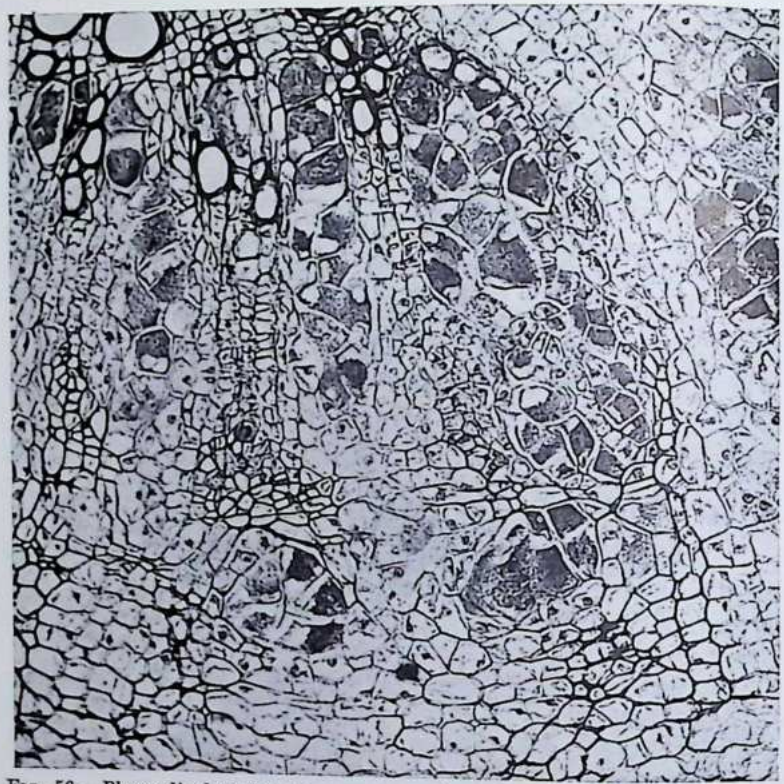


FIG. 56.—*Plasmodiophora brassicae:* cross section of young root of *Brassica oleracea* with developing plasmodium. Fixed with formalin-aceto-alcohol; stained by a triple combination.

CHYTRIDIALES

Members of this order, commonly called "chytrids," are regarded as the lowest of the fungi by some authors (Fitzpatrick 1930).

The plant body is unicellular, or with only a weakly developed mycelium. Most of the species are parasitic. In some species the entire cell becomes transformed into a reproductive body; in other species, the cell becomes differentiated into a globose fertile portion and a rhizoidal vegetative portion. A simple and quick method of obtaining chytrids is to place fresh or dry pine pollen in pond water. The pollen presumably carries zoospores. The grains may be removed after 2 or 3 days and examined.

Rhizidiaceae.—The unicellular parasitic plant body is differentiated into a walled fertile portion, which lies external to the host, and a naked branched rhizoidal portion. The species, of which there are both marine and fresh-water types, grow upon other aquatic fungi, on algae, or on pollen grains. Whole mounts are probably the only kind of permanent preparations that can be made; most of the published observations appear to have been made from living material.

Olpidiaceae.—The entire unicellular plant body, which lies completely within the host cell, is fertile. Treatment is predicated by the nature of

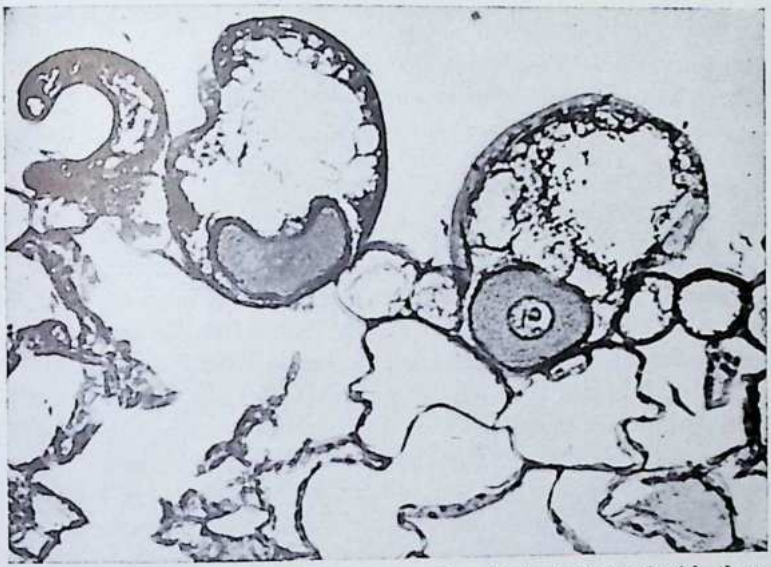


FIG. 57.—*Synchytrium amsinckiae:* cross section of *Amsinckia* leaf with three uninucleate summer spores. Fixed with formalin-aceto-alcohol; stained with iron hematoxylin and fast green.

the host; whole mounts should be made when possible. Iron hematoxylin is the preferred stain for nuclear details.

Synchytriaceae.—Most of the species are parasitic in the epidermal cells of Angiosperms. The whole of the plant body lies within the epidermal cell, and all of it goes into the formation of an aplanospore which in turn is transformed into zoospores.

Synchytrium is the best known of the Chytridiales. Its range is cosmopolitan. Navashin's fluid, preceded or not by a 10-minute immersion in Carnoy's fluid, appears to give very good fixation; formalin-aceto-alcohol causes some plasmolysis. Cut out portions of infected leaves, fix, embed, and section transversely at 10μ. Iron hematoxylin gives an excellent nuclear stain but care should be taken not to leave the cytoplasm, which is somewhat dense, overstained (Fig. 57).

Cladochytriaceae.—In *Physoderma* are placed the species which cause merely a discoloration or slight thickening of the infected parts of the host, while those which produce galls or cause pronounced deformations are placed in *Urophlyctis*. In both genera, sections of portions of the host bearing the parasite are required. In selecting material of *Urophlyctis*, choose the youngest possible infections, since spore formation begins early in the development of the malformations.

Woroninaceae.—The family is composed of unicellular parasites, each of which infects a single cell of the host. The entire body becomes fertile during reproduction. The species infest primarily *Saprolegnia*, *Achlya*, *Pythium*, and similar aquatic fungi, and a species of *Woronina* inhabits filaments of *Vaucheria*. Whole mounts of the host, if carefully stained with iron hematoxylin and critically examined under high-power lenses, should reveal the parasite if it is present.

Blastocladiales

The plant body is a true mycelium. Anisogamous sexual reproduction is known in one of the two genera comprising the order.

Allomyces has received considerable attention and is an easily studied, but not so easily collected, form (Hatch 1935). To induce production of sexual mycelia, dry sterile agar cultures of mycelia at either 40 to 45°C. for 1 to 2 hours, or at room temperature for a few weeks to several months. Add distilled water to the dried culture to soften the agar, then cut out portions of the agar 1 cm. square, and inoculate four halves of hemp seed cotyledons in aqueous culture. The sexual mycelia will have developed sufficiently in three or four days. Gametogenesis may be observed in hanging-drop cultures. Or the sexual mycelia may be fixed with a medium chrom-acetic or other fluid which on trial gives good fixation. For staining, the Feulgen reaction is preferable.

Monoblepharidales

Included in the Monoblepharidales are the only fungi in which active sperms are retained. All the species in the two genera are aquatic saprophytes, commonly found on twigs. There is little mention of the order in the literature, although material is easy to obtain (Sparrow 1933) and it would appear that whole mounts are easily prepared by the general methods. Growth of mycelium and production of zoospores are abundant on pea broth (Couch 1936).

Anguillulales

The parasitic mycelium is small, sparingly branched, and grows within a single host cell. Fresh water members of the Chlorophyta principally

are attacked. Whole mounts of the host may be made; staining should be in iron hematoxylin, very carefully differentiated.

Saprolegniales

The mycelium is well developed. The reproductive organs are terminal on the hyphae: asexual reproduction is by biflagellate zoospores; sexual reproduction is oögamous. Gemmae are also present in some species.

Saprolegniaceae.—*Saprolegnia* can generally be obtained by dropping dead flies, bees, meal worms, ant eggs, small dead fish, etc., into pond water. Within a few days to a week the host should be well covered with mycelium. Egg masses of *Triturus* and frogs are often attacked by *Saprolegnia*. A portion of the mycelium can be transferred to peptone agar (1% peptone and 1% agar); the outer ends of the resulting mycelium can be transferred to a fresh culture dish and a pure bacteria-free growth obtained. Pea decoction (1 pea to each 20 cc. of water) or soybean agar are excellent culture media. *Saprolegnia* and related genera are very sensitive to the minutest traces of copper, consequently glass-distilled water should be used in making up culture media.

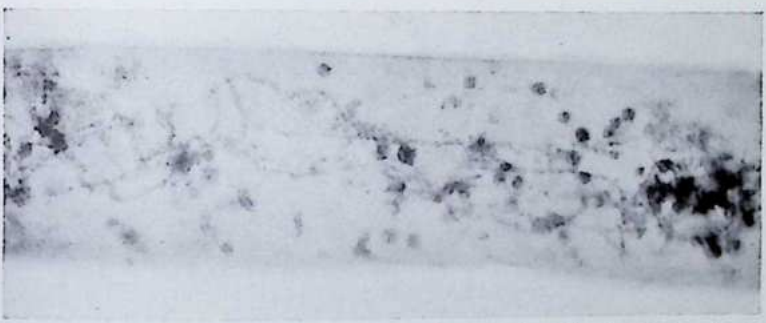


FIG. 58.—*Pythium gracile* endophytic in *Vaucheria terrestris*. Fixed with formalin-aceto-alcohol; stained with iron hematoxylin and dehydrated with hygrobutol.

Two methods of inducing the formation of oögonia and antheridia have been described. One is to transfer highly nourished mycelium to a 0.1% solution of leucin; the sex organs should appear in about 24 hours. The other method is to cut ordinary corn into small pieces, boil for 20 minutes, and place the cooled corn in Petri dishes. Nearly cover the corn with pond water. The sex organs may appear in about 4 days after inoculation.

There are nine other genera resembling *Saprolegnia* in a general way (Coker 1923). All may be treated alike microtechnically. Many species are well fixed in 10% aqueous formalin, particularly if mitochondrial investigations are being made. Formalin-aceto-alcohol has also proved

satisfactory. For nuclear details, iron hematoxylin is the only satisfactory stain, but it must be very carefully differentiated, as the nuclei may lose their stain before the cytoplasm has become cleared of stain.

Dehydrate and mount the material very much as if it were *Spirogyra* (page 110), using the hygrobutol method.

Leptomitaceae.—The mycelium, unlike that in the preceding family, is constricted at intervals. Treat material as if it were *Saprolegnia*.

Pythiaceae.—The species are aquatic parasites, some causing "damping-off" of seedlings. *Vaucheria* is also frequently invaded by *Pythium*, whose presence is readily revealed in whole mounts of the host (Fig. 58). Cultivation may be on corn- or pea-decoction agar (Diesmann 1927). Fixation may be with 1% chrom-acetic or with formalin-aceto-alcohol, staining with iron hematoxylin, not too far destained, with a counterstain of fast green to aid in showing up the mycelium.

Peronosporales

All species assigned to the Peronosporales are parasitic on land plants. Asexual reproduction is by the production of conidiosporangia that either form biflagellate zoospores or germinate directly. Sexual reproduction is oögamous.

Peronosporaceae.—Species with a branched sporangiophore, each bearing a single conidiosporangium, are assigned to the Peronosporaceae.

Since the mycelium and sex organs are intercellular, sections of the host are necessary for these structures. For developing sporangiophores sections are also needed, but for the mature fructifications whole mounts are more satisfactory. If the affected part of the host is the leaves, mounts of portions of a leaf with the parasite attached are easily made according to the general methods described above.

Many of the genera can be cultivated artificially. The following nutrient solution produces a good growth of all species of *Phytophthora* (Leonian 1930):

Protease peptone	2 g.
Dibasic potassium phosphate	0.5 g.
Magnesium sulphate	0.5 g.
Succinic acid	0.2 g.
Dextrose	5 g.
Distilled water	1 liter

Or the following, used in Petri dishes at the rate of 15 cc. in each, is also excellent:

Dry malt extract	5 g.
Dibasic potassium phosphate	0.5 g.
Magnesium sulphate	0.5 g.
Agar	20 g.
Distilled water	1 liter

Autoclave both at 10 pounds pressure for 15 minutes. The optimum temperature for growth is 25°C.

General staining methods may be used.

Albuginaceae.—The sporangiophores are unbranched and form a palisade-like layer beneath the epidermis of the host. Conidiosporangia are formed successively. There is but one genus, *Albugo* (*Cystopus*). Only sections are of any value. Portions of the infected host may be fixed in a medium chrom-acetic fluid or in formalin-aceto-alcohol. Sections should not be over 8μ in thickness. Stain with iron hematoxylin, and counterstain with orange G, or if preferred, a quadruple combination might be tried.

Mucorales

The black bread mold is one of the commonest fungi that one encounters. The generally unseptate mycelium is well developed and branches freely. Asexual reproduction is generally by means of aplanospores produced in sporangia; sexual reproduction is essentially isogamous. There are both affirmation and denial that nuclear fusion occurs during the formation of the zygote.

Members of the Mucorales occur so commonly and are so easy to obtain in culture that they have acquired a prominent position in botany courses. Methods of cultivating will be given in some detail in the following discussion.

Rhizopus, *Mucor*, and most of the other genera are difficult or impossible to immerse in, or to dampen with, water on account of the great difference in surface tension. Alcohol should not be used for lowering the tension, but a somewhat innocuous medium should be tried. A soap solution or a 0.5% aqueous solution of gelatin may be poured over the mass of mycelium until most of the air has disappeared in the form of bubbles, washed out with water, and finally placed in the killing solution.

Mucoraceae.—Some 10 genera are included in the family, of which those most likely to come to the technician's attention are *Rhizopus*, *Phycomyces*, *Zygorhynchus*, and *Mucor* (Fitzpatrick 1930. Heinrici 1930). The presence of a columella in the sporangium distinguishes the Mucoraceae from the other families in the order. *Rhizopus* and *Mucor*, which are often confused, may be readily differentiated: in *Mucor* the sporangiophores arise directly from the mycelium; in *Rhizopus* the sporangiophores arise in a fascicle from a node on the stolon, opposite a tuft of rhizoids. In Petri dish cultures *Rhizopus* mycelium grows over the underside of the lids, forming sporangiophores; *Mucor* does not attach itself to the lids. Some of the mucors are pathogenic. In addition to the spores produced in sporangia, round bead-like blackish chlamydospores may sometimes be found on the mycelium. The chlamydospores should not be mistaken

for zygospores. When grown in poorly aerated liquid media, spherical yeast-like oïdia may be produced. These oïdia may reproduce by budding.

All Mucoraceae are very easily grown on culture media. Sterilized bread, horse dung, prune agar, or almost any of the sacchariferous agars form convenient cultural media, or the following synthetic medium may be employed:

Distilled water	1 liter
Sucrose	30.0 g.
Ammonium chloride	6.0 g.
Magnesium sulphate	0.5 g.
Monobasic potassium phosphate	0.5 g.

However, it is difficult to remove material from solid media, in an undamaged condition, suitable for slide-making purposes. A simple method of securing abundant and easily removed material is available. Prepare a broth or decoction, whether synthetic or from such articles as potatoes, prunes, or malt extract. Add agar to about a two-thirds portion of the fluid, sterilize, and pour into Petri dishes as usual, but do not fill the containers more than half full. Also sterilize the remaining one-third portion. As soon as the agar has solidified, pour a layer of the liquid portion over the agar to a depth of not over 2 mm. Inoculate. The dishes must, of course, be kept level. When the desired degree of growth has been attained, the entire mass of fruiting mycelium may be lifted off, washed in water, and placed in the killing fluid.

Most of the Mucoraceae grow satisfactorily at room temperature. Species of *Rhizopus*, however, can be roughly divided into three groups, depending upon the temperature at which they grow most luxuriantly: 20 to 22°C., 30°C., 35°C. The intermediate group contains the most vigorous species. The culture vessels may be kept in the dark, but *Rhizopus* seems to grow better in moderate light.

In some species, or in entire genera, the mycelium is homothallic, in others heterothallic. In the latter species the two distinctive types of mycelium are classified as "plus" (the more vigorously growing strain) and "minus." In the homothallic species zygospores are produced when neighboring filaments from the same plant fuse. In the heterothallic species zygospores are developed only when filaments of opposite strains come into contact (Fig. 59). The zygosporic stage will hardly be encountered in ordinary cultures since it occurs very rarely in nature. Cultures of known plus and minus strains are available in practically all universities which have a good mycologist on their staffs, or they may be secured from the American Type Culture Collection. To produce zygospores, prepare a prune decoction agar (or use moistened bread), and inoculate

one end with the plus strain and the opposite end with the minus strain. The spores will develop where the two mycelia come into contact.

In genera, such as *Sporodinia*, which possess only one type of mycelium instead of two, the zygosporic stage is more easily secured. *Sporodinia* may be cultivated on bread soaked in prune juice, on slices of beet or carrot roots, or on prune decoction agar. Zygospore development should commence in about four days.

Formalin-aceto-alcohol fixes the Mucoraceae splendidly, but the amount of formalin may need to be increased to twice the usual volume. Allow the material to remain in the killing fluid for at least two days.

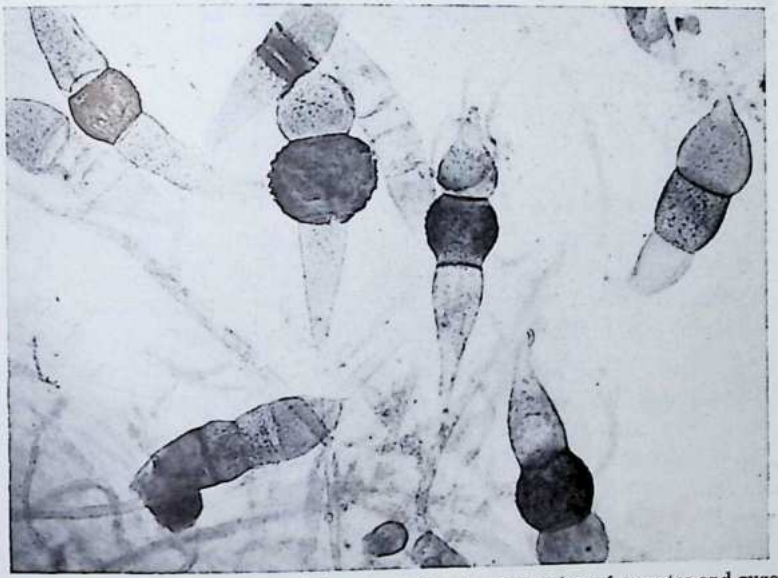


FIG. 59.—*Rhizopus nigricans:* whole mount showing formation of gametes and zygotes. Fixed with formalin-aceto-alcohol; stained with safranin and fast green. (*From a preparation by Dr. Geo. H. Conant.*)

Harris' hematoxylin with a counterstain of fast green brings out nuclear details of sporangial development beautifully. Iron hematoxylin may be used, but the results are not always satisfactory because the dense cytoplasm too often remains overstained. After they have begun to become darker, the zygospores are impossible to stain intensely. Safranin and fast green has been successfully employed on material containing mostly dark zygospores. For whole mounts the hygrobutol method has been the most serviceable one.

Pilobolaceae.—The best-known members of this family are the species of *Pilobolus* inhabiting horse dung. Freshly dropped dung may be placed under a bell jar and abundant mycelium should appear within

a few days. The sporangia are numerous and large. Treat as described for the Mucoraceae.

ENTOMOPHTHORALES

Some of the Entomophthorales are saprophytic; others are parasitic on various insects, particularly flies. The thallus ranges in size, according to the species, from the vegetatively nonhyphal type to a profusely branched mycelium. Asexual reproduction is by means of conidiosporangia that germinate directly; sexual propagation is by the fusion of aplanogametes of equal or unequal size.

Basidibolaceae.—*Complectoria* is an intercellular parasite in the prothallia of ferns. Whole mounts of the prothallia should reveal the parasite if it is present, but staining must be rather precise. Sections of the prothallia to show the zygospores are easily made if the methods for the treatment of the prothallia as described under the Pteridophyta are followed.

Entomophthoraceae.—*Entomophthora* (*Empusa*) is the largest genus in the order, consisting entirely of insect parasites. The host is invaded by the mycelium and the fungus usually develops to maturity in from five to eight days after infection. Special cultural methods are required for artificial cultivation (Sawyer 1939). There are two methods of making permanent preparations. One is to kill and fix the entire insect, first puncturing the abdomen and thorax and using a suction pump if the material does not sink into the killing fluid. Then wash, stain (preferably in a hematoxylin), dehydrate, and infiltrate with balsam. With a needle and fine-pointed scissors, dissect out small portions of the insect bearing the fungus, and mount in balsam. The second method is to kill and fix the infected insect as before, then, after washing out the killing fluid, to soften the chitinous exoskeleton of the insect so that it can be sectioned after embedding. Potassium hypochlorite (eau de Javelle) may be employed as the softening agent, but sodium hypochlorite appears to be preferable as being less violent in reaction. Dilute the commercial solution of either reagent with 4 to 6 volumes of water, and immerse the insects for about 24 hours, or until the chitin becomes sufficiently softened. Wash with water, and treat cautiously with a weak solution of acetic acid if precipitates appear to have formed. Then proceed to paraffin infiltration following tertiary butyl alcohol dehydration.

ASCOMYCETAE

The Ascomycetae are so called because sexual reproduction is by means of spores formed within an ascus immediately subsequent to meiosis. Other types of spores are also produced, but always asexually.

Morphology.—The Ascomycetae include both saprophytic and parasitic species. Some groups lack a mycelium, some have a slight mycelial growth, a few others possess a loosely interwoven mass of hyphae, but most often the mycelium is more or less developed into a pseudoparenchymatous structure of definite and often characteristic form. The hyphae are incompletely septate; there is a central perforation through which the protoplasm of one portion may stream into that of the adjoining portion. Each septated portion is generally uninucleate, but in a few genera, such as *Pyronema*, it may be multinucleate. The mycelium may be homo- or heterothallic.

Sources of Material.—The commonest and most easily found of the Ascomycetae are the so-called "laboratory weeds," *Penicillium* and *Aspergillus*, the cup fungi and the powdery mildews. The latter are abundant in many localities; *Taphrina* can be found on almost any unsprayed peach tree shortly after the leaves appear; in short, no matter where one may be located, some ascomycete or other can be found without much difficulty. As a last resort, material can be purchased from the botanical supply concerns.

Most Ascomycetae, as ordinarily found, are too old for other than display or herbarium purposes. It is a difficult matter to find sexually reproductive phases.

Cultivation.—Cultures of most of the Ascomycetae may be grown with a minimum of bother since their requirements are either simple, or, if specific, are readily provided. Specific directions are cited below for a number of the more interesting genera.

Little work, however, has been done on the nature and development of very many species.

Fixation.—In general a weak or medium chrom-acetic fluid or formalin-aceto-alcohol serves satisfactorily. So much surface is usually presented for direct contact with the killing fluid that perfect fixation is the rule rather than the exception. The only precaution to observe is to dehydrate with a well-graduated series of fluids.

Staining.—For general morphology a triple or quadruple combination serves well. For nuclear details, whether on material to be mounted whole or in sections, nothing surpasses iron hematoxylin; a counterstain is generally undesirable, but if one is wanted, a weak counterstaining with erythrosin or fast green will assist in revealing the ramifications of the mycelium of parasitic species in the host tissues.

Sexual Reproduction.—In most of the Ascomycetae an antheridium is developed so that it lies opposite an ascogonium (the female sex organ). The ascogonium in some species consists of more than one cell; if not broadly rounded at the apex, the distal end may be prolonged into a trichogyne. In other Ascomycetae the sex organs are not paired. In

these species the antheridium is generally replaced by a spermogonium, which greatly resembles a pycnidium and in which spermatia are produced. In some species spermatia are not produced in a definite spermogonium. The ascogonium commonly has a multicellular trichogyne. Material must be very carefully collected if the sex organs are to be studied, and must be at a far earlier stage of development than seems to be generally realized.

After fertilization the resulting zygote develops either directly into an ascus or indirectly into one to numerous asci. The first method is characteristic of the Protoascomycetae; the second of the Euascomycetae. There are in addition irregular methods of ascus formation in some species.

The entire group of asci and their enveloping hyphae form an ascocarp. There are three general types of ascocarps: the cleistocarp, which always remains closed; the apothecium, open and more or less bowl-shaped; and the perithecium, with a pore-like opening at the apex.

Among the most suitable genera in which to find the sex organs, provided sufficiently young material can be secured, are *Phyllactinia*, *Sphaerotheca*, *Pyronema*, and *Venturia*.

Protoascomycetae

In this group the zygote develops directly into an ascus. The asci are borne singly on the mycelium and are never in an ascocarp or surrounded by sterile tissue (Dodge 1935).

Endomycetales

Saccharomycetaceae.—The family includes all the various types of true yeasts which form endospores. So little is known concerning the yeasts that it is extremely difficult to identify unknown forms (Heinrici 1930). Only a few species have such striking characters that they are instantly recognized. Some genera exhibit so many intergrading types and certain species are so variable in certain of their characteristics that even specialists on the yeasts have trouble distinguishing between them. Consequently, unless one is merely seeking to find yeasts in the different types of situations where they occur, it would be better to obtain a culture of known identity from a reliable source. The American Type Culture Collection lists innumerable genera and species.

To show multiplication by true budding, use *Saccharomyces ellipsoideus* (found naturally on grapes) or *S. cerevisiae* (compressed yeast). These forms may be induced to produce spores, as described below, but it will be found much simpler to employ a species in which the spores are regularly formed. For this purpose, *Schizosaccharomyces octosporus* (Fig. 60) or some species of *Zygosaccharomyces* serve excellently.

Yeasts may be grown in liquid media or on nutrient agar; the spore-forming species should be cultivated on nutrient agar only. Malt extract broth (Difco), plain or solidified with agar, is preferable, but one may use grape juice diluted with an equal volume of water or a 5 to 10% aqueous solution of molasses.

Spores can often be found on the surface of cakes of commercial yeast kept in the refrigerator for a week, but, on the whole, spore production in most yeasts is a matter of great uncertainty. It sometimes occurs if the culture is kept overnight in the refrigerator (or at 9 to 13°C.), then transferred the next morning to an incubator kept at 25 to 30°C.; germination of the spores will begin after 2 to 3 hours, or at most after 7 hours. Another method is to make a weak carrot infusion agar and to add a little calcium sulphate just before tubing; tube, sterilize, slant, and inoculate (Heinrici 1930). Or use Gowodkowa's medium (Maneval 1924):

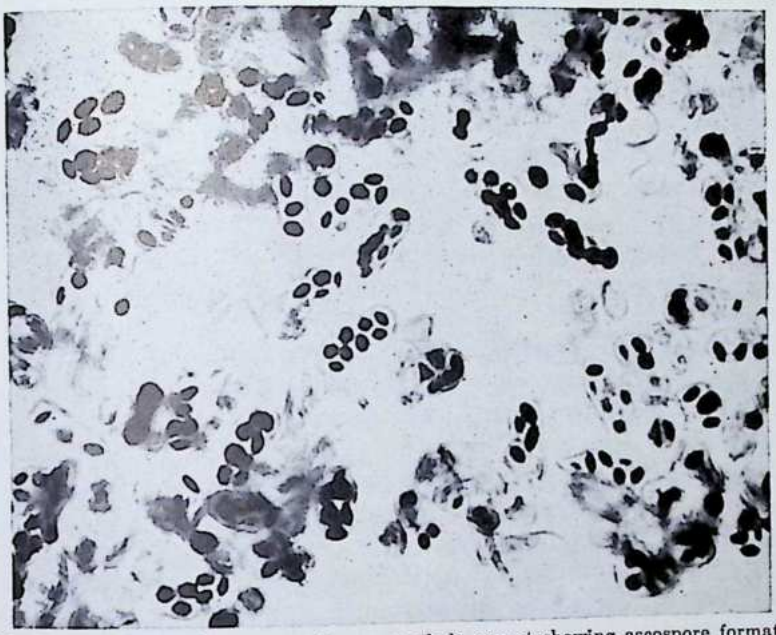


FIG. 60.—*Schizosaccharomyces octosporus:* whole mount showing ascospore formation. The staining is darker in the photomicrograph than on the slide. Pure culture suspension dried to slip, stained with iron hematoxylin and fast green.

Dextrose	0.25 g.
Sodium chloride	0.50 g.
Beef extract	0.30 g.
Agar	1.5 g.
Distilled water	100 cc.

Most workers simply dry the yeast cells to chemically clean slides preparatory to staining. Place 1 drop of water on the slide, mix in a small amount of culture by means of a platinum loop, and, after the water has evaporated, pass the slide through a flame eight to ten times to fix the cells. Better adhesion of the cells to the slide may be secured by first smearing a thin film of Mayer's adhesive, diluted 1:10 with water, over the slide. Still another method is to smear with full strength Mayer's, smear the yeasts on the adhesive (with the finger or a scalpel), and fix the moist film with Bouin's fluid. Basic and acid fuchsins are the dyes most commonly used for staining such smears, and there are innumerable modifications. The slide may simply be placed in a 1% aqueous solution of the dye for a minute; wash off excess stain, and let the preparation dry. A slightly better method is to stain in a stronger solution of the dye for 1 minute, wash with 5% aqueous tannic acid for 20 seconds, then wash with water slightly acidulated with hydrochloric acid, dry, and mount in balsam. A 1% aqueous solution of methylene blue employed for 20 seconds is also good, as is a mixture of equal parts of 1% aqueous solutions of acid fuchsin and methyl green.

The most precise method is to fix a large volume of culture with a weak chrom-acetic fluid, wash thoroughly, and stain with iron hematoxylin and fast green or orange G; run up as if for whole mounts. It will be necessary to use a centrifuge between changes of fluids. Fixing fluids containing alcohol or formalin should be avoided, as they appear to cause flocculation of the chromatin. Mitochondrial fixatives give excellent preservation. The mitochondria consist of long, wavy bodies. Vital staining has been recommended (Heinrici 1930); suspend some of the cells in water under a coverslip, and put a drop of 1% aqueous solution of neutral red at one edge. Dead cells will stain a uniform red, but in living cells only the volutin vacuoles and granules will take up the dye. The former stain light pink, the latter deep red.

Torulaceae.—Placed in the Torulaceae are the so-called "false yeasts" which do not form spores. They reproduce by budding and never form a mycelium. They are difficult to identify. The red forms are very common and are found particularly upon dairy products and on grapes. Technically, they may be treated exactly like the true yeasts.

Euascomycetae

There are some fifty times as many species of Euascomycetae as of Protoascomycetae. The sori are developed on ascogenous hyphae which arise from a zygote. The asci are produced, generally in large numbers, in an ascocarp.

In the following discussion only those families containing species of fairly common distribution in the United States are included.

ASPERGILLALES

The asci are irregularly distributed in a closed ascocarp. A number of saprophytic species of economic importance are included in the order.

Gymnoascaceae.—The peridium is made up of loose floccose hyphae. Species occur on earth, manure, feathers, cadavers, etc. Whole mounts of small fructifications can be prepared, but sections should be made of the ascocarps.

Aspergillaceae.—The family is by far the most significant one in the order. The ascocarps are small, sessile, and remain closed. Of the 13 genera, only the following are likely to come to the attention of the technician: *Thielavia*, *Penicillium*, and *Aspergillus*.

Thielavia basicola appears in wet weather to cause a root rot of numerous Angiosperms, being especially serious on *Nicotiana tabacum*. In the earlier stages the two types of conidia are abundant and the ascocarps appear after the host is killed. Sections of roots at various stages of growth may be prepared according to general methods; a quadruple stain is good.

Aspergillus is more widespread in the tropics than elsewhere but is nevertheless easy to secure. It frequently appears on bread on which *Rhizopus* has been grown, at about the time the latter has run its course. The *Aspergillus* will appear more quickly if the bread is moistened with grape juice or 10% aqueous sucrose rather than with water. Place the slice of bread on slightly dampened filter paper in a suitable moist chamber, after exposing the bread to the air for about 2 hours, and keep at a temperature between 25 and 37°C. Microscopical examination of the growth which appears in a few days will be necessary to determine its identity. *Aspergillus* is usually green in color, but *Mucor* and *Penicillium* also have a greenish coloration. If this procedure is unsuccessful, cultures are obtainable from the supply concerns. Mildewed herbarium specimens are a likely source for *A. herbariorum* or a related species, which are excellent ones in which to induce perithecial development. Once *Aspergillus* has been secured, cultures may be transferred to a simple medium prepared by steaming 10 g. of either wheat or rice bran with 10 cc. water in a 400-cc. Erlenmeyer flask, stoppered with a cotton plug. The proportions of bran and water may be varied to suit requirements. Czapek's medium is the standard for growth studies on *Aspergillus* (Thom and Church 1926). Whole mounts of the conidial stage are easily made by standard methods; stain with either iron or Harris' hematoxylin and fast green. Acid fuchsin also stains well. The conditions under which perithecia formation may be induced are imperfectly understood, but the availability of an abundance of easily assimilable carbohydrates appears to be the principal prerequisite. Agar media seem to be prefer-

able: one might solidify either a 40% solution of sucrose in prune juice (obtainable in larger grocery stores), or a 20% solution of dextrose or glucose in grape juice, in suitable containers and sow spores thereon. The culture should be kept at a temperature of at least 25°C. The perithecia are carried above the surface of the substrate and are loosely borne in networks of hyphae. Cut out 15-mm. squares of the agar containing either sex organs or perithecia, kill in a medium chrom-acetic fluid, and embed the entire mass in paraffin (the agar gives no trouble).

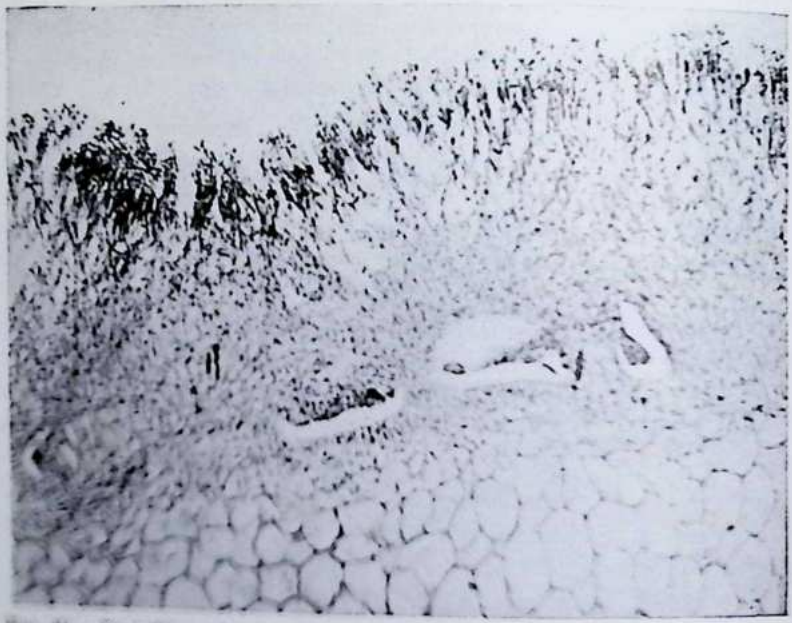


FIG. 61. *Penicillium glaucum*: transverse section of orange rind with the parasite growing in situ. (Only sections can show the penetrating mycelium and the nuclei in the aerial mycelium and spores. Fixed with formalin-propiono-alcohol; stained with safranin and fast green.

Microtome perpendicular to the surface of the agar at 10μ; stain with iron hematoxylin and fast green, or substitute safranin for the hematoxylin.

Penicillium is more easily obtained than is *Aspergillus*. It appears more often than does the latter genus in aging *Rhizopus* cultures on bread. Perhaps the best source, however, is decaying oranges which have been kept slightly damp. Portions of the orange rind may be cut out, fixed with formalin-propiono-alcohol, embedded, sectioned at 10μ, and stained with safranin and fast green or a triple combination (Fig. 61). Details are more clearly revealed in this fashion than by whole mounts: the penetrating mycelium, for example, may be followed out. Most species grow well on saccharine media of the type recommended for *Aspergillus*.

gillus. Perithecia are not often produced, and the species that form them belong mostly to one section of the genus (Thom 1930). Some of these species are known to be heterothallic and consequently produce perithecia only when two different mycelia intermingle.

Erysiphales (Perisporales)

The species are almost all parasitic, usually superficially so. This indicates that sections of portions of the host bearing the parasite in various stages of development should invariably be made.

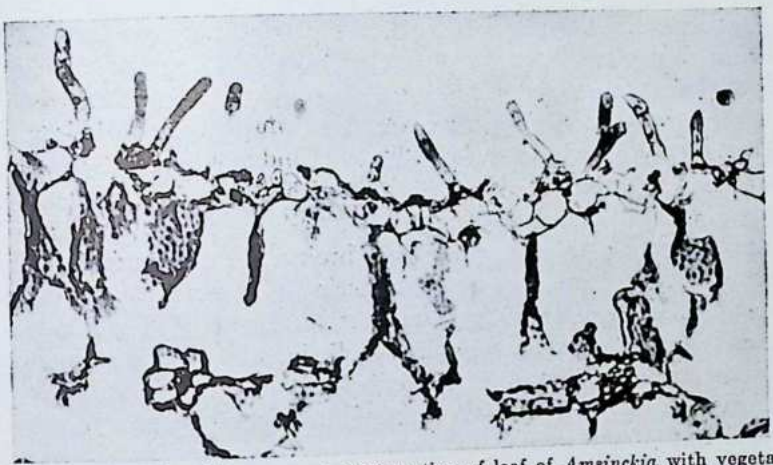


Fig. 62.—*Erysiphe cichoracearum:* cross section of leaf of *Amsinckia* with vegetative hyphae of parasite. Fixed with formalin-propiono-alcohol; stained with iron hematoxylin and fast green.

Erysiphaceae.—This is a widespread and common family, constituting the so-called "powdery mildews." The most prominent representatives are noted below. The life history of only *Erysiphe* has been completely investigated.

Sphaerotheca pannosa and *S. humuli* are very common on leaves of *Rosa*, generally occurring in the conidial stage; the perithecial stage is rare. Material of both host and parasite becomes rather hard during dehydration, but soaking under water remedies the difficulty. Staining is good by quadruple methods, or safranin and fast green may be used.

Erysiphe cichoracearum is cosmopolitan, infecting chiefly Asteraceae and Cucurbitaceae, and is excellent for the vegetative hyphae, haustoria, and conidial development (Fig. 62). Fix portions of the host leaf in either formalin-aceto-alcohol or Navashin's fluid, section transversely at 10μ, and stain with iron hematoxylin. The cytoplasm of the hyphae retains the stain more deeply than does that of the host. A counterstain is inadvisable. *E. aggregata*, which is not so widely prevalent as the

other species, is the most favorable one for the development of the ascocarps. They begin to appear as the growing season of the host is ending. Stems are better than leaves; fix short portions in a medium chrom-osmo-acetic fluid, microtome the host transversely at 10μ, and stain with iron hematoxylin and orange G for critical cytological details, or with a triple combination for general structure. A series of developmental stages are usually obtained in a few sections. *E. graminis* is common on various Poaceae but is not very satisfactory for sections.

Microsphaera alni occurs on numerous hosts in the northern states but is usually collected on *Syringa* leaves. Leaves bearing perithecia and their peculiar appendages may be fixed with 8% aqueous formalin for 24 hours, this washed out, then stained with 1% erythrosin in equal parts of water and methyl cellosolve slightly acidulated with acetic acid, dehydrated with hygrobutol or dioxan, and infiltrated with balsam; the perithecia may be cut off with a small scalpel and mounted independently, or portions of the leaves bearing the perithecia may be cut out and mounted. Great care should be taken at all stages not to handle the material roughly lest the appendages be broken off. However, sufficient pressure may be exerted on the perithecia just before the coverslip is applied in order partially to force out some of the asci. The leaves may be fixed in formalin-propiono-alcohol, sectioned at 11μ, and stained with any desired combination for detailed studies.

Phyllactinia coryleae occurs on a multitude of hosts. It may be treated like *Microsphaera*.

Phacidiales

Of the three families in this large, mainly saprophytic order, only the following occurs in the United States.

Phacidiaceae.—*Rhytisma* is the best known of the 18 or so genera. It produces conspicuous black areas on the leaves of *Acer*. Conidia are produced before the leaves turn yellow, while the ascocarps begin development after abscission of the leaves. Remove portions of the leaves bearing infected areas, fix with formalin-propiono-alcohol, soak under water for several days after embedding, microtome in the transverse plane of the leaf at about 10μ, and stain with a triple combination.

Pezizales

Members of the order are distinguished by their typically dish- or saucer- to cup-shaped apothecia lined with a layer of sori. The apothecia in size may be barely visible, up to bodies 10 cm. in diameter; in consistency they vary from fleshy or gelatinous to leathery, horny, or cartilaginous.

Helotiaceae.—*Sclerotinia fructigena* commonly occurs on mummified drupes and pomes; the apothecia arise from sclerotia produced either within or upon the latter. Cut off the sclerotia, and fix with formalin-propiono-alcohol; as the sclerotia are rather tough bodies, some soaking under water will be necessary. Stain with a triple combination.

Among the other genera, cut the apothecia away from the substrate, and treat as described below for *Peziza*.

Mollisiaceae.—*Pseudopeziza* is the most important genus, occurring as leaf parasites. *P. medicaginis* is common on *Medicago sativa* and

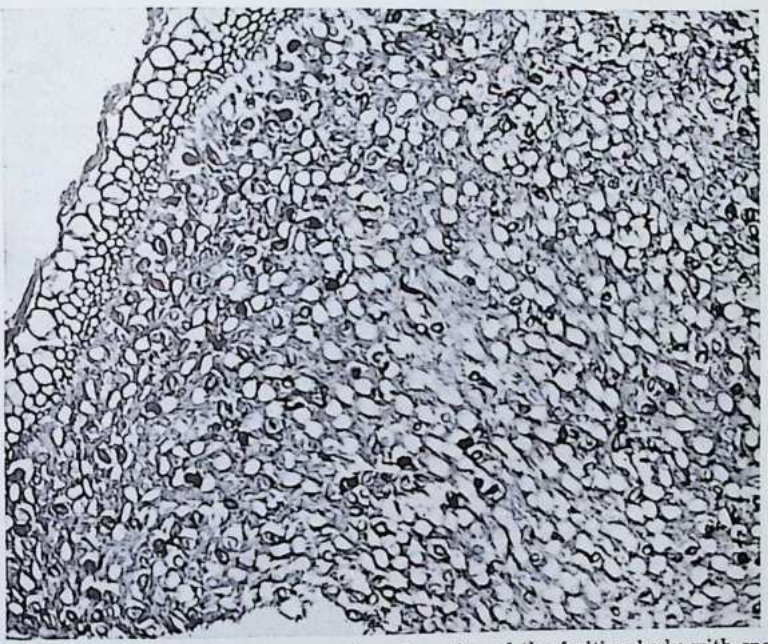


FIG. 63.—*Pyronema confluens:* cross section of portion of the fruiting body with spores. Fixed with 1 % chrom-acetic; stained with iron hematoxylin and fast green.

related species. The very small apothecium develops subepidermally, breaking through only at maturity, consequently sections of the leaves are indicated. *Fabraea maculata* resembles *Pseudopeziza*, occurring on the leaves of pear and quince which have wintered naturally.

Pyronemaceae.—*Pyronema confluens* has long been of interest to botanists. It is not an easy plant to collect, save by experienced mycologists. It is a saprophyte and generally is to be found on soils which have been burned over, sometimes on partially burned, damp wood, or on soils which have been sterilized by heat or steam. The older fructifications are immediately recognizable by their bright rose-red color; the apothecia become confluent in groups. The sex organs constitute the most impor-

tant phase, but the great difficulty is to collect the plants when they are young enough for this stage. Growth is quite rapid: when grown in artificial culture (Seaver 1909), the antheridia and oögonia begin to appear in four to five days after the spores have been sown, and the ascocarps are mature within the next five days. The plants are very sensitive to carbon dioxide, and strong light is required for the optimum growth. Spores may be sown on sterile earth; put the culture in the light at about 25°C.; fructifications will appear within a week. The following is a still better method. Fill the lower half of a Petri dish with the following nutrient medium:

Agar	2 g.
Inulin, pure	2 g.
Monobasic potassium phosphate	0.05 g.
Ammonium nitrate	0.05 g.
Magnesium sulphate	0.02 g.
Ferric phosphate	0.001 g.
Distilled water	95 cc.

Place the dish inside another dish of somewhat greater diameter but of the same height, and fill the space surrounding the inner Petri dish with the same medium minus the inulin. Inoculate the inulin agar with spores or a small portion of mycelium of the *Pyronema*, and cover the whole. Fruiting will occur in a few days on the inulin-free portion. Fix material with a medium chrom-acetic fluid, microtome in the vertical longitudinal plane at 8μ, and stain with iron hematoxylin (Fig. 63).

Pezizaceae.—Members of the family are of two sorts: hypogeous and epigeous. Very little has been described for the technique treatment of the hypogeous types. The epigeous genera, including *Peziza*, *Aleuria*, and related genera, form their brightly colored fructifications on the surface of the ground and are found mainly in damp woods—in the summer and autumn in the Eastern states and in the winter and spring on the Pacific Coast. To secure the youngest stages of ascocarp development, it is often necessary to dig beneath the surface near where mature apothecia occur and to search carefully. Fix with a medium chrom-acetic for the younger stages or with formalin-aceto-alcohol for the older ones, and microtome vertically at 10μ. Young stages should be stained with iron hematoxylin, other stages with the same stain or with safranin and anilin blue.

Tuberales

The Tuberales include hypogeous plants consisting of a colorless mycelium and ascocarps that are more or less completely enclosed apothecia. Nearly all species described from the United States are from California: they are found under trees, mostly *Quercus* and *Sequoia*, and in

shaded locations under humus. They occur at depths of from 8 to 30 cm.; consequently they must be dug up by means of a digging rake with long prongs. As the external surface in many species is mucilaginous in nature, they are covered with debris and may easily be overlooked because of their resemblance to clods or small rocks. The apothecia are more or less globular, or flattened slightly in some species, smooth, warted, or convoluted, and they range from 3 mm. to 10 cm. in diameter. Slabs should be cut from opposite sides of small specimens, and larger ones should be reduced to convenient smaller portions for fixation. Formalin-aceto-alcohol is excellent, or a medium chrom-osmo-acetic fluid may be used. The bodies always become considerably hardened during dehydration and require soaking under water before they can be sectioned easily. The sex organs have never been found, but it is not difficult to secure sections showing young ascogenous hyphae. Microtome this stage at 8μ, the later stages at 10 to 12μ. Stain all stages with iron hematoxylin; apply a counterstain of fast green on those for the ascogenous hyphae, but omit on later stages. Other stain combinations have been unsatisfactory.

Helvellales

Members of this saprophytic order are characterized by a subterranean mycelium and an aerial, usually stalked, ascocarp. The latter assumes many characteristic and sometimes brightly colored shapes. *Helvella*, *Morchella*, and *Gyromitra* are the more prominent genera and are of wide distribution, preferring cooler and damp woods. The sex organs have never been described, but there is a possibility that they have been replaced by a fusion of paired nuclei in certain vegetative cells. Portions of the hymenium of the ascocarp may be cut out, fixed with a chrom-acetic fluid or with formalin-propiono-alcohol, and embedded so that sections may be cut perpendicular to the external surface. Microtome at 11μ; stain with iron hematoxylin or safranin and anilin blue.

Exoascales

The asci are arranged parallel to one another in an open, loosely palisade-like layer.

There are a large number of species of *Taphrina* (*Exoascus*), all of them being aggressive parasites affecting leaves, twigs, and fruits to cause all sorts of malformations. *T. deformans*, which causes peach leaf curl, is the species with which the technician is most likely to deal (Fig. 64). It first appears on the leaves of *Prunus persica* shortly after they unfold in spring and produces yellowish and red discolored malformations. Remove portions so that each is made up about equally of infected

and uninfected tissues, fix with formalin-propiono-alcohol, microtome transversely at 10μ, and stain with either safranin and fast green, a quadruple combination, or iron hematoxylin, although the latter does not always react sufficiently well. If material is unavailable locally, it may be purchased from the supply concerns.

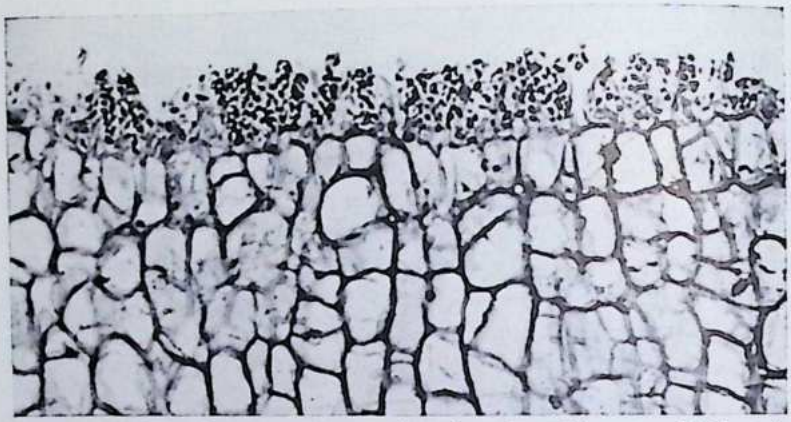


FIG. 64.—*Taphrina deformans:* cross section of portion of hypertrophied peach leaf with the parasite growing on the surface. Fixed with formalin-aceto-alcohol; stained with safranin and fast green.

HYPOCREALES

The species are characterized by soft and brightly colored (white, red, yellow, violet, or brown) perithecial walls, which do not become hardened during dehydration. There are hundreds of species, including many formerly included in *Fusarium* of the Fungi Imperfecti, whose technique treatment is essentially similar.

Each genus usually produces at least one form of free-borne conidia, and, in some, several different kinds of conidia are present. The asciferous stage is often more or less suppressed. Many species which produce asci do so only during the second growing season. Most of the species are saprophytes, some are plant parasites, and others attack insects.

Neurospora, which has been the subject of important genetical researches, belongs in the Hypocreales. Cultures may be obtained from investigators on this plant. Related genera, such as *Melampsora*, are also under controlled cultivation. Whole mounts of such genera are readily prepared according to general methods.

Species of *Nectria* are both saprophytic and parasitic, even within the same species, as in *N. cinnabarina*. It is usually found in wounds of hardwood trees. The mycelium does not penetrate sound tissues; consequently pieces of only the infected part of the stem need be removed for sectioning.

Epichloe typhina is abundant on various grasses; the somewhat fleshy stroma completely ensheathes the stem and becomes a bright orange in color. Microtome short sections of the stem transversely after first soaking under water for several days. Stain by a triple or quadruple method.

Claviceps abounds wherever rye and, to a lesser extent, wheat, oats, and other grasses are grown. There are a number of biological races. The ovary of the host is invaded at the time of flowering, and this organ is soon covered with conidiophores on the surface. The mycelium becomes metamorphosed into a compact sclerotium which matures at the time the grains become ripe. The following spring those sclerotia that have not become too far desiccated germinate to produce capitate outgrowths. The stroma (capitate portion) of each outgrowth contains numerous perithecia in which the sex organs and later the asci are produced. The sclerotia and outgrowths may both be fixed with a strong chrom-acetic fluid or in formalin-aceto-alcohol. The sclerotia may be sectioned at 10μ either transversely or longitudinally; the outgrowths should be microtomed in the vertical longitudinal plane at the same thickness. Fully mature sclerotia, however, become as hard as stone and probably cannot be sectioned; they should be fixed just before they commence hardening. A triple combination will stain both clearly, but iron hematoxylin is better for the sex organs and ascogenous hyphae.

Hypomyces, parasitic on the underside of the fruiting bodies of the Agaricaceae, is noteworthy for the extensive development of gemmae. Remove portions of the host bearing the parasite for fixation; microtome vertically.

Cordyceps is parasitic on various insects and their larvae and is most abundant in the tropics. The fructifications are usually large enough to be cut off and embedded separately.

Sphaeriales

This order is a very large one, embracing about 11,000 species, mostly plant parasites. Little cytological work has been done on the majority of species; consequently there is a dearth of technique information. The peridium of the perithecia is darkly colored and leathery, hard, or carbonaceous and always becomes brittle after embedding has been completed.

The family Mycosphaerellaceae has been most extensively investigated, with special emphasis on the damagingly parasitic *Venturia inaequalis*. Infestation of young leaves or blossoms is caused by either conidia or ascospores developed the previous season. The hyphae are confined to the region between the cuticle and epidermis of the apple and other pomaceous plants, except those of the pear. Conidia are then

formed, and the acervulus becomes exposed. Portions of the infected host may be fixed with formalin-propiono-alcohol, sectioned transversely at 10μ, and stained with a triple combination. The sex organs are produced in October, and the ascospores mature in February in warm locations or as late as May in regions where spring comes late; the perithecia appear on the undersides of leaves or on decaying fruits and are most abundant when protected by humus or sod. Perithecial stages are difficult to section; methods applicable to woody stems should be used.

Xylaria is a cosmopolitan genus, generally inhabiting dead wood and producing cylindrical, clavate, or branched fructifications. Conidia are first formed in the autumn, followed in most species by perithecia the following spring. Fix portions of the fructifications, and section transversely at 10μ. Stain preferably with iron hematoxylin.

Dothidiales

Little technique information is available on the more than 1200 species, of which only a few are plant parasites. They are distinguished by their firm black sclerotium-like stromata. The perithecia occur in vast numbers in the external layer of the stroma, embedded in the undifferentiated mycelium.

Plowrightia morbosa, which occurs on the branches of both wild and cultivated species of plums and cherries, is the species most commonly studied. The parasite requires two years for growth to be sufficiently advanced for the production of conidia in the spring. Shortly before the conidia disappear, perithecial development commences, and asci are produced during the following winter. The stroma are rigid and brittle, and only methods similar to those intended for hard woody stems can be used successfully. Pieces of infected stem should be reduced to convenient small portions, and these should be microtomed in the transverse plane of the stem. Either a triple or a quadruple stain combination may be employed.

Phyllachora graminis may be encountered. The stromata are jet black in color. It is readily amenable to treatment, but some soaking of embedded material under water will be necessary.

Laboulbeniales

The Laboulbeniales are minute ectoparasites which produce their fructifications only on the chitinous integuments of living insects. They do not give rise to fatal epidemics, as does *Cordyceps;* their own existence ends with that of the host. Most of the species are limited to definite insect genera, and even to definite areas of the insect's body.

Botanists in general have given very little study to the Laboulbeniales, mostly because they are hard to find in the United States, and partly

because of the great difficulty of fixing the material. The plants are surrounded by a thin, homogeneous, impermeable membrane. Studies for the most part have been made on living material or on whole mounts of material removed from the host. Powerful fixing fluids and strongly penetrating stains should be employed, followed by any gradual balsam-infiltrating method.

BASIDIOMYCETAE

The Basidiomycetae get their name from the fact that the spores, or basidiospores, are borne on a special one- or four-nucleate structure known as a basidium. Many species produce other types of spores in addition to basidiospores.

The group includes the mushrooms, puffballs, rusts, and smuts.

Morphology.—The mycelium, which is always multicellular and freely branched, may either consist of a single mass of hyphae or develop into a characteristic macroscopic body in which definite tissues are differentiated.

Sex organs are not produced in the Basidiomycetae. There are, however, two distinct cytological phases: the haplophase (gametophyte), in which the cells are uninucleate, and the diplophase (sporophyte), in which they are binucleate. The change from diplophase to haplophase occurs during the development of the basidium. The two divisions of the life cycle may be combined in the same mycelium or segregated on separate mycelia. In the former type of mycelium the earliest cells are uninucleate, those later developed are binucleate. The diplophase arises when a conjugation tube is formed between two uninucleate cells and the contents of one cell migrate into the other. The nuclei, however, do not fuse. The conjugation takes place at various stages of development and between different types of uninucleate cells.

Diplophase mycelia may produce spores, particularly in the rusts, which upon germination always develop into mycelia with binucleate cells. Only in the production of basidiospores does meiosis occur. Not much work, however, has been done on the nature and development of the mycelium of the fleshy fungi (Hein 1930) and of other types.

Sources of Material.—The mushrooms and fleshy fungi, as a rule, are easily collected in woods and fields in most regions during the rainy season. At other times recourse must be had to preserved or fixed material, or a visit may be made to a commercial mushroom growing establishment for *Psalliota campestris*. For most general purposes this cultivated form is quite satisfactory. The Uredinales and Ustilaginales must be collected at the appropriate time of year for each stage in the life cycle. The botanical supply concerns are fairly well stocked with material prepared for slide-making purposes.

Cultivation.—Methods of growing various phases in the life cycle of the more significant species are given in the following discussion.

Preservation.—To preserve the fleshy fungi, soak in a mixture of 2 parts formalin to 1 part liquid phenol, and, after superficial drying, suspend the specimens over strong ammonia until they set solid without drying (Ewart 1933). The final appearance is somewhat like that of candied fruit; the fungi keep indefinitely, are not attacked by insects, and may be handled without damage. The impregnating material can be removed by soaking the specimen in water or alcohol.

If preservation in a purely liquid medium is desired, use 10% aqueous formalin to which is added 10% sucrose to assist in preserving the colors.

All fleshy forms may be dried over gentle heat, then stored in boxes with naphthalene flakes to prevent insect depredations. The parasitic forms may be preserved by methods suitable for the preservation of the host.

Eubasidii

The basidia develop directly from terminal vegetative cells of the diplophasic mycelium and are arranged in continuous or discontinuous hymenia borne on a macroscopic fruiting body.

Methods of arranging the orders differ considerably (Gaumann-Dodge 1928, Killermann 1928, Stevens 1921, etc.), but the one most recently proposed is being followed here (G. M. Smith 1938, based on Killermann 1928).

Agaricales

The basidia are exposed, nonseptate, and club-shaped. A few genera are parasitic on woody plants, the rest are saprophytic. The fructifications usually develop as centrally stipitate pilei. The substance of the fructifications is usually fleshy or fibrous, rarely tough, leathery, or horny, but they generally become more or less hardened during technical treatment.

Exobasidiaceae.—*Exobasidium*, a widespread genus, is parasitic, mainly on the leaves of *Vaccinium*, *Rhododendron*, and related genera. It is peculiar among the Agaricales in that it does not form a definite fructification. The affected host areas are red and gall-like: these may be cut out, fixed with formalin-propiono-alcohol, embedded, soaked under water for about a week, and sectioned at 10μ in the transverse plane of the host. Stain critically in iron hematoxylin, without a counterstain, for cytological details. The cytoplasm of the parasite is so dense that it is difficult to differentiate by ordinary staining procedures.

Hypochnaceae.—*Hypochnus*, representative of a group of mainly European genera, is parasitic on a variety of cultivated plants. *H. ochroleucus* forms a brown felty growth along the twigs and petioles of the host. It is occasionally abundant enough to be removed for treatment as whole mounts, but sections are required to show the development of the basidia.

Thelephoraceae.—The fructification of this very large family of saprophytes is usually leathery or membranous, sometimes woody. A species of *Thelephora* causes a root rot on oak trees resembling that of *Armillaria*. The sterile mycelium of *Corticium vagum solani* (commonly known as *Rhizoctonia*) is parasitic on an amazing variety of hosts. The sterile mycelium turns yellowish with age and forms brownish to black sclerotial structures. The hymenophore, which frequently entirely surrounds the green stems of the host near the ground, is a dark network of hyphae which change to grayish-white during sporulation. Practically no definite technical information is available, but general methods as determined by the nature of the material should be applicable.

Clavariaceae.—All but one of the genera are saprophytes; about half possess small simple hymenophores, and the remainder have large branched hymenophores. The fructification is fleshy or waxy in most of the genera, but a few are cartilaginous, horny, or leathery and hairy. *Clavaria*, a very widespread genus growing in the humus of damp woods, has numerous colorful species commonly used for laboratory study. Many species harbor a symbiontic alga. The fleshy hymenophore may be divided into suitable portions, fixed with a chrom-acetic fluid or with formalin-propiono-alcohol, and sectioned either transversely (the better plane) or longitudinally. The hymenial layer is so compact that sections should not be over 8μ thick. Stain with either iron hematoxylin or safranin and fast green.

Hydnaceae.—The spore-bearing body is very variable in texture; it may be fleshy, cuticular, leathery, corky, felty, or woody. In any event, the body invariably becomes brittle after technical treatment. Most of the species grow on rotting wood, forming shelf-like or resupinate sporophores. The hymenium of *Hydnum*, common in woods, is beset with subulate spines, which carry the basidiospores. A group of these spines may be removed, fixed, sectioned transversely at 8μ, and stained with iron hematoxylin.

Polyporaceae.—The so-called "pore fungi," including the genera *Boletus* and *Polyporus*, make up this family.

Boletus possesses a fleshy, capitate, centrally or laterally stipitate sporophore, with a hymenium on the undersurface composed of tubes that are adnate to each other. To show basidiospore formation, remove

rectangular portions of the hymenium long before it is fully mature, embed, section transversely at 10μ, and stain with safranin and fast green. The other genera most closely related to *Boletus* are more or less fleshy (*Fistulina*) or leathery and have the tubes separate.

Polyporus is a genus of over 500 species. The sporophore is usually annual and may be simple or compound, stipitate or shelf-like, with the pores developing from the base toward the outer margin. Several species, such as *P. sulphureus*, are the cause of heart rot in native and cultivated trees. Apparently the only preparations that are made of these fungi are sections across the tubes. The sporophores are rather thick, fleshy when young, becoming leathery or corky as they mature. Material may be treated as indicated above for *Boletus*, but it is occasionally difficult to secure material that shows active spore formulation. The other genera may also be treated like *Boletus*.

Agaricaceae.—This is the largest family, whose 9,000 species have been described as being of "fatiguing regularity." A single common and easily collected species therefore will be described in detail, *viz.*, *Psalliota* (*Agaricus*) *campestris*.

The fruiting body—the so-called "mushroom"—is a stipitate fleshy sporophore. This mature stage is not quite so interesting as the initial developmental phases. It is not possible to collect these stages in the field under ordinary conditions; it will be necessary either to visit a commercial mushroom-growing establishment or raise them on artificial media. Spores remain viable for at least a year if kept quite cold. In tap water, germination occurs in about 24 hours. It appears to be advisable to germinate the spores in sterile tap water first and then to transfer to suitable receptacles containing various media, such as sterilized dung, dung extract agar, malt extract agar, soil mixed with shredded horse dung, or corn-meal agar. The young "buttons" will appear in about 2 to 3 months. In case the medium shows signs of drying out, add sterilized water cautiously by means of a pipette. Remove the young buttons carefully, and place in the fixing fluid: it is advisable to cut slabs from opposite sides to facilitate penetration, but this is a very delicate operation since the material does not cut easily. If the material was grown on agar, cut out small blocks of agar bearing the buttons. The killing fluid should be a weak or medium chrom-acetic, and the time of fixation should be short (one investigator states that the finest cytological fixation was obtained from material that remained in the fixing fluid for 5 minutes). Closed stages require several hours, open button stages about an hour. A suction pump, flowing gently, should always be used. The material has always become excessively hardened by xylol and chloroform dehydration methods; consequently these fluids should be avoided. It would be better to employ an essential oil, such as that of cedar,

bergamot, or origanum. All changes should be most gradual because of the fragile nature of the individual hyphae. Embed in a hard paraffin, and section buttons longitudinally until the prelamellar chamber is fairly well developed, after which transverse sections through this region may also be microtomed. Sections should never be over 8μ thick; 6μ is the optimum thickness. If fully mature sporophores are wanted, the spores should have been sown in large Erlenmeyer flasks on a substrate of broth or horse-dung decoction plus beech or spruce sawdust. The fruiting bodies begin to develop when the substrate becomes exhausted without completely drying out. Iron hematoxylin, both with and without a counterstaining of fast green, is the most commonly used stain, but the following combinations have also been employed: aqueous safranin followed by crystal violet in clove oil; aqueous pararosanilin plus a trace of phenol, followed by fast green; for photographic purposes, mordant with 5% aqueous tannic acid, then stain with pararosanilin. A triple combination has been suggested, but care must be taken not to overdo the orange G.

Lycoperdales

The group commonly known as the Gasteromycetes composes this entirely saprophytic order. Many well-known fungi are included: puffballs, stinkhorns, bird's-nest fungus, earth stars, false truffles, etc. Several are characterized by foul odors. The basidia are permanently surrounded by sterile tissue, or become exposed only after development has been completed.

There are 11 families and over a thousand species (Coker and Couch 1928), which differ considerably in external characteristics, but all may be treated alike technically, at least in the earlier developmental stages. Technical methods are the same as described above for *Psalliota*.

Dacromycetales

The Dacromycetales are minute, inconspicuous, gelatinous saprophytes found on decaying wood and leaves. The order is characterized by the freely exposed, nonseptate, Y-shaped basidia. Growth is quite slow.

The fruiting bodies of *Dacromyces* are scarcely over 3 mm. in diameter and are yellow to orange in color. Cut them from the substrate with a scalpel, microtome in the vertical plane at 6μ, mounting all sections serially in order, and stain with iron hematoxylin.

Tremellales

The order consists of a very few parasitic species and a number of fleshy or gelatinous saprophytes found on fallen branches and decaying

wood. The basidia are fully exposed and septate in a nearly vertical plane.

The gelatinous types are extremely difficult to fix adequately. Strictly fresh material should be collected in the field, and placed immediately in the killing fluid. Large portions should be reduced to smaller pieces before fixation. Either of the following fluids may be used (Whelden 1934):

(1)	Chromic acid	7	g.
	Glacial acetic acid	7.5	cc.
	Distilled water	1	liter
(2)	Saturated picric acid in 70% ethyl alcohol	100	cc.
	Saturated aqueous mercuric chloride	5	cc.
	Glacial acetic acid	7	cc.

Microtome in the vertical plane; for stages up to fusion of the two nuclei in the youngest basidia, cut at 4μ, thereafter at 8 to 10μ. Stain with iron hematoxylin and counterstain very lightly with either orange G or fast green.

Auriculariales

The Auriculariales are gelatinous saprophytes with irregular and expanded or capitate fruiting bodies. They become horny when dry. The species grow mainly on stumps or fallen trees, and are most abundant in the tropics. Reduce the fructification to convenient portions, fix with a weak chrom-acetic fluid, microtome vertically at 8μ, and stain with iron hematoxylin. A counterstain is not desirable because of the dense cytoplasm.

Hemibasidii

The life cycle alternates between a haplophase and a diplophase. The basidia are produced by the germination of a special resting spore. The life cycle is polymorphic. All species are parasitic.

Uredinales

The special one- to several-celled spore, the teleutospore, which produces the basidiospores, is formed by the metamorphosis of the terminal cells of subepidermal hyphae.

The teleutospores are binucleate: if no other cells of a binucleate type are produced, the species is described as microcyclic; if one or more additional types of binucleate spores are developed, the species is described as macrocyclic. Species that are macrocyclic include, in addition to teleutospores and basidiospores; (1) binucleate aecidiospores originating upon a haplophasic mycelium and producing a diplophasic mycelium upon germination; (2) binucleate uredinospores borne upon a diplophasic

mycelium and producing a diplophasic mycelium upon germination; and (3) uninucleate spermatia borne upon a haplophasic mycelium, which may possibly produce a haplophasic mycelium upon germination but generally assist in the initiation of a diplophasic one. Microcyclic species usually produce only teleutospores and basidiospores but occasionally develop spermatia. The entire vegetative mycelium is haplophasic in some species, but in others certain portions may be haplophasic and others diplophasic.

Melampsoraceae.—Little technical information is available concerning the majority of the species.

Cronartium ribicola forms "blisters" on stems of species of *Pinus:* these constitute the uninucleate aecial stage. Pines which show infection with this usually fatal parasite should be examined closely, and the youngest possible branches which appear to have the sites of early growth stages may be removed in short lengths and treated as described for *Pinus* stems (page 426). The writer has never seen preparations of the aecial stage which were adequately fixed or had a really satisfactory stain differentiation. Triple combinations have usually been used, but such are apparently the worst combinations that might be selected. The uredosori and teliosori are produced on the leaves of wild and a few cultivated species of *Ribes.* During recent years a systematic destruction of plants of *Ribes* has been under way in an effort to check the spread of this destructive fungus; consequently material of the telial and uredinial stages is becoming increasingly difficult to procure. If it is available, fix with formalin-propiono-alcohol, microtome at 10μ in the transverse plane of the *Ribes* leaf, and stain with safranin and fast green.

One species of *Pucciniastrum* commonly occurs on *Hydrangea* and another on *Agrimonia.* Only the uredinial and telial stages are present. Treat as for *Cronartium.*

Biological specialization is unusually complicated in *Melampsora.* The aecial and spermatial stages occur on a wide variety of gymnosperms and Angiosperms; the uredinial and telial stages occur in abundance on *Populus* and *Salix.* Material may be dealt with as described for *Cronartium* stages on *Ribes* leaf.

The aecial stage of *Coleosporium solidaginis,* a macrocyclic rust, occurs on *Pinus,* the uredinial and telial stages on *Aster, Solidago,* and the cultivated aster, *Callistephus.* It is a very common rust. The usual methods are applicable.

Pucciniaceae.—Teleutospores are either disposed singly, mostly stalked, not connected together, occasionally one-celled; or united to form an umbrella-like head on a compound stalk.

It is needless to say that *Puccinia graminis* is the most extensively investigated and most widely studied species in the family, although

there are numerous other species in the genus (Arthur 1934, Holway 1905–1924). *P. graminis* is most abundant, in all its stages, in the plains states and the wheat-growing provinces of Canada. Persons located in other regions will experience difficulty in collecting material and identifying it accurately; in such cases, the safer course is to obtain material from the botanical supply concerns, which have gone to considerable trouble to stock really good material.

The haplophase occurs on the leaves of *Berberis* and *Mahonia*, and the aecial and spermatial sori are frequently present in great abundance.

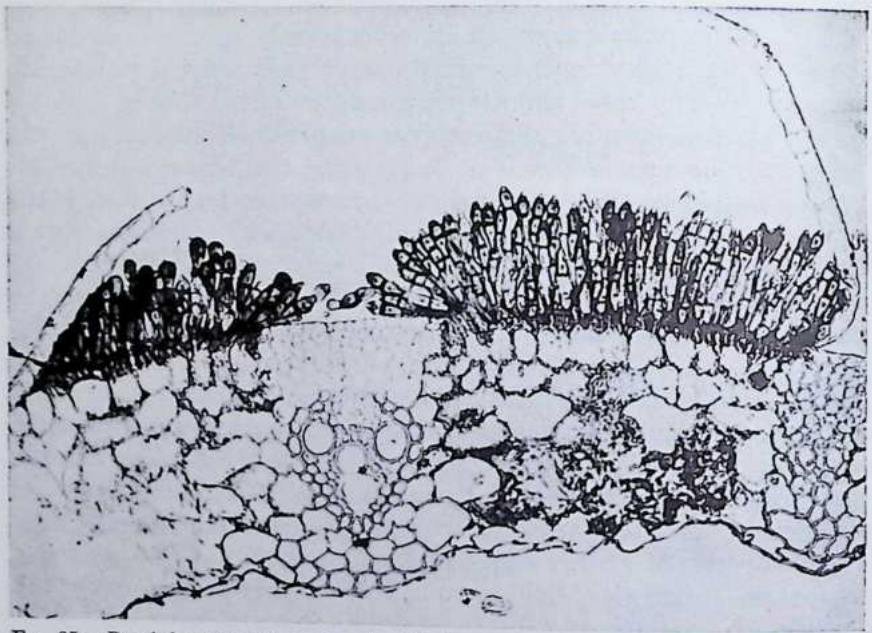


FIG. 65.—*Puccinia graminis:* section of a telial sorus on wheat leaf. Fixed with formalin-aceto-alcohol; stained with a quadruple combination.

Fix portions of the leaves with formalin-propiono-alcohol, soak the embedded material under water for two or three days, and microtome in the transverse plane of the leaf at 10μ. Stain with iron hematoxylin, and counterstain with orange G; this is the only combination which satisfactorily differentiates the two nuclei in the aecidiospores, but no basic stain is known which always gives perfectly clear differentiation of the entire spermogonium (pycnium). The pycnia are evidently developed before the aecidia; at least they are more abundant on leaves which show the younger stages of aecidia formation. The pycnia are usually formed on the upper side of the leaves, the aecidia on the abaxial side.

The diplophase consists of a number of biological races and occurs parasitically on the leaves and stems of many grasses and cultivated cereals such as *Triticum*, *Secale*, *Hordeum*, and *Avena*. The reddish-brown uredosori appear in late spring, followed by the development of teleutospores in late summer. The latter usually first appear in the uredosori, then in teleutosori. Both uredospores and teleutospores are binucleate, but the former are unicellular with four to five germ pores, and the latter are two-celled with a single germ pore to each cell. Portions of the host containing diplophasic stages may be fixed with formalin-aceto-(or propiono-) alcohol. The host tissues are hard to section; the use of 10% hydrofluoric acid for a day or two has been recommended, but it does more damage to the fungus than it softens the host tissues. Soaking the embedded material under water for a week or longer is the better procedure. Sections should be 10μ in thickness. A quadruple stain is recommended; a variety of colors is produced, but all structures are clearly differentiated—the invading hyphae should be stained green to indicate that the combination has been correctly applied (Fig. 65).

Uredospores germinate promptly when they come into contact with a suitable host plant. The teleutospore must overwinter or be subjected to prolonged freezing temperatures before it will germinate. Most authorities state that it germinates within a few hours, but others claim that months are required for basidiospores to be produced. The teleutospores of some species other than *P. graminis* should probably give better results if one desires to prepare whole mounts of the basidiospores. Germinate the spores in sterile water; kill at the optimum time with a medium chrom-acetic, then stain with Harris' hematoxylin and fast green or with a carmin stain, dehydrate with hygrobutol or dioxan, and infiltrate with balsam. Handle the material with extreme care to avoid breaking off the basidiospores.

Other species of *Puccinia* may be treated exactly as described above for *P. graminis*, but the nature of the life cycle of each species should first be ascertained.

Kunkelia (*Caecoma*) *nitens* has frequently figured in research investigations. It is one of the two orange rusts occurring on *Rubus*. Sections of the so-called "caecome" stage are easily prepared. The uninucleate spores may be germinated on sterile tap water in Petri dishes; the temperature should be kept below 25°C. To show the basidiospore development, attach the germinated spores to clean slides by means of Mayer's adhesive, fix in a weak chrom-acetic fluid, harden in 70% ethyl alcohol, then stain by a triple combination, dehydrate, and mount in balsam.

Phragmidium commonly occurs on wild species of *Rosa* and contains a great many species. Prepare sections as described for the diplophasic

stages of *Puccinia*. The very large genus *Uromyces* may also be treated as for *Puccinia*.

Ustilaginales

The special spore producing the basidium is a chlamydospore formed by direct metamorphosis of an intercalary cell of a diplophasic mycelium. The order contains the so-called "smuts."

Ustilaginaceae.—The basidium formed by the germination of the chlamydospore becomes transversely divided into uninucleate cells, each of which may produce an indefinite number of basidiospores.

There are about a dozen genera, but only *Ustilago* is sufficiently well known. There are over 200 species in this genus.

U. avenae infects the leaves of *Avena*, then the mycelium grows through the leaf and stem tissues until it reaches the ovaries, where it produces sori. Sections of the infected portions of the plant may be prepared without difficulty. The spores produce a well-developed promycelium in about 24 hours after being sowed in sterile tap water, and this may be transformed into whole mounts by the hygrobutol method. *U. levis* is not easily distinguished from *U. avenae* and occurs on plants of the same host as the latter; the sori are formed in the spikelets. *U. hordei* occurs on *Hordeum vulgare* and produces the sori in the spikelets. Prepare longitudinal sections of the young spikelets to show the development of the spores. The latter germinate readily in water and produce abundant epibasidia. The equivalent species on *Triticum* is *U. tritici* and occurs wherever wheat is raised. The chlamydospores should be germinated in plain water; they do not produce epibasidia in nutrient solutions.

The corn smut, *U. zeae*, is widely prevalent. The sori, no matter where produced on the plant (they are usually found in the ovules, which become enormously hypertrophied), are generally prominent. If a stand of *Zea mays* should show indications of the presence of *U. zeae*, young ears may be opened and ovules showing early stages of development removed and fixed individually. It is difficult to make out the details of chlamydospore development, which takes place somewhat late in the growth of the sori. Kill with formalin-aceto-alcohol, section at 10μ in any plane, and stain with safranin and fast green. The chlamydospores germinate poorly in water, hence weak nutrient solutions should be used. In such media the conidia are freely produced by the basidiospores.

Tilletiaceae.—The basidium does not become transversely divided into uninucleate cells; a definite number of basidiospores are produced at the distal end of each basidium.

Urocystis cepulae produces its sori on the leaves and occasionally in the bulb of *Allium cepa*. General methods for sections are satisfactory. The chlamydospore is completely surrounded by sterile cells; the latter are tinted, while the viable spore is reddish-brown. It remains viable in the soil for many years. Upon germination in water, the basidiospores arise on a short promycelium and then give rise either to conidia or to a new infective mycelium.

In *Entyloma* the chlamydospores enter through the stomata, germinate in the stomatal chamber, and produce the basidiospores on tufts of promycelia which emerge through the stomatal opening. Sections of the host should therefore be prepared.

Tilletia includes the stinking bunts, common on *Triticum* in the Pacific Northwest. The bunts include two species, *T. foetans* and *T. tritici*, which are rather similar in appearance. The host can be infected only within the first six to eight days after the seeds are sown, but it is not possible to determine definitely whether infection has occurred until the wheat flowers appear. The mycelium can be found near the growing point of the host stem apex at almost any stage; longitudinal sections of this portion therefore can be prepared and stained with a quadruple combination. The chlamydospores may be germinated in water to show the development of the basidia and the primary and secondary conidia. If desired, whole mounts may be made of these structures by standard methods.

FUNGI IMPERFECTI

The Fungi Imperfecti constitute a wholly artificial group which serves as a more or less temporary repository for species in which a perfect stage such as ascus, basidium, or zygote has not been discovered. There are some fungi which never form spores of any sort: these are also included in the present group.

Mycelia Sterilia

Sclerotia, rhizomorphs, and various other forms of mycelium occur. Technique treatment consequently depends upon the form assumed by the mycelium. The general methods for parasitic species are readily applicable since most of the species are plant pathogens.

Sphaeropsidales

Members of this order are mostly leaf-spotting fungi, though some grow on fruit and stems, causing rot, cankers, and blight.

The conidia are produced in pycnidia or in various modifications of such a structure. The pycnidia are usually tough structures, occasionally becoming black. hard, and very brittle. Others are waxy. The

fructifications are easily embedded, but microtoming is frequently exceedingly difficult. The embedded material should be soaked under water before sectioning is attempted. Staining may also be difficult on dark-colored forms; differential acidification should be resorted to. Quadruple stain combinations are excellent.

Moniliales

Many of the species or forms formerly included in this order have been transferred to the Ascomycetae or Basidiomycetae but the perfect stages have not yet been found for even closely related forms; consequently the same genus is to be found in two very different fungal groups. The Aspergilli and *Penicillium* are examples. The very diverse order contains a multitude of forms of which most are saprophytes.

Moniliaceae.—The hyphae occur in more or less fragile, loose cottony masses; they and the conidia are clear or bright colored.

Oïdium and *Monilia* are frequently confused. The present tendency is to use the name *Oïdium* for those forms that produce free cells by a disarticulation of the mycelium itself and to refer to *Monilia* those species that produce free cells by budding from the mycelium.

Oïdium lactis is an omnipresent mold, especially troublesome in the dairy industry. It probably causes the ripening of Limburger cheese. In milk and artificial liquid-culture media it produces a white, firm felt-like mass. When grown on solid media, the mass is firmly adherent. In older cultures the aerial hyphae bear conidia. The conidia- or oïdia-bearing mycelium may be worked up like *Rhizopus* or *Mucor* (page 329). *Oïdium dermatitidis* causes the so-called "blastomycosis" disease in mankind. The organism, which can be cultivated on Sabouraud's agar if not too heavily contaminated with bacteria, assumes all forms from a budding yeast-like oïdium stage to a typical mycelium. Technical treatment therefore depends on the form, but on the whole permanent mounts are best made from a considerable quantity of the organism worked up in bulk. Staining may be with iron hematoxylin or acid fuchsin.

Melanconiales

The so-called "anthracnose" diseases are produced by species placed in the Melanconiales. The mycelium is internal in the host; true pycnidia are never developed. The conidia are variable, being borne on conidiophores which form a stratum in immersed or erumpent black or light-colored acervuli. Many important plant diseases are caused by members of the order. General methods may be employed, conditioned by the nature of the material.

LICHENES

Lichens occur everywhere under very variable environmental conditions, and material should therefore be readily accessible. The lichens, however, have a well-deserved reputation for being formidable subjects from the technique standpoint. This is due to their tendency to become excessively hardened after dehydration, to buckle or wrinkle during the microtoming, and to be difficult to stain sharply. They greatly resemble the Rhodophyta in these respects.

The lichen plant consists of a fungus and an alga in more or less intimate association, and these two components are the same in every plant of a given lichen species. The algae are mostly referable to other free-living species, but none of the fungi can be so referred. The fungi in all save three genera belong in the Ascomycetae, the exceptions to the Basidiomycetae.

Collection and Preservation.—Lichens ordinarily occur where there is considerable moisture. They make their greatest growth during rainy seasons, then become desiccated when dry weather arrives, and become revived when the rains again come. Crustose species should be cut away from the substratum for fixation, but for preservation a portion of the substratum should also be removed. Foliose species usually must be cut from the substratum by clipping through the rhizines, while fruticose species are simply lifted off. Plants too large to be run up in their entirety should be reduced to smaller portions.

Most collectors simply dry the lichen thalli, either with or without pressure, then store in envelopes or small boxes. The plants may be preserved in formalin-aceto-alcohol to which are added about 0.2% cupric sulphate and about 5% glycerin.

Fixation.—Formalin-propiono-alcohol is the only fixing fluid which the writer finds worthy to be recommended. Dehydration should be by very gradual stages, but the length of time in each change should be short rather than prolonged. Xylol as a dehydrating medium should be avoided.

Microtoming.—Practically all species should be sectioned, ordinarily in a transverse plane, at 10μ. If the material has become hardened, soak the embedded pieces under water for two or three days. When the sections are placed on the flood of the adhesive, they will buckle considerably and may even become loosened from the paraffin matrix, but one should not be dismayed by such mishaps—simply proceed as usual, keeping the sections arranged in as natural a position as possible. After the sections have dried to the slides, coat with a dilute celloidin solution, as it is extremely difficult to retain many species on the slides during the staining.

Staining.—Safranin and fast green have commonly been used by technicians recently, as a substitute for the unsatisfactory cyanin and erythrosin of the older botanists. Fairly good differentiation is usually secured (Fig. 66). The best staining, however, is to be gotten from a quadruple combination. When correctly applied, the various portions of a foliose lichen such as *Sticta pulmonaria* should be colored as follows: upper cortex light purple; algal layer: cytoplasm pale reddish-orange, nuclei bright red; medulla orange; lower cortex bright purple; rhizines purplish-orange; pycnidia bright green, spores orange; asci purple, spores darker, nuclei red; paraphyses orange-brown.

FIG. 66.—*Physcia* sp.: cross section of thallus with longitudinal sections of two apothecia. Fixed with formalin-propiono-alcohol; stained with safranin and fast green.

Classification.—Many genera form such characteristic thalli that they are easily recognized as such, but species determination is a difficult task, especially in the large group Cladonieae. A manual covering all species found in the United States is available (B. Fink 1935), as are various locality lists (*e.g.*, Torrey 1934).

In this connection, mention should be made of two tests used to distinguish species of *Cladonia*, which are apparently applicable to other genera. In one, a 5% aqueous solution of potassium hydroxide is applied to dry material: in some species there is no reaction; in others, various color reactions are produced. If a reaction occurs, it indicates the presence of a bitter-tasting substance, fumarprotocetraric acid. In the other test, a small amount of paraphenylenediamine (paradiaminobenzine) is dissolved in a watch glass in alcohol. The proportions have not been determined exactly; the efficiency of the solution is judged by testing it first on a very bitter-tasting species—if the reaction occurs, the

solution, which is stable for only a short time, is all right. If the bitter acid is absent, there is no reaction or a pale permanent-yellow stain results. If the acid is present, a yellow color quickly appears and deepens to either orange, orange-red or brick-red, according to the species, as the solvent evaporates.

Whole Mounts.—Thalli of species in which the association between the two components is loose can be made into whole mounts by standard procedures. The most satisfactory stain will have to be determined by experiment on the species concerned. Erythrosin, acid fuchsin, and other acid stains may be tried. The thallus should be dissected to a slight extent just before the coverslip is applied.

CHAPTER XXVII

BRYOPHYTA

The Bryophyta, although cosmopolitan, reach their greatest development in the tropics. Species preferring the temperate zone are sometimes to be found in widely scattered localities. *Funaria* is unquestionably the most widespread of all, with *Ricciocarpus natans* a close second. Mosses grow far north of the Arctic Circle, and species of *Riccia* have been reported from desert regions: in short, Bryophyta of one sort or another occur everywhere and, at the proper growth seasons, are readily accessible to the technician. If unfamiliar with field collecting of Bryophyta, one can, as a last resort, always find *Lunularia* or *Marchantia* and various mosses in any commercial greenhouse. Professional botanists usually despise materials from such a source, but they are very useful to the technician.

Since general technical methods are essentially similar for the entire phylum, these will be presented first. Structural peculiarities which demand special treatment, particularly with regard to planes of sectioning, will be mentioned under the families or genera concerned.

Collecting.—Some species are easily collected or preserved in the field, but it is tedious work getting other species into a suitable condition for embedding and sectioning. Aquatic forms like *Ricciocarpus natans* are simply dropped into the killing fluid, but if the rhizoids are long and abundant they should be trimmed with scissors. Practically all other forms require reduction to smaller portions. In the case of those that adhere firmly to the substrate, a large chunk of substrate should be dug up, the whole brought to the laboratory, and the plants there cut off, by means of a scalpel, as close to the substrate as possible. It is of little use trying to wash the plants free from the substrate since so much grit will adhere that the sections will be torn and the microtome knife may be badly nicked. Species which grow in large masses on the trunks of trees, such as *Porella*, should be fixed immediately after removal since they dry out very rapidly; the desired portions can be removed later.

It should always be borne in mind that most Bryophyta are essentially semiaquatic organisms and must be handled accordingly. They should never be allowed to become dry before being killed. Some species can, of course, survive desiccation better than others, but material that

has been allowed to dry out and then revived by soaking invariably gives poor preparations. Genera such as *Riccia, Fossombronia, Sphaerocarpos,* and various mosses can be removed together with generous portions of the substrate, placed in trays, pans or shallow pots, kept watered and in a cool and partly shaded place, and grown on until ripe spores are produced. This cannot be done with epiphytic species.

There are few Bryophyta that cannot be preserved entire in formalin-aceto-alcohol and the desired portions removed at any future time for further treatment.

Fixation.—Formalin-aceto-alcohol and formalin-propiono-alcohol have proved to be entirely satisfactory for all stages of both sporophytic and gametophytic development over the entire range of the Bryophyta. For special purposes, particularly for critical cytological studies, a chrom-acetic or chrom-osmo-acetic fluid, with the proportions of each reagent carefully adjusted by experiment, may sometimes be necessary. On the other hand, a few investigators claim that only Carnoy's fluid will give satisfactory fixation of chromosomes in the Bryophyta (see *e.g.*, Lorbeer 1934, whose methods might be followed by those particularly interested in the cytological aspects).

A water suction pump should always be used on the Bryophyta since the tissues contain considerable air. The proper procedure in getting rid of air, and consequently obtaining perfect infiltration with paraffin, is to extract just enough air at the time of killing to cause the material to sink, then after 24 hours or longer to pump out all remaining air possible. If one attempted to exhaust all the air at the beginning of fixation, some plasmolysis would result, and certain structures would actually be collapsed. The sporophytes of *Polytrichum*, for example, will be crushed inwardly. If only part of the air is exhausted, the fixing fluid will ordinarily so harden the tissues that the remainder of the air can later be extracted with no damage resulting.

Embedding.—Certain investigators contend that the delicate thalli of some species must be embedded in paraffin of a low melting point, preferably not over 43°C. Experiment has shown, however, that this was necessitated because rather weak killing fluids were used, and these did not bring about sufficient rigidity of the tissues to permit paraffins of higher melting points and coarser structure to penetrate without causing some damage. With more modern methods of dehydration and infiltration, no damage is likely to occur, and a medium Parlax permits perfect infiltration. The older sporophytes of some mosses, notably those of *Funaria* and *Polytrichum*, have a tendency to become incredibly hardened; consequently they should be embedded either in a paraffin of around 62°C. or in hard Parlax, which can be further hardened by cooling with ice water at the time of microtoming.

The time in the paraffin oven should be short, particularly if the killing fluid contained chromic acid, because the heat renders the tissues of a few species somewhat brittle.

The great majority of the Bryophyta are sectioned at 10μ, both for general morphology and for development of the sex organs and sporophytes. Very young antheridia and archegonia should be cut at 6μ; this thickness should also be used for details of sperm formation in the antheridia and for the early sporogenous cells of the sporophyte. Those who claim that sections 1 and 2μ thick are the only useful ones are in a class with microscopists who consider the diatoms to be the only objects worth examining.

Orientation of the objects for microtoming is always a difficult proposition. Suggestions regarding specific forms will be given in the following discussion, but there are nevertheless some species (*Riccia* and *Anthoceros* in particular) which one can only microtome with general reference to the orientation of the thallus and trust to luck to get perfect median longitudinal sections of the reproductive organs. This is not so discouraging as it sounds, however, since the proportion of desirable sections is actually quite high.

Staining.—The most precise staining for antheridia and archegonia on all Bryophyta is to be obtained with iron hematoxylin and a counterstain of fast green or orange G. The greatest care must be exercised during the destaining because most species give up the stain with unusual rapidity. Sometimes only a few seconds in a 1% ferric ammonium sulphate solution are permissible. In those species whose gametophyte is a thallus and whose sporophyte is wholly or mostly embedded therein, the same stain combination is preferable. In other types this combination generally is to be selected for the antheridia, but not always for the archegonia and sporophytes. It is excellent for the sporophytes of *Funaria* but poor on those of *Polytrichum*. With the latter, safranin and fast green are superior. The archegonia of *Marchantia* and some other genera are notoriously difficult to stain sharply; Harris' hematoxylin preceded by differential acidification, and followed by counterstaining with orange G, is the only combination which gives clear results.

Most commercial technicians use a triple combination, but the net effect is a gaudy splash of colors which generally fade completely in two or three years. In the hands of experienced technicians, such combinations can be manipulated to give beautiful and useful results, but the novice should first get considerable experience by using the stains on more suitable objects, such as anthers or root tips.

On sections of stems and leaves of the mosses safranin and fast green or a triple combination will be satisfactory.

Whole Mounts.—The prime requisite to making satisfactory whole mounts of the thalli, flat portions, or leaves of any of the Bryophyta is to secure adequate fixation of the material, since the thin and delicate structures have a pronounced tendency to curl up tightly during the later stages of dehydration or during the infiltration. A strong fluid is better than a weak one, and an alcoholic better than an aqueous fluid.

Stain with Harris' hematoxylin, counterstain with fast green, and follow the gradual hygrobutol method. Many technicians stain in the

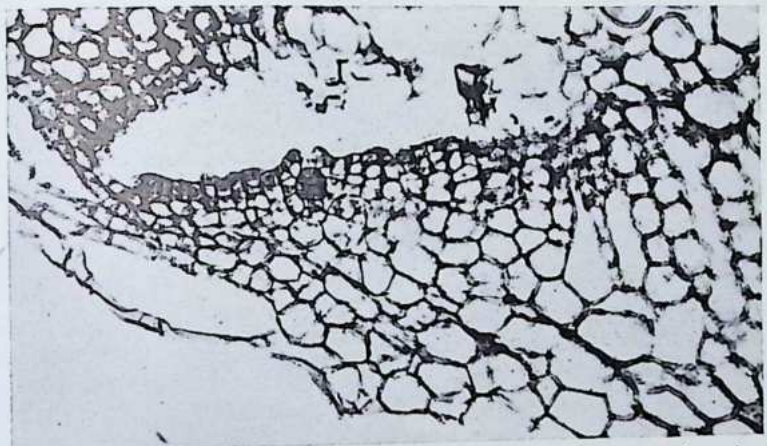


FIG. 67.—*Riccia glauca:* cross section of thallus with longitudinal section of a two-celled and an older antheridium. Fixed with formalin-aceto-alcohol; stained iron hematoxylin and fast green.

fast green alone, but the slight additional effort required for the hematoxylin will be more than repaid by the clearer internal differentiation. Mount the material as soon as the balsam becomes thick enough. If they are available, mount in depression slides.

HEPATICAE

MARCHANTIALES

Ricciaceae.—In this family the sex organs are developed in a longitudinal strip running the entire length of the thallus. There are five genera, and all but six of the species are included in the largest genus, *Riccia*. This genus is nearly cosmopolitan. *Ricciocarpus natans* and *Riccia fluitans* are the only aquatic species. *R. natans* is more common in the eastern part of the United States, with terrestrial species of *Riccia* more abundant in the Far Western states.

Ricciocarpus natans commonly grows in pools and ponds which are dried up during part of the year. It commences growth along the edges of the body of water as a terrestrial plant and becomes floating after the

rising water level causes the plants to become detached. In some regions, however, the plants always remain on muddy banks and are said to become unusually large in such situations. Development of the sex organs commences while the plants are still small and attached to the substrate; for the best preparations for the origin of the antheridia it is necessary to collect material at this time or shortly after the plants become floating. After the plants have been floating for a week or longer, it will be nearly impossible to get very young antheridia. Microtome the thalli transversely at 10 to 12μ; examine portions of the ribbons under

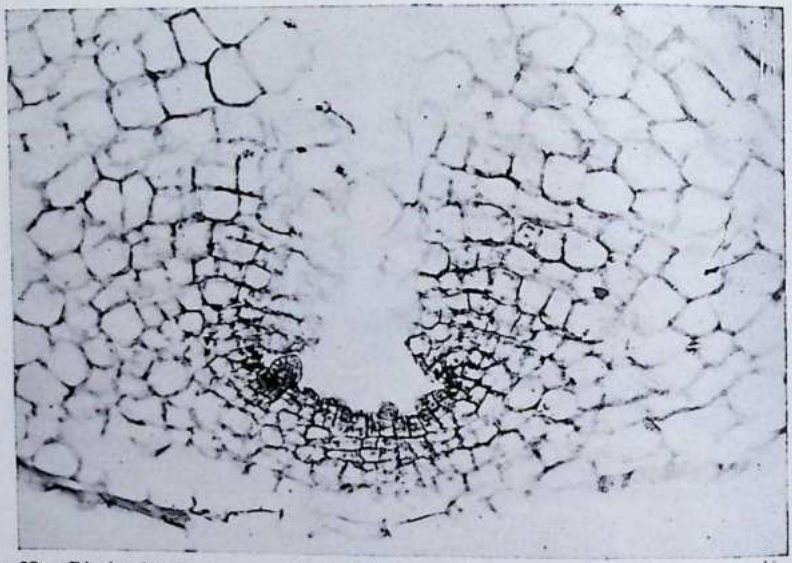


FIG. 68.—*Riccia glauca:* cross section of thallus with longitudinal sections of two young archegonia. Fixation and staining as in Fig. 67.

the microscope, and select those that show median sections of the sex organs, embryos, and sporophytes. If the thalli are cut longitudinally, median sections of the sex organs cannot be secured. For the apical cells, cut off most of the sides to within 1 mm. of the central depression, and section perpendicular to the flat surface at 10μ.

Riccia fluitans always grows submerged but is very rarely found in a fruiting condition. Whole mounts of the thalli are useful.

The terrestrial species are quite firmly attached to the substrate, and it will be necessary to cut through the rhizoids horizontally to get the plants off without too much adhering grit. Most of the species of *Riccia* are homothallic, but two are heterothallic, *viz.*, *R. bischoffii* and *R. curtisii* (McAllister 1916, 1928). Transverse sections of entire plants may be cut, at an optimum thickness of 10μ, for all developmental stages (Figs.

67, 68). Since the sex organs are commonly close together, a few sections will afford a number of stages. The transverse sections will frequently show the apical cells, which occur in a transverse row of three or more attached laterally to one another, but vertical longitudinal and horizontal longitudinal sections may also be cut. The latter will afford fine views of the sex organs and sporophytes in transverse section.

Meiosis takes place in the sporocytes, and it is not at all difficult to catch this phase in some species, notably *R. glauca* and *R. sorocarpa*. One haploid chromosome is extremely small in nearly all species. Stain meiotic stages critically with iron hematoxylin.

Targioniaceae.—*Targionia* is abundant in the coastal region of California and Oregon, and widely distributed in other states. The principal character distinguishing *Targionia* from the other Marchantiales of the United States is the enclosure of the archegonia and sporophytes by an involucre.

The antheridia occur on short lateral branches. Cut these off, and microtome in the vertical longitudinal plane at 10μ. The archegonia originate very close to the apical cell. They are strictly dorsal in position but appear to be on the ventral side because of the overgrowth of the thallus. While the presence of sporophytes can always be determined by looking for the characteristic purple-colored involucre, the presence of archegonia can definitely be told only by sectioning. The tips of the thalli should be cut off, about 3 mm. from the apex, and most of the lateral wings of the thallus eliminated. Section in the vertical longitudinal plane at 12μ. The archegonia are rather few in number. Ordinarily only one of them develops into a sporophyte, but it is not uncommon to encounter two equally well-developed sporophytes or a sporophyte and an embryo. The sporophytes are rather difficult to stain sharply.

Marchantiaceae.—All Marchantiales in which the archegonia are borne on a stalked archegoniophore belong in this family. Some genera have a stalked antheridiophore; in other genera the antheridia are partially or completely embedded in the thallus, and may be localized or produced continually or intermittently.

Two genera reproduce vegetatively by means of gemmae. In *Marchantia* these are developed in cup-shaped depressions, and in *Lunularia* in crescent-shaped cups. To demonstrate the origin and growth of the gemmae, select thalli in which growth of the gemmae has not progressed to the point where they break loose easily. Cut out portions bearing receptacles, and microtome in the vertical longitudinal plane of the thallus at 10μ. Stain with iron hematoxylin and fast green or a triple combination. Whole mounts of mature or germinating gemmae are easily prepared: fix with formalin-aceto-alcohol, stain lightly with

alcoholic fast green, dehydrate with hygrobutol, and infiltrate with dilute balsam.

Marchantia is the most widely distributed genus and if not readily available locally (Evans 1917), material can always be secured from the supply concerns. The antheridia are borne on a stalked receptacle which can be distinguished from the archegoniophores in that it is flat on top and has no involucres. The earliest stages of antheridial development are to be found when the receptacle first becomes definitely recognizable at the apex of the thallus. Do not try to remove such young receptacles from the thallus, but cut out narrow transverse portions of the thallus bearing the receptacles. Fixation has always been excellent with formalin-propion-alcohol. Section the thalli at 8 to 10μ in the transverse plane, which will give longitudinal sections of the receptacle and antheridia. After the stalk becomes 3 mm. or more in height, the receptacle may be removed from the thallus and sectioned longitudinally. The youngest antheridia occur at the periphery of the receptacle. The archegonia likewise appear when the archegoniophore is barely recognizable as a "button" at the apex of the thallus. At this time they point upward but rapidly become turned over, to point downward, by an upward growth and swelling of the central region of the archegoniophore. There are usually eight archegonial receptacles on each archegoniophore in *Marchantia*. An involucre (a "ray") is developed between each receptacle after the periphery of the archegoniophore has become inverted. By the time the involucres are readily recognizable, most of the archegonia are mature and may have already been fertilized. However, in vigorously growing plants, archegonia continue to be produced until the first two or three produced in each receptacle contain young embryos. The very youngest archegoniophores should be manipulated and fixed as has been described for the young antheridiophores; even the somewhat older stages are more easily oriented for microtoming if left attached to narrow portions of the thallus. Section at 9 or 10μ for the younger phases and 11 or 12μ for the mature archegonia. If sectioned exactly parallel to the longitudinal axis of the stalk, most of the archegonia will be medians. Stain with Harris' hematoxylin, first applying differential acidification, and counterstain with orange G. Up to the time that the sporogenous cells and elaters are differentiated in the young sporophyte, entire archegoniophores may be sectioned. These stages should be stained with safranin and fast green. As the sporophytes begin maturing, the archegoniophores should be cut into portions which appear in triangular outline when observed from above, by cutting through the involucres with a very small scalpel. If one attempted to section an entire archegoniophore bearing mature sporophytes, very few of the latter will be in median section. Microtome the sporophytes

longitudinally at 10μ; stain in a triple combination, although safranin and fast green will do just as well, the elaters having a particular affinity for the green. Meiosis occurs in the sporophytes but is an extremely difficult stage to obtain.

Conocephalum is a larger plant in all respects than *Marchantia* and is frequently to be found where the latter does not grow (Fig. 69). Sterile plants can be distinguished from those of *Marchantia* by the absence of gemmae cupules. Treat exactly as described for *Marchantia*.

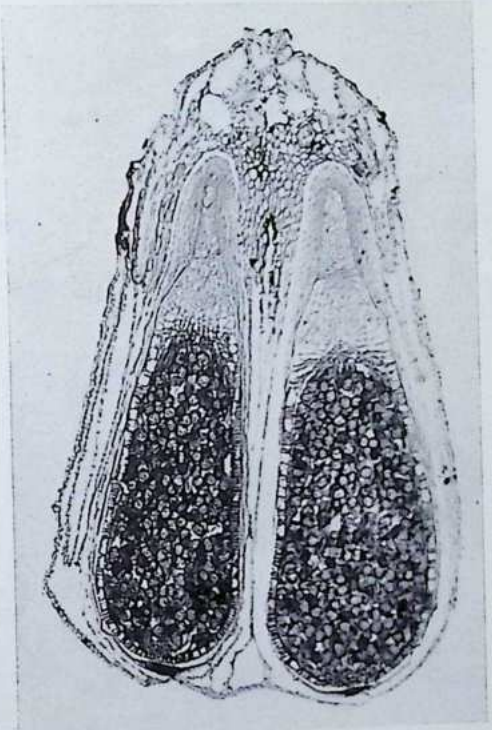


FIG. 69.—*Conocephalum conicum:* median longitudinal section through two mature sporophytes. Fixed with formalin-aceto-alcohol; stained with iron hematoxylin and fast green.

Lunularia, whose sole species, *L. cruciata*, is an advent from Europe, is common in and around greenhouses, and will almost invariably be found only in the gemmiferous condition.

Asterella is very common in Oregon and California, *Cryptomitrium* less so. The antheridia are borne in more or less rounded, cushion-like receptacles a short distance back from the apex of the thallus; these areas are easily recognizable in both monoecious and dioecious species. Cut out portions of the thallus bearing such regions, and microtome transversely at 10μ. Treat the archegoniophores as in *Marchantia*.

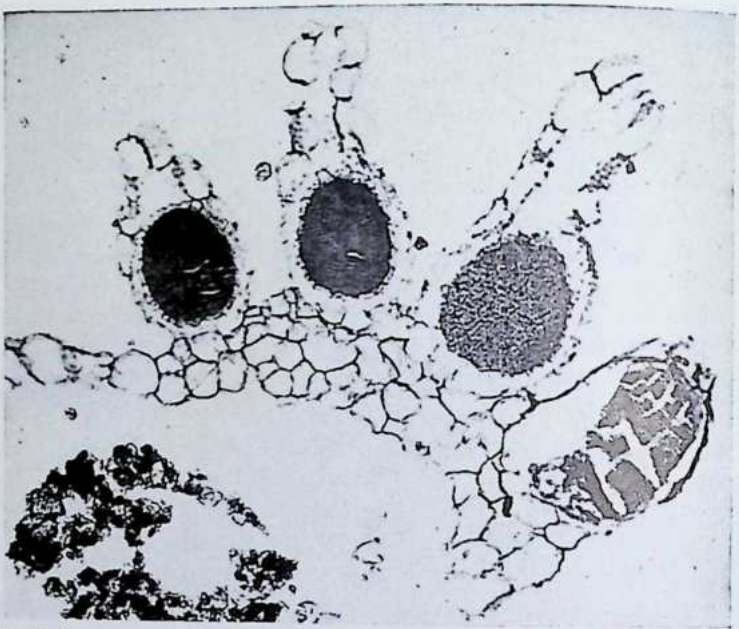


FIG. 70.—*Sphaerocarpos cristatus:* median vertical section of thallus with antheridia. Fixed with formalin-aceto-alcohol; stained with iron hematoxylin and fast green.

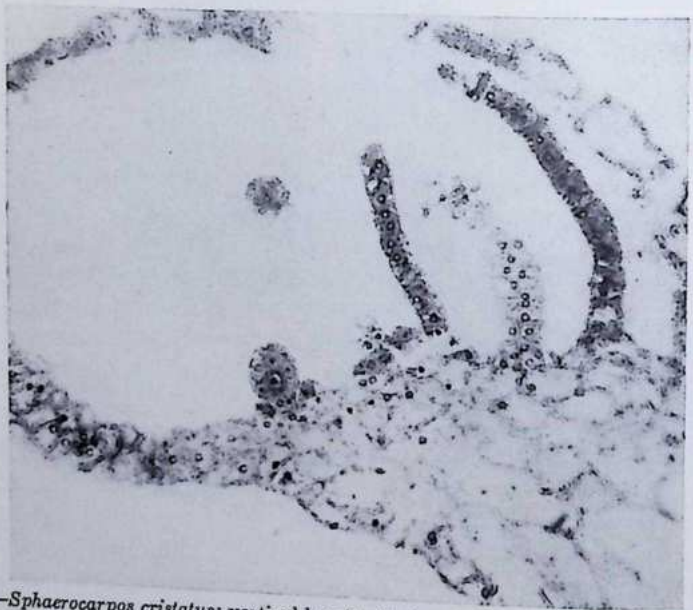


FIG. 71.—*Sphaerocarpos cristatus:* vertical longitudinal section of gametophyte with young archegonium. Fixation and staining as in Fig. 70.

There are about a dozen other less well-known genera. The directions cited for the better known genera in the family will serve as guides for the treatment of these less known genera.

Sphaerocarpales

Sphaerocarpaceae.—*Sphaerocarpos* occurs in the Gulf and Pacific Coast regions; in central California it is, in very wet seasons, so abundant

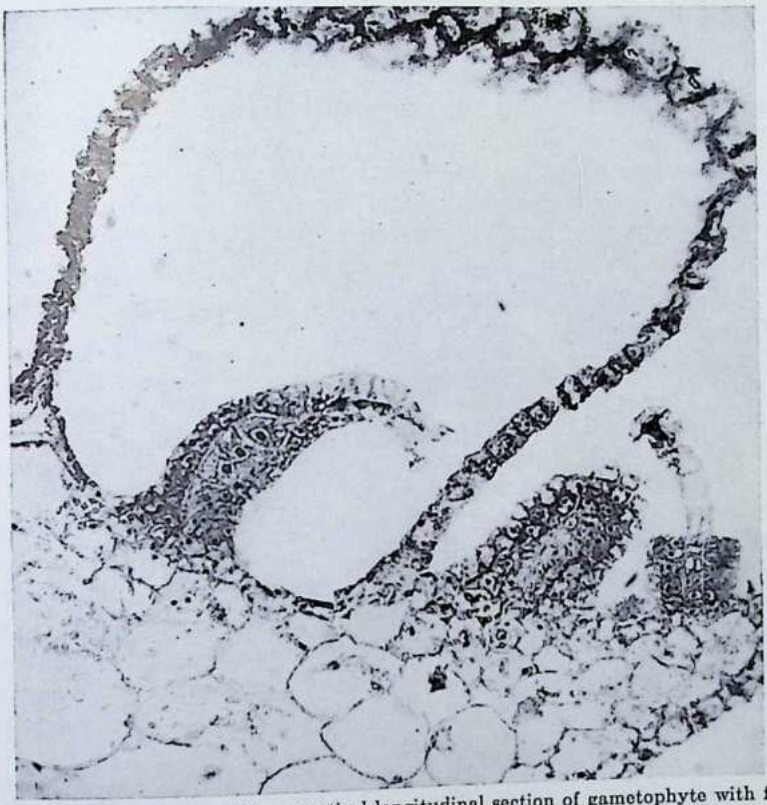


Fig. 72.—*Sphaerocarpos cristatus:* vertical longitudinal section of gametophyte with four-celled embryo. Fixation and staining as in Fig. 70.

that it covers acres of bare ground or soil that had been plowed the preceding summer. It is readily grown from spores and has been the subject of significant genetical investigations. Preserved material can be obtained from the supply concerns. When collected in the field, the best procedure is to cut through the rhizoids, wash thoroughly in running water to get all possible grit out of the involucres, then fix the plants without attempting to separate the colonies to any great extent. The species are all heterothallic, and the antheridial plants are often difficult to recognize in the earliest growth stages. They are not so abundant as

the archegonial plants. In many species they can be distinguished by their purplish color. Fixation should be with formalin-aceto-alcohol; chromic acid solutions have generally given atrocious results. Microtoming should be done with safety razor blades in a suitable holder, as plenty of trouble will be encountered with grit which has lodged within the involucres or among the rhizoids and it will nick knives badly. Microtome in the vertical longitudinal plane at 10μ for all stages. Series of stages in the development of the antheridia, embryos, and sporophytes are very easy to obtain, but good archegonia sections are rare (Figs. 70, 71, 72). Iron hematoxylin with fast green is the only combination that can be recommended. If a triple combination is used, only a few seconds in the safranin should be allowed.

Riellaceae.—*Riella* grows entirely submerged in pools; one species is known from Texas and South Dakota. In case material should be available, section parallel to the flat wings. Sporophytes are readily recognized, but one will simply have to section considerable material to get the sex organs. Staining has been found to be extremely difficult. Harris' hematoxylin has given fairly good results.

Jungermanniales

By far the larger number of genera and species of Hepaticae are included in the Jungermanniales, but little information of value from the technique standpoint has been published.

The apical cell in the order is always solitary and is easy to find in some species, but in others it is difficult to cut sections in the proper plane.

Both true gemmae and other vegetatively reproductive bodies sometimes called "gemmae" are present. Gemmae cannot usually be collected so abundantly as in *Marchantia*, consequently whole mounts are impracticable. Sections of the gemmiferous branches or receptacles are more useful. The gemmae are frequently to be found in sections primarily intended for other structures.

If the gametophyte is thallose, the antheridia are borne either on special branches or dorsally along the midrib of the main axis. If the gametophyte is foliose, the antheridia occur in the axils of the leaves and may be solitary or as many as four in each axil.

In both thalloid and foliose genera the archegonia occur either on the main axis or on lateral branches and originate close to the apical cell.

The Jungermanniales are separated into two suborders, in one of which the apical cell is transformed into an archegonium (Acrogynae) and in the other it is not (Anacrogynae).

Acrogynae

The number of foliose Jungermanniales is very large, but remarkably little technique information has been reported for the group. There is

scarcely anything in the literature concerning even so common a genus as *Porella*. There are also very few descriptions of the development of the antheridia and archegonia. Some species are homothallic; others are heterothallic, with the sex organs produced upon either the main axis or on short lateral branches; but many species, particularly those from the tropics, apparently never fruit. The species in most of the genera

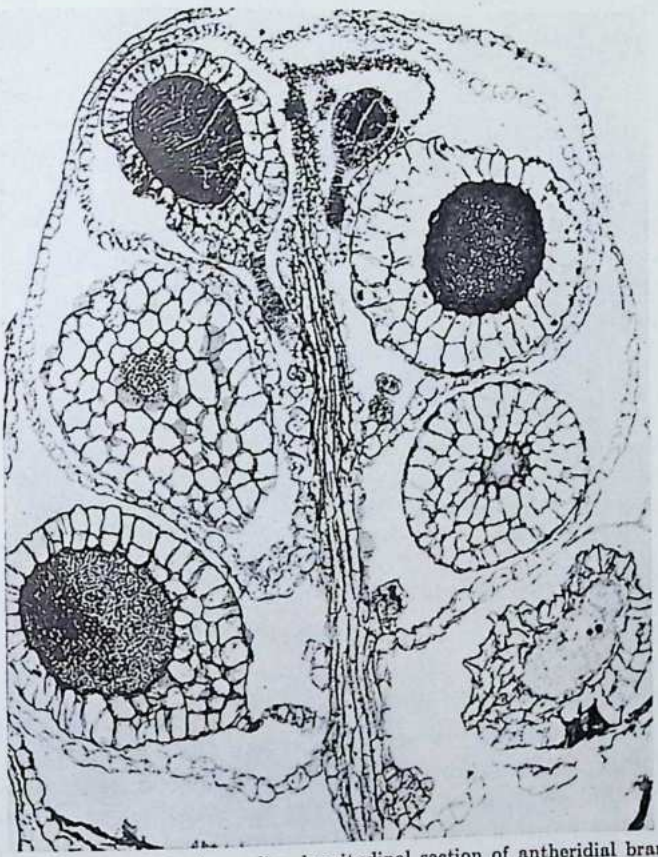


FIG. 73.—*Porella navicularis:* median longitudinal section of antheridial branch with antheridia in several developmental stages. Fixed with formalin-aceto-alcohol; stained with iron hematoxylin and fast green.

are extremely difficult to identify, since splitting of both species and genera has been carried to such an extreme that only a specialist of long experience could understand the involved taxonomic literature.

Porella is the only genus with which the technician is likely to work. Plants of this genus are common on the bark of trees, principally *Alnus*, and sometimes form extensive mats. Others grow on shady banks of creeks. The sex organs occur on side branches which are easily recognized even in the earliest developmental phases. Remove these branches,

fixing each sex and the sporophytes separately. A strong fixing fluid is necessary; formalin-propiono-alcohol has given far better results than chrom-acetic fluids. Microtome parallel to one flat surface at 8μ for antheridia and 10μ for archegonia and sporophytes. It will be almost impossible to get perfectly median sections of the older and mature antheridia because the organs are usually pushed around by the stalks,

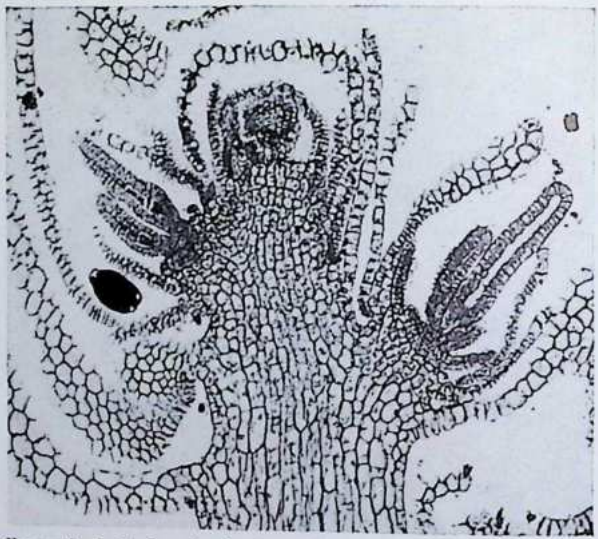


FIG. 74.—*Porella navicularis:* longitudinal section of archegonial branch with archegonia. Fixation and staining as in Fig. 73.

which do not have sufficient room for straight growth and hence become twisted (Figs. 73, 74).

Anacrogynae

Riccardiaceae.—Most of the genera are thallose. *Riccardia* and *Metzgeria* are common in coastal Oregon and Washington, less so in California; the first genus also occurs in the northeastern states. In *Riccardia* the sex organs are produced on short lateral branches; in *Metzgeria* the branches arise from the ventral side of the thallus. Remove these branches, embed separately, and microtome in the vertical horizontal plane.

Codoniaceae.—A few genera are foliose, but most of them are thallose. The family is immediately recognized by the globose sporophyte borne on a relatively long seta.

Pellia is a well known and widespread thallose genus. It has two species in the United States. *P. epiphylla* is monoecious, and *P. neesiana* is dioecious. The latter is especially abundant in coastal Oregon, forming dense mats. Only material taken from moist habitats, or material which is damp at the time of collection, should be fixed. For the anthe-

ridia and archegonia cut the thalli into portions not over 5 mm. in length, fix with formalin-aceto-alcohol, and section in the vertical longitudinal plane. Since both sex organs are somewhat obliquely oriented, with the apices directed toward the apex of the thallus, transverse sections are valueless. As soon as the sporophytes become recognizable, but before the seta commences rapid elongation as the sporophyte matures, cut out narrow longitudinal portions of the thallus together with the involucre enclosing the young sporophyte, embed these portions on their sides and microtome parallel to one side, thus obtaining longitudinal sections through the curved portion of the seta. After the sporophyte emerges from the involucre, cut it off through the seta at any convenient point. Meiosis takes place comparatively late in the development of the sporophyte, but it is difficult to catch this important phase. The spores germinate while still enclosed in the capsule, and they are shed as six- to nine-celled ovoid masses. The spores at the discharging stage are so gorged with reserve foodstuffs that thin sections are necessary—8μ is about right—and staining must be critically controlled. Iron hematoxylin is excellent, but a triple stain will better reveal the beautiful mitotic figures occurring during spore germination.

Fossombronia, also widespread, generally occurs in patches of a few to 35 to 50 plants intermingled with other bryophytes. The plants are distinctly foliose and may be homothallic or heterothallic. The presence of antheridia, archegonia, or embryos generally cannot be told from the outward appearance of the plants. The latter are more readily removed from the substrate than are most other terrestrial bryophytes. Fix the plants entire, microtome in as nearly a vertical longitudinal orientation as possible, then examine the ribbons under the microscope, and pick out the satisfactory sections. Those species which are more prostrate than erect may sometimes give good sections when microtomed transversely, but longitudinal sections are preferable. As soon as the sporophytes emerge, they may be handled separately.

Calobryales

Members of the order are seldom to be encountered. They are so much like the mosses (Eubrya) that they may be treated similarly.

Anthocerotae

Of the Anthocerotae found in the United States, *Anthoceros* is widespread in the northern states and west of the Mississippi River, being especially common in coastal California, and *Notothylas* has been found in Texas and California.

These and the other genera of the Anthocerotae are superficially much alike and can be dealt with by technical methods common to all. The

plants grow on steep banks along roads, especially where there is dripping water, near waterfalls, on rocks and boulders in damp situations, and on rotting logs in the tropics. Some species have such a peculiar dark green color that they can readily be distinguished by this character from the thalli of other Hepaticae, with which they frequently grow intermingled.

The gametophytes are dorsiventral thalli. They are homothallic in all genera. Both antheridia and archegonia are completely embedded in the thallus, as are the embryos and the youngest stages in sporophyte development. It is difficult to determine from external examination what stages might be present, up to the time that the sporophytes are well along in development, and it is impossible to know whether sex organs are present without making sections. The gametophytes are easily separated from the substrate.

A single apical cell is present, but it is a matter of sectioning and examining literally hundreds of sections to locate it.

The thalli may be fixed entire in formalin-aceto-alcohol, a medium chrom-acetic, or in Navashin's fluid. The writer's experience leads to a preference for the fluid first mentioned. Many investigators have complained that excessive brittleness results following dehydration with xylol and similar fluids; if the tertiary butyl alcohol schedule is followed, there should be no brittleness.

Microtome in the vertical longitudinal plane; 8μ is usually the optimum thickness, but since the older archegonia are rarely oriented exactly perpendicular to the dorsal surface of the thallus, it would be better to cut at 10μ if these are particularly desired. The ribbons should be stretched in water, examined under the microscope, and the satisfactory sections picked out, since about three-fourths of the ribbon from a given thallus will be found devoid of sex organs. One must search very carefully for the youngest archegonia, as they are easily passed over. Sometimes several archegonia and a series of stages in the development of the antheridia can be found in a single section from a large thallus. If *Nostoc* colonies occur in sections otherwise lacking desirable structures, these may be mounted separately as many instructors find them valuable in courses on the algae. For the early sex organs and the *Nostoc* colonies, iron hematoxylin and fast green are superior; for later stages a triple combination might be substituted if preferred. It is entirely a matter of chance that one obtains stages in the older embryo showing the differentiation of the archesporium (such as those shown by Campbell 1918, Fig. 70*E*; G. M. Smith 1938, Fig. 46*H*) and any technician who takes pride in his art will feel considerable excitement, as did the writer, when this stage is found for the first time. As soon as the sporophytes have attained a length of about 2 mm. above the surface of the thalli, the latter should be trimmed away from each sporophyte in such a manner as to

leave a narrow zone around the foot. These portions should be embedded with the sporophyte flat, so that it can be microtomed longitudinally. It is actually difficult to get perfectly median longitudinal sections of the entire sporophyte, since the foot and involucre are curved and the capsule is sometimes bent as it emerges from the involucre. Spores are produced early and develop rapidly; since the basal cells of the sporophyte remain embryonic, there is a continuous production of spores and elaters so long as the gametophyte remains capable of nourishing the sporophyte. Maturing portions of long sporophytes should be bisected and embedded in bunches of four or more for sectioning both transversely and longitudinally. The younger stages should be sectioned at 10μ and stained with iron hematoxylin and fast green; the older stages at different thicknesses, from 8 to 12μ, and stained with safranin and fast green or a triple combination.

MUSCI

The plants commonly known as mosses belong in this homogeneous group. In general the methods cited in the introductory paragraphs to the Bryophyta are sufficient for the Musci, but additional details for various structures are given below.

Protonema.—The spores of most mosses are viable for several years. Unruptured capsules are the best source for spores for germination purposes. The culture medium found to be most consistently satisfactory is made up as follows:

Distilled water	1 liter
Ammonium nitrate	1 g.
Potassium sulphate	0.5 g.
Magnesium sulphate	0.5 g.
Calcium sulphate	0.5 g.
Ammonium phosphate	0.5 g.
Ferric sulphate	0.01 g.
10% aqueous potassium hydroxide	A few drops

Sterilize in an autoclave for from 30 minutes to 1 hour at 5 pounds pressure. Pour sufficient of the medium into sterile Petri dishes. Open the capsules by means of sterile needles, and scatter the spores as evenly as possible over the surface of the medium. Keep the cultures in bright or diffuse light, at room temperature (averaging 20°C.). The time required for germination varies from a few hours to a week. The protonema will usually grow rapidly and branch profusely but will rarely produce gametophoric buds in liquid media. To induce growth of the gametophores, obtain some soil from the habitat of the species, if possible, otherwise use any good loam to which some wood ashes have been added. Put in 2- and 3-inch clay pots, soak with the nutrient

solution, then sterilize in an autoclave at 15 pounds pressure for 3 hours. After sterilizing, place the pots in earthenware plates in which water to irrigate the plants may be kept. Place the whole in a Wardian case or a similar container to avoid contamination by air-borne spores, and to keep the plants in a humid atmosphere. The protonemata may be removed from the culture dishes by means of pipettes and transferred to the surface of the pots. In controlled growth studies, care should be taken to place only a single protonema in each pot.

Whole mounts of the protonemata at any stage of growth are easily made. Kill and fix with formalin-propiono-alcohol or a weak chrom-

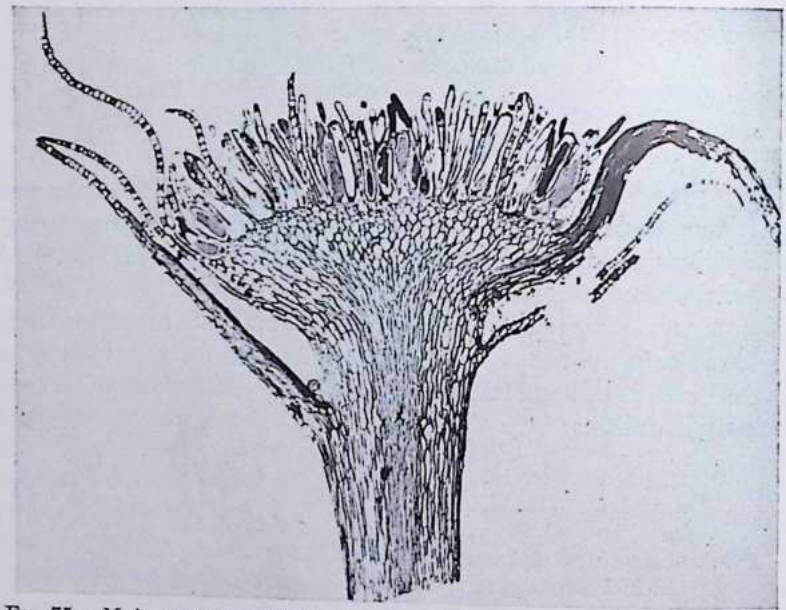


FIG. 75.—*Mnium affine:* longitudinal section through central portion of an antheridial head with antheridia in various stages of development. Fixed with formalin-aceto-alcohol; stained with iron hematoxylin and fast green.

acetic fluid, stain with iron hematoxylin, counterstain lightly with fast green, and follow a gradual hygrobutol method.

Gemmae.—A considerable number of Eubrya produce multicellular gemmae in groups at various points on the aerial portions. Solitary subterranean gemmae, known as "bulbils," are produced on the rhizoids of either the protonemata or the sporophores. When borne at the tips or along the midribs of leaves, detach the latter, and mount entire. The same procedure may be followed in the case of gemmae borne on the branches or at the stem apices. If the plants are not too large, those bearing subterranean gemmae may be mounted entire, otherwise the rhizoids may be cut off. Fix all types of material in a weak chrom-acetic

fluid, wash thoroughly, stain critically with Harris' hematoxylin, counterstain with fast green, and infiltrate with balsam by a gradual hygrobutol method. The bulbils of some species have a distinctive bright color that is not affected by fixation or staining; they may even be mounted unstained if fixed with formalin-aceto-alcohol and taken directly through hygrobutol into balsam.

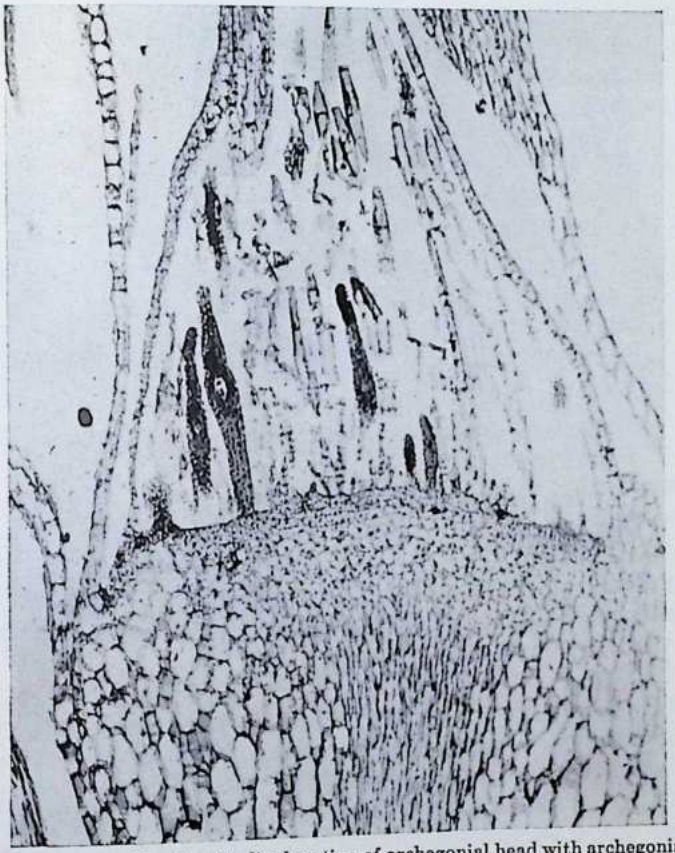


FIG. 76.—*Mnium affine:* longitudinal section of archegonial head with archegonia. This is a typical section, illustrating the difficulties in obtaining perfectly median sections of the archegonia. Fixation and staining as in Fig. 75.

Antheridia and Archegonia.—Tips, which by dissection and examination under a high-power binocular microscope are known or strongly suspected to contain the sex organs, are snipped off by means of fine-pointed forceps and dropped into the killing fluid (Figs. 75, 76). For ordinary preparations the more robust species are sufficiently well fixed by formalin-aceto-alcohol, but for critical studies of spermatogenesis a weak chrom-osmo-acetic fluid is needed. The following formula may be recommended:

1% aqueous chromic acid	180 cc.
2% aqueous osmic acid	25 cc.
Glacial acetic acid	12 cc.
Distilled water	210 cc.

For the archegonia, a 0.25% chrom-acetic heated to 30°C. is excellent. The most satisfactory stain combinations are safranin and fast green, and iron hematoxylin, without a counterstain or with fast green. The archegonial heads must be very carefully oriented for microtoming in order to obtain perfectly median longitudinal sections.

Sporophyte.—The sporophytes of some genera give no trouble, but those of others are likely to be exasperatingly difficult. Formalin-aceto-alcohol is usually much better than a chrom-acetic fixing fluid, and the dehydration should not be rushed. For the earlier stages, up to and including the stage where the spores are rounding up, iron hematoxylin is the preferable stain, but safranin is excellent for maturing spores and for the structure of the sporophyte.

It is interesting to follow out the origin of the plastids during sporogenesis (Senjaninova 1927). First fix in Lewitzky's fluid for two to three days:

10% aqueous formalin	90 cc.
1% aqueous chromic acid	10 cc.

then transfer to

1% aqueous chromic acid	30 cc.
2% aqueous osmic acid	8 cc.

for nine days. Wash thoroughly, dehydrate, and embed. Microtome the sections at from 2 to 5μ. Bleach, then stain with iron hematoxylin.

Sphagnobrya

Sphagnum, the peat moss, grows in moist places where the water is quite acid and is restricted mainly to the North Temperate Zone. The sex organs have been reported to be difficult to find, but this probably came about because searches for material were made at the wrong time of year. Some species are heterothallic and others homothallic. In the Middle West and on the Atlantic Coast, the antheridia begin development in August; the heads of the antheridial plants are decidedly globose and vary in color from yellow-brown to red-brown or almost black. The archegonial heads appear early in September, are flatter on top than the antheridial heads, and may be distinguished by the yellow-brown to red-brown conspicuous bud in the center of the head. The archegonia are fully mature early the next spring, and fertilization takes place at this time. Plants with sex organs are said to be more readily found than are those with sporophytes, but this may be only a regional difference.

Remove the short lateral branches bearing the sex organs individually, as it will be much less trouble to obtain median sections by sectioning

these smaller portions than by attempting to microtome the entire mass. Little has been reported regarding the proper fixation of the sex organs in *Sphagnum*, but it appears that a 1% aqueous chrom-acetic fluid diluted with three times its volume of water and heated to 30°C. gives excellent fixation (Bryan 1915). Formalin-aceto-alcohol has been found wholly satisfactory for the sporophytes. The lateral branches are wider in one direction than in the other; microtome parallel to one flat surface at 10μ for the earlier stages and 12μ for later development. The long and somewhat twisted stalk of the antheridia will make it difficult to secure perfectly median sections of the entire organ. Iron hematoxylin usually, but not always, stains brilliantly; a counterstain in fast green is desirable. Safranin and fast green may also be used.

Andreaeobrya

In *Andreaea* the cespitose plants are strictly saxicolous and are usually found in cold regions or on high mountains but descend to sea level in New York state. Their color is dark brown to black. The plants are readily distinguished by the longitudinal dehiscence of the mature capsule into four valves. The gametophytes are usually homothallic, but the antheridia and archegonia are borne on separate branches in terminal groups.

Material is generally quite brittle, consequently special methods, involving the use of hydrofluoric acid, are required. The younger portions, including those bearing the sex organs, should not give much trouble if fixed with formalin-aceto-alcohol, embedded in a hard paraffin, and then soaked under water for some time before being microtomed.

Eubrya

The true mosses are among the commonest and most widespread plants, occurring almost everywhere. They are to be found in cold and warm, fresh and brackish waters; in caves; on rocks, bare ground, tree trunks, on flower pots in greenhouses, and in other habitats too numerous to mention. Most mosses are gregarious in habit and are but little mixed with other plants; consequently material as a rule can be collected in great quantity with ease.

Despite the fact that there are many thousands of species of Eubrya, the differences are of such minor importance that almost any available moss will provide satisfactory material for the technician. It will be found, however, that material of the same species from widely separate localities will react more or less differently to the identical technical treatment. Material of *Polytrichum commune* from Wisconsin is somewhat difficult to work with, the tissues having a tendency to collapse and become hardened; that from central California is less troublesome, not shrinking but becoming hardened; while material from the coniferous

forests of Oregon is a delight to work with and gives no trouble whatever, except for the older stems. Some species may prove to be objectionable because the sex organs are too few in number: *Funaria hygrometrica*, which is extremely common, has few antheridia and fewer archegonia in comparison with *Polytrichum* or *Mnium*, and in *Buxbaumia* there is generally only a single antheridium and but one archegonium. Presumably most species are heterothallic, but this condition may be more apparent than real. In the homothallic species, the antheridia and archegonia may either be freely intermingled in the same head, in separate groups in the same head, or in separate branches on the same stem.

All factors considered, *Mnium* and *Polytrichum* (or *Pogonatum*) are preferable for the sex organs, and these two genera together with *Funaria* for the sporophytes. For bulbils *Leptobryum pyreforme* has proved to be excellent, but on examination other species may be found to be equally satisfactory.

Beautiful fixation has always been obtained with either formalin-aceto-alcohol or formalin-propiono-alcohol. Chromic fluids may be used, but they always cause brittleness and discolorations that are hard to bleach.

The antheridial and archegonial heads are always microtomed in the longitudinal axis. The first should not be cut at more than 10μ if details in spermatogenesis are desired; 12μ is thick enough for general purposes. Before the archegonial heads are placed in the killing fluid, all leaves surrounding them should be carefully snipped off with fine-pointed scissors. At best, it is difficult to orient these heads to get most of the archegonia in median longitudinal section. The archegonia are always long and are commonly borne on elongated stalks, and as they get older, the jacket portion becomes bent and even convoluted. In practice, one might try microtoming at 12μ; if the younger stages appear to be satisfactory and at least a quarter of the nearly mature archegonia have been sectioned through most or all of their length, then that thickness may be considered as the optimum for that material. A series of developmental stages of the sporophyte is far more useful and instructive than merely sections of the mature capsule. Both transverse and longitudinal sections should be prepared. In the latter the development of the peristome may be followed out. Some mosses lack a peristome (*e.g.*, *Pleuridium*, which is common on both coasts). In some species the sporophyte is extremely difficult to section at all times (*Funaria*), and more or less shattering must be expected; in others the capsule may become too hard to section, although earlier stages give no trouble. To avoid these difficulties in large measure, all air must be exhausted during or following fixation, infiltration must be very thorough, and embedding should be in a hard paraffin.

CHAPTER XXVIII

PTERIDOPHYTA

The ferns are a large, diverse, and extremely interesting group, and in many respects they tax to the utmost the skill and ingenuity of the technician. Indeed, they are exceeded only by the Rhodophyta in the problems that they present. The beginning technician would do well to acquire considerable experience on less difficult groups before tackling the Pteridophyta. *Ophioglossum*, *Isoetes*, and *Marattia* are the only genera all the parts of which section easily and give no trouble with the staining; consequently, if material of one of these genera should be available, the beginning should be made on it. Thereafter progress toward the more difficult genera can be started, taking up such as *Botrychium*, *Azolla*, *Lycopodium*, and *Psilotum*, and progressing from these to the Filicales and Equisetales.

Detailed directions for the various subclasses, orders, or species will be given, since they differ considerably in the nature of the treatment required. A number of general manuals are available, which contain information of value to the technician (*e.g.*, Ogura 1938, Verdoorn 1938).

Stelar Types.—The ferns have played a prominent part in the development of the stelar theory. On the supposition that examples which will illustrate each of the several types of steles in the stems and rhizomes (G. M. Smith 1938) might be required, the following list has been prepared.

Haplostele: Lycopodium cernuum (mixed type), *Selaginella kraussiana*, *Lygodium palmatum*, *Hymenophyllum*.

Actinostele: Psilotum, *Isoetes*, *Lycopodium phlegmaria*, *L. inundatum*.

Plectostele: Lycopodium adpressum, *L. clavatum*, *L. volubile*, *L. tristachyum*.

Polystele: Polypodium, *Filix fragilis*, *Pleopeltis simplex* (probably most species of the Polypodiaceae).

Amphiphloic solenostele: Dipteris (old plants), *Marsilea vestita*, *Dicksonia punctiloba*, *Marattia* (older stem), *Pilularia*.

Ectophloic solenostele: Ophioglossum (rare), *Schizaea* (older parts), *Botrychium virginianum* (rhizome).

Dictyostele: Ophioglossum (usual condition), *Osmunda* (older parts), *Ceratopteris* (older stems), *Botrychium virginianum* (stem), *Helminthostachys*.

Polycyclic solenostele: not known in any North American pteridophyte. Occurs in rhizome of *Matonia pectinata.*

Polycyclic dictyostele: Pteridium (older rhizome).

Mixed types (recapitulation): *Matonia pectinata, Cyathea, Gleichenia.*

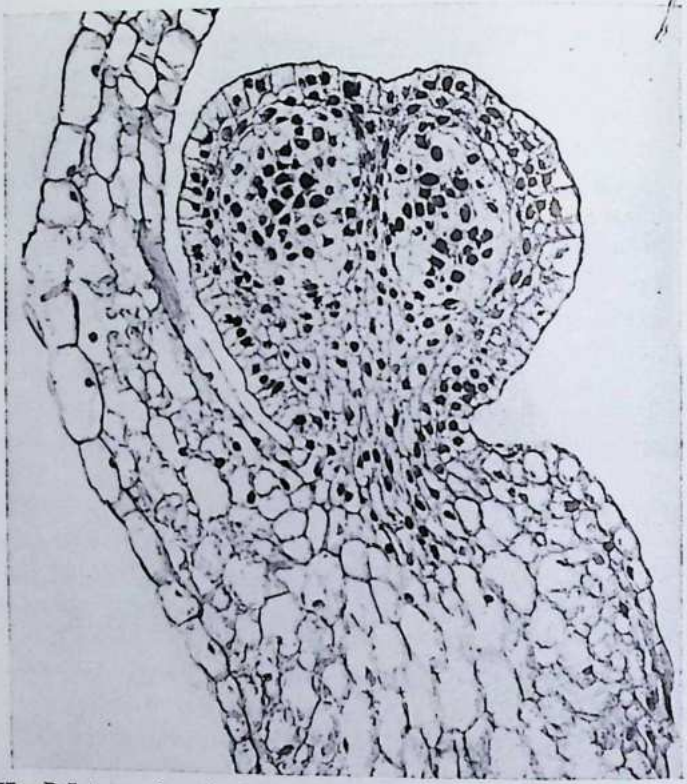


FIG. 77.—*Psilotum nudum:* longitudinal section from near stem apex with two young sporangia containing spore mother cells and enclosed by the bract. Fixed with formalin-aceto-alcohol; stained with safranin and fast green.

PSILOPHYTINAE

PSILOTALES

There is one family with two genera, *Psilotum* and *Tmesipteris.* The latter occurs only in the South Pacific, but the former is widespread in the tropics and subtropics and may even be found in fern collections as it grows readily under cultivation. Anatomical material should be obtainable from the supply concerns. The younger sporangia and stems section easily, but the older sporangia and stems, as well as the rhizomes, become greatly hardened, and embedded material must be soaked under water for some time, whereupon it cuts without difficulty. All stages fix excellently with formalin-aceto-alcohol, but the dehydration and

infiltration must be very gradual and prolonged; at least a day should be allowed for each stage of a closely graduated series of fluids. For some unknown reason, most parts of the plants, especially the older stems and rhizomes, have a marked tendency to shrink badly during the dehydration process. Gametophytes are unobtainable. All parts give beautiful staining effects with either safranin and fast green or a triple combination (Fig. 77).

LYCOPODINAE

Lycopodiales

The order includes two living genera: the monospecific *Phylloglossum* from Australasia, and the cosmopolitan *Lycopodium*.

Species of *Lycopodium* occurring in the United States are usually trailing or shrubby terrestrial plants, but a few are epiphytic with either erect or pendulous sporophytes. The tropical species are mostly epiphytic and attain larger dimensions than do those from temperate climates. Although *Lycopodium* should be present in most of the states, it rarely occurs in abundance on the Pacific slopes and in the Southern states, and colonies are rather sporadic and difficult to locate. If definite locations are not known, herbarium specimens in a local collection should be examined and notes made of the exact localities. In Canada and the New England and Northern states it becomes as common as a weed. *Lycopodium* is well represented in most fern collections, but the species may be difficult to determine. Plants are easily obtained from nurserymen. If all these sources fail, either preserved or embedded material can always be procured from the botanical supply concerns. The tropical species are less likely to give trouble during the microtoming as they have softer stems and strobili.

Root.—The root of *Lycopodium* has the typical differentiation characteristic of vascular plants (Fig. 78). Fix with formalin- aceto-alcohol, microtome at 10μ, and stain with safranin and fast green. The younger roots section readily, but if trouble is encountered in older roots from the hardening of the xylem, they may be soaked under water for two or three days.

Sections of older stems frequently show the adventitious roots, which grow down through the cortex before emerging. Among the species which have this feature are *L. serratum* and *L. pithyoides*.

Stem.—Apical cells are not known to be present in the stem of *Lycopodium:* instead, there is a mass of meristematic tissue. The terminal portions of the stems may be detached about 5 to 8 mm. from the apex, fixed in formalin-aceto-alcohol, embedded, and sectioned longitudinally at 10μ. These will show the apical meristem, the develop-

ment of the leaves, and the early differentiation into epidermis, cortex, and stele. Serial transverse sections are also useful.

There is great variation, even within the same species, in the organization of the vascular tissues. A series of preparations of stem sections of as many species as could be obtained will form a collection of inestimable value in any study of plant anatomy. Contrary to the common

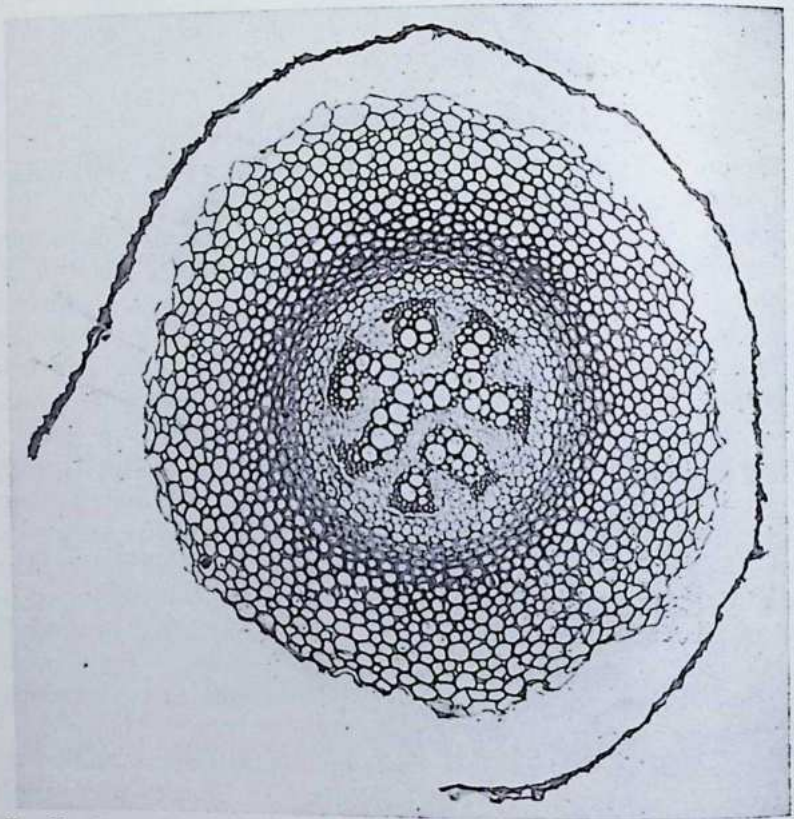


FIG. 78.—*Lycopodium clavatum:* cross section of a rhizophore with exfoliated epidermis. The heavily sclerized region of the inner cortex causes great difficulty during microtoming. Fixed with formalin-propiono-alcohol; stained with safranin and fast green.

impression, it is not at all difficult to microtome perfect sections of even the oldest stems. The stems of all species become more or less hardened during the dehydration; consequently it is better to treat them all alike. Cut the stems into portions not over 7 mm. in length, fix with formalin-aceto-alcohol, dehydrate with tertiary butyl alcohol (being sure to use the alcohol-paraffin oil mixture immediately preceding infiltration: this is the critical step), then after embedding, expose one end of each piece of material under water for about a week for softer portions and longer for the tougher pieces. Microtome at 12μ, and stain with safranin

and a counterstain of either fast green, Harris' hematoxylin, or anilin blue (Fig. 79).

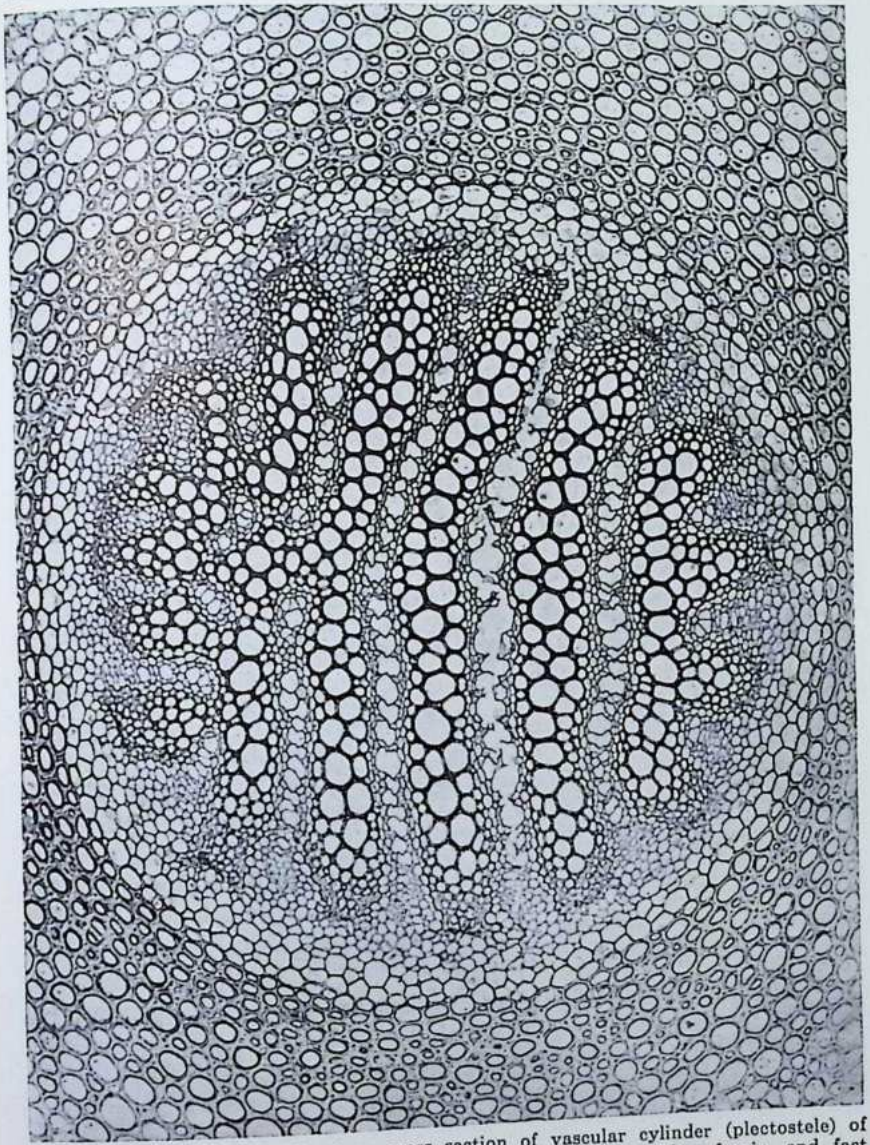


FIG. 79.—*Lycopodium clavatum:* cross section of vascular cylinder (plectostele) of mature stem. Fixed with formalin-propiono-alcohol; stained with safranin and fast green.

Leaf.—The younger stages of leaf development are to be found in sections of the stem apices. A single vein is present. If the leaves are sufficiently closely appressed to the stem, the two may be worked up

as a unit. If the leaves are over 1 cm. in length or are inserted at an angle, they should be removed. Cut off short portions at both ends. Embed in bunches or layers. There should be no trouble with the microtoming, even though the leaves of most species become rather brittle.

Strobilus.—Strobili usually appear in June and mature in October. The strobili in the various species differ greatly in external form, but there is little internal difference. Insofar as the technician is concerned, the strobili of any one species are as useful as those of another. In most of the species occurring in the United States, the fertile region is so definitely delimited from the sterile portions that it becomes a simple matter to distinguish between the two. In some tropical species there are alternating sterile and fertile zones, and in others sporangia are found in the vegetative region subtended by foliage leaves.

In northern regions, *L. clavatum*, *L. inundatum*, *L. dendroideum*, and *L. obscurum* are the native species which have the most satisfactory strobili. In the first two species the strobili become so long as they approach maturity that they must be bisected. The sporangia are progressively developed from the base of the strobilus toward the apex, and in most strobili not too far along in development a fine series of stages are to be found.

For ordinary purposes formalin-aceto-alcohol fixes very well; but for critical stages a fairly strong chrom-acetic or chrom-osmo-acetic fluid would be preferable. There may be a little plasmolysis at certain later stages of spore development; to avoid this, individual sporangia will have to be dissected out of the strobilus. If the sporophylls are large, they may either be trimmed off with small scissors or slabs may be cut from opposite sides of the strobilus. The strobili should not be bisected longitudinally; if this is done, they will curl up tightly. The strobili may be sectioned transversely if desired, but longitudinal sections are preferable. In either case the sections may be cut at 10μ for all stages.

When sections of strobili are mounted on the slides, it will generally be observed that a milky substance runs out of them. This is due to the fact that the spore mother cells and spores float in a viscous fluid; the fluid is only partially coagulated during fixation, and the uncoagulated portion becomes dissolved in the water on which the sections float. The matter, fortunately, is of no moment since the spores are retained in position and the dissolved matter does not become discolored by stains.

Staining will occasionally prove to be troublesome. The earlier stages and those involving maturation of the spores stain beautifully with safranin and fast green. The intermediate stages are those that give the most difficulty. Iron hematoxylin may be tried, but the

differentiation must be carefully controlled, especially when the chromosomes are involved.

Gametophyte.—Innumerable attempts to germinate the spores of *Lycopodium* have been made, but most trials have been unsuccessful. Two difficulties are attendant upon such attempts: (1) spores of certain species do not germinate until after the lapse of from three to eight or more years; (2) the young gametophytes, especially those which are subterranean, cannot be carried beyond the 4- to 10-celled stage unless they become infected with the characteristic symbiontic fungus. It has been suggested that the first difficulty can be removed if some method, possibly chemical, can be found to break the resting period. As for the second difficulty, no one apparently has made a special study of the fungus; at least, no information concerning its identity is available, although it probably is an Ascomycete.

In some species the gametophyte is only partially embedded in the substrate, the aerial portions containing chlorophyll and bearing the antheridia and archegonia. Some species have spores which germinate immediately upon being shed, the sex organs are soon produced and the young sporophyte is established the same season. No gametophytes of this type are apparently known which do not harbor a fungus.

The other type of gametophyte is subterranean, tuberous, and entirely lacks chlorophyll. There are usually definite areas containing the fungal symbiont. Spores producing this type of gametophyte require several years for germination, and the latter develop very slowly, sometimes taking 16 years or longer for the development of the sex organs. The gametophytes are buried at depths of from 0.5 to 6 cm. Their probable presence can be inferred by the finding of a few very young sporophytes. They are generally found at some distance from the parent plants and usually grow in the shade (Degener 1924; Spessard 1917, 1922; Stokey and Starr 1924).

The majority of the localities in the United States where gametophytes have been found are in Massachusetts, but others have been discovered in Michigan and New Jersey. These are all regions where rains occur during the summer. Whenever the gametophytes are found, they usually occur in abundance.

Formalin-aceto-alcohol has given perfect fixation of the aerial type of gametophyte. Microtoming should be in the vertical longitudinal plane at 11μ. If the sex organs are at all present, they usually occur in great abundance; the archegonia more so than the antheridia. Iron hematoxylin is the only stain which has afforded satisfactory results with the sex organs; fast green may be used as a counterstain, but orange G is better on some species. For the embryo and young sporophyte a triple combination might be tried.

Selaginellales

The order includes one living genus, *Selaginella*, which differs from *Lycopodium* in that it is heterosporous. The North American species of *Selaginella* are found in habitats ranging from damp shade to xerophytic conditions. Some species are terrestrial and others epiphytic; most of those occurring in the United States belong to the former group. *Selaginella* is commonly planted in greenhouses as a ground cover and in pots for table decoration. *S. kraussiana* is the species usually employed as a ground cover. Tropical species are frequently to be found in greenhouse fern collections, and these are the best source of material, especially of the strobili, since they are less refractory than the native species. The xerophytic species should be avoided, except for special purposes.

Root.—The roots are very small, are adventitious, and are usually borne at the ends of the rhizophores. They present no technical difficulties.

Rhizophore.—This structure is generally considered to be a part of the stem, but its anatomical structure is similar to that of a root. It is easy to manipulate, but occasionally becomes so hard that some soaking of the embedded material under water is required.

Stem.—The stems of most species become too hard and brittle to be sectioned without the expenditure of the utmost ingenuity. The stem tips, whose apex may be occupied by either a mass of meristematic tissue or a single apical cell, according to the species, gives no trouble, and may be treated as described for the corresponding parts of *Lycopodium*.

As in *Lycopodium*, the tropical species of *Selaginella* have stems which are easier to microtome. *S. kraussiana* is the easiest of all, but the steles are of relatively small extent.

Stems of some species may be easier to section in celloidin, but considerable success has been had after dehydration with tertiary butyl alcohol and prolonged soaking in water. Fix with formalin-aceto-alcohol or a strong chrom-acetic, microtome at 11μ, and stain with safranin and anilin blue.

In the stems there is a space of considerable extent between the steles and the inner face of the cortex; the space is bridged by trabeculae which are easy to demonstrate in some species but not in others.

Leaf.—The leaves are so small and so appressed to, or parallel with, the stem that sections of at least the younger stems show their structure adequately.

Strobilus.—The strobilus of *Selaginella* is one of the most difficult of all plant organs to microtome. Up to the time that the walls of the megaspores become thickened in the younger strobili, the latter section

without difficulty, but after that stage the megaspores cannot be cut, except in rare instances, without shattering. A few technicians claim that embedding in celloidin removes the difficulty, but others have had no success with this procedure. The best that can be done is to fix in formalin-aceto-alcohol or a medium chrom-osmo-acetic fluid, taking care to exhaust all air; to dehydrate over a considerable period (allowing each change to react for at least a week) by the tertiary butyl alcohol method; to leave in the paraffin oven for several weeks or even months; and to embed in a hard Parlax or a paraffin melting at 58°C. After embedding, the exposed material must be soaked under water for months (see also Slagg 1932). The strobili of an unidentified species from Jamaica sectioned easily after 45 days' immersion; *S. douglasii* from Oregon required 8 months' immersion, but even then there was some shattering. All methods designed to soften the spore coats by chemical means are worthless.

In most species microsporangia and megasporangia are borne, usually rather irregularly, in the same strobilus, but in others only one kind of sporangia are to be found in a strobilus. The distribution of the sporangia follows four types of arrangement but occasional exceptions may be encountered (Mitchell 1910): (1) a single large basal megasporangium subtended by an especially large leaf; (2) several basal megasporangia with microsporangia above; (3) strobili wholly either megasporangiate or microsporangiate; (4) megasporangia and microsporangia disposed indiscriminately.

The strobili should be microtomed longitudinally. The claim has been made that, in order to cut through the stalks of the sporangia, the plane of sectioning should be diagonal, from corner to corner; the strobilus is square when viewed in transverse section. This may be true of some species, but it does not appear to be so in others. The strobili of some species are curved; consequently they can only be sectioned parallel to either flat side. The thickness for general purposes may be from 10 to 12μ. It is difficult to keep the sections on the slides; hence they should first be coated with a thin layer of celloidin and taken through carbol-xylol for deparaffining, thence to 95% alcohol, and to the staining. For the stages up to the rounding up of the spore mother cells, iron hematoxylin is preferable; thereafter either a triple combination or safranin and fast green will be preferable.

Gametophyte.—Both microspores and megaspores commence development into the respective gametophytes before being shed from the sporangia. In most species development is not wholly completed at the time of shedding. The stage of development at the time of shedding of the megasporangia is far more variable than that of the microspores. If the spores are not sufficiently advanced before shedding, it is a simple

matter to sow the spores on filter paper or plaster of Paris blocks placed in Petri dishes and moistened with distilled water or a weak nutrient solution. After several days, spores may be removed daily and fixed. It should also be possible to obtain sporelings in the same manner. Sections to show the development of the androcytes should be cut at 4μ; those for the archegonia and embryos at 8μ. Safranin and fast green constitute an excellent stain combination, but in the megagametophytes the cell contents acquire a very intense color from the safranin. Triple combinations may be used, but tend toward gaudiness. Young sporelings are easily mounted entire, stained with Harris' hematoxylin and fast green, and dehydrated by a gradual dioxan or hygrobutol method.

Isoetales

The single living genus, *Isoetes*, is essentially aquatic, and the locations where it might be expected can be found in any flora or in monographs (Pfeiffer 1922). It is common over the United States but may be easily overlooked because of its strong resemblance to grasses and sedges. The plants are herbaceous, consisting of ligulate leaves borne upon a corm-like stem, which is so short as to be externally indiscernible, and roots borne upon a massive rhizophore below the stem.

Root.—The roots may be snipped off, fixed with formalin-aceto-alcohol, microtomed transversely at 12μ, and then stained with safranin and fast green. Sections invariably look as if the roots were badly disorganized, but this is the natural condition. The root tips are not easy to collect; longitudinal sections will reveal the apical meristem.

Stem and Rhizophore.—The two should be worked up together, since it is useless to attempt to separate them. Take plants which have just begun to produce sporangia, trim off the roots 1 or 2 mm. from the rhizophore, and cut through the leaves just above the apex of the stem (the apex has the shape of an inverted cone). Provided they are not too large, even older plants may be used. Fix in formalin-propiono-alcohol. In some species the stem is bilobate; in others it is trilobate (Fig. 80). To show the ramifications of the interesting vascular system, serial sections, both longitudinal and transverse, should be cut and all mounted in order. For this purpose microtome at 15 or 16μ. Safranin and fast green are the most adequate stain combination.

Leaf.—Sections of young plants, prepared as noted in the preceding paragraph, will show the structure of the leaves. However, there is no reason why portions of the leaves cannot be removed from the plants and run up independently.

Sporangia.—The outer leaves on the stock are sterile, then come the megasporophylls, next the microsporophylls, and near the center are the younger leaves with the youngest stages in sporangia development.

However, plants will be found in which the older sporangia contain only either megasporophylls or microsporophylls. The earlier ontogenetic development is alike in both types.

Sections of young plants for the stem and rhizophore are very likely to show the sporangia also. The material intended for demonstration

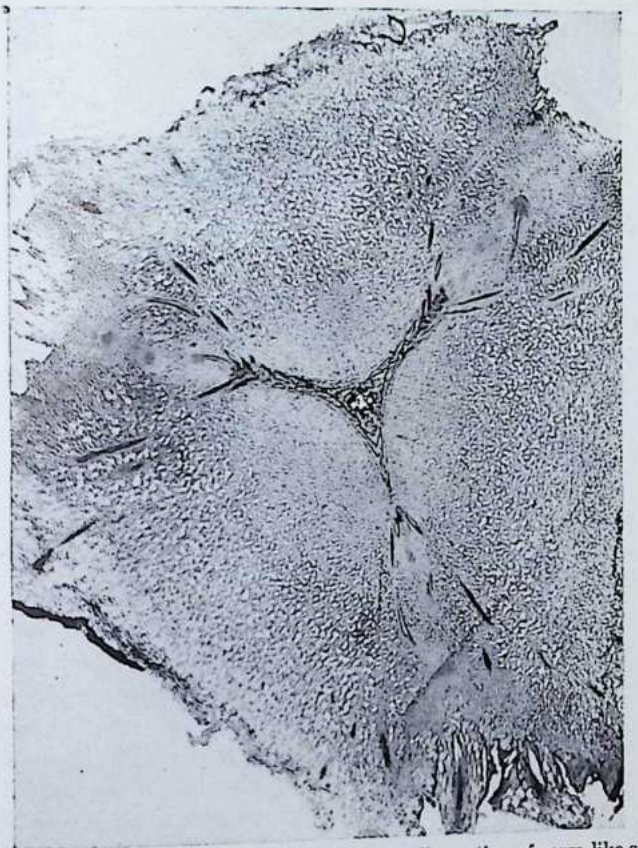


FIG. 80.—*Isoetes nuttallii:* cross section through middle portion of corm-like stem, showing ramifications of the vascular system. Fixed with formalin-propiono-alcohol; stained with safranin and fast green.

of the sporangia should be prepared as described for the stem, but for the earlier stages the sections should not be over 10μ in thickness. While safranin affords an excellent basic stain, iron hematoxylin may occasionally prove to be more satisfactory. Unlike most aquatic plants, *Isoetes* is easy to stain.

The megaspores, unlike those of *Selaginella*, give no trouble during the microtoming; the real difficulty is to fix them properly. A fixative of strong penetrating power apparently is required, and formalin-aceto-

alcohol appears to be as good as anything. A triple combination of iron hematoxylin plus orange G gives good results.

Gametophyte.—Germination of the spores begins immediately after liberation from the sporangia, provided suitable conditions are present. However, large numbers of the spores are incapable of germination; in the case of the megaspores, one will have to undertake the tedious job of separating the sound from the shriveled ones previous to fixation.

Bring portions of the soil with young megaspores and young plants to the laboratory, place in suitable containers, and keep well watered. It appears probable that the megaspores at least remain viable for about a year. Transformation of the microspores into antheridia occupies about a week; the period elapsing from germination of the megaspore to the well-developed embryo stage is about a month. To secure a series of developmental stages, collections of germinating spores should be made at frequent intervals during the respective periods. The microgametophytes should be sectioned at 5μ, the megagametophytes at 7 to 8μ. It is impossible to orient the megagametophytes and younger embryos in the proper position for sectioning; hence one can only cut blindly, but the number of usable sections is surprisingly high.

EQUISETINAE

Equisetum, the only living genus of the Equisetinae, is well known to every botanist, but there appears to be a general impression that it is one of exasperating difficulty to the technician because the plants are encrusted with silica. It seems not to be realized that the most interesting and critical parts of the plants are not encrusted, or at most only superficially so, and that microtoming of these parts is actually very easy. The only serious difficulty is that all parts have an exceptionally weak affinity for basic stains. *Equisetum*, on the whole, is a genus well worth the technician's efforts.

Equisetum

Equisetum is a nearly cosmopolitan genus and affects a variety of habitats. Most of the species prefer aquatic or damp, shaded situations but others are almost xerophytic. Fresh material from which to collect material for sectioning is sometimes frequently lacking, very small

descend to more than a meter. This rhizome in many species is the only active means by which the plants spread over new territory.

Root.—The adventitious roots are borne at the nodes of the rhizome and in most species function for only one season. Therefore, roots which plainly appear to have been formed recently should be collected; the old roots are shriveled and not worth collecting. Root hairs are abundant and conspicuous in the finished preparations. There is an apical cell at the tip of the root. The roots are not silicified and are easily sectioned. Fix the tips in a chrom-acetic or chrom-osmo-acetic fluid and the older portions in formalin-aceto-alcohol. Microtome the former longitudinally at 8μ, the latter transversely at 12μ. Staining is usually fine with a triple combination.

It is sometimes possible to obtain longitudinal sections of the origin of the roots by sectioning young rhizome nodes transversely.

Rhizome.—There is a large apical cell, with three cutting faces, at the tip of each rhizome. The apical cell of the rhizome is generally in better condition, its divisions are easier to follow out, and it stains better than is the case with the apical cell of the vegetative branch. The latter is very apt to have the external wall indented, and it is difficult to section it in a satisfactory plane. The lateral apical cells of the vegetative stem are, in fact, usually in a better condition for study than is the terminal apical cell. The tip of the rhizome is massive in many species; slabs from opposite sides should therefore be cut off, and it would be well to examine the sections under the microscope since only those very close to, or containing, the apical cell are worth mounting. Fix in chrom-acetic or formalin-aceto-alcohol and microtome at 8μ.

The structure of the subterranean and aerial portions of the sporophyte is nearly identical. The rhizomes are only slightly silicified; consequently if the aerial portion is too difficult to section, the rhizome may be substituted. Otherwise, it would be preferable to utilize the aerial branches. Fix portions of the rhizome internodes in formalin-aceto-alcohol, section at 14μ transversely, and stain with safranin and fast green.

Aerial Branches.—These are of two types in most of the species. The fertile branches, when present, are flabby, not much silicified, and each is terminated by a strobilus. The sterile, or vegetative, shoots are formed and appear aboveground after the fertile branches have, as a rule, completed growth.

In the other type the fertile and vegetative branches are combined into one organ. The strobili are borne at the termini of the lateral branches. In addition, there are one or two evergreen perennial species.

The vegetative shoots differ in the amount of silicification according to the species. Some are slightly, others heavily encrusted. The

former can generally be sectioned, after the blocks have been soaked under water for several weeks, without evident tearing, but the heavily encrusted types must be treated with hydrofluoric acid. Fix with formalin-aceto-alcohol, wash with two changes of 70% alcohol, then treat with 50% hydrofluoric acid in 70% alcohol for two days, finally washing thoroughly with 70% alcohol, and proceeding to the dehydration. The dehydration and infiltration should be very thorough. Safranin and fast green are as good a stain combination as any; triple combinations have not been satisfactory.

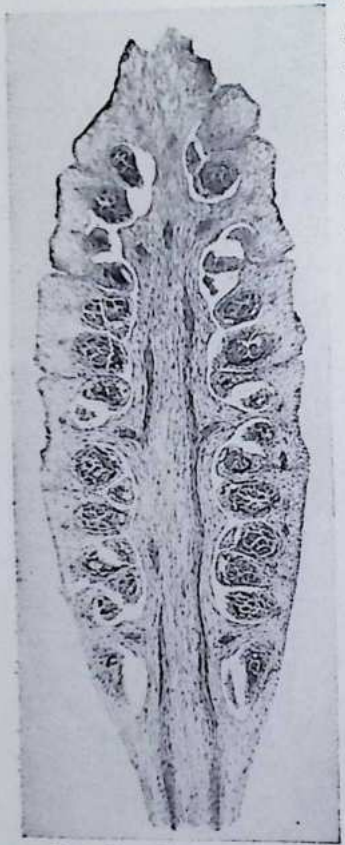


FIG. 81.—*Equisetum arvense:* median longitudinal section of a young strobilus with spore mother cells. Fixed with formalin-aceto-alcohol; stained with iron hematoxylin and fast green.

There is an apical cell at the terminus of each branch. That at the end of the main axis is the one which is meant when the "apical cell" is mentioned in most texts. This cell invariably looks better in drawings than it does in the actual preparation, as it is rare that this cell is either perfectly fixed or sectioned in exactly the right plane. The external wall is generally indented. The apical cell of the rhizome, as was mentioned above, is better organized and is easier to fix. The apical cells of the primordial branches are just as useful as the terminal apical cell, although few botany instructors seem to be willing to admit the fact. *E. arvense* is one of the best species for apical cells. Stem tips that have just emerged from the ground may be cut off about 6 to 8 mm. below the tip, fixed with a medium chrom-acetic, and microtomed longitudinally at 8 to 10μ. Stain with safranin and fast green.

Leaf.—The leaves are small, chaffy or scale-like, and more or less united to form a sheath around the base of the internode. In most species they are not worth the trouble required to section them, since they are heavily encrusted with silicon.

Strobilus.—The young strobilus is first recognizable at the apices of the subterranean rhizomes in June or July; the sporogenous cells are differentiated by the middle of August (Fig. 81); meiosis occurs late in the same month, but the spores are not naturally shed until the follow-

ing March or April. However, if strobili are brought into the laboratory and kept in a warm place, they can be induced to shed their spores any time after mid-September. In those species in which the strobili are borne on the termini of vegetative shoots, they appear in early spring, develop rapidly, and the spores are ready for shedding by mid-spring.

The strobili of all species are easily fixed and sectioned at all stages of development. In those species in which the strobili originate on the rhizomes, dig up the latter carefully, wash thoroughly under running water, and cut off the rhizome apices. If the particular colony is known to produce strobili, the latter are fairly certain to be present at the rhizome apices. Fix the earlier stages in Navashin's fluid or in a medium chrom-acetic; for the later stages following meiosis, formalin-propiono-alcohol is good. Section at 10 to 12μ. The younger stages should be microtomed longitudinally, but transverse sections may also be made of the later stages to show the relation of the sporangiophore to the central axis. For all stages up to meiosis, stain with iron hematoxylin but omit counterstaining. The later stages are difficult to stain adequately; a quadruple combination may be attempted.

Gametophyte.—The mature spores with their characteristic elaters are easily mounted entire in their natural colors. The simplest method is to mix a small quantity in a drop of melted glycerin jelly on a slip and to add a coverslip carefully so as not to let any of the medium extend beyond the periphery of the coverslip, then ring with Duco or other ringing material. More substantial mounts may be made by placing fresh spores in 95% ethyl alcohol for 15 minutes, then pour off the alcohol, replace with Euparal or Diaphane diluted several times with 95% alcohol, and place in a cool part of the paraffin oven. The alcohol evaporates quickly; with a pipette draw up a small quantity of the mixture, put 1 drop on a slide, and add a circular coverslip 18 mm. in diameter. Avoid jarring or shaking the spores, else the elaters become detached. By either method the preparations will keep for at least five years.

The spores of some species retain their viability for only 1 hour after being shed, while those of others may remain viable for a few days. Prothallia are easily raised artificially, provided fresh spores, secured from strobili that are about ready to discharge, are used. Boil shredded sphagnum for about 1 hour, then pack tightly in sterilized moist chambers, of a suitable type, to the depth of 1 or 2 inches. Press out the surplus water. Cover the receptacles until cool, then sow the spores on the surface of the sphagnum. Keep the moist chamber covered, and place in a north window. No special precautions to prevent the appearance of fungi or algae need be taken, since it is possible to control these organisms with potassium permanganate. If they should appear, add enough

crystals of the permanganate to distilled water to give the latter a purplish color. Pour over the affected areas, then drain off all excess solution. The solution may be allowed to remain for as long as 10 minutes in cases of bad infection, as the permanganate appears to benefit rather than to harm the cultures. If only a few algae are present and they do not seem to crowd the cultures, they may be allowed to remain. It will be approximately a month after germination when the first sex

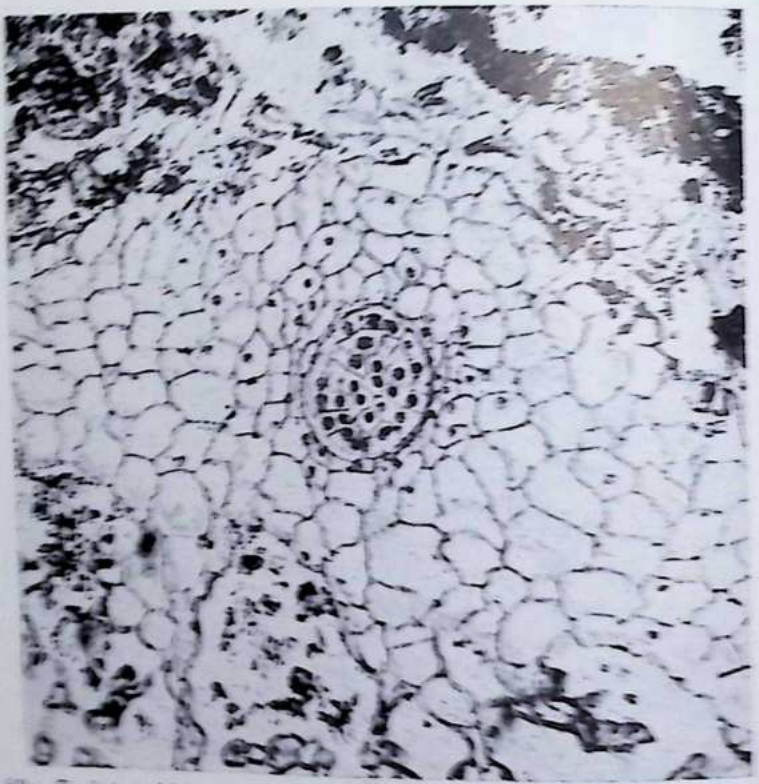


FIG. 82.—*Equisetum debile*: longitudinal section of young embryo. Fixed with formalin-acetic-alcohol; stained with safranin and fast green.

organs (usually the antheridia) are recognizable. The cultures can be kept growing for years.

Equisetum arvense is the species whose spores are most easily germinated in the above manner. The gametophytes are more like those of the Filicinae and whole mounts may be prepared in exactly the same manner (see page 407). Other species produce cushion-like protballia that are too thick for whole mounts. *E. laevigatum* is an example of such species, but *E. debile* from India is better known. These massive protballia must be embedded and sectioned. They usually grow in mud. It is impossible to get rid of all the enveloping mud; if the mud

does not contain grit that might nick the microtome knife, do not bother about it, but proceed to embed prothallus, mud and all. Fix with formalin-aceto-alcohol or a medium chrom-acetic fluid, and microtome in the vertical plane at 10μ. If the gametophytes are in good condition (which is not always the case), a fine series of developmental stages of antheridia, archegonia, and embryos is easily secured (Fig. 82). The question whether the gametophytes of *Equisetum* are homothallic or heterothallic has not been settled; in any event, most of the gametophytes in the majority of species will be found to be homothallic. In those species, like *E. debile*, with the cushion type of gametophyte, the nature of each gametophyte can be determined only by sectioning. It will sometimes be necessary to search very carefully for the archegonia. Even if the gametophytes are sectioned blindly in the vertical plane, a very large number of embryos and young sporophytes can nevertheless be secured. All stages are difficult to stain. One might try placing the slides with sections attached, after bringing them down to water, in 1% aqueous chromic acid for 1 hour, rinsing thoroughly with water, and then staining with safranin and fast green. It was nearly impossible to differentiate the hematoxylins on all materials which were available to the writer.

Gametophytes of *Equisetum* have been found in many localities, most of those in this country being in Nebraska.

FILICINAE

The American ferns have been rather thoroughly studied taxonomically and can be readily identified from popular manuals, floras, and special monographs. The ferns are widely distributed over the country, but most of the species are included in the Polypodiaceae. There are numerous dealers in native plants who advertise their local species; if mature specimens are ordered, they are usually in a condition for immediate fixation, except occasionally for the sporangia. All the botanical and most public gardens possess extensive fern collections; if the head gardeners are approached, they are almost always willing to permit one to collect stems, leaves, and sporangia. Material of many tropical species is purchasable from the botanical supply concerns. Altogether, it is not a difficult matter for the technician to obtain, from one or another of the sources just mentioned, suitable material of any desired type.

One is likely, however, to encounter trouble in securing stages of sporangial development. It will be necessary to learn something of the life history of the species concerned, particularly with regard to the time of year when the sporangia are first formed. This period differs considerably even within the same family. Those species which have the sori at the periphery or on the abaxial side of the vegetative leaves

commonly produce sporangia intermittently or continuously throughout the year, especially when in cultivation indoors. Those which produce the sporangia on special fertile spikes (as in *Struthiopteris*) usually have the sporangia already formed as these spikes begin to appear from the crown. Sporangia may develop slowly or rapidly, and sometimes may even turn out to be sterile. Different species in the same genus may be more suitable from both technical and teaching standpoints and more useful than others (*e.g.*, *Polypodium linearе*, a Hawaiian species, is the best of all the numerous species in the genus for the origin and development of the sporangia).

As has already been intimated, the ferns belonging to the Leptosporangiatae are very difficult technically. The stems of most species are rigid; the leaves are tough and leathery; the rhizomes in many species are massive and surrounded by heavily sclerified leaf bases; but if the proper precautions are observed, it is entirely possible to cut perfect sections as thin as 10μ of even the most formidable specimens. However, material that has been kept in plain alcohol for years is worthless as the vascular tissues have become almost completely disorganized.

Certain precautions need to be observed when collecting fern material in the field. If roots and rhizomes of purely terrestrial forms are to be collected, the plants should first be loosened all around by means of a spading fork. Any roots or rhizomes that appear to be too long to be dug out in their entirety should be cut off as far from the base as possible. Avoid all pulling or stretching, as it is very easy to cause the vascular bundles to become separated from the surrounding tissues. Each clump after removal from the soil should be thoroughly washed in a near-by stream or under a water faucet, whereupon the desired root or rhizome portions may be cut off with a large, sharp scalpel. If they are not to be fixed immediately, they may be wrapped in damp newspapers for transportation to the laboratory. Epiphytic species are easy to manipulate, it merely being necessary to free them of adhering mosses, liverworts, etc.

Eusporangiatae

The group is so designated because the sporangia develop from more than a single cell. The antheridia are embedded in the gametophyte.

Ophioglossales

The order includes three genera of perennial herbs. One genus, *Helminthostachys*, ranges from India through Malaysia north to Japan. *Ophioglossum* and *Botrychium* are world-wide in distribution, but are more common in the Northeastern states than west of the Rockies in the United States (R. T. Clausen 1938). Both occur usually scattered in colonies consisting of few plants, although in some especially favorable

localities extensive stands are known. The plants are found in a variety of habitats—moist meadows, shady fields, grassy thickets, rich swamplands, and even on sandy beaches. If not already known, locations

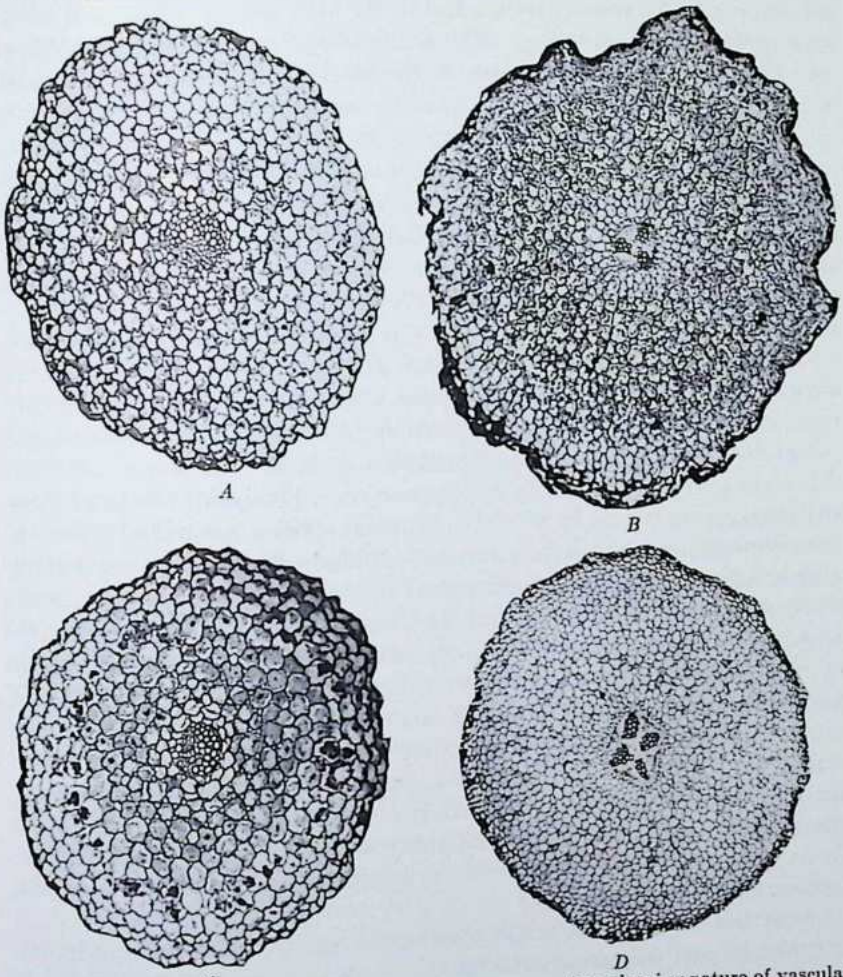


FIG. 83.—Roots of the Ophioglossales, in transverse section, showing nature of vascular cylinder and comparatively large cortex: *A*, *Ophioglossum vulgatum*, *B*, *O. pendulum*; *C*, *O. reticulatum*, *D*, *Botrychium virginianum*. Fixed with formalin-aceto-alcohol; stained with safranin and fast green.

where material can be obtained are difficult to find; it would be well to check herbarium specimens whose labels bear precise collection data. If material cannot be collected in the field, the botanical supply concerns are usually well supplied with excellent material.

The genera will be treated together in the following discussion, since technical methods apply equally to all.

Root. The adventitious roots are comparatively large, somewhat fleshy in a few species, and usually gorged with starch (Fig. 83). Cut them into portions 5 to 8 mm. in length, fix with formalin-aceto-alcohol, microtome transversely at 12μ, and stain with safranin and fast green or a quadruple combination. The material rarely becomes so hardened as to require soaking; in any event, the blocks should not remain under water for longer than a day.

There are no root hairs.

Stem. The stem is short, subterranean, and upright, although it is dorsiventral in some tropical epiphytic species. In some species of *Ophioglossum* the stem is so short that one can only trim off the roots and petioles and run up the whole mass. In old plants of *Botrychium* the stem may become so long that it has been called a "rhizome."

The stem is easily manipulated, even when quite old. Section transversely at 12μ, and stain with safranin and fast green. If necessary to soak, do not leave the material under water after it becomes whitish-opaque, or it cannot be cut at all; two days' immersion is long enough.

The stem apex is particularly worth attention. There one will find the buds of the leaves which will appear successively for the next three (or four) years. The buds of the tropical species are naked; those of the temperate one are closely covered with hairs which may occasionally give trouble. There is a single apical cell, but it is extremely difficult to locate it and to be certain of its identity. In removing the spikes from the plants, cut them off carefully so as not to cut through the petiole of the oldest one, lest it be lost. Wash the buds very thoroughly, preferably under a small jet from a thick rubber tube attached to a faucet, so as to wash out all the grit. Fix with formalin-aceto-alcohol, imbed with care, cut down, and section across the flat face of the bud. Examine sections before mounting, and discard those that do not go through all four buds. Stain preferably with a quadruple combination. It is best to select buds in which the leaves are not yet unrolled, but it will be possible to find the origin of the leaves even in buds in which the leaves are unrolled.

Leaf and Petiole. Both are very easy from the technical standpoint, and ordinary methods are applicable.

Fertile Spike. To obtain the very youngest stages, the plants must be carefully watched. In early spring the plants may be dug up and the buds examined for the earliest stages, and, after this, collections may be made at regular, frequent intervals in order to secure a complete series of developmental stages. The spikes are readily fixed, sectioned, and stained at all stages. Both transverse and longitudinal sections should be prepared; the optimum thickness is 10μ. Safranin and fast green give a brilliant stain, but perhaps iron hematoxylin may be preferred for the stages up to meiosis. The clusters of sporangia in *Botrychium*

should be reduced to smaller proportions as soon as they become large enough.

Gametophyte.—In both *Ophioglossum* and *Botrychium* gametophytes have frequently been found (R. T. Clausen 1938). They are usually subterranean in habit but may be located so near the surface that small lobes appear aboveground and develop chlorophyll. No one has succeeded in artificially raising gametophytes from spores beyond the 13-celled stage. The gametophytes, if one should be so fortunate as to find them, may be fixed in a medium chrom-acetic or in formalin-aceto-alcohol. If dorsiventrality can be distinguished, microtome in the vertical longitudinal plane at 10μ; otherwise try transverse sectioning. Staining is better with iron hematoxylin than with coal-tar dyes for the sex organs, but for general purposes a quadruple stain may be attempted.

Marattiales

All species are tropical; material consequently is hard to obtain. Leaves with synangia attached should be available from the supply concerns, but other organs probably are not. In any event, *Marattia*, the principal genus, is one of the easiest of all Pteridophyta with which the technician might work. Despite their large size, the stems section with surprising ease in paraffin, but the mature synangia may require some softening under water. If material of *Marattia* is unavailable, either *Danaea* or *Angiopteris* will serve equally well. The latter genus might be found cultivated in conservatories or large fern collections. The leaves and sporangia of these two genera are more rigid than those of *Marattia* and will require softening under water for a week or longer. Some care must also be taken to avoid overstaining.

Leptosporangiatae

The plants which are more commonly understood when the term "ferns" is mentioned are included in the Leptosporangiatae.

The Leptosporangiatae are very widely distributed, but the majority of the species are tropical. They range in size from the great tree ferns of the tropics and subtropics to the tiny floating *Azolla;* from the fragile "filmy ferns" (*Trichomanes* and *Hymenophyllum*) of humid rain forests to the tough and leathery xerophytic species of the deserts. In the United States, however, most of the species are found in places where the soil does not dry out completely during the rainless seasons, as in moist woods, the shady sides of canyons and ravines, in fresh-water swamps and marshes, and in deforested lands. Interesting species are to be found growing under ledges and between boulders on high mountains. Aquatic species are not so widely distributed: there is one species of *Azolla* on the East coast and another in the West; *Marsilea* is commoner

but less readily found, and *Ceratopteris* occurs in the regions immediately adjacent to the Gulf of Mexico. Such a wealth of material is available to the technician that the question becomes one of making a satisfactory choice of the species with which to work. Each species, as a rule, is more favorable in some respects than in others, but all have their individual disadvantages. That is, each has an inherent technical problem more or less peculiar to itself. These problems will be specifically mentioned in the appropriate place in the following discussion.

The only characters which are constant in distinguishing one family from another are those of sori and sporangia (E. M. Smith 1898, Verdoorn 1938). Almost every general and suitably local flora has a section devoted to the Leptosporangiatae, and in addition there are several popular manuals, all of which will be useful in identifying local species. Warning should be given, however, that hybridization is rampant in certain genera, and many species moreover are decidedly polymorphic.

Methods common to all Leptosporangiatae will be described first, and these will be followed by fuller discussions under each family.

Root.—The roots in general offer no special technical difficulties, but those of some species are wiry (*Filix*) and consequently require soaking under water. For the root tip and its prominent apical cell, fix with a medium chrom-acetic, section some tips longitudinally and others transversely at 10μ, and stain preferably with safranin and fast green. For older roots, use formalin-aceto-alcohol, and microtome transversely at 12μ.

Rhizome and Stem.—The rhizomes and stems of certain species offer very difficult technical problems, but the extreme beauty and great utility of these structures are more than worth all the trouble involved. Remove subterranean rhizomes carefully and wash free of adhering soil. The stems of some species are covered with old leaf bases, which should be trimmed away as much as possible since they interfere with smooth sectioning. Any rhizomes or stems that can be cut across with a sharp scalpel can be microtomed after embedding, even if the portions should become hardened during the dehydration and infiltration. The portions should not be over 1 cm. in length. The rhizomes of certain species (*e.g.*, *Pteridium*) are filled with a mucilaginous substance whose dissolution during dehydration will cause more or less plasmolysis. Fix with formalin-aceto-alcohol, embed in a hard paraffin, and microtome transversely (and also longitudinally if desired) at 12μ. All hard pieces of material should be well softened under water, but any that are congested with starch should not be left in the water too long, otherwise hydrolysis of the starch occurs, and the material cannot be sectioned. Safranin and fast green afford superb differentiation; Harris' hematoxylin or crystal violet may be substituted for the

fast green. Triple combinations are usually too gaudy, but quadruple methods are excellent.

The stems and rhizomes of the Leptosporangiatae are exceptionally valuable in studies on the various types of steles to be found among vascular plants. The stelar type in the young stem is frequently very different from what is to be found in much older stems; even in the same stem it may differ at various levels. In some species of *Osmunda*, for example, the portion of the stem first developed may be protostelic, but the portions later developed may be dictyostelic. It is therefore necessary to take into account the age of the plant when the material was collected and also the portion of the stem that was selected.

Leaf.—In those species in which the leaves are all one type, it is the common practice to make sections of the leaves and sporangia simultaneously. Microtoming is usually in such a direction (transverse or longitudinal) as to go through the largest possible number of sori. Where the leaves are dimorphic, the structure of the fertile leaf is sufficiently well shown in sections primarily intended for the sporangia, but the sterile leaves must be worked up independently. The vegetative leaves differ considerably in structure, but particularly in the extent to which the cell contents are revealed. Practically nothing can be found in mature leaves of *Pteridium* and *Adiantum*, as it is almost impossible to stain them differentially; those of *Polypodium* or *Cyathea* are far less troublesome. The leaves of the filmy ferns are only one cell in thickness.

To obtain the earliest stages in leaf development, when the activity of the apical cells is especially prominent, select young, tightly coiled fronds which have just emerged from the crown; those which are about 1.5 to 1.8 cm in diameter are excellent. *Pithyrogramma* is one of the best genera for the purpose. Microtome across one flat side of the circinate frond at 12μ. Safranin and fast green are excellent, but care should be taken not to overstain with the basic stain since it is sometimes not easy to differentiate.

Sporangia.—The sporangia are as a rule borne in sori of different types arranged in various positions on the leaf blade. In some sori the sporangia develop simultaneously, in others they are formed in basipetal succession, and in still others they are produced in an irregular sequence (Fig. 84). When the sori are borne on the abaxial sides of the leaves, at the periphery of the leaf, or at its apex (as in *Hymenophyllum*), it is a simple matter to watch the plants for the first appearance of the sori, and a series of developmental stages is easily secured. In some of the species in which the sporangia develop simultaneously, leaves bearing the earliest sori up to the mature spores can be found on the same plant. Species with special fertile spikes are more difficult to find with the earliest stages: the sporangia are already formed when the

coiled fronds emerge from the crown of the plant and develop rapidly as the coils unroll.

For sections of the leaves and soil, fix portions with formalin-acetic-alcohol, microtome in the plane which permits passing through as many soli as possible (scattered as in *Cyrtomium*), or transversely (arranged in a linear series as in *Woodwardia*), or longitudinally when arranged in widely spaced rows, as in *Polypodium*) at 11μ. If the leaves have become brittle or if the sporangia contain mature spores, soaking under water becomes necessary. Safranin and fast green or a quadruple combination stain adequately.

types of annulus: rudimentary, equatorial, vertical, apical, and oblique. Simply place the sori in hygrobutol for several hours, give a change, then infiltrate with weak balsam, and evaporate down to a mounting consistency. A more refined method would be to fix first in formalin-aceto-alcohol, replacing this gradually with hygrobutol, and infiltrating with balsam. Staining is not required. Small portions of leaves bearing sori may also be mounted entire, with the sori and sporangia remaining in their natural positions. Fix with formalin-aceto-alcohol; staining is unnecessary, but if one is desired, wash out the fixative with 85% alcohol and apply a strong solution of fast green in equal parts of methyl cellosolve and 95% alcohol for several hours or overnight, then wash out with two or three changes of equal parts of the two fluids just mentioned, and infiltrate with highly diluted balsam, which should be brought to a concentrated condition quickly. Mount the leaf portions, sori side up, in culture slides. The leaves will become very brittle in a short time—no method that circumvents this annoying propensity is known—therefore they should be mounted as soon as possible.

Gametophytes.—Spores of all Filicales are easily grown in artificial culture. The gametophytes are rarely found in first-class condition in nature, and then only where the habitat does not become dried out. In cultures, moreover, one may inspect the plants from time to time and thus keep an accurate check on the growth stages.

Three general methods of cultivating prothallia are in use:

1. A shallow pot or fern dish is half filled with broken pottery chips or fine gravel. Over this place a layer of rich loam to within 2 cm. of the top, then follow with a layer of clean plasterers' sand about 5 mm. deep. Water completely, and sterilize. When cool, scatter the spores over the surface of the sand, and cover the container with a pane of glass. Set in diffuse sunlight.

2. Procure a new porous clay flowerpot, wash thoroughly, and pack tightly with damp sphagnum. Invert and place in a convenient pan or saucer and sterilize. When cool, put sterile water to a depth of about 2 cm. in the saucer. Scatter the spores over the sides of the pot, cover the whole with a battery jar, and place in diffuse sunlight. Turn the whole around occasionally so that all sides will become evenly illuminated, otherwise the prothallia will acquire abnormal shapes.

3. The third method is better adapted for research purposes since it affords complete control over the cultures. Sow the spores on a film of nutrient agar (use Knop's or Pfeiffer's solution) about 5 mm. deep in small Petri dishes under strictly aseptic conditions. For purposes of cross-fertilizing or producing hybrids, each individual immature prothallium may be transferred to a separate Petri dish, which transplantation should be performed before the archegonia appear. Fertilization

may be effected when the archegonia are open, by flooding the culture with nutrient solution and placing a pteridium with mature antheridia in the solution. The solution is permitted to remain for about 12 hours and is then poured off, and the antheridial pteridium removed. The sporophyte will be visible in a week or two; after the root and rudiculus have become well developed, the young sporophyte may be transferred to soil in a small pot if it is desired to grow the plant to maturity.

The last method is probably the best one to follow if one wishes to obtain an abundance of material of all stages in the origin and development of the sex organs, embryos, and sporophytes. It may be necessary

Fig. 85. Prothallium of *Pteris cretica*, bearing young sporophyte. ×3.

antherozoids is malic acid, which fact may be demonstrated by placing some antheridial fragments in water under a coverslip and placing a drop of a weak aqueous solution of the acid at one side of the cover. The antherozoids will promptly swim toward the acid.

The gametophyte of many ferns is at first either asexual or antheridial. The antheridia may appear even during the filamentous stage, and are usually very abundant. Considerable growth takes place before the archegonia originate, customarily just behind the growing apex.

For sections, fix the gametophytes with 1% chrom-acetic. Microtome them in the longitudinal vertical plane; it is better to try to obtain the archegonia in median longitudinal section and to get the antheridia by chance. For the antheridia alone, 5 to 8μ is thick enough; stain with iron hematoxylin. For the archegonia, 10μ is satisfactory; staining may be difficult, but a triple combination usually gives passable results. For embryos and young sporophytes, section at 10μ, and use safranin and fast green. Cut all sections perpendicular to the anteroposterior axis of the gametophyte.

For whole mounts fix in either 1% chrom-acetic or formalin-aceto-alcohol. Stain with Harris' hematoxylin, which will give brilliant differentiation of the sex organs and embryos (Fig. 85). After differentiating the hematoxylin, upgrade to 85% ethyl alcohol, then leave overnight or even for as long as 24 hours in a strong solution of fast green (dissolved in equal parts of methyl cellosolve and 95% alcohol). Overstaining rarely results; wash with two changes of 95% alcohol, then dehydrate with hygrobutol, and place in diluted balsam. This method is excellent with all developmental stages, but the counterstain may be omitted on young embryos for the sake of greater clarity. In mounting on slides, be sure to orient with ventral side up. The prothallia should be mounted as soon as convenient, since they begin to curl up and become brittle after remaining in thick balsam for more than three days.

Special directions for the gametophytes of the Marsileaceae and Salviniaceae will be given in the appropriate places under these families.

Filicales

Osmundaceae.—The cosmopolitan genus *Osmunda* does not occur west of the Rocky Mountains in the United States. The stem is very difficult to microtome because of the large masses of sclerenchymatous leaf bases; prolonged soaking under water sometimes renders them sufficiently soft to cut. The stelar portion of the stem is comparatively small; the part first formed is protostelic and later portions are dictyostelic. The leaves are tough and leathery. Production of sporangia

from the supply concerns. *Trichomanes* is abundant in Florida and one species extends as far north as Kentucky, occurring on dripping sandstone cliffs. The rhizome has a protostele. The leaves of many species are only one cell layer in thickness; young stems and leaves are very easily sectioned. The sori are marginal, and the sporangia are borne in basipetal succession on a receptacle; the whole is surrounded by an indusium. Pinnae with sori should be removed separately; embed with one flat side down, and section parallel to this flat side at 10μ. Fixation of all organs is adequate with formalin-aceto-alcohol, and sharp staining is given by practically all combinations. The spores are easy to germinate, but cultures will require three or more years before they commence producing sex organs. The monoecious gametophyte is either a profusely branched filament (*Trichomanes*) or an irregularly branched ribbon (*Hymenophyllum*); the sex organs are borne on short adventitious branches.

Cyatheaceae.—The tree ferns are mainly tropical or subtropical plants, but one representative (*Dennestaedtia adiantoides*) occurs in Florida. Specimens of *Cyathea* are common in greenhouse fern collections in the East and are widely planted outdoors in central and southern California. Material is thus relatively easy to collect or to purchase, but stem material is of rather doubtful attainability. Fixations should be in formalin-aceto-alcohol; staining is usually easy but stains giving sharp contrasts should be selected. The sori are already present when the circinately coiled fronds begin to unroll, but it is not difficult to secure the earliest stages of sporangial development. As the leaves develop, they become tougher and are rigid after being embedded; consequently soaking is required. Prothallia develop very slowly.

Marsileaceae.—In the United States *Pilularia* occurs chiefly on the Pacific Coast; *Marsilea* in the same region and along the Gulf Coast. One species of the latter genus has become established in a few ponds in New England. Material of *Pilularia* is difficult to procure, but *Marsilea* is easily raised from sporangia, which can be purchased if not otherwise obtainable.

Marsilea sporophytes have a branched, creeping rhizome, whose ramifications may cover a considerable area. The leaves and roots appear at the nodes. Young nodes may be fixed entire and sectioned in the vertical transverse plane (*i.e.*, as if the internode was being cut transversely); these will provide a great variety of interesting and instructive tissues: origin of the adventitious roots, young leaves, petioles in longitudinal section, and even the origin of the sori and the youngest developmental stages, as well as sections of the rhizome proper. Fix with formalin-propiono-alcohol, use the water suction pump, soak the

embedded material under water for about three weeks, microtome at 10μ, and stain with either safranin and fast green or a quadruple combination.

The vascular cylinder in the rhizome is an amphiphloic solenostele. The broad flat leaves of *Marsilea* should be cut into convenient widths for fixation with formalin-aceto-alcohol, and the awl-shaped leaves of *Pilularia* into short sections.

The youngest sporocarps must be looked for in sections of the nodes. As soon as they can be observed emerging, they should be cut out. They are borne on peduncles inserted just above the bases of the petioles. In *Pilularia* and in many species of *Marsilea* the sporocarps are solitary; in other species of the latter genus they may number as many as 20. As long as they can be cut with a razor blade or sharp scalpel, the sporocarps can be embedded and sectioned. Cut off a small segment at one end to facilitate penetration of fluids, and fix with formalin-aceto-alcohol. Infiltration with paraffin must be quite thorough. Soaking under water appears to be only briefly needed by some species, a much longer time by others. Serial sections are better than a few isolated sections. The sporocarps should be microtomed perpendicular to their flat side—transversely for the younger stages and longitudinally as soon as the sporangia are definitely recognizable. (When nodes are sectioned for the origin of the sporocarps, the latter obviously cannot be oriented in a definite plane, but practically all sections are useful.) The optimum thickness is 10μ. Iron hematoxylin is the only satisfactory stain, and the solution should not have been used before on other slides.

The sporangial initials at the apex of each receptacle become megasporangia, and those lower down develop into microsporangia. It is a fortunate matter for the technician that after the sporocarps become too hard to be sectioned, the stages between this period and the emergence of the sporangia on germination are of little importance.

The sporocarps require a resting period of three months or longer before they can be induced to germinate. The spores remain viable for 30 years or longer. The sporocarps should be filed at one end until the whitish cavity is exposed and may then be placed in large Petri dishes containing sterile tap water (distilled water should not be used). The gelatinous sporophore will be extruded within ½ hour; the perfect ring shape delineated in most textbooks does not always appear. The time of emergence should be noted, since collections should be made at stated intervals beginning as soon as all the sori have emerged. Development proceeds rapidly; that of the microgametophyte requires from 12 to 20 hours, and that of the megagametophyte 14 to 22 hours, both at ordinary room temperature. [At 28 to 30°C. antherozoids appear in about 6½ hours (Lang 1936).]

For the development of the microgametophyte, collections should be made at intervals of 30 minutes for the first 10 hours: at the first ½ hour, the nucleus is in the center; at 1 hour, it begins to move toward one side of the spore; at 1½ hours, it has reached the side and preparations for the first division are evident; at 2 hours the sori begin to disintegrate, but enough spores are still caught in the gelatinous envelope for the sori to be fixed entire—after this time the spores must be taken

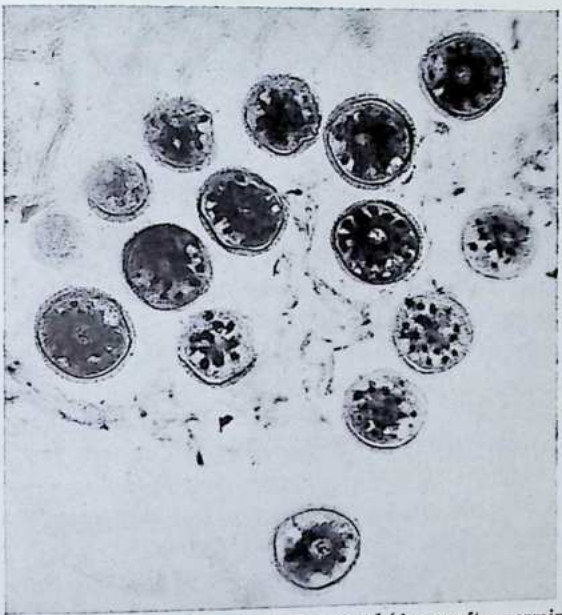


FIG. 86.—*Marsilea vestita:* sections of microspores 2½ hours after germination, immediately before the reduction divisions begin. Killed with Sharp's special chrom-osmo-acetic fluid; stained with iron hematoxylin and fast green.

up with a pipette; at 2½ hours the first mitosis occurs (Fig. 86); and at the end of the seventh hour, the 16 androcytes are fully developed. So close a series of stages has not been worked out for archegonial and embryonal development. Matters here are complicated by the prevalence of parthenogenesis. Fertilization apparently occurs in about 24 to 36 hours. In four or five days the cotyledon has attained a length of 6 to 8 mm.; it has a bright green color.

For the microgametophytes fix with Navashin's fluid made up in the proportion of 13 cc. of Part A, 13 cc. of 4% formalin and 1 cc. of 1% aqueous osmic acid, or with the following (Sharp 1914):

1% aqueous chromic acid	25 cc.
Glacial acetic acid	1 cc.
2% aqueous osmic acid	14 drops
Distilled water	75 cc.

Chromic fluids are in general worthless on the developing megagametophytes, but a fluid which is completely satisfactory on these structures is as yet unknown. Young embryos fix well enough with formalin-propionic-alcohol. It will be tedious changing the dehydrating fluids after the spores have become freed from the soft, but the cautious use of a centrifuge will be of great assistance. For embedding, use small paper trays or porcelain dishes about 5 mm. square, in order to concentrate the material as much as possible. Microtome at 5μ, mount the ribbon serially, and stain with iron hematoxylin and fast green. The starch grains stain so intensely that it is not easy to judge the destaining accurately. Whole mounts of the megaspores with young sporophytes attached are readily prepared: fix with formalin-propionic-alcohol, stain with Harris' hematoxylin and fast green, and follow the hyperbutanol method.

Polypodiaceae.—This family contains the largest number of species and is the dominant one in the United States, especially in the Pacific states.

Technical methods are essentially similar for all species and the general methods outlined at the beginning of the Pteridophyta apply. The one point to bear in mind is that any one species is not so equally good in all respects as another might be. For instance, the rhizome of *Pteridium aquilinium* is easier technically and has better organized tissues than that of *P. latiusculum*. The latter occurs in the Eastern states and the former on the Pacific Coast. The very finest material of *P. aquilinium* comes from the humid forests of the Northwest. All species of *Polypodium* are fine for the stem, but only a few have sporangia which make good slides. *Aspidium* is excellent for the development of the sporangia; *Pteridium* and *Adiantum* are among the worst.

Parkeriaceae.—The sole genus, *Ceratopteris*, which occurs from Florida to along the Gulf Coast, is aquatic. It may be found in a few botanical gardens or living specimens may be purchased from fern specialists or dealers in aquarium supplies. General methods are applicable, but the tissues are so fragile that great care with the dehydration and infiltration is imperative.

Salviniaceae.—Like the Marsileaceae, this family is aquatic and heterosporous, differing in that each sporocarp contains either microspores or megaspores, but not both, and in that the plants float on the surface of the water. There are two genera, *Salvinia* and *Azolla*, both with only a few species each.

Salvinia is mainly an African genus, but *S. natans* is cultivated as an aquarium plant. The sporophyte looks somewhat like a gigantic *Lemna*. The leaves are arranged in threes; of these, two are floating and one is submerged. The former are covered with papillae on the upper surface and are densely matted with brown hairs on the underside. The submerged leaves are finely dissected and give the appearance of being roots, but true roots are absent. In general the methods described

below for *Azolla* apply to *Salvinia*, but it is easier to distinguish the young sporangia in the latter.

Azolla is to be found in small ponds and the quieter parts of slowly moving streams. In the shade the color is a pale green, becoming a distinctive reddish shade in full sun. As a rule, only those plants growing in full sun produce sporangia, which generally appear in late summer or early autumn. If unavailable locally, live plants may be purchased from dealers in aquarium supplies or embedded material (which is preferable to preserved material since the latter is likely to be sterile or minus sporocarps) can be secured from the botanical supply concerns.

The plants may be fixed entire; if there are so many lateral branches that the whole mass is too large, cut them off. The material should be handled carefully since the maturing sporocarps are easily detached. It is very difficult to get the sporophytes to sink into killing fluids. Use a medium chrom-acetic, or formalin-aceto-alcohol, and add to the fluid from 2 to 5% ethyl acetate, which should assist in lessening the surface tension on the plants. Use the water suction pump to remove air contained in the leaves. Embed the plants singly, dorsal side down. Microtome at 10μ in the vertical longitudinal plane (*i.e.*, lengthwise perpendicular to the dorsal surface), as the greatest number and variety of useful sections will be obtained in this fashion. If vertical transverse or horizontal sections are desired, use small portions of the sporophyte; large plants cannot be accurately sectioned in these two planes because the tips are curved ventrally. Examine the ribbons under the microscope, discarding those that apparently have no sporocarps or which do not come sufficiently close to the stem. In *A. filiculoides*, the most extensively studied species, the sporocarp will generally be microsporangial. Experience has shown that an older megasporangium will be found in about 1 out of every 60 plants. The younger megasporangia are relatively easier to find. Microsporangia in all stages of growth are usually abundant. *Azolla* is not easy to stain sharply or brilliantly for all structures simultaneously. It would be better to concentrate on the desired structures and to ignore the others. Safranin and fast green constitute the best general stain combination; for mitoses in the microsporangia, which are easy to obtain, use iron hematoxylin.

To obtain the later stages in the development of the sporangia, especially for the archegonia and embryos, it will be necessary to place plants bearing nearly mature sporangia in a large glass receptacle, which is then placed in full sunlight. After the megaspores have fallen to the bottom of the container, they may be removed and fixed at frequent intervals over a week or longer. It is, however, a gamble whether one will secure anything worth while.

The endophytic alga, *Anabaena azollae*, appears in almost every section.

CHAPTER XXIX

CYCADOPHYTA

CYCADALES

The order includes one family, the Cycadaceae, with nine genera and about 65 species. *Zamia floridana* is the only cycad that occurs naturally within the confines of the United States; it occurs abundantly in certain portions of Florida. Plants of other cycads are commonly grown in large conservatories, particularly those in the parks of the larger cities.

The cycads are strictly dioecious. The male strobilus is always a compact structure in all the genera. The female strobilus may be either a crown of loose sporophylls or a compact cone; all transition types between the two extremes are also to be found.

It is a rather difficult matter to obtain material of the cycads, especially of the reproductive phases. To obtain a series of developmental stages, one would have to make collections over almost an entire year. Some stages in the life history progress so rapidly that daily collections are required, but for other stages one collection a week suffices. A great number of plants are obviously needed to afford sufficient material when a large number of collections are to be made. From cultivated specimens one can usually obtain material at only one developmental stage, except in the case of such as *Cycas revoluta* or *C. circinalis*, when it is possible to secure a series of stages at one time—provided one comes at the right time. Preserved material, or even embedded material, may be secured to a limited extent from the supply concerns. Leaves and perhaps petioles can be readily obtained from a public park or commercial florist upon request, but in such cases one should check up on the identification of the plants. If one can establish contact with a collector in regions where the cycads grow naturally, it might be possible to procure living plants or large quantities of material from which to work.

alcohol fixes very nicely, but Navashin's fluid should be used on root tips. After safranin, anilin blue is a more satisfactory counterstain than fast green.

On all seedlings and on specimens in greenhouses are to be found peculiar apogeotropic (aerial) roots; these produce profusely branched coralloid masses. Such roots contain bacteria in the form of bacteroids, and midway between the vascular cylinder and the epidermis there is an enlarged layer of cells, usually only one-cell wide, in which *Anabaena* grows. The general methods reveal the alga clearly, but for the bacteroids, mitochondrial methods are indicated. A 10% solution of neutralized formalin fixes well. If the usual mitochondrial staining techniques fail to reveal the bacteroids clearly, resort may be had to differential acidification, with subsequent staining in safranin and fast green, since this procedure has worked well in the case of the bacteroids in legume nodules.

Stem.—The stems of most of the cycads are fleshy, with very little wood present; consequently they are easily sectioned. It has been claimed that the stems can be most readily sectioned freehand while fresh, but no trouble was occasioned in microtoming material that had been in formalin-aceto-alcohol for 25 years. After killing and fixing and with or without treatment with hydrofluoric acid, the stems or stem pieces can be embedded in either paraffin or celloidin. Cutting presumably should be easiest with a sliding microtome, even for paraffin material, but the writer had no difficulty microtoming paraffin material that had been in water for a fortnight.

Bowenia and *Stangeria* are tuberous, with all or most of the stem located underground. Some species in *Zamia*, *Macrozamia*, and *Encephalartos* also have subterranean tuberous stems.

Petiole (Rachis).—If stems are unobtainable, petioles of most cycads provide interesting material. In some species the vascular bundles are arranged in characteristic positions. In *Cycas revoluta*, for example, they are disposed in a flaring horseshoe manner when viewed in transverse sections.

The petiole, except when still young, is rather rigid and tough. However, if fixed with formalin-aceto-alcohol, dehydrated with tertiary butyl alcohol, and embedded in hard Parlax or a hard paraffin and the cut ends of the embedded pieces exposed under water until really soft (for months if necessary), microtoming will be found to be easy. Cut at 16 to 18μ.

Leaf.—The leaflets vary considerably in size among the different species. The length may range from 7 to 40 cm., and the width from 1 mm. to 2.5 cm. In each genus the leaf type is characteristic (Lamb 1923). The leaflets of the adult plant are often quite different from those

of juvenile plants. The structure of the mature leaflet is strongly xerophytic. The epidermal cells are thick walled and heavily cuticularized, which adds to difficulties in sectioning leaves. All genera save *Cycas* and *Stangeria* lack a midrib in the leaflets (Fig. 87). In *Stangeria* side veins emanate from the midrib, but such secondary veins are absent in *Cycas*. *Zamia* is easily distinguished by its bipinnate leaves. There is a gland at the base of each leaflet in species of *Macrozamia*.

Young leaves are readily embedded and sectioned. The heavy phlobaphene deposits and other substances in many species do not permit a sharp stain differentiation, but results are generally satisfactory after

FIG. 87.—*Zamia floridana*: cross section of a portion of the leaf. Fixed with formalin-acetic alcohol; stained with a triple combination. The dark areas represent sclerenchyma cells.

either safranin and fast green or a triple combination. Thick, leathery, mature leaves are quite difficult to section, but after prolonged immersion in the softening effect of water, one can usually manage to obtain good sections. The method of tying several mature leaves together and sectioning them fresh in a sliding microtome, as recommended by some technicians, is too crude to merit further discussion.

Staminate Strobilus. In some genera, such as *Dion*, there is a single terminal cone; in other genera, such as *Macrozamia*, the cones are axillary. The strobili vary greatly in size, ranging from 2 cm. in length in a species of *Stangeria* to the huge cone of *Macrozamia denisonii*, which is usually 60 cm. long and 30 cm. in diameter. Measurements, however, are not of great import because the strobilus elongates greatly and rapidly just before the pollen is shed. The microsporophylls are very similar in structure.

The integument and ovules borne on the abaxial (lower) surface of the megasporophyll in pairs of two, three or more, are in a

sorus. The sorus arrangement is like that in typical ferns; in fact, the structure of the microsporangia is remarkably like those of *Angiopteris*. Development of the microsporangium is of the eusporangiate type. The number of microsporangia on a sporophyll varies, being greatest in *Cycas* and fewest in *Zamia*, *Z. floridana* possessing only 25.

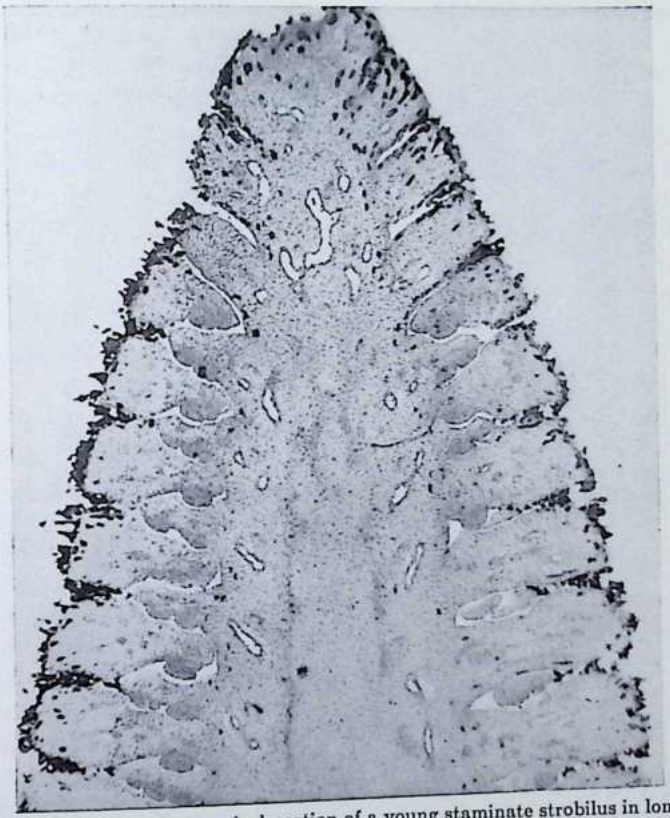


FIG. 88.—*Zamia floridana:* apical portion of a young staminate strobilus in longitudinal section, at the time of origin of the sporogenous cells. Fixed with formalin-aceto-alcohol; stained with safranin and fast green.

The entire staminate cone, as can be judged from the dimensions of the structure as cited above, is too large to be sectioned entire except during the younger stages. Of course, the larger of the young cones can be cut up into convenient portions, but for the later stages the sporophylls should be removed and fixed individually. The sporogenous cells appear first at the base of the strobilus, but the pollen grains mature first at the apex and lastly at the base of the strobilus. Formalin-aceto-alcohol has proved to fix adequately at all stages. For the finer cytologi-

cal details, however, Navashin's fluid, or a modification thereof, should be substituted and only very small pieces of tissue should be fixed.

For staining, almost any combination will give satisfactory results, although iron hematoxylin may be superior for cytological details and safranin with fast green for general morphology (Fig. 89).

After the microsporocytes have rounded up and are more or less free from one another, smears are readily made according to the general

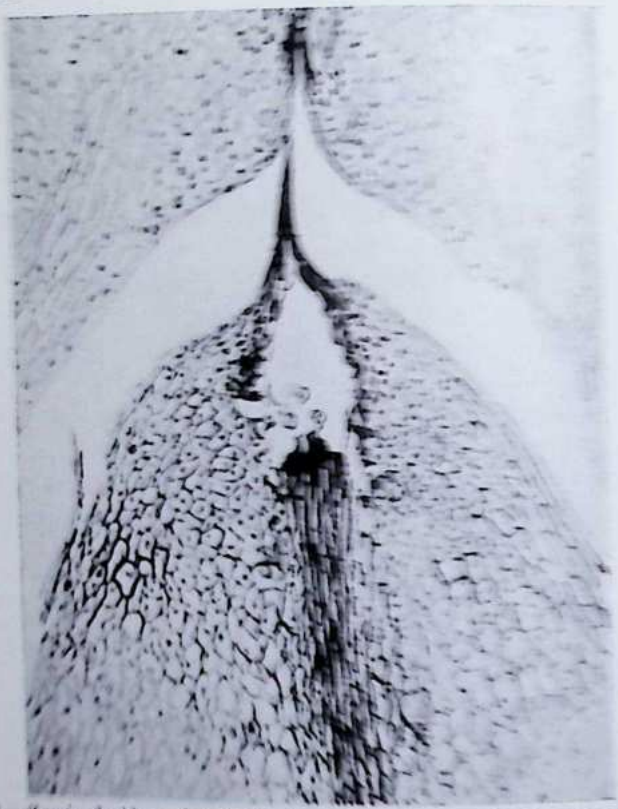


Fig. 89.—*Zamia floridana*: longitudinal section of an ovule through the apex of the nucellus with young microgametophytes. Fixed with formalin-aceto-alcohol, stained with safranin and iodin blue.

methods outlined in Chap. XIII. If the pollen, especially when at the shedding stage, is obtainable in sufficient abundance, better results can be secured if the material is worked up by a whole-mount method. A carmin stain or the Feulgen reaction will give the sharpest staining. Dehydrate with hyperalcohol and infiltrate with balsam.

The microspore germinates before shedding. When shed, the pollen grain consists of a generative cell, a tube cell, and a prothallial cell. The grains are likely to be somewhat shrunken at this time. Before putting them into the boiling fluid, they should be left in water for about 30 minutes to restore turgidity.

The pollen may be germinated in a 5 to 10% solution of sucrose or in various sirups. Only the earlier stages of pollen tube development may be obtained in this manner. The later phases must be studied in sections of the nucellus.

The microgametophyte is a haustorium that penetrates the nucellus and can be recognized as brownish lines radiating from the beak of the nucellus. Cut across the young ovule in such a manner that the cut is just below the lower portion of the nucellus, then remove those portions from the upper half of the ovule in which the pollen tubes presumably lie. Kill and fix in a chrom-acetic mixture in the proportion of about 2 cc. glacial acetic acid to 100 cc. of 1% aqueous chromic acid. The material can be run into paraffin, or it can be stained in bulk, dehydrated, run into balsam, and the tubes with their sperms dissected out of the nucellus just previous to mounting. For the latter purpose, staining may be in aqueous safranin (about 5 hours), Harris' hematoxylin, or carmalum. Transfers in the dehydrating process must be very gradual. Sectioned material is most satisfactorily stained with iron hematoxylin or safranin and anilin blue (Fig. 89).

Records indicate that in the region of Miami, Fla., pollination occurs in late December or early January, the blepharoplasts appear in March and swimming sperms may be found during the first week of June (Chamberlain 1932). The interval between pollination and fertilization is about five months. The body or spermatogenous cell divides immediately before fertilization.

Pistillate Strobilus.—The mature pistillate strobili are such large structures in most of the species that it is impossible to cut sections of the entire strobilus. More or less reduction in dimensions is required. Even the individual megasporangia are so large in some species that they cannot be sectioned entire when mature or nearly so.

Under ordinary circumstances it is impossible to obtain material of the very youngest stages in the development of the megasporangia. In the first place, the external appearance of the stem apices gives no indication whether what is developing beneath the scale leaves is a strobilus or merely a crown of leaves. In the second place, the plants may be too scarce to run the risk of killing them by digging out the stem apex. For the early stages portions of the ovule should be trimmed off on opposite sides, but great care must be exercised not to cut within 2 mm. of the endosperm, which is so turgid that it ruptures easily. A medium chrom-acetic fluid fixes splendidly. Staining may be in a triple combination or with safranin and anilin blue. The same procedure may be followed for all the later stages, including embryogenesis.

CHAPTER XXX

CONIFEROPHYTA

GINKGOALES

The order was once cosmopolitan but is now reduced to a single living representative, *Ginkgo biloba*, which probably no longer exists in the wild condition but is widely cultivated. Trees of *G. biloba* are to be found almost everywhere in the United States, and anatomical material is easily secured at any time. Material for cytological or morphological purposes is more difficult to obtain at just the correct times. Preserved or embedded material is readily purchasable from the supply concerns, except for the critical stages between late free-nucleate female gametophyte and young embryos.

Root. In most plants the root has a diarch vascular bundle, but in the seedlings with three cotyledons it is triarch. The structure of the mature root greatly resembles that of the mature stem. The root is easier to section in paraffin than are young stems but nevertheless requires long soaking under water after being embedded. Safranin and anilin blue or fast green is a good stain combination.

Stem. Two types of branches occur in *Ginkgo*, as in many other conifers: long branches and short spur branches. Long branches always appear first and grow for at least a year before spur branches are developed on them. The anatomy of the two types of branches differs. In the long branch there are a comparatively small pith and cortex, there are fewer resiniferous cavities, and the wood is harder. Sections of spur branches are easily cut in paraffin, but the embedded long branches must be soaked under water for about two months before they can be microtomed easily enough. If they are embedded in celloidin, they must be treated with hydrofluoric acid immediately after fixation. Fix with formalin-aceto-alcohol; staining may be with any desired combination.

The wood is rather difficult to microtome; resort should be had to celloidin embedding preceded by treatment with hydrofluoric acid. Freehand sections may also be cut on a sliding microtome. The vascular cylinder is an endarch siphonostele. Annual rings are present and usually prominent. In the tracheids of the secondary wood there are one or two rows of scattered bordered pits on the radial walls. Both bars and trabeculae of Sanio are to be found in the secondary wood. The medullary rays are almost invariably only one cell wide and rarely over four or five cells in height.

Leaf.—Portions of the leaf are readily embedded and microtomed. In the leaves of the spur branches a palisade layer is absent, but one is to be found in the larger leaves of the long branches. Formalin-propiono-alcohol fixes well; staining is sharpest with safranin and anilin blue, or a quadruple combination may be used.

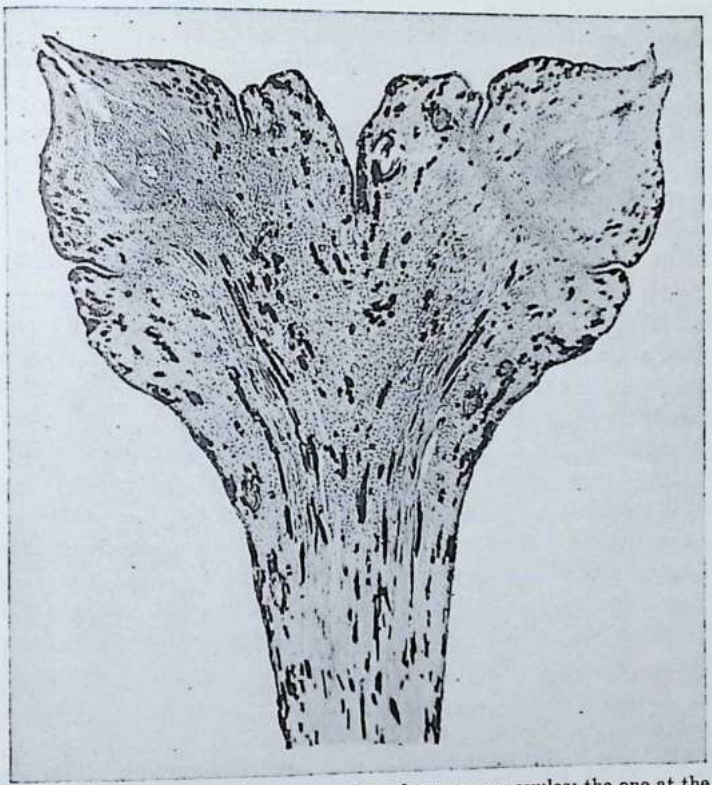


FIG. 90.—*Ginkgo biloba:* longitudinal section of two young ovules; the one at the left is sectioned medianly through the micropyle, pollen chamber and megaspore mother cell. Fixed with formalin-propiono-alcohol; stained with a triple combination.

Two endarch vascular strands occur in the petiole, which may be treated like the leaf, but may require soaking under water before being microtomed.

Staminate Strobilus.—The beginnings of the staminate strobilus are first recognizable early in July by the swelling of the buds on the spur branches of staminate trees. Development proceeds slowly until cold weather sets in, at which time the microsporocytes have become differentiated. The time at which growth is resumed depends upon the locality: in the Palo Alto, Calif., region, this has occurred in mid-January during mild winters, but may be delayed until early March in other

localities. Once development has been resumed, it is very rapid. Meiosis takes place at once; if meiotic figures are desired, one usually has to anticipate the recurrence of growth and make collections before it appears to have definitely started. Fixation should be in a strong chrom-acetic fluid, and only small portions of a strobilus should be fixed. For general morphology of the strobilus even entire strobili may be preserved, and formalin-aceto-alcohol is a better fixing fluid. Iron hematoxylin is excellent for the reduction divisions, but safranin and anilin blue are more useful for general topography.

Each sporophyll of the strobilus generally bears two microsporangia, but as many as four and even seven have been reported. Development of the sporangium follows the eusporangiate scheme. Throughout an entire strobilus there is not so wide a series of developmental stages present at any one time as might be expected, because of the rapidity with which the spores are formed.

Pistillate Strobilus.—The pistillate strobili are borne on the spur branches at the apex. The ovules occur in pairs on long peduncles, but one ovule frequently aborts early in development. The ovules are first recognizable at about the time that the terminal bud on the spur branch begins to swell. To get the earliest stages the tip of the spur may be cut off, the outer scales removed, and the whole embedded and sectioned longitudinally in a plane as nearly perpendicular as possible to the two ovules. When the ovules begin to emerge from the bud, the single integument has partially covered the nucellus. Between the middle and end of April the megasporocyte has appeared, and it is also at this time that pollination occurs (Fig. 90). The megasporocyte undergoes meiosis, a linear quartet of megaspores being the immediate result. Irregularities, however, are not infrequent. In any event, only one megaspore becomes functional.

In the peduncle which carries the ovules there are four vascular bundles, in contrast to the two bundles in the leaf petiole. This fact has led to the interpretation that the peduncle is a stem bearing two sporophylls (the prominent collars at the base of each ovule), each supporting a single ovule. If more than two ovules are present, the number of vascular bundles in the peduncle is twice the number of ovules.

Microgametophyte.—The microspore germinates before being shed from the microsporangium, as in the cycads. When shed, the microspore contains four cells—two prothallial cells, one of which is aborted, a generative cell, and a tube cell. The lower two-thirds of the spore is covered by the exine, the upper third by the intine alone. In the pollen chamber of the ovule the generative cell divides into a stalk cell and a body cell. Two blepharoplasts are developed in the body cell, which

divides to form two sperms. The blepharoplasts eventually undergo metamorphosis into cilia.

Megagametophyte.—The megasporocyte undergoes meiosis, at about the time of pollination, or in late April, to form a linear row of quartets. The lower one becomes functional and enlarges rapidly, and free nuclear divisions take place (Fig. 91). The nuclei are forced against the periphery of the cell by a large central vacuole. At first the mitoses are simultaneous, but later on some nuclei lag behind the others, and finally

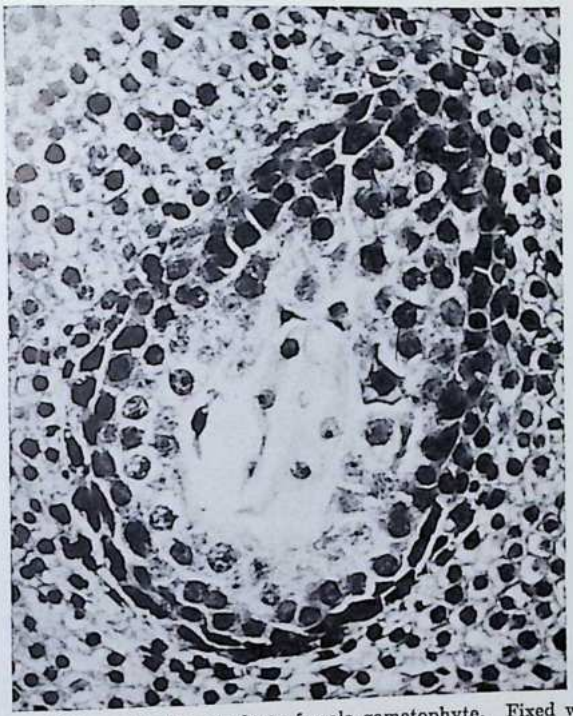


FIG. 91.—*Ginkgo biloba:* early free nucleate female gametophyte. Fixed with formalin-aceto-alcohol; stained with safranin and anilin blue.

a few fail entirely to divide again. These divisions occupy about two months, or until the end of June or very early in July. At this time a delicate membrane develops over the outer surface of the coenocytic protoplasm, and cell walls perpendicular to this membrane are formed between the nuclei. Wall formation progresses centripetally, but, before the gametophyte becomes completely cellular, two or rarely three archegonial initials have already made their appearance at the apex. The two neck cells and the central cell are organized quickly, and the latter commences growth and enlargement. Early in September the ventral canal cell and the egg become differentiated, a definite wall

being formed between the two. Such a wall is not produced after the same mitosis in the cycads.

Fertilization.—Accurate and detailed descriptions of the fertilization process have not been published.

Embryogenesis.—Simultaneous free nuclear divisions follow upon fertilization. Unlike conditions in the megagametophyte, the nuclei are not pushed against the periphery by a central vacuole but are evenly distributed throughout the egg cell. About eight mitoses occur, where-

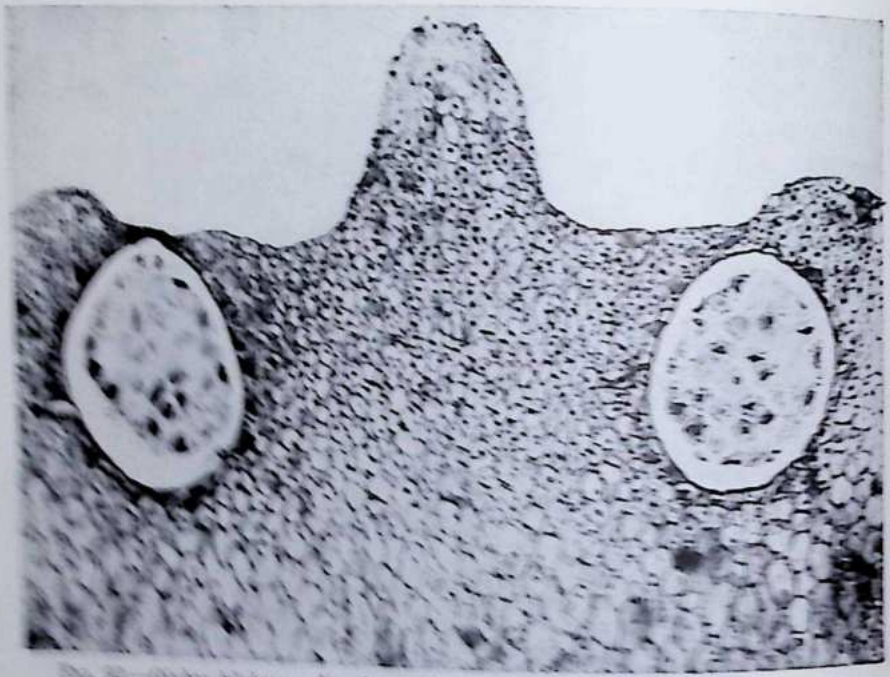


Fig. 92.—Ginkgo biloba: median longitudinal section of nucellus with beak and pro-cambium of beginning of root formation. The typical shrunken appearance of the pre-fixation is common. Fixed with medium chrom-acetic; stained with safranin and malla-lon.

upon walls are simultaneously developed all through the egg (Fig. 92). At first the cells are of the same size, but presently those at the basal end grow rapidly and become small and numerous, those in the central region rather evenly (Chamberlain, 1933). A readily distinguishable suspensor is not developed. The basal portion soon grows rapidly and becomes differentiated into cotyledons, stem, and root. The embryo remains naked in Ginkgo.

At the stage of embryo development as early fixed and sectioned in Figure 92, the cotyledon development has normally progressed to the point where the embryo can be seen with the unaided eye. It is advisable to dissect out the embryo and to work it up independently of other tissues, which will have become so hardened by this time as

section easily. A strong chrom-acetic or even formalin-aceto-alcohol fixes well; safranin and a suitable counterstain may be used. The cells of the older embryo are so small and compact that somewhat thin sections are required; about 10μ is thin enough.

The seeds mature in November and germinate if planted at once. As with the cycads, there is apparently no normal resting period.

Coniferales

Although the greatest natural concentration of the Coniferales is to be found on the Pacific Coast and in the Northern states, conifers nevertheless are to be found in practically every locality. If native species are lacking, introduced forms are freely planted everywhere. Material is thus readily accessible; even reproductive phases are not difficult to collect, but they are unfortunately not always of a suitable nature or desirable type. The most widely distributed genus is *Pinus*, with *Larix*, *Picea*, *Tsuga*, *Abies*, *Thuja*, *Cupressus*, and *Taxus* of secondary degree of distribution, particularly by landscape plantings.

There are 10 families, divided into two groups: Pinares and Taxares (Buchholz 1934, Chamberlain 1935). In the Pinares the seed cone is conspicuous and well developed, woody in most genera and fleshy only in species of *Juniperus*, and the plants are mostly monoecious. In the Taxares the seed cones are mostly small, poorly developed or reduced to only a few scales, and fleshy; the plants are mostly dioecious.

Despite a very extensive bibliography on the Coniferales, it has been claimed that "a complete life-history has not been worked out in any of the genera in this large order" (Chamberlain 1935). *Pinus* has been more extensively investigated than any other genus, and thus more is known about it. In the other genera there has been too much emphasis on either wood anatomy or on reproductive structures, so that in reality only some one isolated aspect of the complete life history has been investigated.

The various organs and reproductive structures will be discussed separately in the following paragraphs.

Root.—Preparations of the mature embryo, in both longitudinal and transverse section, will show many details of the primary root structure. The seeds may be removed from mature pistillate cones and the endosperm with the embryo removed from the seed. It is difficult for fixatives and dehydrating fluids to penetrate the dense endosperm, hence slabs should be cut from opposite sides to facilitate penetration. Seeds may also be germinated and specimens fixed at various stages of development. A strong chrom-acetic or formalin-propiono-alcohol may be employed.

To illustrate the origin of the stele in the primary root and the development of the secondary roots, the seeds must be germinated in fine soil or in sphagnum. Begin fixing portions of roots when the hypocotyl has attained a length of 2 cm. and continue until it is about 6 cm. high. Root hairs are present but occupy a space rarely over 1 mm. in length at the tip and are exceptionally fragile.

Longitudinal sections of the root tip of many species reveal, even by ordinary fixation methods, characteristic features, but the results are far more pronounced if mitochondrial fixatives are employed (Zirkle 1932). The tannin-containing vacuoles are well organized in the root tips of the gymnosperms and form definite patterns. The tips may be fixed in a mixture of 100 cc. of 10% aqueous formalin and 5 g. ferrous sulphate for 48 hours to several days. Wash with water briefly, embed as usual, section at not over 10μ, affix to slides, remove the paraffin with xylol, and mount in balsam without further staining.

Portions of the fully mature root should be dealt with as if they were stem sections, but pieces of younger roots can be cut without difficulty until they are about 5 mm. in diameter. Avoid overstaining with the basic stain.

Stem.—As in *Ginkgo*, long branches and spur branches occur in many of the Coniferales, such as *Abies*, *Cedrus*, *Larix*, and *Pinus*, but the leaves on the spur branches are in fascicles. Structurally, the vascular cylinder is endarch siphonostelic. Annual rings are conspicuous features of the mature wood of all but a very few species. The transition between "spring" and "winter" wood is abrupt in many species, but in others there is a greater amount of "spring" wood than of the other type. "Spring" wood consists of larger, thinner walled, and less hard cells; "winter" wood is compact, with thick-walled cells.

In *Phyllocladus* the spur shoot is borne in the axil of a leaf. It becomes flattened (i.e., a cladode) and has the functions of a leaf. The vascular bundles in these phylloclads are mesarch.

The meristematic stem and young stems up to a year old are easily embedded and sectioned without special treatment. Cut into portions about 1 cm. in length; it is unnecessary to trim off the young leaves along the sides. Fix with formalin-aceto-alcohol, not in an aqueous fluid. Suck the exposed ends of the embedded pieces of material under water for about 10 days, then sections as thin as 10μ are readily cut. Stain with safranin and fast green or substitute Harris' hematoxylin for the green. Quadruple methods are also excellent.

Older stems, up to four years old of all species, that can be cut across with a sharp scalpel can also be embedded in paraffin and sectioned if sufficient pains are taken at all stages, especially with the mixture. However, most conifer stems become too hard, particularly from the

heat of the paraffin oven, to section easily. If sufficiently rigid, young stems may be cut freehand in a sliding microtome. It will generally be impossible to cut sections of even thickness across the entire piece of material, but small pieces in any event are just as serviceable and are apt to be thinner in some portions than in others. Too often the cambial region becomes impossibly crushed. The steam method for hardwood sections may be useful on most conifer woods, but when all is said and done, the celloidin method remains the most satisfactory of all.

Sections of live material cut freehand should be placed in formalin-aceto-alcohol for 24 hours before being treated further. Well seasoned wood of course does not need fixation. Useful stain combinations for both types include safranin and anilin blue or crystal violet or Harris' hematoxylin. The modern safranins stain so intensely in a short time that the long periods recommended by the older authors should be greatly abbreviated, else it will be difficult to differentiate the stain sharply. Special attention should be paid to the clear differentiation of the bars of Sanio and the bordered pits.

Live materials to be embedded in either paraffin or celloidin require fixation. The pieces should be trimmed down carefully in order that the radial longitudinal sections may be microtomed exactly parallel with the rays, and the tangential longitudinal sections precisely tangential to the rays. Formalin-aceto-alcohol is the most useful fixing fluid. Several days in the fluid is better than the usual 24 hours, and the material can be left in the fluid indefinitely if a little glycerin is added to prevent excessive evaporation. The stains mentioned in the preceding paragraph are equally applicable to sections of embedded material, but most workers apparently prefer safranin with Delafield's hematoxylin.

Whether freehand or embedded methods were employed, each slide should bear cross, radial longitudinal, and tangential longitudinal sections. The longitudinal sections should first be examined under the microscope in order to make certain that they are either radial or tangential, as the case may be, and not both radial and tangential.

The woods of all gymnosperms are easily macerated. An examination of such slides will provide an idea of the nature of wood very different from that given by sections. The acids of the macerating fluid sometimes render staining rather difficult if they are not very thoroughly washed out. A basic dye or a hematoxylin may be used, but it is of little avail to attempt double staining.

Leaf.—In the Coniferales the leaves are all simple; no compound leaves occur. Wide variations exist in the size and character of the leaves. Some species have very small scale-like appressed leaves, others have fairly large needle leaves (as in *Pinus coulteri*), and still others possess broad, thick leaves. The leaf of *Sciadopitys* consists of two

needles fused along the posterior margins. Most leaves are rigid and sharp-pointed. A few species have deciduous leaves, but in others the needles persist for two years, or in some species for as long as 14 years.

Leaves of all ages are easily sectioned in paraffin. The youngest leaves are to be found in cross sections of the stem apex, and these are very desirable for comparison with mature leaves. The stem apices are rather soft; after being embedded they need soaking under water for only a day or two. Microtome at 13μ, and stain with safranin and aniline blue or by a quadruple combination. In *Pinus* there are both primary and secondary leaves. The latter are the needle-like leaves and are borne in the axils of the scale-like primary leaves. At the base the leaves are surrounded by a membranous sheath, which is deciduous in those species whose needles have one vascular bundle and is persistent in those with two bundles. The contents of the mesophyll cells in the leaves of *Pinus* and in a few other genera are generally shrunken and tend to overstain.

Species with rigid, appressed, scale-like leaves are difficult to section. The best that can be done is to cut off portions of the young branches, together with what leaves they bear, and, after embedding, to soak under water for several weeks or even months until they cut satisfactorily.

Aqueous killing fluids are quite unsatisfactory. Excellent fixation of all types has been obtained with formalin-propiono-alcohol.

Staminate Strobiles.—Throughout the order the staminate strobilus is a cone or is cone-like. The strobili are dominantly monosporangiate, but bisporangiate strobili have frequently been described. They should be regarded as teratological. Monoecism prevails, but some species and even genera are dioecious. For example, the Juniperineae are dioecious, with the exception of two monoecious species, *Agathis australis* and *Araucaria bidwilli*. So are the Taxaceae, with a few exceptions, and in the Podocarpaceae the principal genus, *Podocarpus*, is dioecious. If one is not familiar with any species of which material is being collected, it becomes necessary to determine whether the specimen is monoecious or dioecious. The staminate strobili generally appear before the pistillate strobili, but the remains of both can usually be found; hence determination of sexuality is easy.

The strobili vary considerably in size, the smallest being found in species of *Podocarpus* and the largest in *Araucaria*. In the Cupressineae the arrangement of the sporophylls is cyclic, in all other families it is spiral. The sporophylls also vary greatly in size, and the extremes are to be found in the same genus (see woodcuts). The sporangia, which generally number 2 but may be as many as 10, are borne on the lower side of the sporophyll.

The very youngest strobili are to be found at the apices of the stems, partially or completely covered by the enveloping scales. Stem apices intended for growth studies and for the origin of the leaves will occasionally show the origin of the staminate strobili if they happen to have been collected at a favorable time. The time at which the strobili originate naturally depends upon the species. In some species, such as old cultivated specimens of *Sequoia sempervirens*, strobili in various stages of development can be found the year round. In most species of *Pinus*, in the central California region at least, the strobili have pushed out of the scales in early September and growth begins in earnest in December. Meiosis usually occurs in early March but is governed to a considerable extent by the severity of the winter months. The strobili in *Cupressus*, in both the native species and in those commonly planted in the region just mentioned, appear much later, and develop more rapidly; the mature microspores are shed from late spring to midsummer. Many forms transplanted from the Southern to the Northern Hemisphere appear to have difficulty becoming readjusted to the reversal in the seasons and produce strobili at the "wrong" time. Altogether, one must keep a close watch on the specimens, provided they are known to be sufficiently mature to produce strobili, and to make collections at appropriate periods in order to obtain a series of developmental stages.

For fixation of the early stages, formalin-aceto- (or propiono-) alcohol has been found quite satisfactory; a triple combination should give good differentiation. Sections should be about 10μ thick. Longitudinal sections are more useful than transverse ones.

Microsporogenesis.—To determine the stage of development in the young strobili, dissect part of a microsporangium from the base of the strobilus in a drop of iron-acetocarmin, and examine under the microscope. If it shows division figures, the whole strobilus may be fixed, whether cut into portions or not. If pollen tetrads are observed, then examine a sporangium from near the apex; if microsporocytes are found, the divisions are somewhere in between. In most species meiosis commences at the bottom of the strobilus and progresses toward the top, but in some species of *Pinus* with small strobili the divisions are nearly simultaneous throughout. It appears that the longer the strobilus, the wider is the variety of stages, ranging from microsporocytes to pollen tetrads. The first division in the microspores, however, seems to occur very unequally, even in the same microsporangium.

Strobili up to fully mature stages are easily embedded and sectioned, but in large specimens equally good fixation cannot be obtained throughout. Penetration of the fluid will be facilitated if slabs are removed from opposite sides. In order to secure the optimum fixation for cytological purposes, the strobilus should be bisected and the individual sporangia

removed by cutting through the stalks with a tiny scalpel. For entire strobili, fix in formalin-propiono-alcohol; for cytological purposes first dip into Carnoy's fluid for a few minutes then transfer to Navashin's fluid. Longitudinal sections of the strobilus are the most useful, but transverse sections should be made for comparison. For the earliest stages 10μ is thin enough; for the later stages 15μ is satisfactory, but for species with pendulous cells 10μ is better; for meiosis and divisions in the microspores, the sections may need to be somewhat thicker. Safranin and fast green are good for the earlier stages, but great care must be taken with the differentiation of the safranin. For the middle developmental stages a triple combination will probably be most useful. The chromosomes in the mitoses in the microspores stain beautifully with iron hematoxylin.

The microspores of many species can be smeared exactly like similar cells of the Angiosperms. After the microspores begin to round up, they become too dry to adhere to the slides and must therefore be treated as if for whole mounts. It is not at all difficult to secure pollen at the shedding stage, if the trees are frequently examined. A wide paper funnel may be fitted over the mouth of a bottle, and the pollen shaken into the paper cone. Several times the amount of pollen that might actually be needed should be collected, to make allowance for losses during staining and dehydration. The pollen will be more or less shriveled, but turgidity is quickly restored if the pollen is placed in water for a few minutes. Fixation may be in either formalin-propiono-alcohol or Navashin's fluid. The material may be either embedded and sectioned or treated for whole mounts. In the latter case, the contents are usually too dense for the nuclei and chromosomes to be stained by the usual methods. Feulgen's reaction, when properly carried out, gives superb staining. Dehydrate by going directly into [illegible] gradually after the staining is completed, thence into highly diluted balsam. To embed the pollen, resort to a slow-speed centrifuge will probably be necessary during the dehydration. Both dehydration and infiltration may be carried out rapidly. Embed by pouring into a small paper tray or embedding dish, which should not be over 3 mm. square, in order to concentrate the material. [illegible] satisfactory methods are either to [illegible] on a [illegible] of cold glass, or to solidify in a small vial, breaking [illegible] and removing the block after the paraffin has hardened. [illegible] when the pollen grain of the Coniferales is [illegible] according to either the genus or [illegible] included in Pinus, Cupressus, and in [illegible] when this is the case, pre- [illegible] In these genera, the two nuclei [illegible] the microspore and produce the

generative cell and the tube cell. In the other species the prothallial cells and generative cell are developed before the microspores are shed. The prothallial cells are recognizable with difficulty in some genera (*Pinus*) but are quite conspicuous in others (*Podocarpus*). In most of the Taxodiaceae and Cupressaceae and in all the Taxaceae prothallial cells are absent. These cells are presumed to be vestiges of the tissues which originally bore the antheridia.

In all the Coniferales the generative cell divides to form two cells; in the Araucariaceae one of these cells aborts, and the other develops gametes. In the other groups one cell assumes a stalk position, with the other and larger cell attached at the other end. The nature or function of the stalk cell is unknown, but in *Microcachrys* (Downie 1928) it has the function of a spermatogenous cell, dividing repeatedly, each time producing a body cell which in turn forms two gametes.

Internal organization in the microspore follows a definite plan. Differentiation of base and apex is the exact opposite of that occurring in *Ginkgo* and the cycads. In the latter the prothallial end of the pollen tube grows down into the nucellus, carrying the whole pollen grain with it. In the Coniferales the entire pollen grain and prothallial end of the pollen tube remain where the former lodged on the nucellus; the tube grows down into the nucellus, carrying at first only the body cell, then the two gametes produced by this cell.

The microspore, in most species, grows straight down from the tip of the nucellus to the archegonia. In *Sequoia*, *Pseudotsuga*, and *Larix* the microspore lodges laterally and thus traverses the nucellus obliquely. In the Araucariaceae, the microspore comes to rest at various locations—on the ovuliferous scale, or in its axil, or on the ligule.

Longitudinal sections of the entire young ovulate strobili almost always show various stages of pollination and the germination of the microspore. If a series of developmental stages of the ovulate strobili are examined, all the different steps may be reconstructed. If the species is a dioecious one, pollination and fertilization obviously will not be found in ovulate strobili if staminate trees are not present in the immediate neighborhood.

Growth of the microgametophyte, generally called "the interval between pollination and fertilization," occurs over a highly variable period, and may even be sporadic or with a resting interval, but commonly requires months to a year or longer. The time of pollination is a highly variable factor, but the duration of microgametophytic growth is rather constant for the species. In other words, if the time of pollination is on record from previously observed instances, the period required for fertilization to be effected can be computed with a high degree of accuracy from the following table, citing instances recorded in the literature.

Pinaceae: *Abies balsaminea*, 4–5 weeks; *Cedrus deodara*, 8 months; *Pinus*, 13 months in most species; *Pseudotsuga*, 2 months; *Tsuga canadensis*, 6 weeks; *Picea excelsa*, 1 month.

Araucariaceae: *Araucaria braziliana*, 6 months; *Agathis australis*, 1 year.

Sciadopitaceae: *Sciadopitys*, 14 months.

Taxodiaceae: *Sequoia sempervirens*, 6 months; *Taxodium distichum*, 3 months; *Cryptomeria japonica*, 3 months; *Cunninghamia*, 3 months.

Cupressaceae: *Libocedrus decurrens*, 2 months; *Juniperus communis*, 12½ months; *J. virginiana*, 7 weeks; *Actinostrobus pyramidalis*, 3 months.

Podocarpaceae: *Dacrydium intermedium*, about 3 months.

Taxaceae: *Taxus baccata*, 1 month in some localities to twice as long in others; *Torreya taxifolia*, 5½ months.

Since the species most likely to be available in the United States are included in the above list, it serves as a fairly reliable guide in making collections. It should be borne in mind that in those species in which a long period elapses between pollination and fertilization there is generally a rapid initial development of the microgametophyte. The latter grows down as far as the free part of the nucellus and there remains until growth recommences.

As has already been noted above, stages in pollination and growth of the microgametophyte are to be found in sections of the ovulate strobili. The same technical methods required by the latter are generally equally satisfactory for stages in the development of the sperms, but somewhat more care is required with the staining. It would be better if the paraffin sections were examined under the microscope immediately after mounting and before the water has entirely evaporated under the sections (after drying it is too difficult to observe structures clearly), and those that appear to contain the desired stages may be picked out and stained particularly for microgamete formation. Safranin is usually good enough, but if a critical stain is desired, iron hematoxylin may be applied provided the sections are not over 12μ thick. A triple combination may sometimes come out sharply enough.

Microgametes.—Microgametes of the Coniferales are of two types: naked nuclei or highly organized cells. The latter type is presumed to be the most primitive form since it resembles the sperms of the cycads and *Ginkgo*, but differs in that blepharoplasts which produce cilia are lacking. These well-developed sperms occur in the Cupressaceae, Podocarpaceae, Taxaceae, and Taxodiaceae. They are produced in pairs and are equal in size in most of the genera but are greatly unequal in *Cephalotaxus*, *Taxus*, and *Torreya*. In *Cupressus arizonica* (Doak 1932) and *C. goveniana* several small sperms are present. The sperms are naked nuclei in the Pinaceae.

Obtaining slides showing the microgametophyte with the microgametes is almost wholly a matter of chance. Material collected at about the time that fertilization is presumably to be effected is most likely to show these structures. A large number of ovulate strobili will have to be sectioned, and the apex of every ovule should be carefully scrutinized. Any satisfactory slides that might be found are of great intrinsic value.

Ovulate Strobilus.—The point and time of origin of ovulate strobili are quite unpredictable in most species. Getting the very earliest stages is a complete matter of chance since it is impossible to distinguish between purely vegetative buds and ovulate buds. The best that can be done is to preserve a large quantity of suspected buds, to embed and section them, and to examine the sections under the microscope before staining them. If present, the ovulate strobili are easily detected, and the sections containing them can then be stained. The ovulate strobili, in the bisporangiate forms, appear to originate slightly later than do the staminate strobili. After the ovulate strobilus has broken through the enveloping bud, it is, of course, easily recognized.

The ovulate strobilus in the Coniferales is a compound structure, in the sense that the ovules are not carried directly upon the axis of the strobilus, as are the microsporangia. Save for the Cupressaceae and in a few isolated genera, the bracts with their associated structures are spirally arranged on the axis. The strobili vary enormously in size, ranging from the small berry-like cones of *Juniperus communis*, 6 to 8 mm. in diameter, to the huge woody cones of *Pinus coulteri*, which average 40 cm. in length. They are also very variable structurally, but at maturity most of them are hard, woody, and difficult to cut open.

The ovulate strobilus of all genera is readily sectioned in paraffin or celloidin until the ovules are at the late free-nucleate stage. Remove slabs from opposite sides, fix with formalin-aceto-alcohol, dehydrate slowly with tertiary butyl alcohol, embed in a hard paraffin (or in celloidin), and soak exposed portions under water for about two weeks, whereupon longitudinal sections as thin as 10μ should cut smoothly. The tips of the scales will give the most trouble. If one will go to the trouble of trimming off all projecting scale tips, especially from most species of *Pinus*, a lot of exasperation will later be avoided. Transverse sections can be cut just as easily but are of little service other than for general structure of the strobilus. Stain carefully with safranin and anilin blue or a triple or quadruple combination.

Megagametogenesis.—The initial cell of the megagametophyte is the megasporocyte. It arises soon after the origin of the ovule by the periclinal division of a hypodermal archesporial cell into a tapetal cell and the megasporocyte.

In the majority of species the megasporocyte is a single cell, but in some species, such as *Pinus muricata* and *P. contorta*, groups of megasporocytes are regularly formed. Such groups are also present in *Taxus*, where several megaspores may germinate and reach advanced developmental stages, but a similar condition is not known in the species of *Pinus* just mentioned.

It is not at all easy, on the whole, to obtain slides showing the megasporocyte, and it is extremely difficult to get the meiotic divisions whereby a linear quartet of four megaspores is formed. The writer has sectioned and examined several hundred ovulate strobili in many species of *Pinus* but in all that material he has never encountered either a reduction division or quartet, although the stages immediately preceding and succeeding were frequently observed. Scarcely anything has been described in the literature regarding formation of the quartet, but it is known to exist; it is also known that the megaspore nearest the axis of the strobilus becomes the functional one, the three others aborting. Cases are on record in which only three megaspores were apparently produced, the outer cell formed following the reduction division failing to undergo the second mitosis.

The functional megaspore enlarges and, in all the Coniferales, embarks on a period of free nuclear division. The extent of this period, and consequently of the number of nuclei produced, depends upon both the size and the shape of the swollen megaspore. In long and narrow cells the period is much shorter than in those that are nearly spherical. Low numbers of nuclei occur in *Taxus* (256) and high numbers in *Juniperus* and *Pinus* (2000). The free-nucleate stage is comparatively easy to obtain in slides, but careful fixation is required. If entire strobili are fixed for this stage, there will be a little plasmolysis, the term here meaning that the thin layer of protoplasm with its nuclei is wrenched away somewhat from the periphery of the megaspore. The ovules should be carefully cut away from the ovuliferous bract and fixed separately in a strong chrom-acetic fluid. Since the tissues dry out rapidly, one should work fast, and material not being worked upon should be kept between damp cloths. A great deal of the unpleasantness involved in working with pitchy cones can be avoided if leather gloves are worn. After imbedding, section the ovules perpendicular to their flat sides; if cut parallel to the flat sides, only oblique sections are produced.

[illegible] commences at the apex of the megagametophyte [illegible] and when more or less completed, the [illegible] is directly related to the type of the [illegible] are separated by successive [illegible] in contact with one another. In the first

group the number of archegonia is small, and they are arranged more or less in a circle around the center. *Pinus* is of this type, the number of archegonia ranging from two to not more than six. In the species (common in Taxodiaceae and Cupressaceae) with an archegonial complex,

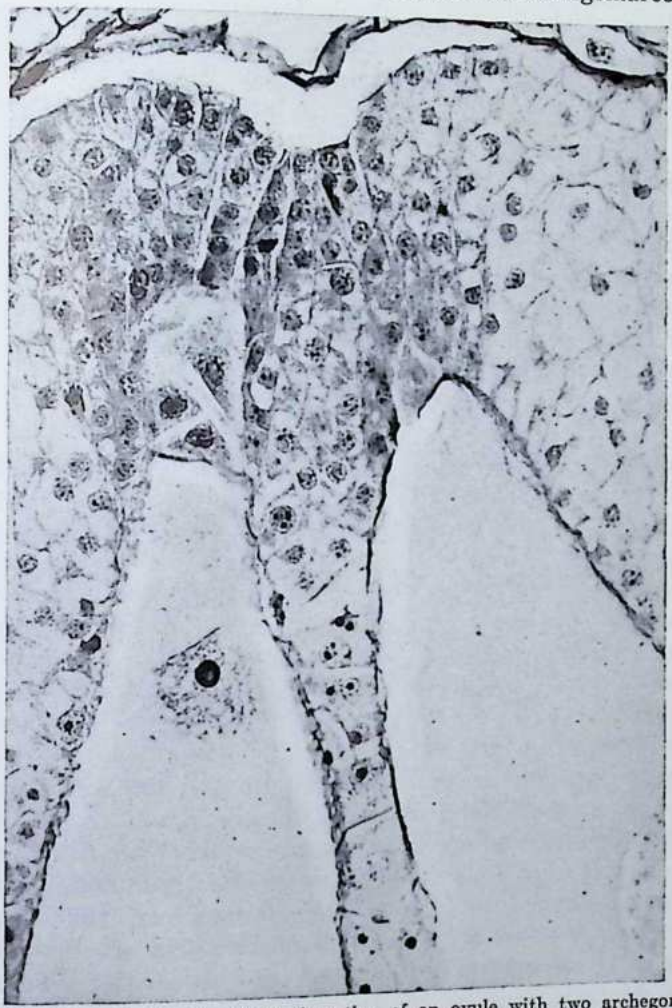


FIG. 93.—*Pinus laricio:* longitudinal section of an ovule with two archegonia; one at left with neck cells, ventral canal cell and egg nucleus. Fixed with strong chrom-acetic; stained with safranin and anilin blue.

the number of archegonia ranges from 6 to 200. *Sequoia sempervirens* has both types of archegonial plexus, while in *Torreya taxifolia* only a single archegonium usually occurs. The archegonia in their youngest stages are extremely difficult to find in most species, and the sectioning and staining of considerable material are generally required. Among the

pines. *P. laricio* is the most favored species. *Libocedrus decurrens*, *Thuja*, and *Juniperus* are good, but the genus with the largest number of archegonia (*Widdringtonia*) neither occurs nor is cultivated in the United States.

Division of the nucleus of the archegonial initial occurs about a week after the cell is first recognizable. This results in the ventral cell and the primary neck cell. The latter soon divides, but the ventral cell requires about a month in which to become very greatly enlarged, and in which then divides to form a ventral canal nucleus or cell and the egg nucleus (Fig. 3B). This division evidently proceeds rather slowly since it is commonly found in slides. Beginning with this stage, it will be found advisable to cut sections somewhat thicker than before; 10μ is about right. Material should have been collected daily as soon as the archegonia are known to have begun growth, since development is so rapid that important stages would be missed in case a critical study of the life history was being undertaken. As all the ovules in a given strobilus develop nearly simultaneously, the ovules from any one strobilus should be kept separate.

Fertilization.—The term "fertilization" is here taken in its broad sense, to include all phases from the arrival of the microgametophyte immediately above the megagametophyte, up to and including the first zygotic mitosis. The actual union of the male and female nuclei, to which the term has by some been restricted, is more properly called "syngamy." The latter term, nevertheless, has in turn a fairly broad meaning since it includes both cases of actual nuclear fusion (as more commonly occurs in Angiosperms) and those in which there is no fusion of chromatin.

The probable occurrence of fertilization may be determined by the external appearance of the ovulate strobili (Burkholz 1938): this stage occurs at about the time, or just shortly before, the strobili have reached their maximum size, following the period of rapid enlargement. It has been claimed that this general size rule is infallible, with the exception of *Taxus*, in which fertilization occurs long before the ovule is fully grown, and certain of the Podocarpaceae, in which fertilization takes place before the ovules themselves have attained their full size.

The microgametophyte reaches the megagametophyte while the latter is in various stages of development, which differ according to the species. In *Torreya*, for example, the megagametophyte is still in the early free-nucleate condition, while in *Pinus*, at the other extreme, the archegonium has almost reached maturity. There is a direct relation between the stage of megagametophytic development and the nature of the microgametes: where the latter are highly organized cells, the earlier the stage of development.

Fertilization phenomena differ according to the family:

Pinaceae: At the tip of the microgametophyte a pore is formed by rupturing, and the contents are ejected with considerable force. Sperms, stalk, and tube nuclei are all discharged into the egg.

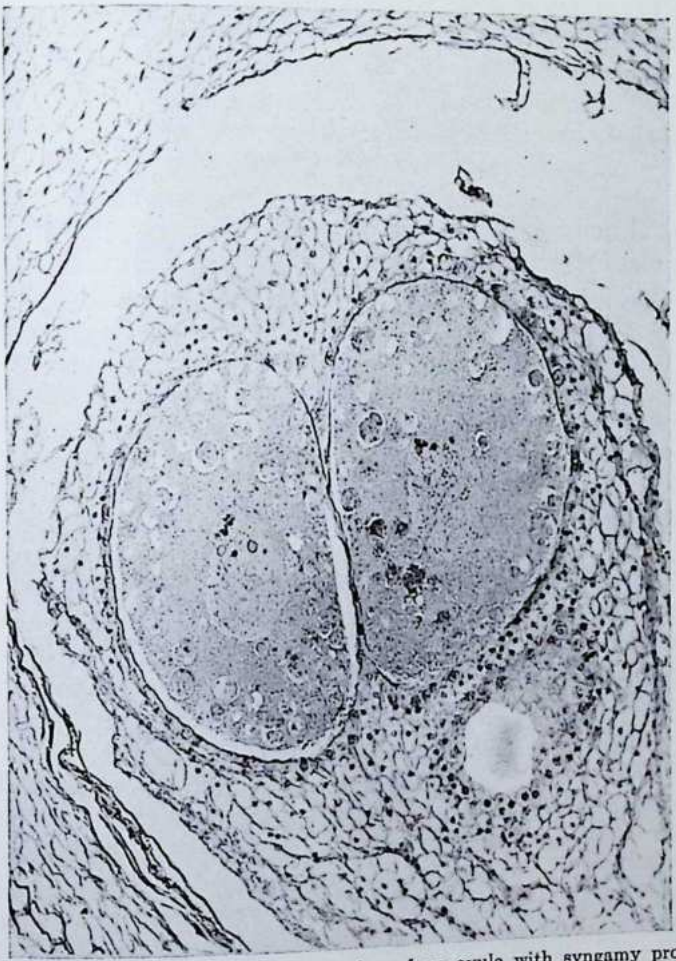


FIG. 94.—*Pinus laricio:* longitudinal section of an ovule with syngamy progressing simultaneously in two adjoining archegonia. Fixed with strong chrom-acetic, stained with safranin and anilin blue.

Sciadopitaceae: In *Sciadopitys* (with scattered archegonia), both sperms enter.

Taxodiaceae: In all genera (with archegonial complexes) only one sperm enters.

Cupressaceae: In *Juniperus* only one sperm enters, but the second may follow it.

Araucariaceae: Both sperms and some of the smaller nuclei (stalk, tube, and prothallial, which all look much alike at this time) enter the egg.

Podocarpaceae: Although both sperms and stalk and tube nuclei enter, all save one sperm nucleus remain on top of the egg and disorganize.

Taxaceae: In *Taxus baccata* only one sperm enters, but in *T. canadensis*, sperms and stalk and tube nuclei all enter. *Torreya taxifolia* resembles *T. baccata* in behavior.

Cephalotaxaceae: In *Cephalotaxus* both sperms enter, but one remains at the top and there degenerates.

The interpretation of structures within the megagametophyte during fertilization and syngamy is fraught with great danger. It is extremely easy to make misinterpretations, especially as there are so many bodies having the appearance of nuclei. If in doubt as to the identity of any structure, the coverslip may be removed from the slide, the sections bleached free of stains, and Fuelgen's reaction may be applied. This will generally give a specific reaction to the nuclei and chromosomes directly concerned with fertilization and particularly with syngamy.

Accounts of syngamy are meager (Beal 1934, Hutchinson 1915). Various stages in fertilization and syngamy may be found by chance. Species of *Pinus* appear to be most suitable, since fixation and staining are not at all difficult (Fig. 94). If a stage close to syngamy is found in any ovule, the other ovules from the same strobilus are very likely to yield earlier or later stages. Longitudinal sections of the ovule should be cut at about 14μ; safranin will give a clear chromosome stain and may be followed with a counterstain of anilin blue. Since the spindles are very prominent, especially during the postsyngamic phases, particular attention should be paid to clear staining of these structures.

Embryogenesis.—In all Coniferales, with the exception of *Sequoia sempervirens*, free nuclei are formed immediately after the division of the syngamic nucleus. In *Sequoia* walls are formed after the first and subsequent mitoses. The free nuclei are produced in the center of the archegonium; they then migrate to the bottom of the archegonium, and wall formation commences at the conclusion of the last mitosis in such a manner that there are two or more tiers of cells with an average of four cells in each tier. The next mitosis occurs in the cells of the upper tier, followed by nearly simultaneous divisions in the lowermost tier. Depending upon the species, the lowest or the next to the lowest tier of cells goes into the formation of the embryonal organs—root, stem, cotyledons, and leaves.

The number of free nuclei and also the number of tiers of cells is somewhat variable:

Pinaceae: Usually 4 free nuclei and 4 tiers.

Araucariaceae: 32 free nuclei in *Araucaria braziliana*, 32 and 64 in *Agathis australis;* 3 tiers.

Podocarpaceae: *Phyllocladus* has 8 free nuclei, *Podocarpus* 16.

Cephalotaxaceae: *Cephalotaxus* has 8 free nuclei and 3 tiers.

Taxaceae: *Austrotaxus* has 8 free nuclei, *Taxus* has 16 and occasionally 32, but *Torreya* has only 4.

Cupressaceae: Usually 8 free nuclei and 3 tiers.

Taxodiaceae: Usually 8 free nuclei and 3 tiers.

These early stages are readily found if the material has been collected at the opportune time. The ovules at this stage are broader in one direction than in the opposite plane; they should be microtomed at 12μ parallel to the broader surface. Stain critically with safranin and anilin blue. Mitotic figures are frequent. After the tiers of cells are developed, it is very common to find more or less plasmolysis, which is probably due more to partial desiccation of the tissues than to bad fixation. Some technicians claim that this difficulty can be remedied by soaking either the cones or the ovules in water before fixing, but from the writer's experience the remedy seems to be worse than the disease.

The most significant stages during embryogenesis are those occurring after the organization of the proembryo and preceding the differentiation of the embryo into the various tissues and organs. Sections of entire gametophytes were formerly used for the study of these stages, but such sections are inadequate in that they never present complete pictures and many details are so obscured that their presence can hardly be detected. It is, for example, impossible to make out much of the phenomenon of cleavage polyembryony, or to differentiate between primary and secondary suspensors. For these stages whole mounts of the dissected suspensor-embryo system are required. It takes but a little practice to prepare relatively satisfactory mounts which are not only intrinsically valuable in themselves but are very useful for purposes of instruction.

Living material is always preferable to preserved or fixed gametophytes (Buchholz 1929, 1938); the writer has had extremely poor success with fixed material. The dissections, in the case of live material, should be made under water or preferably under a 15% sucrose solution, but equally good results may be obtained if the dissection is done under formalin-propiono-alcohol or under 6% formalin in 50% alcohol. The beginner will find the chances of the cells rupturing to be less in a fixative than in plain water. The ovules should be removed from the strobili; if a number are being removed at one time, they should be placed between damp cloths. In dissecting large, pitchy cones, such as those of *Pinus*, it will be less messy to wear a pair of stout leather gloves and to remove 50 or so ovules at one time. The hardened seed coats and membranes

are next removed, and the gametophytes are placed in a watch glass or either half of a small Petri dish containing the sugar solution or fixative. The container is then placed on the stage of a binocular dissection microscope; both reflected and transmitted illumination are ordinarily necessary for clear observation.

Pinus, since material of this genus is most easily collected and the gametophytes are large and thus more easily manipulated, will be taken as an example in the following discussion of the technique of removing

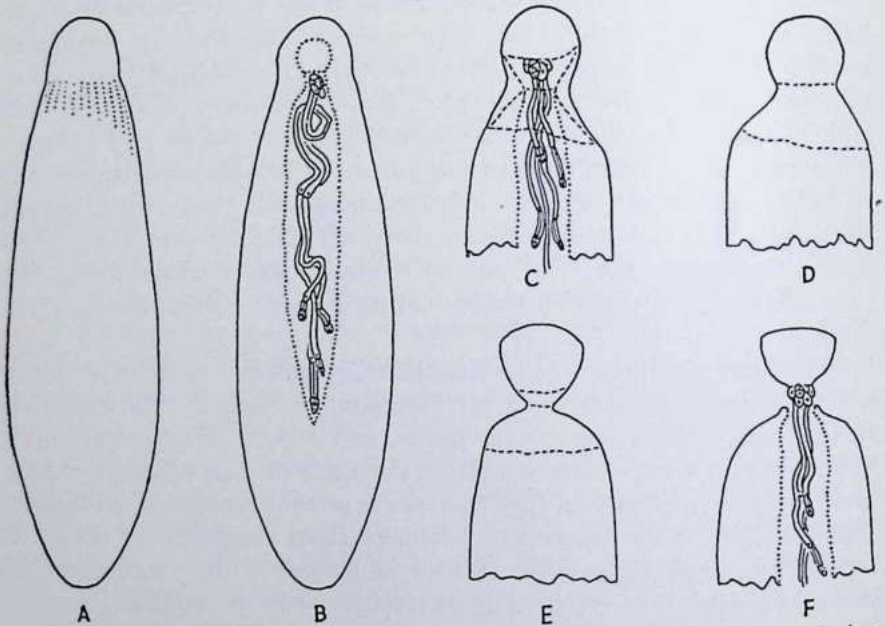


FIG. 95.—Dissection of *Pinus* gametophyte (based on *P. ponderosa*): *A*, external view before operation is begun; *B*, optical section showing extent of corrosion cavity—note that it is narrowest at the neck; *C*, optical view showing how deep the preliminary cuts should extend; *D*, the operation nearly completed; *E*, cuts extended almost to corrosion cavity and operation completed; *F*, remaining tissue broken and suspensors partially withdrawn. (*Drawing by Mrs. Carl F. Janish.*)

the suspensors and embryos. In Fig. 95*A* is shown the gametophyte removed from the testa and ready for operation; Fig. 95*B* is a median longitudinal section through the gametophyte, designed to show the average extent of the corrosion cavity occupied by the suspensors and embryos at the time; Fig. 95*C* is a combination of the two in optical section and shows (by dotted lines) the points at which cuts are to be made or portions of gametophytic tissue are to be removed. The anterior end of the gametophyte appears more or less like a cap; consequently the position of the transverse incision extending completely around the gametophyte is easily determined. For making the incisions a very small cutting instrument with narrow, sharp blade is required. Some

technicians use a needle flattened and sharpened to give a spear-shaped end; others use a scalpel with a spear-pointed end, of which only one edge is sharpened. Hold the posterior half of the gametophyte, as gently as possible, with weak-springed forceps, then, with the gameto-

FIG. 96.—*Pinus ponderosa:* dissected whole mount of proembryos and suspensors. Although the preparation is brilliantly stained, this object does not photograph well. Fixed with formalin-propiono-alcohol; stained with Harris' hematoxylin and fast green; dehydrated with hygrobutol and infiltrated with balsam.

phyte submerged in the fluid and the operation carried out under the microscope, cut away wedges of tissue at the anterior end, completely around the gametophyte—in somewhat the same way that one cuts a log in two with an axe. The nearly completed operation is shown in Fig. 95*D*. Complete the lateral incision with great caution, lest the suspensors be cut into, by making a short, straight cut into the corrosion

cavity, as shown in Fig. 95*E*. Grasp the anterior cap with another pair of weak-springed forceps, and pull gently by means of successive jerks. The suspensor-embryo complex usually comes out in a few moments (Fig. 95*F*). During later stages of embryo growth, some of the embryos may become wedged into the gametophyte at various points and may readily become broken off. If the suspensors pull straight without the embryo becoming dislodged, make a longitudinal incision down the center of the gametophyte. A succession of short, jerky pulls is generally more effective than a steady pull, and embryos are less likely to become broken off. If the embryo complex still does not come out without breaking off, wedges of gametophytic tissue may be cut away from all around until the complex has been sufficiently loosened, but this should be done only as a last resort since there is danger of cutting off embryos. If the embryos were dissected in sugar solution, they should be transferred immediately to a fixing fluid, in which they may remain indefinitely. The embryos should be washed with water before being stained. As the basic stain, Harris' hematoxylin diluted one-half with 50% alcohol or safranin is equally satisfactory; for counterstaining, orange G may be used after the hematoxylin, and fast green after the safranin. The older technicians mounted the embryos in Venetian Turpentine, a slow and erratic procedure; they later dehydrated through 25 and 50% ethyl alcohol and then mounted in diaphane. By far the better method, however, is to employ the gradual hygrobutol method (Fig. 96).

For paraffin sections of the same stages, remove the gametophytes as before, then cut off slabs from opposite sides, taking care not to expose the corrosion cavity. Place in either formalin-aceto-alcohol or a medium chrom-acetic fluid. Dehydrate slowly, and infiltrate with paraffin over a period of several days. Microtome parallel to one of the cut surfaces at 10 or 11μ. The ribbons are likely to give some trouble, as they have a tendency to cling to the knife and other objects. If this happens, only the most careful handling will help matters. Serial sections are more useful than the usual two or three to a slide. Staining is precise with safranin and fast green or a triple combination, but iron hematoxylin and orange G may be used if desired.

In the upper portion of the corrosion cavity of *Pinus* and some other genera there will usually be found a number of rosette embryos. These, as well as all save one of the primary embryos, become aborted.

When several archegonia are present and an embryo arises from each whose egg is fertilized, simple polyembryony is the result. When each fertilized archegonium gives rise to several embryos, the phenomenon known as "cleavage polyembryony" occurs. The embryos resulting by cleavage from a single zygote constitute an embryo system; when several embryo systems are confusedly intermingled, the entire

mass is known as an "embryo complex." Among all these embryos, the one possessing the greatest growth vigor is the one that develops to maturity; it can frequently be recognized during early stages by its straighter, stouter, and more rigid suspensor. The salient features of the embryology in the different families, as far as known, may be summarized (Buchholz 1929) as follows:

Pinaceae: Indeterminate cleavage polyembryony in *Pinus, Cedrus, Tsuga;* each rosette cell may put out a suspensor with a multicellular embryo (*Cedrus, Pinus*), or the rosette cells abort early (*Tsuga*). *Abies, Pseudotsuga, Picea,* and *Larix* form only one embryo from each archegonium. Rosette cells are present, soon collapsing; early growth apical. *Abies* undergoes cleavage in a few cases. No rosette cells in *Pseudotsuga.*

Araucariaceae: Highly specialized; probably a group of polyembryonic initial cells rather than a single embryo, *i.e.*, a compound embryo.

Sciadopitaceae: Cleavage polyembryony. Rosette cells none, few or many. Apical cell present.

Taxodiaceae: Cleavage polyembryony in *Taxodium* and *Cryptomeria.* Rosette tier probably absent; growth apical.

Cupressaceae: Cleavage polyembryony; rosette embryos probably present (*Juniperus, Biota, Libocedrus*). Determinate cleavage polyembryony in *Dacrydium.* Primary and secondary embryos resulting from cleavage polyembryony in *Chamaecyparis.* Single embryo in *Thuja;* growth distinctly apical; apparently no rosette cells.

Podocarpaceae: Lowest proembryonic cells binucleate in all species investigated; several types of embryonic development; cleavage polyembryony in some species; rosette cells present or absent; sometimes only one embryo from each archegonium; apical growth occasional.

Cephalotaxaceae: Considerable similarity to conditions in preceding family, but proembryonic cells are uninucleate. Rosette embryos present. Distinctive deciduous cap cells present.

Taxaceae: Simple polyembryony; rosette cells rare, absent in *Torreya;* growth apical; tendency toward cleavage.

For the later stages in the development of the single embryo, gametophytes may be treated as for the earlier stages up until such time as the embryo fills most of the corrosion cavity. After this period it is better to dissect out each embryo and to fix it separately. For mature embryos of *Pinus* and certain other genera, seeds (which may be purchased from a seedsman) may be soaked in water for two or three days to restore turgor, then the embryos may be dissected out. Fix with formalin-aceto-alcohol, section at not over 12μ, and stain with either safranin and fast green or a triple combination.

The order of appearance of the various regions in the embryo is as follows: plerome tip of root, calyptoperiblem, stem tip, cotyledons and

hypocotyl, epidermis, procambium, resin passages, leaf primordia, protoxylem elements in the cotyledons.

Gnetales

The order contains three genera, *Ephedra*, *Welwitschia*, and *Gnetum*, generally combined into the one family, Gnetaceae. The two last-named genera do not occur naturally in the United States, but species of *Gnetum* may rarely be found in cultivation in botanical gardens. *Ephedra*, on the other hand, is one of the characteristic plants of the Southwest, and it is widely distributed elsewhere.

It is so doubtful whether material of *Gnetum* and *Welwitschia* would be available that no further description of these genera will be presented. In any event, the various structures will require similar treatment to that described below for *Ephedra*.

Ephedra occurs abundantly in certain parts of Arizona, New Mexico, and California; in the last state it reaches as far north as the Panoche Pass, west of Fresno. The plants are so characteristic that once some have been seen, the genus can always be readily recognized thereafter. They form straggling, rough, xerophytic shrubs rarely over 2 meters in height. In color they are grayish most of the year, becoming a bright green in the younger portions in late winter and early spring, but some Asiatic species when brought into cultivation become mesophytic and remain a vivid green. Anatomical material can usually be obtained from the botanical supply concerns, but care should be taken to specify that it is for slide-making purposes, since "preserved material" is worthless.

Contrary to the statements in some texts (*e.g.*, Chamberlain 1932), *Ephedra* is easy to cultivate, except where it might be snowed under for long periods. The Asiatic species are more amenable than the North American species; *E. distachya* and *E. alata* have flourished and produced great quantities of staminate and pistillate strobili in central California. Transplanted plants of the Californian species can be obtained from nurserymen specializing in desert plants in that state.

Root.—A persistent tap root is present, but the lateral roots furnish more satisfactory material for slides. Root hairs are easily obtained from young cultivated plants but are hard to find on plants growing in nature. In the root tip there is little differentiation into growth regions; consequently slides of this part of the root are not of so much usefulness as are sections of older regions.

Younger roots may be fixed in formalin-propiono-alcohol and microtome easily. Both transverse and longitudinal sections should be made; safranin and anilin blue is a satisfactory stain combination, but others may be experimented with.

Stem.—Young stems, a few months old, section easily in paraffin and afford most instructive preparations (Fig. 97). They reveal clear evidence that photosynthetic activities are carried on within them. Longitudinal sections of the first two to four nodes will show the meristematic region at the base of each internode, also the development of the abscission layer. The beginning of the vascular system will be

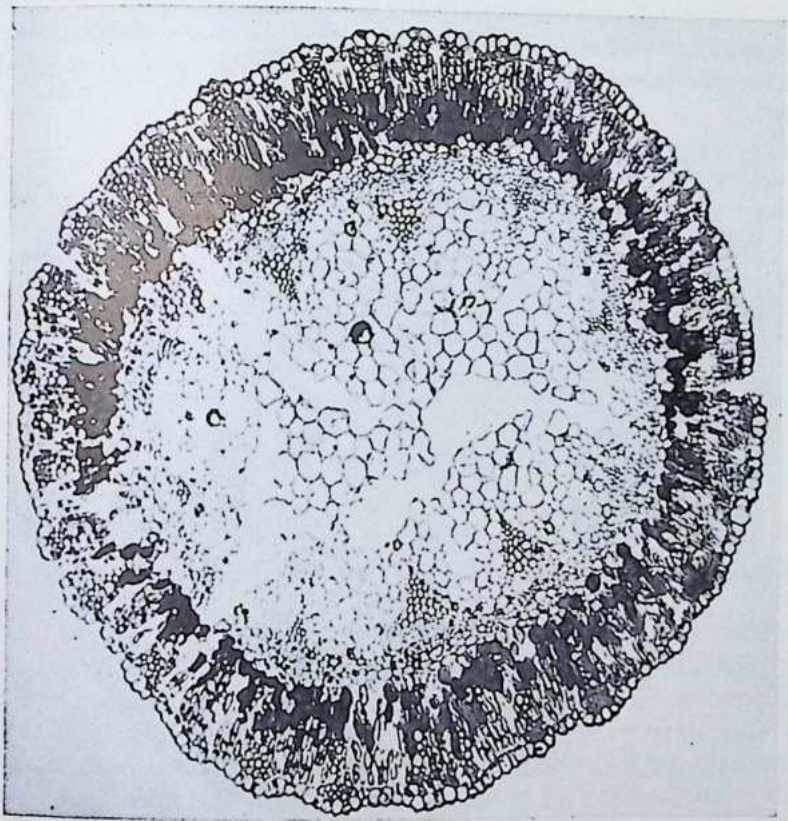


FIG. 97.—*Ephedra alata:* cross section of a young stem, showing assimilating tissue, dark tannin-containing cells and gaps in central region. Fixed with formalin-aceto-alcohol; stained with safranin and fast green.

encountered at about the fourth internode. Microtome young stems at not over 12μ, and stain with safranin and anilin blue (or fast green, with caution, may be used).

Older stems become hard and rigid, but those of many species can be sectioned in paraffin following prolonged softening under water of the embedded material. The tissues are so compact that sections thinner than 12μ are necessary. Very old wood is not required to show the characteristic structure elements; stems two to three years old show everything clearly. Older wood requires treatment with 50% hydro-

fluoric acid for two months, following which dehydration should extend over a period of about two weeks, and the time in the paraffin oven should take a week. Since the wood exhibits both angiosperm and gymnosperm characters, sectioning and staining should be carried out with this feature in mind.

Leaf.—Most species possess only small scale-like leaves, but *E. foliata* from India has distinct leaves 1 cm. long (Fig. 98). In *Gnetum* the leaves are broad and externally greatly resemble those of Angiosperms. The leaves of *Welwitschia* are the most characteristic feature of that genus.

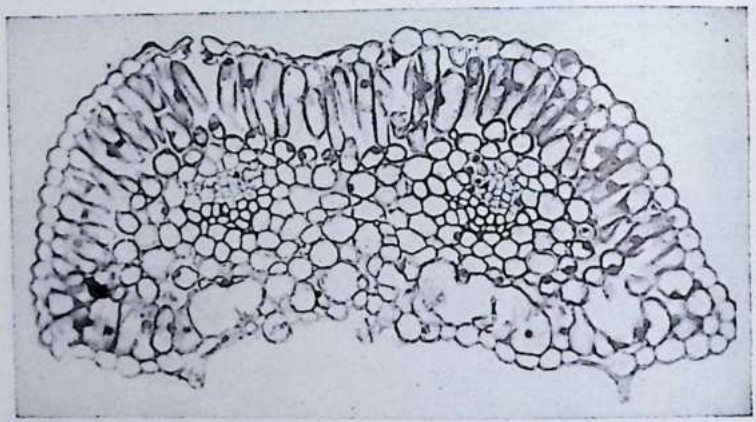


FIG. 98.—*Ephedra foliata:* cross section of leaf. Fixed with formalin-aceto-alcohol; stained with safranin and fast green.

Staminate Strobilus.—Exceptions may occur with cultivated specimens, but in most species the staminate strobili originate in December, the microsporocytes are to be found early in February, and meiosis occurs early in March (or a little later if the weather has been unusually cold). At the time of shedding in mid-April, the microgametophyte contains two prothallial cells, one of which is naked and the other is cut off by a wall, a stalk cell, a body cell, and tube nucleus (Land 1904).

The staminate strobilus at all stages is well fixed with formalin-aceto-alcohol, but for the meiotic figures the strobili should be dissected somewhat and fixed in a strong chrom-acetic fluid. Longitudinal sections at 10μ are most satisfactory. Staining may be in safranin with a suitable counterstain; iron hematoxylin may be used on thin sections for cytological details.

Germination of pollen grains may be observed by sowing them in 10% saccharose. In the ovules the grains come to rest at the bottom of the pollen chamber very close to the archegonia, into which the microgametophyte grows directly (Land 1907).

Ovulate Strobilus.—The ovulate strobilus originates at about the same time as the staminate strobilus but does not grow with equal rapidity. The two may be distinguished in that the staminate strobilus is shorter and broader. The integuments and archesporial cell appear about the first of March, and the reduction divisions occur within a few days. Free-nuclear divisions then take place in the functioning basal megaspore, covering a period of about 20 days. Wall formation next sets in, and the archegonial initials appear about Apr. 1. The average number of archegonia is two, but there may be three in some species (Maheshwari 1935).

Excellent fixation of all stages in the growth of the ovulate strobilus is given with formalin-propiono-alcohol. The bracts should always be removed, and from the older flowers the outer integument should be dissected away. The material at all times should be handled very carefully to avoid crushing. Sections should be microtomed rather thin, 10μ being the optimum thickness. Safranin and fast green may give good staining, but it appears that iron hematoxylin differentiated with picric acid is superior for nuclear details.

Fertilization.—Pollination occurs about Apr. 1, but the exact date is greatly dependent upon climatic conditions. Fertilization may occur within 10 hours after pollination; consequently it is a difficult matter to catch this stage.

Embryogenesis.—Eight free nuclei arise following fertilization; of these, from three to five develop into proembryos. Only one embryo attains maturity.

For the earlier stages in embryogenesis prepare the material as for older stages in archegonial development. For the later stages remove all superfluous tissue possible, and cut slabs from opposite sides of the gametophyte. The earlier stages may be sectioned at 12μ, but later stages should not be over 10μ. Safranin and fast green have given excellent staining of all stages.

CHAPTER XXXI

ANTHOPHYTA

To the technician there is little difference between the dicotyledons and monocotyledons; consequently the Angiosperms will be dealt with by structures rather than by the phylogenetic sequence which has been followed thus far. The dicotyledons, on the whole, are more difficult than the monocotyledons by reason of their greater structural complexities, but the technical methods nevertheless are essentially similar for the two groups. If materials intended for research purposes are disregarded, the problem is one of finding the plant whose root tips, leaves, or whatever the structure may be, are most suitable from a particular standpoint. It is possible in most cases to find equally satisfactory species from both groups.

ROOT TIPS

The manner of treatment of root tips depends entirely upon the purpose for which they may be wanted. The method to be followed if the somatic complement of chromosomes is to be counted is entirely different, for example, from the one to be used if it is merely the general structural details that are wanted.

Methods of obtaining tips, however, are the same, no matter what the ultimate purpose for which they are to be used. It is not a simple matter of going out into the garden and digging up plants, for, as a matter of fact, it is a very rare occasion indeed that any can be secured in this way. The tips should be specially grown according to the method most suitable for the plant concerned.

Securing Root Tips.—Bulbs which produce roots quickly, as of the *Allium cepa* and *Hyacinthus* type, may be germinated as follows: Bottles, such as milk or cream bottles, with mouths of a diameter just wide enough to hold the bulbs by their bases are the most convenient. Otherwise obtain some wide-mouthed jars of about 1-quart capacity. If a suitable place in total darkness is not available, paint the jars on the outside with black paint. Fill the jars to the top with a suitable nutrient solution (Knop's or Pfeiffer's); the solution must be thoroughly aerated, otherwise the roots will bend upward. For the wide-mouthed jars suitable holders may be provided by cutting stiff cardboard into squares wide enough to project about 2 cm. beyond the mouths, then cut circular holes from the centers of the cards of a size sufficient to allow the root ends of the bulbs

to project to a depth of about 2 cm. into the nutrient solution. To prevent the cards from rotting, dip them into hot paraffin until they are saturated, then remove, and cool. Insert the bulbs into the jars, then set the whole in a dark place at ordinary room temperature. The tips of *Allium cepa* are sufficiently long for removal in four days; those of *Hyacinthus* require about 10 days. The type of hyacinth used should be selected with care because most of the varieties on the market are either triploids or have irregular chromosome numbers. The diploid variety Yellow Hammer is best of all, with Gertrude as second choice. Other diploid varieties of *H. orientalis* include: Garibaldi, Cardinal Manning, Flora, King of the Yellows, and Roi des Belges. Excellent triploid ($3n$-24) varieties include: King of the Blues, Lady Derby, Grand Maitre, and Lord Balfour.

All other bulbous plants should be started in clay pots; they require a longer time than the onion and hyacinth to develop roots and do not produce so satisfactory tips in aqueous media as in soil. Moreover, the longer period required for development is prone to allow excessive growth of bacteria and aquatic molds which penetrate the tips and spoil them. Peat is sometimes used in the pots and is excellent for all bulbs and tubers whose roots are not covered with slime: *Tulipa, Erythronium, Hyacinthus, Crocus, Narcissus, Lilium, Gladiolus,* and tuberous species of *Anemone* and *Ranunculus* may be planted in peat. Shred the peat finely and pack tightly into 6-inch pots. In order to allow as much room as possible for the roots, insert the bulbs or tubers so that the crown is just below the surface. Allow a single large bulb (as of *Tulipa* or *Lilium*) or two or three smaller bulbs (*Crocus* or *Anemone*) to each pot. Finely sifted garden loam, free from sand, may also be used. Place a piece of broken pottery over the hole in the bottom of the pot, and insert the bulbs with the crown barely emerging from the surface of the soil. Soak the pots thoroughly with water, and set aside in a cool, dark place for at least two weeks. Some bulbs will require about a month before the root tips are large enough for removal. Take care not to let the pots become dry. To remove the roots, knock the entire mass out, wash quickly but thoroughly under running water to remove all soil and other debris, then cut off the tips with a scalpel, and place in the killing fluid with the least possible lapse of time.

From annual species which grow very rapidly in the seedling stage, root tips may be secured by germinating the seed in fine soil in any suitable container; as soon as the first leaves are well developed, remove the seedlings from the soil, insert individually in paraffined cardboard squares, using nonabsorbent cotton to hold the stem in position, and place the cards over the mouths of jars filled with nutrient solution. Great quantities of fine tips should be available in about a week's time.

If it is desired to grow the plants to maturity, they may then be replanted in clay pots and later transferred to the open garden.

Seeds of perennials and slow-growing annuals should be planted in suitable seedbeds (pots or boxes), transplanted to 2-inch pots (which should then be placed in a large shallow box with the interstices packed with damp moss) if the mature plants are of small stature, or into 6-inch pots if they grow to a large size. Fine soil without sand or organic fertilizer should be used as the medium. In from two weeks to a month the space between the soil and the pot becomes filled with roots and root tips; few tips, if any, will be found within the soil. Place the base of the stem between the index and middle fingers of the left hand, invert the pot, and knock the edge against some solid object in order to cause the mass of soil and roots to come free. The tips may then be snipped off by means of fine-pointed forceps and placed in the killing fluid. The plant may be reinserted in the pot and grown on until buds are produced; in this way both the monoploid and diploid chromosomal complements of the same plant may be investigated.

Treatment of Tips for Cytological Purposes.—This subject is discussed in Chap. XIV. In many species there are definite periods when mitoses occur with maximum frequency and other periods when they are at the minimum. In *Allium cepa* the maximum periods are between 11:30 P.M. and 12:30 A.M., and 12:30 P.M. and 1:30 P.M.; the minimal periods are around 7 A.M. and 3 P.M. In *Vicia faba* the maximum period is from 7 P.M. to 1 A.M.; in *Pisum sativum* from 9:30 P.M. to 1:30 A.M. However, in most plants mitoses can be found in material collected at any time if a large number of sections from a good many roots are searched.

Treatment for Anatomical Purposes.—The tips should be fixed as for cytological purposes, *i.e.*, preferably with Navashin's fluid. Sections may be cut in any desired plane; since the chromosomes are of no particular significance, the thickness should be uniformly 10μ for all species. If a single stain is desired, Harris' hematoxylin is superb; safranin and fast green give an excellent double stain, and any triple combination should be useful.

ROOT HAIRS

Root tips grown in water or in nutrient solutions rarely have root hairs; those taken from plants grown in pots sometimes have root hairs, but these are usually not in a very satisfactory condition. A few plants have roots that are abundantly covered with root hairs that are well shown in sections of the roots. Among such plants are nearly all species of the Cruciferae and Leguminosae, Euphorbia, Brassica, Solanum,

Aster, *Eupatorium*, and *Gleditsia*. Roots which soon become woody or fleshy are ordinarily poor for root hairs.

Some roots are better sectioned longitudinally for the hairs, particularly if the hairs are turned toward the tip, but others should be microtomed transversely. In either case the sections should be somewhat thicker than usual: from 12 to 16μ should be about right. A triple combination will give the most satisfactory staining.

By far the best method of demonstrating the origin and growth of root hairs is to prepare whole mounts of root tips. The following species are excellent: *Raphanus sativus* and other members of the Brassicaceae, *Hordeum vulgare*, *Secale cereale*, *Avena sativa*, and other cereals and grasses. Sow the seeds thinly on damp filter paper in large Petri dishes or similar moist chambers. In three or four days the roots will be between 1 and 1.5 cm. in length. Cut off the roots at this time, if they are abundantly covered with hairs, but avoid removing with older portions that are over 1 mm. in diameter. Fix with Navashin's fluid or with a fluid giving a basic fixation image, wash thoroughly, then place in either Mayer's carmalum or Grenacher's borax carmin for 24 hours. Wash briefly with distilled water, dehydrate with hygrobutol or dioxan, infiltrate with highly diluted balsam, and evaporate the latter to a mounting consistency. If the roots are over 0.5 mm. thick, they should be mounted in depression slides. The hairs are well shown by this method, as are any incipient lateral roots.

ROOTS

An amazing variety of roots are to be found in the Anthophyta: fleshy, woody, tuberous, fibrous, etc., which may be subterranean, aerial, or aquatic in habitat. The structure, however, is relatively uniform, and the vascular anatomy is of the same general type. Some roots are easy from the technical standpoint, but most of them are beset with difficulties of one sort or another.

In digging up plants in order to obtain the roots, great care needs to be exercised to avoid strains, since it is extremely easy to cause separation of the tissues in different regions, as between xylem and cortex. The roots should be washed thoroughly under running water before being cut into sections about 1 cm. in length and placed in the killing fluid. Grit that lodges in wounds and other breaks, in the exfoliating epidermis and in other places, is a very common cause of torn ribbons and bad nicks in the microtome knife. Use a sharp scalpel for cutting the roots into portions. Formalin-aceto-alcohol and formalin-propiono-alcohol fix nearly all roots perfectly, but a strong chrom-acetic fluid may also be used. Small fibrous roots should be embedded in bunches; it is difficult to locate the tiny sections of single very small roots in the com-

pleted preparations. If a few trial sectionings on the microtome reveal that some elements in the root have become too hard to cut, the material should be soaked under water for several days to a month or longer. Roots which contain much starch should be watched and sectioned as soon as they acquire a whitish-opaque appearance; if left under water too long, it becomes impossible to microtome them.

Origin of the Root.—The origin, structure, and development of the young root must be sought during the growth of the embryo. This can be found in any suitable plant, preferably one from the monocotyledons. *Zea mays* is most extensively used: obtain young pistillate inflorescences whose stage of development is about a week or 10 days after fertilization. Remove the ovules individually. The embryo is readily recognizable; cut slabs from the ovule on each side of the embryo perpendicular to the flattened surfaces, fix with formalin-aceto-alcohol, and microtome at 10μ in the longitudinal plane perpendicular to the flat sides. The young seed sections easily at this time, but afterward soaking under water will be necessary. Safranin and fast green or a triple combination both stain sharply, but the latter should be employed only if it is desired to color the starch grains. *Canna indica* has a large embryo which is also good for root origin. Among the dicotyledons, *Capsella* has been most commonly employed, but it is a poor example. The whole process of root origin is easy to follow out in the Cruciferae; *Lepidium* may be recommended, as it grows with almost everywhere. One might try soaking seeds in water overnight, then removing the testa, and killing the seeds before the roots have emerged.

Secondary Roots.—The origin of secondary (lateral) roots occurs about 1 cm. back from the tip. The best material for this is to be found in perennial plants or trees growing with their bases submerged in water: *Salix pentandra* and other willows, *Sagittaria* and related genera, *Typha* (the rhizome is even better than the root), and *Pistia* are all excellent, but the roots of completely submerged plants are poor. Seeds of *Tropaeolus* and *Vicia faba* germinated on filter paper quickly produce roots which should show the origin of the lateral roots at a distance of from 1.5 to 2 cm. back from the tip. Kill and fix in a strong chrom-acetic fluid or in formalin-propiono-alcohol, section at 8 to 10μ in either plane, and stain with safranin and a suitable counterstain.

TUBERS, RHIZOMES

The majority of tubers are comparatively easy from the technical standpoint. The main precaution to be observed is with regard to the mucilaginous nature of the cell contents of many tubers and rhizomes. A fixative which will not cause excessive cytoplasmic shrinkage should be used; if acetic acid is an ingredient, the least possible amount should

be used. Most tubers and rhizomes contain starch, some (such as those of *Solanum tuberosum*) in great abundance. For this reason a triple or quadruple stain combination is indicated.

Mature tubers are less useful, except possibly under certain circumstances, as when the origin and development of the phellogen are particularly wanted, rather than younger examples. The latter possess most of the structures of the mature tuber and reveal more detail in a much smaller extent of tissue. Tubers of the type of *Beta vulgaris*, *Daucus carota*, and *Pastinaca sativa* are in the optimum condition when about 15 mm. in diameter, and it is easy to cut sections across the entire structure at this stage. If sufficiently young tubers of the type of *Ipomoea batatas* or *Solanum tuberosum* are unavailable, cut off triangular wedges from large specimens, which should preferably have been freshly dug. Specimens from grocery stores are likely to have been so vigorously scrubbed that the epidermis is badly broken.

Rhizomes in general are less troublesome than roots, but those of certain types may possess heavily lignified tissues which will require softening under water. That of *Iris*, which has an unusually prominent endodermis, is an example. Large, fleshy rhizomes, like those of *Zingiber* or *Nymphaea*, should be treated like tubers.

STEM APICES

Longitudinal and even transverse sections of stem apices are useful for a variety of purposes: such sections generally show how the cells which make up the structure of the stem originate; the development of leaves; the origin of lateral branches; the protective covering of the delicate meristematic tissues; and other features. The cells are generally small and isodiametric (probably 14-sided) and have delicate walls, large nuclei, and tiny vacuoles. Mitochondria are occasionally present, but most other cell inclusions are absent. Mitoses are abundant, but, because of crowding, the chromosomes usually cannot be counted. In most woody plants the stem tips are covered with bud scales; these scales are sometimes permeated with resinous or sticky substances which interfere with staining.

Stem apices, on the whole, are easy subjects technically; the one outstanding difficulty is to orient them for sectioning in such a plane that perfectly median longitudinal sections are obtained. Nonmedian sections are of little or no value. Hard scales and excess leaves should always first be trimmed away, then the apices may be cut off from the stem about 8 to 10 mm. back from the tip, save that greatly elongated types (as those of *Lonicera* and *Veronica*) should be about 12 to 15 mm. in length.

Fixing fluids of strong penetrating power are necessary. Formalin-propiono-alcohol has given perfect results, but those who so prefer may use a strong chrom-acetic fluid. Air should be exhausted under a suction pump, otherwise the material is certain to float while in the paraffin oven.

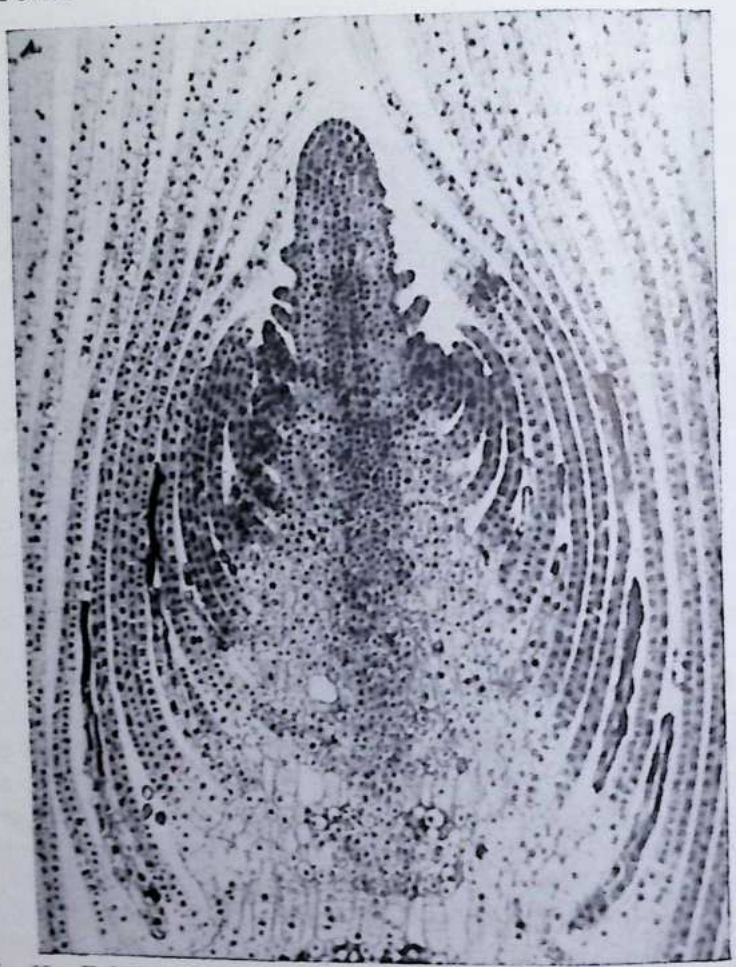


FIG. 99.—*Hydrilla verticillata:* perfectly median longitudinal section of the stem apex, with meristematic region and developing leaves. Fixed with formalin-aceto-alcohol; stained with safranin and fast green.

Elodea stem apices are more generally used than any other type; the related and much larger *Hydrilla* from the tropics is even better and is also likely to have either staminate or pistillate flowers present (Fig. 99). Some people prefer *Hippurus* or *Myriophyllum*, but both are rather difficult to stain sharply. These plants are employed primarily for the origin of the leaves. Among herbaceous and semiwoody plants, vines and scandent species are particularly useful. Among them may

be mentioned *Aristolochia, Lonicera, Ampelopsis, Parthenocissus, Vitis, Clematis, Wisteria, Passiflora, Dolichos, Asparagus,* and *Cobaea.* The majority of the species in these genera have terminal buds that are laterally flattened, which makes for greater ease in orienting for microtoming, since it is merely necessary to cut parallel to either flat surface. Other herbaceous plants which have broad apical meristems are preferable to those in which this region is narrow and of slight extent. Excellent types are to be found in *Coleus, Syringa, Ricinus, Rosa,* and *Veronica.* It will be noted that most of these genera have opposite leaves; plants with alternate leaves usually are not very satisfactory for the apical meristems. These materials should all be collected in early spring when growth is most rapid. The terminal buds of woody plants, on the other hand, should be collected in late winter or in early spring before they have expanded. The outer scale leaves, which are generally too hard to be sectioned easily, should first be removed. Practically any woody plant will provide suitable material; those that are deciduous are somewhat better than those that are evergreen. Certain groups present peculiar difficulties which require considerable ingenuity on the part of the technician to circumvent. The buds of the Ericaceae, for instance, are saturated with phlobaphene compounds which interfere with both fixation and staining. The xylem in a few other types becomes so heavily lignified at an early stage that soaking of the embedded material under water becomes necessary.

Single and double staining methods should be employed. Triple and quadruple combinations are of little service; there is, for example, generally nothing for the violet to stain and the orange G of quadruple combinations completely overshadows the green. Either Harris' or Delafield's hematoxylin used alone is frequently excellent, as are safranin and fast green. Foster's tannic acid method, when correctly used, is superb for differentiation of the embryonic cells.

STEMS

Freehand Sections.—All stems which are sufficiently rigid may be cut either freehand or in a sliding microtome in the fresh condition. The older generations of botanists employed the freehand method almost exclusively, then graduated to celloidin embedding. The celloidin method still remains supreme, but the newer methods of embedding in paraffin plus soaking under water have enabled technicians possessed of patience and a high degree of manipulative skill to produce thin, perfect sections which can be mounted on slides for staining and do not require handling as loose sections.

If the sections are cut freehand, fix them for 24 hours in formalin-aceto-alcohol, then stain with safranin. The time in the safranin depends

upon the amount of lignification present. If scanty, the optimum time is 24 hours or longer; if abundant, shorten the period to 2 hours. It is difficult to differentiate heavily lignified tissues that have been overstained. If an aqueous counterstain is to follow, wash the sections thoroughly with water, then differentiate with water slightly acidulated with hydrochloric acid until the sections have a deep pink color generally, with the lignified portions a bright red. Again wash thoroughly with water to remove all acid, then apply the counterstain. Harris' or Delafield's hematoxylin is excellent, or a 1% aqueous solution of fast green may be substituted. After excess counterstain has been washed out, or the hematoxylin differentiated in the usual fashion, dehydrate by the gradual addition of hygrobutol or dioxan over a period of about 45 minutes, then infiltrate with dilute balsam, which should then be concentrated quickly. Mount the sections as soon as practicable, as they will become brittle in time and may crack when flattened.

If an alcoholic counterstain is to follow the safranin, wash the latter out with water, then cover with 70% ethyl alcohol nearly saturated with picric acid for about 10 seconds. Remove, and replace with a change of 85% ethyl alcohol, then apply the counterstain. Anilin blue and fast green are the dyes most commonly used, and these work better if first dissolved in methyl cellosolve and mixed with an equal volume of 95% alcohol. The period depends on the material: some types stain quickly, others slowly—the sections should be allowed to remain until thoroughly stained since there is little chance of overstaining. Wash with two changes of 95% alcohol, then commence adding hygrobutol gradually, and finally infiltrate with dilute balsam. Xylol should always be avoided since it hardens sections excessively and causes them to curl up tightly.

Paraffin sections of woody materials may be placed in xylol to dissolve the paraffin, then brought down to alcohol or water for staining, later treated as if they were freehand sections. Materials which are difficult to retain on the slides should be treated in this fashion.

Celloidin Sections.—Directions for manipulating celloidin sections are given in the chapter describing the Celloidin Method.

Paraffin Sections.—All meristematic, herbaceous, and young woody stems can, and should, be sectioned in paraffin. Fix with either formalin-aceto-alcohol or formalin-propiono-alcohol, and follow preferably the tertiary butyl alcohol method. Infiltration with paraffin should be thorough; some materials may need to be left in the oven for a week or longer. Embed in a hard paraffin or in Parlax. After cooling, cut the required pieces out of the block, trim roughly, expose the end which is to be microtomed, then place under distilled water plus a small crystal of phenol in a small bottle. The important point is to judge the period of immersion accurately; this requires some practice. The optimum

time for young stems is overnight; for most herbaceous stems, the same length of time suffices; for harder and older stems a few days are required; for woody stems a week to two months may be necessary. As the tissues absorb water and become softened, they acquire a whitish-opaque appearance. The stems are ready for sectioning as soon as this appearance extends for their entire length. There is great danger in overimmersion; stems of the *Pelargonium* type, which have an extensive pith, should not be left in the water for more than 36 hours. If stems are immersed too long, it becomes impossible to section them, since the tissues are torn

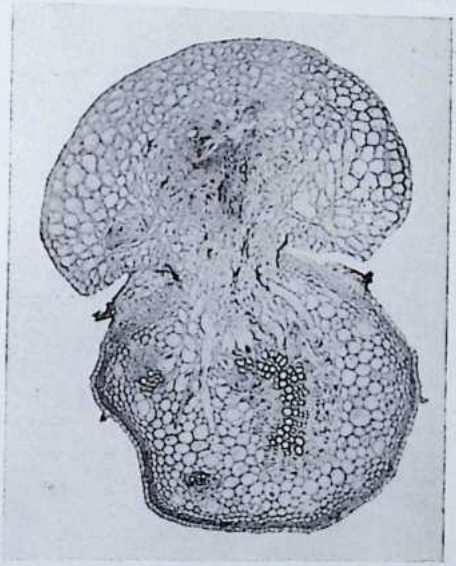


FIG. 100.—*Cassytha filiformis:* cross section of the parasitic stem, with the haustoria penetrating the stem of *Passiflora foetida*. Fixed with formalin-aceto-alcohol; stained with safranin and fast green.

rather than cut by the knife. The thickness at which the sections are cut depends upon the nature of the tissues. The tissues of some stems are composed of huge numbers of rather small cells; such stems should be sectioned at 10 to 12μ. Other stems are made up of relatively few, large cells and should therefore be cut at thicknesses of from 14 to 24μ. A few trial sections, examined microscopically with or without staining, should reveal the optimum thickness. The one great difficulty in microtoming stems is to cut the cambium region smoothly and without wrinkling; the cambial cells are the weakest structurally, and if proper care is not taken, there will be more or less buckling at one or more points. Another source of trouble is tearing of sclerenchyma and collenchyma cells, which may be in small isolated groups in some stems (as in *Aristolochia*, Fig. 101), or in localized regions (as at the corners of the square stems of the Labiatae).

Sections of many stems come off the slides easily during the staining or dehydration. For this reason, stem sections should always be taken through celloidin during the deparaffining. As a further precaution in obstinate cases the sections on the dry slides may be coated with a 1% celloidin solution with a camel's-hair brush, and this allowed to dry.

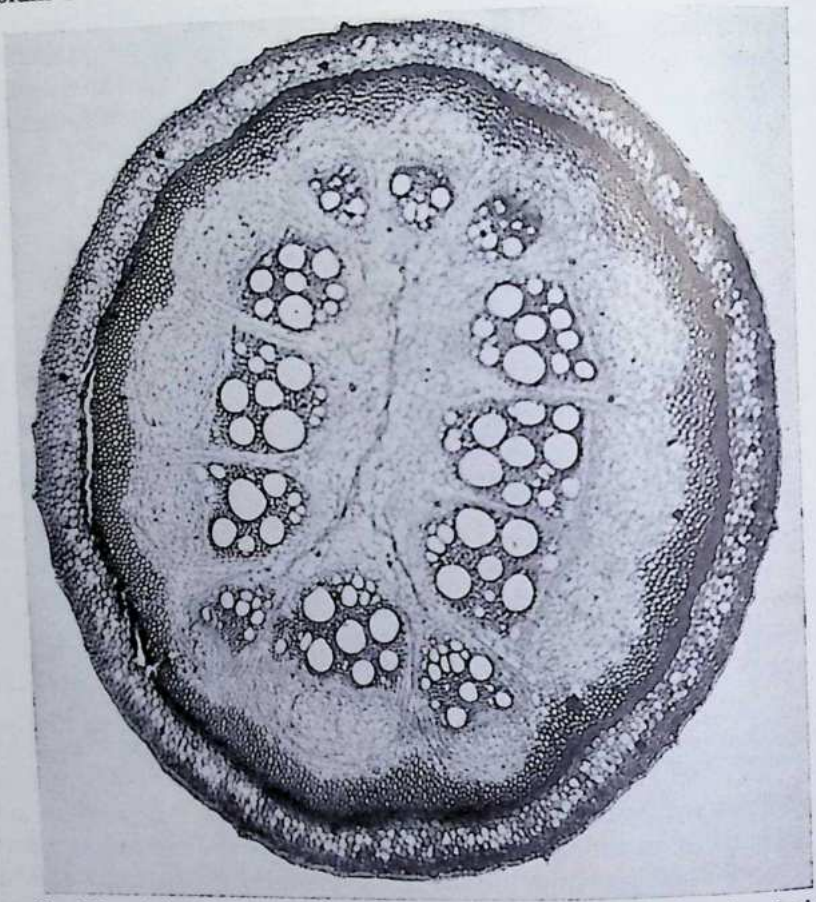


FIG. 101.—*Aristolochia braziliensis:* cross section of an older stem. The breaks in the cortex at the left are caused by release of torsion and are faults that can not be entirely avoided. Fixed with formalin-aceto-alcohol; stained with safranin and anilin blue.

Thereafter it is necessary to avoid any fluid that is a celloidin solvent: deparaffining may be with carbol-xylol, thence to 95% ethyl alcohol and down to water, and dehydration may be done in the reverse order, except that the slides are passed through pure xylol immediately before mounting in balsam.

Staining of stem sections is to a considerable extent a matter of individual preference. A great variety of combinations is available. The simplest is safranin and fast green. Others include safranin and

Harris' or Delafield's hematoxylin; malachite green, methyl green or crystal violet and erythrosin or acid fuchsin; or safranin and anilin blue or crystal violet. Quadruple combinations are more useful than triple combinations since the orange G of the latter tends to overstain. The basic stain should be selected with regard to the structures which are particularly wanted to be shown to the best advantage.

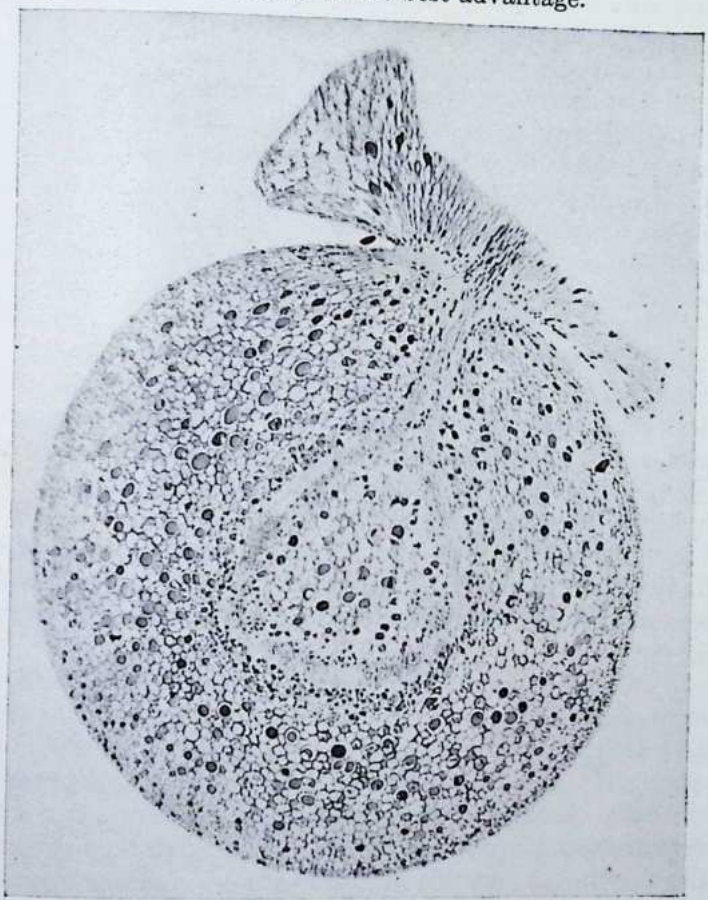


FIG. 102.—*Sedum praealtum:* cross section of succulent stem with leaf trace. Fixed with Sass's acetone mixture, dehydrated with acetone and tertiary butyl alcohol; stained with safranin and fast green.

Herbarium or other dried material of small stems, leaves, etc., may be restored to a reasonably satisfactory condition for embedding and sectioning by being cut into 4-mm. lengths, placed in absolute alcohol for 24 hours, then taken through graded alcohols down to distilled water. Next transfer to an 8% aqueous solution of potassium hydroxide for a week at room temperature, then wash in 15% aqueous glacial acetic acid, using several changes. Wash thoroughly with water, dehydrate, and embed.

Vascular System.—The ramifications of the vascular system may be followed out by perfusing the living plant with basic (not acid) fuchsin (Gourley 1930). Prepare the staining solution by dissolving 50 mg. basic fuchsin in 2 cc. of 95% alcohol and diluting this with 100 cc. tap water. Remove the plants from the soil (the plants should be of a somewhat succulent type), wash the roots free of adhering debris, immerse the roots in the staining solution, and cut off part of them. If the stain does not show signs of penetrating within 12 hours, recut the roots. The vascular system, even to the minute veins in the leaves, should be well stained in from 24 to 48 hours. The plants may then be removed from the solution and the roots washed thoroughly with water, followed by weak alcohol to remove all excess stain.

The plants or portions thereof may then be boiled in water or a very dilute solution of potassium hydroxide in order to secure partial dissociation. The parts are next placed under a binocular microscope and dissected to the desired extent.

Or the plants may be cleared by placing them in a large test tube or similar container and passed successively through 50, 70, 85, 95%, and absolute alcohol, then cleared through 3 parts absolute alcohol and 1 part xylol, equal parts absolute alcohol and xylol, 1 part alcohol and 3 parts xylol, and two changes of pure xylol. The material should remain in each change for about half a day.

Other methods have also been suggested (Camp and Liming 1931, Simpson 1929, Stebbins 1938, Varrelman 1938). They are essentially similar to the one described above but do not include prestaining of the vascular system.

Fresh, dried, or preserved material may be boiled in water for 2 to 3 minutes, then bleached for one to three days in a mixture of equal parts of concentrated ammonia and hydrogen peroxide, or 2 parts of the former to 1 part of the latter (Stebbins 1938). The higher the degree of oxidation, the more peroxide is used. Next transfer the material to 95% alcohol, and harden for from 1 to 12 hours. Although staining is unnecessary, this may be done at this juncture with 1% aqueous crystal violet. The original schedule called for passing through three changes of normal butyl alcohol (allowing about 2 hours in each), passing through equal parts of normal butyl alcohol and xylol, next through pure xylol, then to mount in balsam; this long procedure may be simplified by the use of hygrobutol or dioxan. All cell contents are removed; the walls appear transparent (without staining), and lignified xylem strands stand out sharply.

The transpiration stream may also be traced with light green, using the dye, which is nontoxic, in the proportion of 1 g. to each liter of distilled water (Harvey 1930).

Macerated Stem Tissues.—All stems may, by proper maceration methods, be dissociated into the component cells. The term "maceration" unfortunately connotes, in the minds of many people, an entirely erroneous conception. It is taken to mean that the tissues are so altered that nothing can really be recognized. All that actually happens is that the middle lamellae, which bind the cells together, are dissolved and the cells are freed more or less completely from each other. If the material has not previously been fixed, there may be some plasmolysis of the cytoplasm of the more delicate cells, but this is not a matter of any great importance.

Jeffrey's method is recommended. In order to master the method correctly, a semiwoody stem should first be experimented with. Young stems of *Tilia*, *Aristolochia*, *Liriodendron*, *Pyrus*, or similar plants with soft wood may be cut into portions about 2 cm. in length, then split into slivers about the size of toothpicks. Stain with safranin after maceration and subsequent thorough washing. It is advisable to use a centrifuge cautiously in making changes of fluids. The stem of *Pelargonium* is probably the best of all herbaceous types for maceration; it affords a great variety of interesting cell types.

Slides of macerated tissues are very useful and furnish the only means by which the entire topography of complete, uncut cells may be followed out. A conception of the nature of cells quite different from that given by sections is obtained.

Sectioning Hard Woods.—Special methods have been devised for the easy sectioning of mature woody stems, which have long been a nightmare to the average technician (page 104).

Abscission Layer.—Abscission layers occur at the junction of the stem with petioles and pedicels. Some of the more suitable plants in which to demonstrate the abscission layer include petioles of *Coleus*, *Salix*, and *Populus* and pedicels of *Lycopersicum*.

Lactiferous Ducts.—Stems which exude a white or yellowish fluid when cut open may be presumed to contain either latex vessels or latex cells. The following families contain numerous species with either or both types of lactiferous ducts: Asclepiadaceae, Euphorbiaceae, Papaveraceae, Caricaceae, Musaceae, and Moraceae.

Starch grains are frequently abundant in latex. For this reason a triple or quadruple stain combination is indicated. Formalin-propiono-alcohol is an excellent fixing fluid; the sections should be between 12 and 15μ in thickness.

Resin Ducts.—Ducts in which resins, oils, gums, and other substances are secreted are present in many families. They occasionally form extensive branching systems, but in certain families (as in the fruits of the Umbelliferae) they are localized. Most fixing fluids dis-

solve the secretory substances; consequently the presence of such substances cannot be employed to indicate the presence of resin ducts. It would be better to cut freehand sections of fresh material and to employ microchemical tests.

Internal Glands.—These structures are of rather rare occurrence in the Anthophyta. Young stems of any species of *Citrus*, particularly of lemons, are the most useful. The lysigenous cavities are very prominent and readily recognized following the customary methods. Bud scales that are sticky on the external surface should, after being sectioned, have glands present.

Lenticels.—Species of *Sambucus* are commonly used for the demonstration of lenticels in woody stems, but the most satisfactory stem

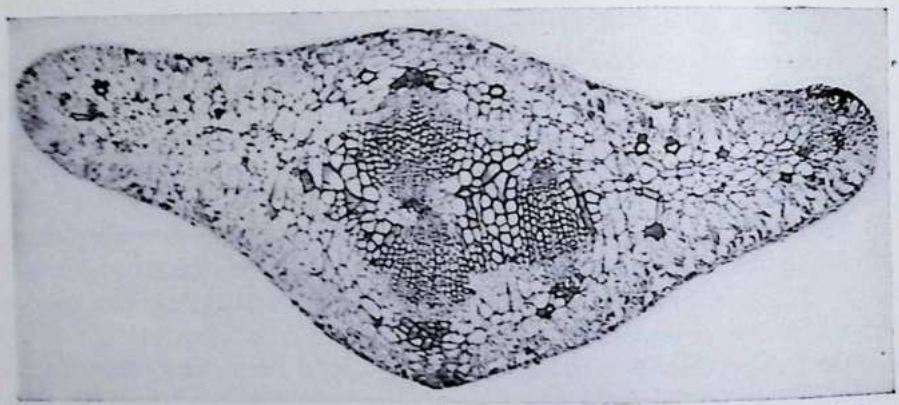


FIG. 103.—*Viscum articulatum*: cross section of the stem of a parasitic dicotyledon. Fixed with formalin-aceto-alcohol; stained with crystal violet and erythrosin. (*From a preparation by Dr. Panchanan Maheshwari.*)

for the purpose has probably not yet been found. *Menispermum*, *Forsythia*, *Aesculus*, and *Pyrus* and many other plants have also been used.

Selected portions of the stem or cortex containing the lenticels may be removed and fixed with formalin-aceto-alcohol. The sections should be cut in the transverse plane of the stem and should not be over 12μ in thickness. The cell walls are thick in the outer regions and take stains intensely because of their heavy suberization.

Tyloses.—Many semiwoody and woody stems contain tyloses in their vessels. They are conspicuous in certain species of *Vitis* (Fig. 104), *Aristolochia*, *Menispermum*, *Aesculus*, *Robinia*, *Juglans*, *Sassafras*, *Rhus*, *Catalpa*, *Quercus*, and *Populus*. They are less common and more difficult to find in herbaceous plants, having been reported from *Coleus*, *Rumex*, *Asarum*, *Convolvulus*, and *Cucurbita*. Formation of tyloses may be induced by wounding.

The walls of the tyloses are generally so thin and delicate that some plasmolysis must be expected. In rare instances the walls may become thick and lignified. Methods commonly used on stems will reveal the tyloses with sufficient clarity.

Cladodia.—These structures, variously also called "phylloclads" and "cladophylls," are stems, usually more or less flattened, which are adapted for carrying on photosynthetic activities in the absence of the usual type of leaf. All species in the family Ruscaceae and the genera *Phyllanthus* and *Asparagus* possess cladodia. *Semele androgyna*, a

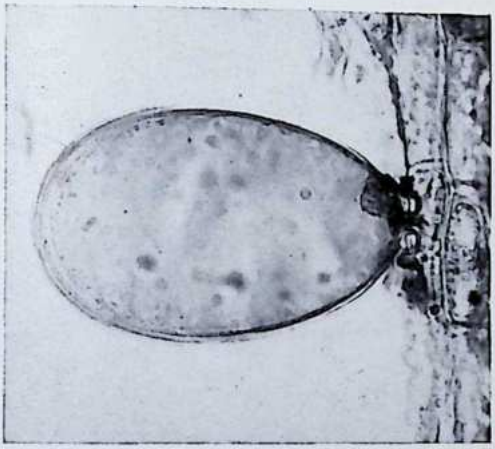


FIG. 104.—*Vitis vinifera:* longitudinal section of a tylose through the stalk. Fixed with formalin-aceto-alcohol; stained with safranin and anilin blue.

member of the Ruscaceae, is widely cultivated in greenhouses and furnishes excellent material.

Material should be treated as if it were thick, leathery leaves; it is difficult to section cladodia easily. The tissues are compact; hence thin sections are desirable; 10μ is about right. Safranin and fast green or a quadruple combination are both excellent for staining.

LEAVES

The origin and earliest stages of leaf development are to be found in sections of leaf buds or stem apices. The cells which become the leaf initials are usually several cell generations removed from the initials of the main axis. The leaf initials are first recognizable as meristematic protuberances. Growth is at first apical, but this phase is of relatively short duration, and growth thereafter is general throughout the leaf tissues. In the Poaceae and in certain other monocotyledons with long linear leaves, such as *Iris* and *Allium*, intercalary meristems are of common occurrence but of brief duration.

Leaves of all types are rather erratic in their reaction to technical methods. The underlying reason is undoubtedly that of photosynthetic activity, less so to minor structural variations. The marked differences between the leaves of monocotyledons and dicotyledons are also a frequent source of technical troubles. The leaves of most monocotyledons possess parallel veins and have been considered to be phyllodia by many botanists. The majority of dicotyledonous leaves, on the

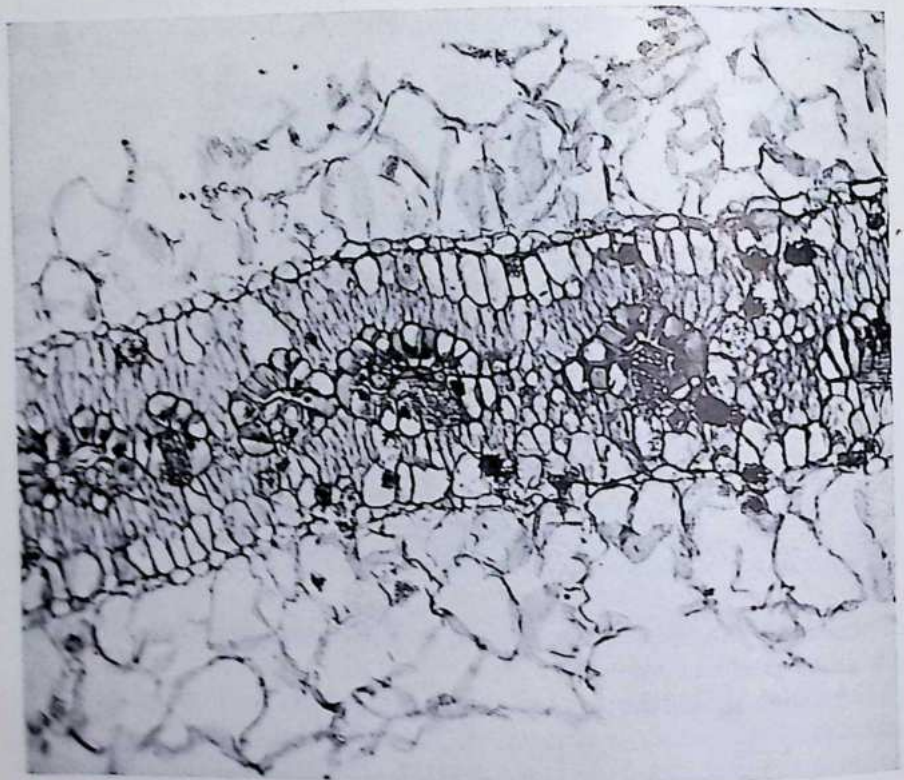


FIG. 103.—*Atriplex hymenotyra*: cross section of portion of a densely trichophyllous isobilateral leaf. Fixed with formalin-propiono-alcohol; stained with safranin and fast green.

other hand, are dorsiventral. Isobilateral leaves are not common among dicotyledons and are generally correlated with habitat factors. Those of plants growing in regions of intense light and heat, as in Death Valley, for example, are usually isobilateral in structure even though they appear externally to be dorsiventral (Fig. 103). The most prominent technical difficulty with leaves is to get sufficient sharpness and contrast with stains. It may be recalled that it is practically impossible to obtain differential staining with the leaves of many Pteridophyta; the situation is not quite so annoying with the Anthophyta, but it is sometimes bad

enough. The chloroplasts are the structures most difficult to stain adequately in the majority of cases. The blame for poor differentiation does not always rest upon the staining procedure employed; sometimes it belongs to the fixing fluid, occasionally to the dehydration method. *Rosa* leaves are among the best for the chloroplasts; the more commonly used *Syringa* is poor.

For some reason, possibly because the older botanists used the handiest plant in their gardens, the leaf of the lilac, *Syringa vulgaris*, is more universally employed than any other to demonstrate the structure of a "typical" dicotyledonous leaf. Objections on one ground or another that it is not entirely a suitable type nevertheless do not prevent it from being recommended as a test object with which the technician can gain experience in the manipulation of leaves. Similarly, leaves of the corn, *Zea mays*, may be utilized as a representative of monocotyledonous leaves.

Manipulation.—Perfect examples should be selected for fixation. Diseased, damaged, senescent, or wilted specimens should be avoided. Evergreen leaves may be collected at any time, but deciduous types are at their prime in spring and early summer.

There is considerable difference of opinion among technicians as to the most satisfactory killing fluid for leaves. Many claim that strong chrom-acetic or medium chrom-osmo-acetic fluids are the only ones which give completely adequate fixation. These claims were made previous to the introduction of the newer methods of dehydration and infiltration and can no longer be wholly justified. Chromic fluids are objectionable mainly on the ground that leaf portions over 3 or 4 mm. in any direction are never completely and evenly fixed throughout. Formalin-propiono-alcohol, if followed by tertiary butyl alcohol dehydration, gives almost perfect preservation of all elements except mitochondria and permits sharper staining than has been obtainable after chromic fluids. This method also has the somewhat strange virtue of not hardening the leaves to the extent that soaking under water is required; sections as thin as 8μ are easily microtomed.

If the leaves are fixed in the middle of a sunny day, most of the stomata will be open; in late afternoon or after dark, they are mostly closed. Most mature leaves have the stomata permanently open. Very few leaves contain starch grains; if present, they are usually in or close to the vascular bundles, and are more likely to be found in leaves fixed just before daybreak. *Pellionia* is the most conspicuous example of a plant whose leaves contain starch.

Leaves, no matter how small, should never be fixed entire but should always have opposite sides or ends cut open. This also applies to submerged aquatic leaves other than those of the dimensions of *Elodea*

leaves. The epidermal layers of the leaves of all but a few species are cutinized, some weakly, others rather heavily. This prevents the penetration of all liquids, hence the necessity for open areas.

The majority of leaves are sectioned transversely, across the veins. If the leaf is not over 18 mm. in width, it should be transected into portions about 5 mm. deep. Leaves over 18 mm. wide should be cut into strips lengthwise about 12 mm. wide and the strips next cut into transverse portions 5 mm. deep. The strips should always include the midvein or in the case of unusually large leaves one of the principal secondary veins. It will be understood that portions prepared as specified are to be microtomed vertically along the longer edge. If longitudinal sections are to be cut, *i.e.*, parallel to the midvein, then the portions should be longer in the lengthwise direction of the leaf. The two types of leaf portions should never be mixed together but be embedded separately, and record of their nature should be kept; it is virtually impossible to determine their type from embedded material.

Scissors should not be used for cutting leaves since they compress the tissues. Place each leaf on a hardwood board or piece of stiff, smooth cardboard and make quick, straight cuts with a sharp scalpel.

As soon as the portions have been placed in the killing fluid, exhaust the air under a water suction pump, but take care not to overdo the process. There will be less danger of plasmolysis if only part of the air is first exhausted and the remainder removed the next day, after considerable hardening of the cytoplasm has occurred.

As stated above, the tertiary butyl alcohol method has given perfect results. Some technicians carry out the preliminary dehydration with glycerin: if an aqueous killing fluid was used, first wash out the fluid thoroughly, then place in 10% aqueous glycerin in a flat dish, and let remain until the water has evaporated completely and the solution has the consistency of pure glycerin. Wash out the glycerin thoroughly with 95% ethyl alcohol, and complete dehydration with absolute alcohol. Pass successively through mixtures of absolute alcohol and xylol in the following proportions: 3:1, 1:1, and 1:3 (about 4 hours in each). Finally give two changes of pure xylol, and embed in paraffin as usual. Or the glycerin may be washed out with tertiary butyl alcohol and then infiltrated with paraffin. If an alcoholic killing fluid was used, wash out with plain alcohol of the same percentage as that in the fluid, place the material in aqueous glycerin in which the percentage of water equals that of the alcohol in the killing fluid, and thicken the glycerin by evaporation. Wash out, and proceed as usual.

Very succulent leaves, such as those of *Sedum*, *Bryophyllum*, *Aloe*, and *Echeveria*, are troublesome because of the very thin cell walls and excessive water content. If they show no shrinkage during fixation

they may be brought into paraffin by the regular tertiary butyl alcohol schedule, provided the changes are made over extended periods. Acetone dehydration is often successful (Sass 1932).

Most leaves should be embedded singly, but some types may be embedded in bunches, one leaf portion being laid on top of another. The tougher and more difficult types should always be treated singly.

Many types of leaves become hardened during the dehydration and infiltration and consequently require a brief soaking under water previous to microtoming. The process should never be prolonged since the leaves have a tendency to shrink sufficiently to become loose in the paraffin matrix, and the sections then fall out as each section is cut. However, the greatest source of trouble in microtoming is the midvein. Some midveins contain as great a quantity of lignified tissue as a small stem and are even more rigid; hence particular attention should be paid to this aspect at all times.

The stain combination most generally employed is safranin and fast green. A quadruple combination is frequently very good, but there is rarely any structure with a special affinity for the violet part. Many people prefer to use either Harris' hematoxylin or Mayer's haemalum alone; these stain practically all structures sharply in various shades of purple and do not give color differentiation between diverse structures; consequently they should be used only if one is familiar with the anatomy of leaves.

Leaf Types.—Leaves are usually classified according to the familiar taxonomic types, but this classification is wholly useless to technicians since they are primarily interested in anatomical structure. The following rough classification into anatomical types has been devised in order to assist the technician in selecting species to show particular features of the anatomy:

Typical dicotyledonous leaves, with one palisade layer (the preferred types for elementary instruction): *Ligustrum ovalifolium, Syringa.*

Typical dicotyledonous leaves, with two or more palisade layers: *Quercus, Pyrus, Citrus, Polygonum, Hedera, Rosa.*

Malacophyllous types: *Begonia, Tradescantia fluminensis, Scoliopus.*

Sclerophyllous types: *Banksia, Hakea.*

Typical, with large hypodermal cells below the upper epidermis: *Piper nigrum, Peperomia reflexa, Ficus elastica, Rosa. Arachis* has large hypodermal cells above the lower epidermis.

Little differentiation and poor internal organization; not to be recommended: *Beta, Zantedeschia, Amsinckia, Montia, Sedum, Chrysanthemum, Pelargonium.*

Dicotyledons with structure resembling that of typical monocotyledons: *Eschscholtzia, Portulaca oleracea, Eichhornia, Atriplex hymenelytra.*

Isobilateral dicotyledons: *Ranunculus aquatilis* (aerial), *Aptenia, Prosopis, Larrea, Eucalyptus* (adult), *Encelia, Dianthus caryophyllus.*

Typical trichophyllous dicotyledons: most desert species belong in this category, *e.g., Salvia apiana, Encelia farinosa, Atriplex hymenelytra.*

Typical monocotyledonous leaves, no differentiated mesophyll: *Zea, Avena sativa.*

Xerophytic monocotyledons, inrolled type, heavily lignified and cutinized: all bamboos, such as *Phyllostachys; Ammophila, Spartina.*

Monocotyledonous phyllodia: *Amaryllis, Typha, Semele.*

Monocotyledons with structure resembling that of typical dicotyledons: *Anthurium andraeanum, Allium, Trillium.*

Aquatic types, with aerenchyma: *Trapa, Castalia, Neptunia.*

With conspicuous external glands: *Potentilla glandulosa, Dionaea, Drosera.*

With conspicuous lactiferous ducts: *Ficus carica, Jatropha, Euphorbia.*

With conspicuous internal glands: *Citrus, Ruta, Ficus elastica, Eucalyptus.*

Phyllodia.—Petioles which become flattened to carry on photosynthesis are known as "phyllodia." Most species of *Acacia* have phyllodia, at least in mature plants. In *Coccoloba* the base of the petiole is dilated into a phyllodium. Treat phyllodia as if they were cladodia (page 463). Considerable care must be taken with the differentiation of stains, as the tissues very readily overstain.

Leaf Epidermis.—In order to show the number, disposition, and surface anatomy of stomata, the epidermis of the leaf may be peeled off, stained, and mounted in balsam. The epidermis does not peel equally well from all types of leaves; in fact, the number which can be utilized is quite small.

The most useful type is *Graptopetalum* (*Byrnesia*) *weinbergii,* one of the Crassulaceae. The leaves are large, grayish-green, and the entire epidermis from either side may be stripped in one operation. Take fresh leaves, cut off both ends and a narrow strip along each side, then insert the edge of a scalpel under one lower end, and peel off the epidermis slowly. It should not be stripped rapidly, or it will curl tightly. Submerse immediately in formalin-aceto-alcohol, taking care not to allow the edges to roll inward. After fixing for 24 hours, wash with a change of 50% alcohol, then stain with Harris' hematoxylin. Differentiate, wash with water, dehydrate with hygrobutol, infiltrate with diluted balsam, then cut into portions about 5 mm. square for mounting. Be sure to mount with the external side up.

If *Graptopetalum* is unavailable, most species of the related *Sedum* are good, particularly those with broad and somewhat flattened leaves.

The epidermis of *Tradescantia virginiana* is also excellent (Fig. 106). Strip as large pieces as possible. Stain in safranin and anilin blue, which gives a striking color contrast. The bulb scales of *Allium* are modified leaves, and it is very easy to peel the epidermis from the inner

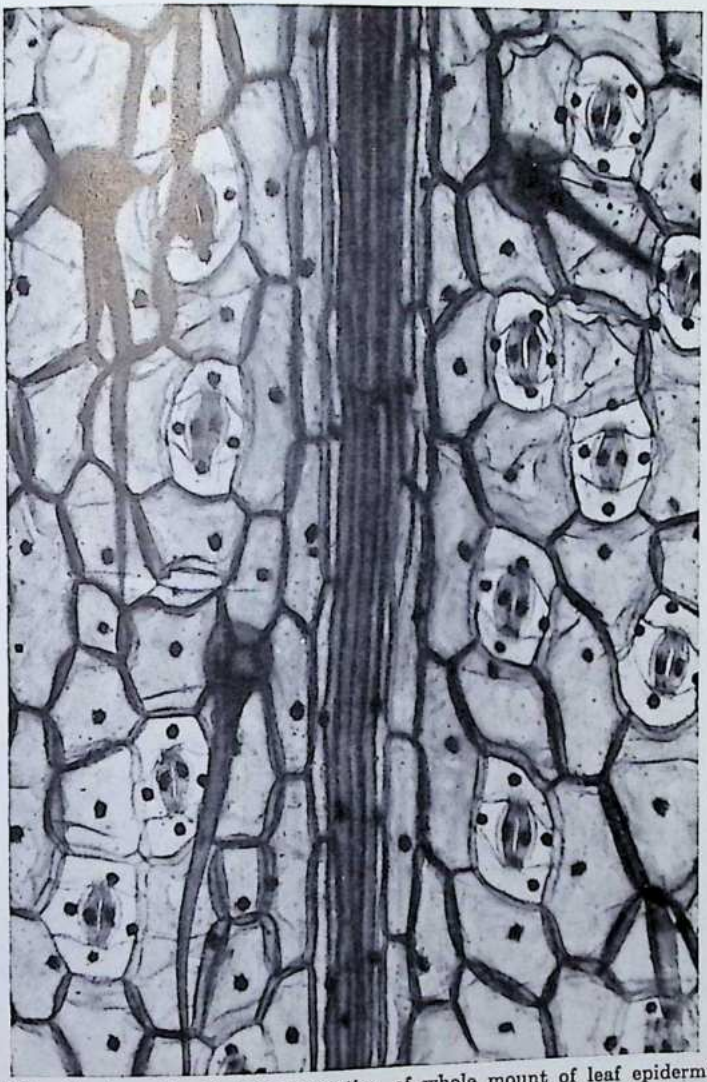


FIG. 106.—*Tradescantia virginiana:* portion of whole mount of leaf epidermis, with stomata, hairs and secondary vein. Fixed with formalin-aceto-alcohol; stained with safranin and fast green, dehydrated with hygrobutol.

surfaces of the scales. This type of epidermis is good only for simple cells and their nuclei; there are no stomata.

With other leaves which do not peel readily, the epidermis can be more or less loosened by blanching with boiling water for a few minutes,

then plunging into cold water, and peeling small portions. Fix as usual, even if the boiling water is in the nature of a fixing fluid.

Whole Mounts of Small Leaves.—To show the venation in suitable small leaves (*Ulmus* and *Fraxinus* are excellent types), stain them in the living condition by Gourley's basic fuchsin method (page 460). Then boil in slightly alkalinized 85% alcohol until all chlorophyll is removed, pass through 95% and absolute ethyl alcohol, place in methyl salicylate (synthetic oil of wintergreen) until cleared, then remove the salicylate with xylol, infiltrate with balsam, and mount.

Another method (McVeigh 1935), adaptable with either fresh, fixed, or dried leaves, is to place them in any one of the common household bleaching solutions (Chlorox, Sani-Chlor, or Purex) containing about 5% sodium hypochlorite. The length of time required for bleaching depends upon the thickness and texture of the leaves. Thin leaves and those of most aquatic plants require 30 to 60 minutes, while thick ones, such as those of *Bryophyllum* and *Sedum*, require 24 to 96 hours. After bleaching, wash thoroughly in running water for several hours to remove the bleaching agent. Next the leaves may be dehydrated with ethyl alcohol and stored in a mixture of 75 parts glycerin and 25 parts 95% alcohol until they become impregnated with glycerin, whereupon they are pliable, easy to handle, and the vascular system is clearly visible. If it is desired to make starch tests, kill the fresh leaves with boiling water, bleach, wash quite thoroughly, then apply the usual iodine-potassium iodide solution.

FLOWERS

The subject of flowers and floral structure is so vast that it cannot be treated at present in any great detail. In any event, the flowers of almost each species present idiosyncrasies peculiar to each; consequently more or less experimentation must be devoted to the one under investigation to determine the precise method of procedure. Reference should first be made to a good general description of the family and order, to the end that the salient structural features be understood (Hutchinson 1926, 1934), after which the general procedure becomes fairly clear in most instances.

An enormous number of types of flowers exist, but a few general features prevail.

Organogeny.—The order of appearance of the different parts composing the entire flower is described as organogeny. At one end of a hypothetical series of floral types are those lacking a perianth (naked or apetalous), and at the other end are those with a perianth of sharply differentiated calyx (sepals) and corolla (petals), with all sorts of modifications in between. The more primitive arrangement of the parts is the

spiral, such as found in the strobilus of more primitive plants, graduating to the cyclic disposition among the more advanced dicotyledons.

In the spiral flower the order of succession is acropetal, meaning that the parts arise successively toward the apex of the receptacle. The succession is sepals, petals, stamens, carpels. In a cyclic flower, if the acropetal succession is maintained, the order of appearance is centripetal. However, the primitive type of succession may be disarranged. In *Capsella*, for example, the order is sepals, stamens, carpels, petals; and in the Asteraceae it becomes petals, stamens, carpels, sepals. In cyclic types there is frequently a definite number of organs in each cycle. In cyclic monocotyledons, for example, the prevalent floral number is three, although there are many spiral monocotyledons with no definite number. Again, in cyclic dicotyledons, the floral number is usually four or five, but there are innumerable spiral dicotyledons with indefinite numbers. All parts of the flower do not necessarily attain a simultaneous cyclic condition, so that exceptions are common.

The first step in preparing slides to demonstrate the organogeny of flowers is to examine the plants and to determine the time and place of appearance of the youngest possible stages. The origin is generally in embryonic regions. In some species an entire year may be occupied by the initial stages of development; this is particularly true of woody plants and many semiwoody perennials. For such types, collections should be begun in the summer preceding the unfolding of the flowers. Most bulbous plants also first develop the flowers during the preceding summer or early autumn. The apical flower buds are, as a rule, recognizable without difficulty but are sometimes not distinguishable from leaf buds when the two types are distinct on the same plant, as in *Syringa*. Cut off the buds, remove all overlapping scales or other protective coverings, fix with formalin-propiono-alcohol, exhaust all air, microtome longitudinally at 10μ, and stain with safranin and fast green, Harris' hematoxylin and erythrosin, or by Foster's tannic acid-iron chloride method. Most buds section without any difficulty, except for tears occasioned by raphides, but a few may need overnight soaking under water. If a research paper is being prepared, one should, obviously, consult published papers on related species or genera, using the indexes to *Botanical Abstracts* (up to 1925), *Biological Abstracts* (after 1925), and the foreign abstracting journals for the purpose.

Whole Mounts of Small Flowers.—Flowers which are of a small size and not too thick when pressed flat may be fixed with formalin-aceto-alcohol, which generally clears them of pigments, washed with 50% ethyl alcohol, stained with Harris' hematoxylin and erythrosin, or with a carmin stain, dehydrated with hygrobutol or dioxan, infiltrated with balsam, and mounted entire. Depression slides should be used if

they are available, otherwise glass threads should be arranged beneath the petals in order to hold the flower level.

The flowers of *Anagallis, Spiraea, Capsella, Erodium,* various native orchids with small flowers (*Ibidium, Liparis, Piperia, Limnorchis*), *Clintonia,* and similar types provide very instructive preparations when mounted so that they are viewed from above. The flowers of various Liliaceae may be cut open and spread out: *Allium* and *Dichelostemma* are excellent. The petals of *Calochortus* may be mounted singly to show the hairs and glands. The florets of the Asteraceae (*e.g., Taraxacum, Hieracium,* or *Erigeron*) may be mounted flat for side views.

Vascular System.—The pedicel is structurally a stem and may be treated like one. The traces which depart to the various organs start from the modified stele in the receptacle; they are similar to those of leaves in origin, structure, and behavior. They pass off successively to sepals, petals, stamens, and carpels according to the manner of arrangement of these organs in the flower. Petals and stamens typically possess a single trace, carpels three traces, and sepals generally the number of traces found in the leaves of the same plant. There are naturally exceptions: in the Magnoliaceae there are three traces to each stamen; petals that are more like sepals have traces comparable in number to those normally possessed by the latter; and in some capsules the number of traces is reduced to one. In most flowers the vascular system is extremely complex, and confusion exists because of the fusion of adjacent traces in the proximal parts.

Sections of buds and very young flowers show more or less of the vascular system, but never in its entirety. To reveal the complete system, the buds or flowers should be stained by Gourley's or a similar method (page 460), cleared, and mounted.

The flowers of the Ericaceae are especially desirable for demonstrating the fusion of bundles in the proximal portions. Young flowers, selected at about the time they have just begun to expand, of any species of *Vaccinium* are excellent, but the much larger buds of *Arbutus menziesii* are even better. Buds of the Ericaceae, unfortunately, are saturated with phlobaphene compounds, which render staining extremely difficult; differential acidification might be attempted, followed by safranin and fast green or a quadruple combination.

Petals and Sepals.—Both of these floral organs are histologically closely similar to leaves in structure, the sepals being the more so. Sections may be prepared exactly as for leaves; the petals never give any technical difficulties, but the sepals of some species may require treatment similar to that needed by tough leaves.

The epidermal cells of petals, as seen in whole mounts, have an extremely interesting structure.

Stamens and Pistils.—Sections of young buds of hermaphroditic plants will reveal both the stamens and the pistils. In monosporangiate flowers, the staminate and pistillate flowers may occur on the same plant (the monoecious condition) or upon different plants (the dioecious condition). In the monoecious plants the nature of the buds cannot always be determined, especially during the youngest developmental stages, from external appearances. The preparation and examination of a considerable number of buds will be necessary. It is usually possible to distinguish the type of flowers of dioecious plants by crushing or dissecting a few specimens and examining for anthers. Occasionally pistillate flowers may contain aborted stamens (staminodia).

Longitudinal sections of the buds are better for the structure and development of the stamens; transverse sections are principally useful for determining the number. Both longitudinal and transverse sections of the pistils will be required, as it is desirable to correlate observations on the one with those on the other. For the later stages in the development of the pistils, especially after the buds attain too large a size for sections of the entire bud, the pistils should be removed from the buds and treated independently. After the style commences rapid elongation, it should be cut off; if sections of the stigmatic surface and the development of the microgametophyte are desired, the styles should be treated individually.

Formalin-aceto-alcohol is an excellent fixing fluid for most plants, but if plasmolysis or separation of cells occurs, resort should be had to a medium chrom-acetic fluid. Safranin and fast green have always given excellent results, but other combinations may also be tried. Special methods for the treatment of styles will be described below under microsporogenesis.

MICROSPOROGENESIS

For the earliest stages of the series of events leading up to the growth of the microgametophyte, very young buds are necessary. The anthers, as a general rule, make their appearance long before the ovules originate in the ovary, and meiosis is completed at about the time the megasporocyte is differentiated in the ovule. The very young anther is a mass of homogeneous cells. The sporogenous cells originate in the layer (hypodermal layer) immediately beneath the epidermis; they are usually recognizable in four distinct regions by their slightly larger size, larger nuclei, and more densely staining cytoplasm. In transverse sections each sporogenous region consists of from one to several cells; in longitudinal section they may form a continuous row in most species (as in the Liliaceae), but in others (as in the Onagraceae) there are alternating layers of sterile cells. Each band of initials divides periclinally

to form two layers of cells. The outer layer is the primary wall layer and the cells later divide to form several wall layers. The innermost wall layer usually becomes part of the tapetum. The inner layer formed after the first periclinal division becomes the primary sporogenous layer, which next divides two or three times to produce the microsporocytes. Meiosis then occurs in the microsporocytes, which process results in tetrads that become microspores.

All these stages, as a rule, are readily obtained, but the reduction divisions are difficult to secure in many species. In *Tritonia*, for example, meiosis will be encountered in only about one out of the hundreds of flowers composing a single inflorescence, and in certain species of *Godetia* thousands of flowers have been sectioned without any showing meiosis. *Lilium* is probably the most favored genus, as it is always easy to secure a complete series of well-fixed and stained stages. *Tradescantia* is also excellent and has large nuclei. In these two genera and in most plants the stage of development is approximately the same throughout all anthers in the same flower at any given time. In a few plants, which have a series of isolated loculi arranged lengthwise of the anthers instead of two or four continuous loculi in each anther, a progressive series of stages occurs, commencing at the distal end and extending downward (as in the Onagraceae), but occasionally development may either proceed simultaneously in all loculi or in a most irregular fashion.

The procedures that are customarily followed in producing slides for all the phases of meiosis are described in the chapters on cytological and smear methods. It is recommended that the beginner start with *Lilium*, as there is practically no chance of failure with this genus. It grows wild in profusion in many localities and is extensively planted in gardens. The so-called Easter Lily, the one most commonly found in greenhouses, should be avoided as the plants are likely to have been subjected to treatments which produce abnormalities. Those species which produce a number of flowers in an umbel are far more favorable than those with very few flowers to the plant, and likewise those with small flowers are somewhat better than those with enormous blossoms. Dissect out the anthers, but do not cut them open, fix with Navashin's fluid or a medium chrom-acetic or chrom-osmo-acetic fluid, and embed in bunches of three or four. Up to the time the microsporocytes begin to round up, sections may be cut in any plane, but after this period transverse sections, at 12μ, are better. If one is unable to collect material locally, it may be obtained from the botanical supply concerns. Blocks tested for definite stages may be procured; they are well worth the high price necessarily charged, since one avoids having to section a quantity of material to find the desired stage.

In the case of other plants the fixing procedure depends upon the nature of the flowers. Most of the Liliaceae and related families may be treated like *Lilium*, but if the buds are too small to be easily handled individually, all that is necessary is to cut off the distal tips of the buds in order to allow fluids to penetrate readily. With compact inflorescences usually only the tips of the larger buds are removed, but the suction pump should always be used to exhaust air. If the buds are covered with chaffy scales or very thick sepals, these should always be removed as much as possible. Buds of the Asteraceae, which usually give a beautiful series of developmental stages, should be cut through between the apex of the pedicel and the base of the receptacle and the outer overlapping scales removed. If the buds (capitula) are over 1 cm. in diameter, they should be bisected longitudinally. Short, broad capitula should be microtomed transversely, other types longitudinally.

By far the best general stain for all stages up to, and including, meiosis is iron hematoxylin; suitable counterstains, if one is desired, include orange G and fast green. Cytologists rarely employ counterstains, as they tend to obscure chromosomal details. For the meiotic chromosomes, a violet stain, used alone, is superb; the violets, however, rarely stain the prophase stages adequately. Triple combinations are most useful on materials difficult to stain with hematoxylin or the violets.

For the tetrad stage nothing is superior to certain genera in the Asteraceae: *Dahlia*, *Coreopsis*, *Crepis*, *Erigeron*, *Cosmos*, and annual species of *Chrysanthemum*. This stage is best stained with safranin and fast green, carefully controlling the safranin so as not to overstain the cytoplasm, or with a triple combination.

For the maturing pollen grain, *Lilium* again is the most suitable material, but *Silphium* is also good. Do not cut the anthers open, lest the loose microspores float out during the dehydration. Microtome at about 8μ. Stain with a triple combination, which will differentiate all structures; or with safranin and fast green, iron hematoxylin and safranin, or any preferred combination. The developing exine and intine, as well as the germ pores, are well shown at this time. If it is particularly desired to differentiate the exine and intine layers, stain the sections for 6 to 10 minutes with 0.5% aqueous Bismarck brown, rinse in water, dehydrate with 95% alcohol, then counterstain with fast green in clove oil-absolute alcohol-methyl cellosolve—the exine is green and the intine brown. The mitosis in the microspore which gives rise to the tube cell and the generative cell, possessed by most species, is easily found in a few species, but in the majority it is obscure and rarely encountered. The finest genus for the purpose is *Trillium*, with *Tradescantia virginiana* as second choice.

In the genera *Acacia, Asclepias, Vincetoxicum,* and in most orchids the mature microspores are united into pollinia, with a common exine. Sections are preferable to whole mounts or smears. The individual microspores are rather small; consequently sections should not be over 10μ in thickness.

Smears.—Methods of preparing smears of microsporocytes are described in the chapter on Smear Methods.

Whole Mounts.—Mature pollen grains embrace a great variety of forms and are particularly noteworthy for their sculpturing; these two characters are of taxonomic importance (Wodehouse 1935).

The simplest method of making whole mounts of pollen grains is to place them in a drop of melted glycerin jelly on a slip and add a coverslip. No staining is needed. Or one may place the pollen on a slide, add 2 to 3 drops of anilin oil which has been tinted with crystal violet only to a pale purple color (Wodehouse 1933). Heat gently over a flame, but not beyond the point where the heated portion of the slide becomes too hot to touch, until the grains become deeply stained. Cool to room temperature, draw off excess oil with filter paper, wash by repeatedly adding xylol and absorbing it with filter paper until all the oil and unabsorbed dye have been removed, then add a drop of balsam and a coverslip. The microspores may also be killed and fixed in any standard fluid, washed, stained with safranin, a carmin stain, or by Fuelgen's reaction (in all cases with a counterstain if desired), dehydrated with hygrobutol, and infiltrated with balsam.

MICROGAMETOPHYTE

Pollen grains may be germinated on glass slides appropriately coated with an adhesive or solidified nutrient solution. The great difficulties are (1) to retain the pollen tubes on the slides during the staining and dehydrating processes and (2) to determine the optimum quantity of nutrient to be added. Sucrose is the nutrient generally employed. The amount varies from 0.5% to as much as 45%. A rough estimate can be made by determining the relative stickiness of the stigmatic exudate of the species whose pollen is to be germinated: the stickier, the larger the amount of sugar required.

Various methods of producing whole mounts of germinating pollen tubes have been proposed (Beatty 1937). One of the simplest methods (Newcomber 1938) is to boil 0.5 g. agar and the optimum quantity of sugar (1 g. may be tried) in 25 cc. tap water or any suitable nutrient solution, cool to about 35°C., add 0.5 g. powdered gelatin, and stir until the gelatin is melted. Keep the solution at about 25°C. in an incubator or on a warming plate. Smear a thin film on a clean slide with a finger, and dust on the pollen. Next place in a suitable moist chamber for

germination, removing occasionally to determine, under the microscope, the progress of the germination.

The most suitable killing fluid is Navashin's, especially since bleaching is not necessary after fixation. The slides should be left overnight for adequate fixation. Stain the chromosomes by any preferred violet method, and counterstain with gold orange in clove oil for about 2 to 4 minutes.

Another method is to fix stigmas on which the microspores have germinated naturally, wash, stain with Mayer's carmalum, dehydrate, infiltrate with diluted balsam, and dissect and crush the stigmas on the slide just before mounting. The stigmas of *Nicotiana* and similar species react well to this procedure.

Stigmas and styles may be run into paraffin, sectioned in the longitudinal plane of the style, and stained by any appropriate method. The hollow styles of *Lilium* are commonly treated in this fashion, but to make certain of the presence of microgametophytes, the stigmas should be pollinated by hand and the pistils removed at the optimum times. The period varies with the species; it may be 24 hours in some, up to 96 hours in others (see also under Fertilization, below).

Numerous methods have been devised for treating styles to reveal the presence, distribution, and rate of growth of pollen tubes (Buchholz 1931, Chandler 1931, Nebel 1931).

MEGAGAMETOGENESIS

In perhaps no other field of botanical investigation are there so many opportunities for making erroneous interpretations as during the development of the megagametophyte. As an instance, there might be cited the case of *Lilium*, whose development was intensely investigated for many decades, yet it was not until a few years ago that the correct sequence of events was described. Insofar as the purely technical aspects are concerned, sources of error may arise because of inadequate fixation, poorly differentiated stains, but above all from using too thin sections. The last fault is the most prevalent one; many embryologists habitually cut sections as thin as 5μ and then attempt to follow out the course of events by means of reconstructions. It is a far better procedure to determine the approximate diameter of the megasporocytes, megaspores, and megagametophytes at their various stages of growth and to adjust the thickness of the sections to correspond to each stage. The megagametophyte of *Lilium* during meiosis averages between 30 and 36μ in diameter; consequently the optimum thickness for sections at this stage is 24μ.

Megasporocyte.—The megasporocyte (megaspore mother cell) originates in most plants after the anthers are well along in development. This cell is usually just at the stage when it begins to expand preparatory

to undergoing the first reduction division at the time that the anthers are beginning to change color (if the mature anthers are other than white in color); the color change also indicates that meiosis has been completed in the microsporocytes. The majority of plants have a single megasporocyte, which is one of two cells produced by a single hypodermal archesporial cell; the other cell produces the layers of cells between the apex of the megaspore and that of the ovule. In a few plants there may be either a group of archesporial cells (quite rare) or two to many megasporocytes (*Godetia, Fuchsia, Calycanthus, Erigeron*). In most plants very young buds are required for the archesporial cells; the megasporocyte develops rapidly in some plants, but fairly slowly in others. Unless one is working with a species whose course of development is unknown, it is the better plan to start with a species with slowly developing buds: *Lilium* is excellent, as are *Fritillaria, Erythronium, Trillium*, certain species of *Erigeron*, etc. The reason for selecting a slow-growing species is that it is thereby possible to obtain a more complete series of stages. Developmental stages are more or less simultaneous in all ovules of a given ovary in most species, but in others, as in *Lilium*, there is a progressive series of closely related stages, with the oldest stages usually at the pedicel end of the ovary.

It must always be borne in mind that the megasporocyte is usually a cell deeply embedded in the ovule, covered with a few (*Lilium*) to many layers of nucellar cells (Vitaceae, Onagraceae), and in addition the ovule may be invested by thin or thick integuments. Ovules of some species have a single integument, others may have an inner and an outer integument. The presence of all these protective coverings means that, if it is not possible or entails too much tedious labor to remove the individual ovules, the ovaries must be reduced to as small portions as possible and that killing fluids of high penetrating power should be employed. If there is considerable free space between the ovules and the inner walls of the ovary, free circulation of the fixing and dehydrating fluids within the ovary is permitted. All superfluous ovarian tissues should be removed, not merely because they might inhibit penetration of fluids, but because they may cause difficulties during the microtoming. The ovarian tissues of the Onagraceae, for example, are full of raphides which tear the ribbons badly, and the ovaries of other genera contain vascular elements that are more or less lignified.

If the nucellar cells consist of only a few layers, a strong chromacetic or Navashin's fluid may be used. It has been found that the use of Carnoy's fluid for a few minutes preceding Navashin's, as is commonly done in the case of anthers, has a distinctly deleterious effect on the subsequent staining. Many technicians have used Carnoy's fluid alone, but on the whole it does not appear to be recommendable. Others

use Bouin's or Allen's B-15 modification thereof. If the nucellus is of considerable extent, then a powerful fluid such as Gilson's or Petrunkevitsch's modification of Gilson's may, with caution in regard to overfixation, be utilized. The mercuric deposits which such fluids leave in the tissues should always be removed, preferably from the sections after they have been brought down to water. The ovules should always be microtomed in the longitudinal plane; it is a simple matter to orient the ovules if they are treated singly, but if entire ovaries or portions thereof are being sectioned, care should be taken to microtome in such a plane that as many ovules as possible are cut lengthwise. If the ovules are oriented approximately parallel to the placenta or placentas (which are usually more or less straight), then section the ovaries longitudinally; if they are attached perpendicularly to the placenta, cut the ovaries transversely. The Liliaceae, for instance, should be microtomed transversely, the Onagraceae longitudinally. If the ovules are inserted haphazardly, one can only cut blindly in either direction, but transverse sections should first be cut since one is more likely to get most of the ovules in longitudinal section in this manner.

The sections of all stages of megagametogenesis and embryogenesis should always be mounted in serial order, and care should be taken that none is lost. (Mount on the slide so that a 24 × 50-mm. coverslip can be used.) This is a most important point: one should make it an invariable practice to examine all the sections of any particular ovule carefully in order that no nuclei or cells are overlooked. Commence at the micropylar end and work toward the chalazal end: if the structure under examination is the mature megagametophyte, for example, look for the synergids, then the egg, next the polar nucleus or nuclei, and finally the antipodal cells. In many species the antipodal cells are evanescent, but vestiges nevertheless should be sought for. Haustoria may also be present; sometimes they are lateral, but they usually occur in the chalazal region and may assume a variety of forms.

A variety of stain combinations have been suggested, most of them being based upon individual preference. The writer's choice has long been for safranin and fast green. Many ovules will take an excellent iron hematoxylin stain, and a triple combination will frequently prove valuable, as starch grains are common and a more precise differentiation of obscure details is often secured. A number of investigators habitually use Harris' or Delafield's hematoxylin or Mayer's haematein. However, it is advisable to employ color-contrasting stains until one has acquired sufficient experience in interpreting structures; then one should be able to use a single stain with confidence.

Megaspores.—The megasporocyte undergoes a short period of rapid growth before the first (reductional) mitosis occurs. This mitosis is

always difficult to secure as it proceeds with comparative rapidity. A wall is laid down in some types, but the nuclei remain free in other types (see below). If a wall is developed, the two cells thus resulting are termed "secondary megasporocytes." If no wall is formed, the megasporocyte becomes metamorphosed directly into the megagametophyte. The second division is equational (with the monoploid number of chromosomes); again, walls may or may not be established. Normally, there is a linear row of four megaspores (the quartet), each of which is potentially functional. However, depending upon the type, either the chalazal —the one nearest the base of the ovule—or the micropylar—the outermost one, nearest the micropyle—megaspore becomes functional and by two or three further divisions becomes the megagametophyte.

The original megaspore cell enlarges but little during meiosis; consequently methods are the same as described above for megasporocytes. Chromosome staining, however, must be critically controlled; the cytoplasm is dense and tends to overstain.

Megagametophyte.—Four well-established types of megagametophytic development may be recognized (Schnarf 1936), and in addition there are minor variations under each type, together with a few problematical types. The principal types may be characterized as follows.

1. *Normal Type.*—This type is considered to be the original one and as typical for the Anthophyta for the following reasons: it involves the largest number of mitoses; megasporogenesis and megagametogenesis are separate processes within it; it is of general occurrence among the Anthophyta, being absent in no group; and, finally, it is impossible to regard any of the other types as the original one from which the normal type may have been derived.

Two features characterize the normal type: the megasporocyte undergoes five divisions before formation of the egg, and the megagametophyte develops from a single megaspore. Furthermore, two phases can be distinguished during the course of development. The first leads to development of the megaspore and consists of two successive divisions which involve conversion of the diploidy of the megasporocyte into the haploidy of the megaspores: this is megasporogenesis. The second phase, usually involving only one megaspore, embraces three successive mitoses which produce the nuclei of the mature megagametophyte: this is megagametogenesis and is characterized by considerable growth and by establishment of the micropylar and chalazal poles.

Immediately after the first division of the nucleus in the functioning megaspore, the daughter nuclei migrate to opposite ends of the cell, and a large vacuole appears between them. Each nucleus divides twice, to form a complex of four nuclei at each end. At the micropylar end two of the nuclei (sisters) are enclosed by cell walls and become the syner-

gids; one of the two others becomes enclosed by a wall (the egg), and the other remains free. At the chalazal end three nuclei become enclosed by walls (or occasionally perish), and the other remains free. The two free nuclei generally fuse more or less completely to form the polar nucleus, which, after union with the secondary male nucleus, becomes the primary endosperm nucleus.

2. *Oenothera Type.*—This type is probably confined exclusively to the Onagraceae. In contrast to the normal type, in which the chalazal megaspore of the quartet is invariably the functional one, the functional megaspore is the micropylar one. The megaspore next below sometimes enlarges and rarely also develops into a megagametophyte. There are four divisions leading up to the organized megagametophyte: after the first, secondary megasporocytes are formed, then a linear quartet of megaspores. Next the nucleus of the functioning megaspore divides, and both nuclei remain at the micropylar end, with a vacuole appearing below them. The two nuclei are arranged one below the other: the upper one divides to produce the synergids, and the lower one produces the egg and a single polar nucleus (which is haploid).

3. *Scilla Type.*—This type of megagametophytic development has been described for a large number of species in widely scattered and unrelated families. It has apparently been derived from the normal type. Four successive mitoses are involved in the transition from megasporocyte to megagametophyte; megasporogenesis and megagametogenesis are not distinct but merged indistinguishably. After the first division (meiosis) in the megasporocyte, a transverse wall is formed, but after the second mitosis no wall is formed, although a purely transitory one might appear. The four megaspore nuclei consequently are situated in two adjacent cells, of which the micropylar one ordinarily degenerates. The remnants of the degenerated cell remain visible for a considerable period. The two megaspore nuclei in the chalazal cell migrate to opposite poles: the micropylar one undergoes two successive divisions which produce two synergids, an egg, and a single polar nucleus; the chalazal nucleus degenerates. Rarely, however, the chalazal nucleus may divide once, to produce a six-nucleate megagametophyte.

4. *Peperomia Type.*—Following the first two mitoses in the megasporocyte, no walls are formed. The four megaspore nuclei thus lie free within the original megasporocyte cell. Two further divisions complete the organization of the composite megagametophyte, which may assume any one of six forms, depending upon the positions and behavior of the four megaspore nuclei:

a. Penaea Form.—This type is restricted to the Penaeaceae and to certain species of *Euphorbia*. No species with this type is known to occur in the United States.

b. Peperomia Form.—This type is restricted to the genus *Peperomia*, which does not occur in the United States.

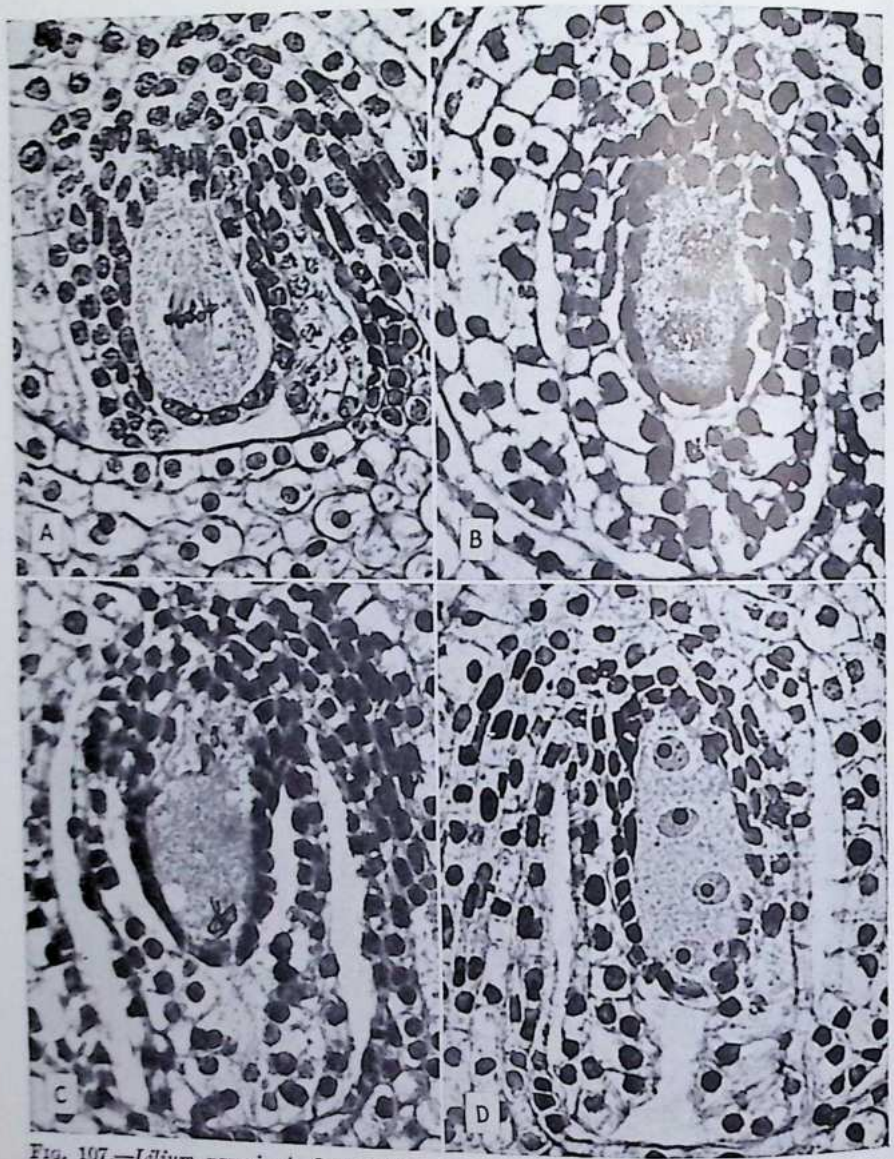


FIG. 107.—*Lilium parryi*: *A*, first division in the megasporocyte; *B*, binucleate megagametophyte; *C*, second division; *D*, first four-nucleate stage.

c. Gunnera Form.—So far as known, this form is restricted to the tropical genus *Gunnera*.

d. Pyrethrum Form.—This form has been described in a single species of *Pyrethrum*, *P. parthenifolium*, now presumed to be a form of

Chrysanthemum parthenium, which occurs from New Brunswick to New Jersey. As a result of meiosis, four free nuclei are produced, arranged in a linear row in the cell, and separated from one another by vacuoles.

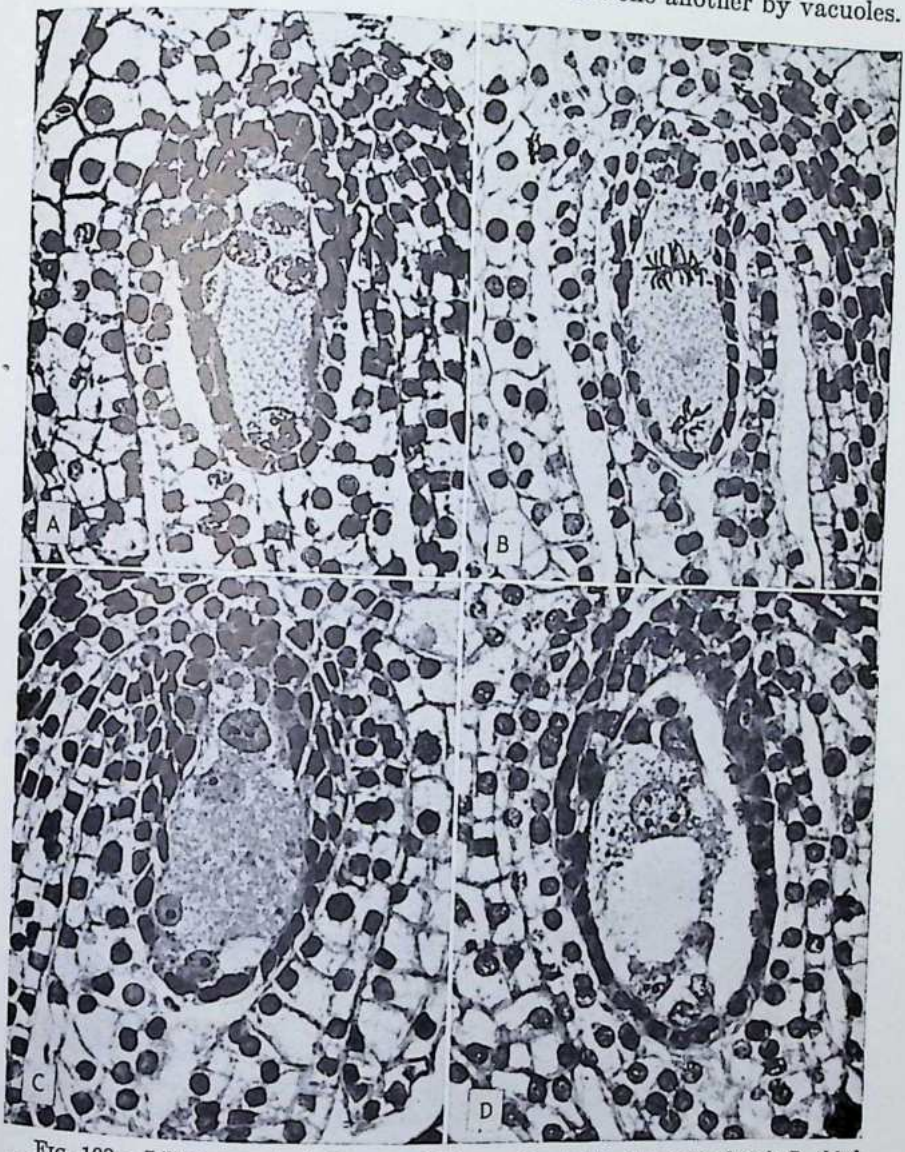


FIG. 108.—*Lilium parryi*: *A*, three nuclei have migrated to the chalazal end; *B*, third division; *C*, second four-nucleate stage; *D*, mature eight-nucleate megagametophyte. (For figure of the fourth division, see Fig. 109.)

They then undergo two successive divisions. The four micropylar nuclei produce the synergids, the egg, and the upper polar nucleus. Of the remaining 12 nuclei, 1 remains free as the lower polar nucleus,

4 become surrounded by a common wall, and each of the 7 other nuclei has a cell wall developed around it.

e. Maianthemum Form.—This type, again, characterizes a single species, *Maianthemum bifolium*, not found in the United States. There are a number of closely related but as yet uninvestigated species.

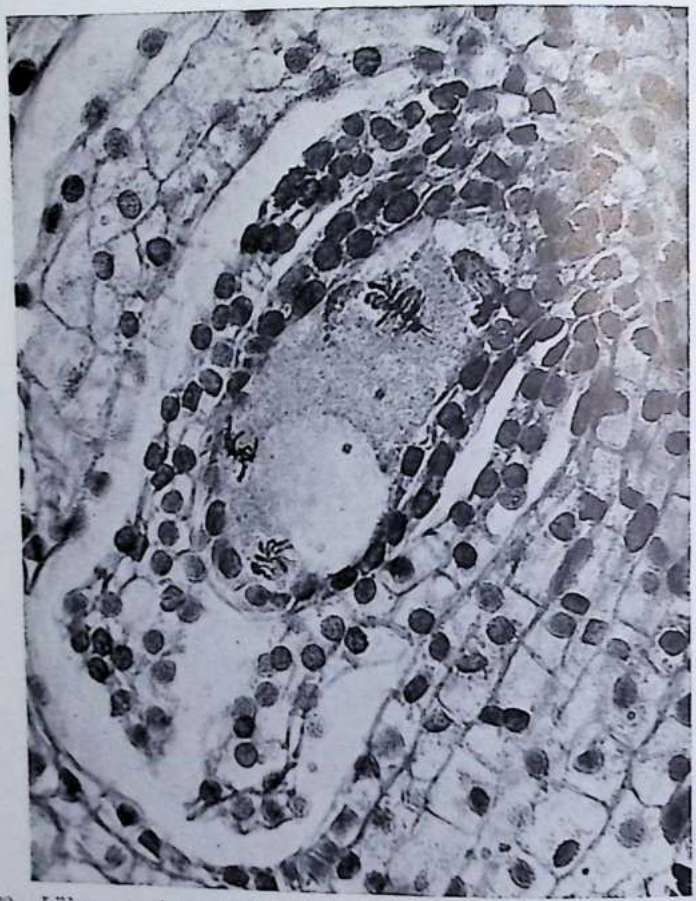


FIG. 109.—*Lilium parryi:* fourth division in the megagametophyte. Fixed with strong chrom-acetic; stained with safranin and fast green.

f. Fritillaria Form.—After meiosis, the four megaspore nuclei are arranged in a linear row down the center of the original megasporocyte. Presently three of the nuclei (the three lower ones) migrate to the chalazal end of the cell. The third division next ensues, during which the spindles of the three chalazal mitoses fuse. There thus result two micropylar haploid nuclei and two triploid chalazal nuclei, and each pair becomes separated by a vacuole. After the fourth division there are four haploid nuclei at the micropylar pole and four triploid nuclei at the chalazal end. The micropylar nuclei produce the two synergids, the egg, and the

upper polar nucleus. The chalazal nuclei form the three antipodal cells and the lower polar nucleus. (The primary endosperm nucleus thus becomes pentaploid after fusion with the secondary male nucleus.) This form characterizes most of the Liliaceae but has been worked out completely in comparatively few species in *Lilium* (Figs. 107, 108, 109), *Fritillaria*, and *Tulipa*. It should be called the *Lilium* Form, but this procedure is dangerous since it might be confused with the older and erroneous so-called *Lilium* Type; consequently the name of the genus in which it was first discovered is being used.

5. *Adoxa Type.*—The older *Lilium* Type nevertheless has some foundation in actuality and has been renamed the *Adoxa* Type. No North American representatives of this type are easily available.

There may possibly be other types which should be recognized as distinct (Schnarf 1936), but the descriptions for the most part have been based upon investigations made many years ago and which should be done all over again by modern methods and in the light of present knowledge of megagametogenesis.

In preparing slides to show the development and organization of the megagametophyte, the same procedure in preparing the material, fixing, and staining as described above for the megasporocytes should be followed. The thickness at which the sections are microtomed is a matter requiring careful consideration and some experimentation; it may vary anywhere from 12 to 30μ. The aim should be to obtain as many ovules as possible in which all the nuclei of any particular stage are present. If individual ovules are being worked with and they are more or less flattened, they should be sectioned parallel to one flat face. If haustoria are present, the ovules should always be so oriented that the haustoria are cut longitudinally. Staining should always be critically controlled; the chances are that there will be either under- or over-staining. It is occasionally difficult to get all regions of the mature megagametophyte equally well differentiated. Contrasting stains are always better than a single uniform stain, since they aid in identifying the nuclei and other structures.

FERTILIZATION

Methods of preparing ovules for fertilization studies are the same as for the megasporocytes. Sections should be a trifle thinner than for the mature megagametophyte, and staining must be critically controlled. Safranin and fast green are a good combination, but the best combination is Fuelgen's reaction and erythrosin. The microgametophyte commonly brings in considerable quantities of foodstuffs, demolished cells, and partially digested nuclei. All this may add confusion when it comes to interpreting what is seen within the megagametophyte, which, upon the

completion of fertilization, becomes the embryo sac. In species in which triploidy and tetraploidy are prevalent, the origin of these features should be watched for during fertilization.

If one collects a complete series of stages in the life history, fertilization is certain to be found, but if the fertilization phenomena alone are sought, it becomes necessary to know something about the time elapsing between pollination and fertilization. This period differs widely among different plants and is conditional upon so many different factors that it is impossible to cite definite hours or days. It is therefore wise to make a series of collections until dissections reveal the presence of young embryos.

The periods intervening between pollination and the time the microgametophyte reaches the megagametophyte are given for some common plants: *Betula*, 1 month; *Carpinus*, 2 months; *Alnus*, 3 months; *Corylus*, over 4 months. *Quercus*, 2 months in some species to 1 year in others; *Fagus*, about 6 days; *Hicoria*, 5–7 weeks. *Polygonum*, about a week; *Fagopyrum*, 18 hours for self-fertilization or more than 72 hours for cross-fertilization; *Pyrus*, 2–4 days; *Phaseolus vulgaris*, 8–9 hours; *Trifolium*, 18 hours in summer to 35–50 hours in autumn. *Oenothera*, 36–72 hours in most species; *Citrus*, 4 weeks; *Convolvulus*, only a few hours; *Nicotiana*, about 2 days; *Datura*, in about 24 hours; *Lactuca*, about 7 hours. *Zostera marina*, 10 hours. *Lilium*, usually 60–72 hours: in *L. auratum*, 7 days, 24–36 hours in *L. grandiflorum*, 96 to 120 hours in *L. martagon*, 120 hours in *L. longiflorum*. *Tulipa*, 8–10 days; *Zea*, 25 hours; *Triticum*, 32 hours to 2 days; *Secale*, 7 hours. Various orchids require from 8 hours to 6 months.

The condition of the petals can sometimes be used as a criterion as to what is transpiring within the ovule, but it is not a reliable one in many plants. In *Lilium*, the Oenotheras, and many herbaceous plants, one is likely to find fertilization if ovaries are removed from flowers whose petals have withered but have not yet fallen.

There are not many plants which are ideal for fertilization studies, and one is generally compelled to section a good many ovules or ovaries before finding what is wanted. But when a good, well-stained preparation has been secured, it is so interesting and instructive that one feels repaid for all the trouble.

EMBRYOGENESIS

The polar nucleus, or nuclei, which may be haploid, diploid, triploid (prevalent condition), or tetraploid (*Lilium*, *Fritillaria*), is usually "fertilized" somewhat in advance of the egg nucleus and invariably divides first.

Endosperm.—The polar nucleus, or nuclei, which lies free in the megagametophyte, customarily in the center or immediately below the egg, is the original source of the endosperm, whether the latter is coenocytic or becomes cellular. Fusion of the secondary male nucleus with the polar nucleus (whether this is a single nucleus or two or more fused nuclei) initiates endosperm growth. There is no definite rule governing endosperm formation, although it is more or less constant for a given species. In long, comparatively narrow embryo sacs, such as those of *Nicotiana*, *Solanum*, and *Verbena*, a cell wall is produced following the first mitosis, and all subsequent mitoses result in walls. When the megagametophyte is large and about as broad as long, the megagametophytic cavity (*i.e.*, the embryo sac) becomes almost filled with coenocytic nuclei which then begin to develop walls, with a single nucleus to each cell, but rarely with two or more nuclei in each cell. *Lilium*, *Reseda*, *Capsella*, and *Ranunculus* are characterized by this type of endosperm growth. Intermediate types of megagametophytes generally contain few coenocytic nuclei; sometimes walls may later be formed, but instances are known where both cellular and coenocytic endosperm exist in different ovules in the same ovary. Mitoses in the endosperm may be simultaneous throughout during the earlier stages in both coenocytic and cellular types; in the latter type the later mitoses are sporadic, and in the former type the mitoses later commence at the micropylar end and proceed in waves toward the chalazal end.

Coenocytic endosperm is invariably evanescent, the substance being absorbed by the developing embryo. Endosperm which is at first coenocytic, then becoming cellular, generally shows some vestiges even during the final stages of embryo maturation, but is not always of a permanent nature. Endosperm which is cellular from the first mitosis onward is of a permanent nature and may even acquire very thick walls and become incredibly hard (Palmaceae, *Diospyros*, *Coffea*, etc.). In general the endosperm type is uniform throughout entire orders among the dicotyledons but is not so uniformly typical among monocotyledons.

Among the monocotyledons, endosperm which is cellular from the first mitosis on is very rare (Lemnaceae). In the Lilaeaceae and Orchidaceae endosperm is entirely absent. The other monocotyledonous families are about equally divided into those which are coenocytic throughout the life of the endosperm and those in which the so-called Helobiales type prevails (this is essentially endosperm coenocytic at first, then becoming cellular). Both types may be found within the same family (Araceae, Potamogetonaceae, and Amaryllidaceae).

Preparations intended for the youngest stages in embryogenesis will always reveal more or less of the endosperm development. Among the more satisfactory types may be mentioned *Nicotiana* for the cellular

type, *Lilium* or *Capsella* for the coenocytic-cellular type, and some member of the Onagraceae for the coenocytic type. All these except *Lilium* also show fine stages in the growth of the embryo. The same fixing fluids as used for embryos will preserve the endosperm. Safranin and fast green are recommended for staining.

Those endosperms that acquire thick walls and become very hard are excellent for showing the nature of plasmodesma (protoplasmic connections). Nearly all palms (Palmaceae) have such an endosperm, as do species of *Diospyros*. Material should not be hard to secure: dates

FIG. 110.—*Diospyros discolor:* portion of a freehand transverse section of mature endosperm, with prominent plasmodesma. Fixed with formalin-aceto-alcohol; stained with iron hematoxylin and orange G.

(*Phoenix dactylifera*) can be purchased at a grocery store, or seeds of related species and numerous other palms may be obtained from nurserymen. The American persimmon, *Diospyros virginiana*, is available at fruit stores from late November into February, but not every fruit contains seeds. The Philippine persimmon, *D. discolor*, is better, but seeds are difficult to obtain (Fig. 110). Fully mature endosperm has better plasmodesma than does immature material. Remove the pulp and testa, and fix the naked endosperm in formalin-aceto-alcohol for about a week. If the endosperm is fixed whole, it is large and rigid enough to be sectioned in a sliding microtome at 10μ. Small portions are just as good as complete sections; consequently it does not matter if the knife has a tendency to spring up. Or the endosperm may be cut into small

portions (4 to 5 mm. cubes), fixed, dehydrated very slowly by the tertiary butyl alcohol method, embedded, soaked under water for two weeks or longer, and cut in a rotary microtome at 10μ. The sections are easily cut, but must be treated as freehand sections as it is impossible to retain them on the slides because of their oily contents. It may be difficult to get good staining unless the oils and fats are first removed. Sections cut either freehand or after embedding are therefore transferred to chloroform (warmed slightly to dissolve paraffin from embedded sections) or ether for a day or longer, then washed successively with absolute, 95, and 50% alcohols for ½ hour in each. Wash with water, and place in 4% ferric ammonium sulphate for 24 hours. Wash thoroughly in water, then stain with 0.5% hematoxylin for 24 hours. Wash again with water, then examine microscopically. If the plasmodesma appear to be properly stained, dehydrate, and mount in balsam without differentiating. Otherwise first differentiate cautiously, being careful not to carry the destaining too far.

Young Embryo.—In the majority of plants it is a troublesome matter to study the young embryo. If the endosperm is cellular, the difficulty is usually to distinguish the few-celled embryos from the surrounding endosperm cells. Main reliance in such cases must be placed upon the counterstain, which has a greater affinity for the cell walls of the embryo than for those of the endosperm: fast green is usually good, but anilin blue is sometimes even better. For later developmental stages the difficulty is that of sectioning them in the correct plane. It should be borne in mind that the cells of embryos are comparatively small and filled with dense cytoplasm; consequently sections should rarely be over 12μ in thickness.

In many plants the embryo grows slowly (*Lilium*, *Nicotiana*, *Oenothera*), in others it develops rapidly (*Capsella*, *Zea*). In any case, a long series of preparations will be required in order to follow out the complete sequence of events. In any one ovary the ovules will all show practically the same stage of development. Fixation has usually been excellent with formalin-propiono-alcohol, and staining is sharp with safranin and fast green, but Harris' hematoxylin, with or without a counterstain of orange G, is also good.

Older Embryo.—After cotyledon development has commenced, the ovules should always be dissected out of the ovaries, unless the latter themselves are too small for easy manipulation, and treated individually. The ovarian walls by this time have become hardened, with extensive lignification and even sclerization, and sectioning becomes increasingly difficult. Formalin-aceto-alcohol may be used for fixation but should be allowed to react for several days; also, the dehydration and infiltration should be gradual. In species which have very large cotyledons or an

extensive endosperm, these structures should be trimmed down as much as possible before fixation; it is annoying to have to cut interminable useless sections on the microtome until the embryo proper is reached. Either safranin and fast green or a triple combination stain beautifully during all the stages of embryo growth.

FRUITS

A fruit is essentially the matured ovary, accompanied or not by various accessory organs, usually containing the seeds. The division of fruits into those which are dry and those which are fleshy is excellent in that it indicates the general technical treatment of each type. The dry fruits are far more difficult to manipulate than the fleshy ones. Botanists apparently have never been much interested in the structure of fruits, being content to leave the problem to pharmacologists. As a matter of fact, one can secure more information regarding the microscopical structure of fruits from a text on pharmaceutical botany than from the average botanical text.

The structure of dry, dehiscent fruits—legumes, capsules, and follicles—is well shown, during at least the earlier stages of development, in preparations of the ovary intended for megagametogenesis and embryogenesis. After the ovarian walls become hard and more or less desiccated, resort must be had to celloidin embedding.

The dry, indehiscent fruits—achenes, grains, nuts, schizocarps, samaras—are even more difficult than the preceding group. The younger stages are, of course, not very troublesome. The most interesting of the indehiscent fruits is the schizocarp, characteristic of the Umbelliferae, and featured by the presence of resin ducts and considerable variation in general structure. The hard walls of nuts require special methods; none of the ordinary sectioning methods gives even passable results. The best procedure is to cut out very small portions, treat with hydrofluoric acid, then embed in celloidin.

Among the fleshy fruits, entire longitudinal and transverse sections of young pomes are readily made until they become about 18 mm. in either length or diameter. The central part of the fruit, to include the core, should be taken. Fix with formalin-propiono-alcohol, microtome at about 11μ, stain with safranin and fast green or a triple combination. Young drupes may be treated similarly until the endocarp becomes too hard to cut with a scalpel.

The berry of *Musa*, *Lycopersicum*, and *Solanum*, the pepo of *Cucurbita* and *Cucumis*, the syconium of *Ficus*, and the hesperidium of *Citrus* are all easily fixed, sectioned and stained at all stages of growth up to the time that the seed coats become too hard to cut readily. The young fruits may be sectioned entire, but as they become larger, wedge-shaped

portions should be cut out. The berries of *Vitis*, *Ribes*, the pepo of *Citrullus*, and similar fruits are composed of such thin walls and contain so much water that it is hard to fix and to dehydrate them. Aqueous fluids should be used. The acetone or glycerin dehydration methods afford excellent results; but whatever the schedule used, changes should always be made over long periods.

Aggregate fruits of a type similar to those of *Fragaria*, *Rubus*, and the Araceae, are easily manipulated until the walls of the carpels become too hard. The walls of the carpels are thick from an early stage in growth; consequently dehydration and infiltration should be prolonged as much as possible. Embed in a hard paraffin or in celloidin. Multiple fruits may be treated as a unit or cut into smaller portions; one usually obtains the most satisfactory sections by microtoming perpendicular to the long axis of the entire fruit.

SEEDS

As a general rule, it is a nearly impossible feat to make sections of entire mature seeds. The trouble is to get fluids and embedding media to penetrate. Seed coats may be cut into small portions and treated as if they were hard woody tissues.

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Numbers in boldface indicate pages on which special directions for treatment of the genus or species concerned are given, or on which formulae, procedures, or methods are described in detail.

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