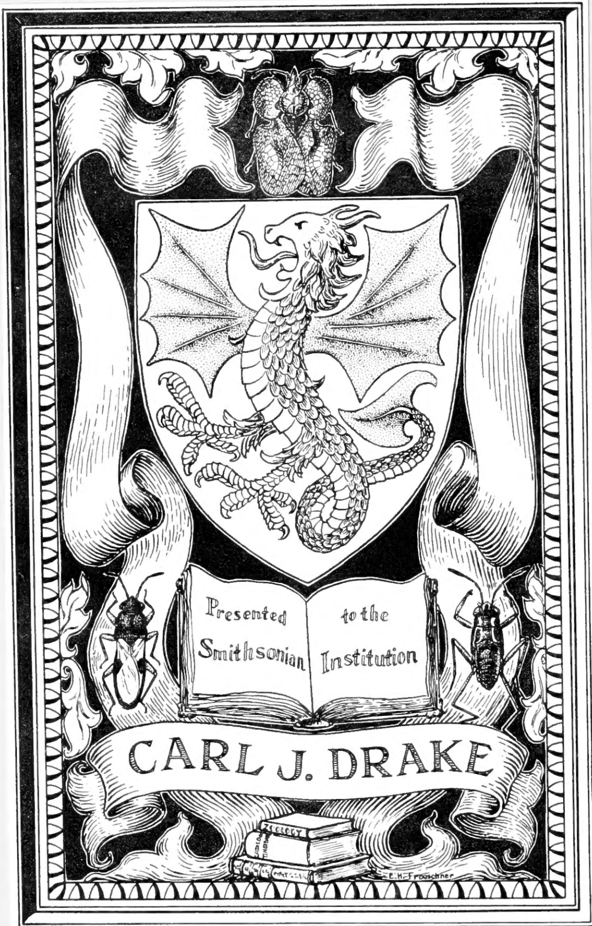
ANIMAL MICROLOGY


Presented to the
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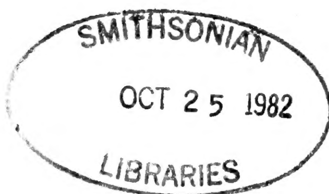
ANIMAL MICROLOGY

PRACTICAL EXERCISES IN MICROSCOPICAL METHODS

BY

MICHAEL F. GUYER, Ph.D.

Professor of Zoölogy in the University of Cincinnati



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PREFACE

For the past ten years it has been a part of the writer's duties to give instruction in microscopical technique, and it has seemed to him that there is need for a series of practical exercises which will serve to guide the beginner through the maze of present-day methods, with the greatest economy of time, by drilling him in a few which are thoroughly fundamental and standard. The book is intended primarily for the beginner and gives more attention to the details of procedure than to discriminations between reagents or the review of special processes. The student is told what to do with his material, step by step, and why he does it; at what stages he is likely to encounter difficulties and how to avoid them; if his preparation is defective, what the probable cause is and the remedy. In short, the book attempts to familiarize the student with the little "tricks" of technique which are commonly left out of books on methods but which mean everything in securing good results.

A very brief, non-technical account of the principles of the microscope is inserted (Appendix A) with the idea of giving the student just enough of the theoretical side of microscopy to enable him to get satisfactory results from his microscope. The microscope is so ably treated in the excellent works of Gage (*The Microscope*) and Carpenter (*The Microscope and Its Revelations*) that the writer feels himself absolved from any further responsibility in this matter.

The aim of the entire book is to be practical: to omit everything that is not essential; and, above all, to give definite statements about things. Appended to each chapter is a series of *memoranda* which serve to supply additional information that is more or less pertinent without obscuring the main features of the method under consideration.

In Appendix B the formulae for a number of the most widely used reagents are given with comments upon their uses and manipulation. Following this (Appendix C) is a concise table of a large

number of tissues and organs with directions for properly preparing them for microscopical study.

Inasmuch as every experienced worker has his own "best" method for the preparation of almost any tissue, it is manifestly impossible to give all "best methods" in such a table. The writer believes, however, that the student will find the methods recommended all good ones which will yield satisfactory results.

In Appendix D some directions are given for collecting and preparing material for an elementary course in zoölogy.

It is hoped that the volume will prove of use: (1) as a class textbook; (2) as a guide to the independent individual worker (teacher, physician, college or medical student, or novice); (3) as a reference book for teachers, in the preparation of material for courses in elementary zoölogy, histology, or embryology.

In the matter of expressing his obligations the writer is at a loss to know just what to do. Many of the methods in microscopical technique have been handed down tradition-wise from one worker to another until their origin is unknown; they are the accumulated experiences of several generations of workers. Furthermore, many points have been absorbed, as it were, by the writer, from fellow-workers in the Universities of Chicago, Nebraska, and Cincinnati, respectively; consequently the obligation cannot be specifically expressed. Where the name of the originator of a method is known, due credit has been given. The books to which the author is most heavily indebted are the volumes of Gage, and Carpenter, already mentioned, Lee's *Microtomist's Vade-Mecum*, Whitman's *Methods in Microscopical Anatomy and Embryology*, Hardesty's *Neurological Technique*, Foster and Balfour's *Elements of Embryology*, Minot's *Laboratory Text-book of Embryology*, Huber's translation of the Böhm-Davidoff *Text-book of Histology*, Stöhr's *Text-book of Histology*, Mallory and Wright's *Pathological Technique*, Bausch's *Manipulation of the Microscope*, and the *Journal of Applied Microscopy*. Grateful acknowledgment is also made to the various manufacturers of microscopical instruments and appliances for the loan of most of the cuts which have been used in this volume.

M. F. G.

CONTENTS

PAGE

INTRODUCTORY 1
Apparatus and Supplies Required, 1; General Rules, 5.

CHAPTER I. PREPARATION OF REAGENTS 7
Practical Exercise, 7; Memoranda, 13.

CHAPTER II. GENERAL STATEMENT OF METHODS 15
Killing, Fixing and Hardening, 16; Washing, 17; Dehydrating, 18; Preserving, 18; Staining, 19; Clearing, 21; Mounting, 22; Imbedding, 22; Affixing Sections, 23; Decolorizing, 24; Bleaching, 24; Corrosion, 24; Decalcification and Desilicidation, 24; Injection 25; Isolation of Histological Elements, 25; Normal or Indifferent Fluids, 25; General Scheme for Mounting Whole Objects (*In Toto* Preparations) or Sections, 26.

CHAPTER III. KILLING AND FIXING 27
Cautions, 27; Alcohol Fixation, 28; Fixing with Gilson's Mercuro-Nitric Mixture. 28; Fixing with Erlicki's Fluid, 29; Formalin as a Fixing Reagent, 29; Memoranda. 30.

CHAPTER IV. SIMPLE SECTION METHODS 33
Free-Hand Section Cutting, 33; Memoranda, 34.

CHAPTER V. THE PARAFFIN METHOD; IMBEDDING AND SECTIONING . 37
The Method, 37; Memoranda, 43; Tabular Statement of Difficulties Likely to Be Encountered in Sectioning in Paraffin and the Probable Remedy, 46.

CHAPTER VI. THE PARAFFIN METHOD: STAINING AND MOUNTING . . 49
Staining with Hematoxylin, 49; Double Staining in Hematoxylin and Eosin, 51; Double Staining in Carmine and Lyons Blue, 51; Staining with Heidenhain's Iron-Alum Hematoxylin, 52; Bordeaux Red and Iron-Hematoxylin, 53; Staining in Bulk before Sectioning, 54; Paraffin Method for Delicate Objects, 54; Memoranda, 55.

CHAPTER VII. THE CELLOIDIN METHOD 59
The Method, 59; Staining Celloidin Sections in Hematoxylin and Eosin, 62; Memoranda, 62.

CHAPTER VIII. THE FREEZING METHOD 67
The Method, 67; Memoranda, 69.

CHAPTER IX. METALLIC SUBSTANCES FOR COLOR DIFFERENTIATION . 71
A Golgi Method for Nerve Cells and Their Ramifications, 71; Memoranda, 72; Silver Nitrate Method for Nerve, 73; Gold Chloride Method for Nerve Endings, 74.

CHAPTER X. ISOLATION OF HISTOLOGICAL ELEMENTS. MINUTE DISSECTIONS **75**

Dissociation by Means of Formaldehyde, 75; Isolation of Muscle Fibers by Maceration and Teasing, 75; Maceration by Means of Hertwig's Fluid, 76; Minute Dissection and Mounting of Various Parts of Insects, 77; Memoranda, 78.

CHAPTER XI. TOOTH, BONE, AND OTHER HARD OBJECTS **79**

Sectioning Decalcified Tooth, 79; Sectioning Decalcified Bone, 79; Sectioning Bone by Grinding, 80; Memoranda, 80.

CHAPTER XII. INJECTION OF BLOOD AND LYMPH VESSELS **81**

Red Injection Mass, 81; Blue Injection Mass, 81; Injection with a Syringe, 81; Memoranda, 84.

CHAPTER XIII. OBJECTS OF GENERAL INTEREST: CELL-MAKING, FLUID MOUNTS, "IN TOTO" PREPARATIONS, ETC. **87**

Turning Cells, 87; Mounting in Glycerin (Water Mites, Transparent Larvae), 88; Killing and Mounting Hydra, 89; Mounting in Glycerin Jelly (Small Crustacea, etc.), 89; Mounting in Balsam (Flat Worms, Mosquito, Gnat, Aphid), 90; Opaque Mounts (Beetles, Wings of Moths and Butterflies, Head of Fly, Foreleg of Dytiscus), 92; Memoranda (Including Directions for Mounting Other Forms), 93.

CHAPTER XIV. BLOOD **97**

Examination of Fresh Blood, 97; Effects of Reagents, 97; To Demonstrate Blood Platelets, 97; Stained Preparation of Fibrin, 97; Crystals of Blood, 97; Cover-Glass Preparations (Dry), 98; Rapid Method, 99; Enumeration of Blood Corpuscles, 99; Observation of the Blood Current, 101; Inflammation, 101; Memoranda, 101.

CHAPTER XV. BACTERIA **105**

Bacterial Examination, 105; Cover-Glass Preparation from Fluid Media, 105; Staining and Mounting, 106; Bacteria in Tissues, 107; Methylen Blue Stain for Bacteria in Tissues, 107; Gram's Method for Bacteria in Tissues, 107; Hanging-Drop Preparations, 108; Memoranda, 108.

CHAPTER XVI. SOME EMBRYOLOGICAL METHODS: THE CHICK; SECTIONS AND WHOLE MOUNTS; OTHER FORMS **113**

The Chick, 113; Series of Chick Embryos for a Course in Embryology, 115; Measuring the Length of Embryos, 116; Orientation of Young Chick Embryos, 116; Preparation of Material for the Embryology of Teleosts, 117; Preparation of Material for the Embryology of Amphibia, 118; Early Stages of the Mammalian Embryo, 119; Older Stages of the Mammalian Embryo, 120; Maturation, Fertilization, and Segmentation in Mammals, 121; Artificial Fecundation (Echinoderms, Amphibia, Teleosts), 122; Early Cleavage in Living Material, 123; Polar Bodies, Fertilization and Early Cleavage in

Ascaris (Sections), 123; Directions for Orienting Serial Sections, 123; Orientation of Objects in the Imbedding Mass, 124; Human Embryos, 126.

CHAPTER XVII. RECONSTRUCTION OF OBJECTS FROM SECTIONS . . . 127
Reconstruction in Wax, 127; Geometrical Reconstruction, 129.

APPENDIX A. THE MICROSCOPE AND ITS OPTICAL PRINCIPLES . . . 133
Optical Principles, 133; Images, 135; The Simple Microscope, 136; The Compound Microscope, 137; Defects in the Image, 138; Nomenclature or Rating of Objectives and Oculars, 144; Some Common Microscopical Terms and Appliances (Alphabetically Arranged), 145; Manipulation of the Compound Microscope, 157.

APPENDIX B. SOME STANDARD REAGENTS AND THEIR USES 161
Fixing and Hardening Agents, 161; Stains, 170; Normal or Indifferent Fluids, 187; Dissociating Fluids, 187; Decalcifying Fluids, 189.

APPENDIX C. TABLE OF TISSUES AND ORGANS WITH METHODS OF PREPARATION 190

APPENDIX D. PREPARATION OF MICROSCOPIC MATERIAL FOR A COURSE IN GENERAL ZOÖLOGY 215

APPENDIX E. TABLE OF EQUIVALENT WEIGHTS AND MEASURES . . . 227

INTRODUCTORY

APPARATUS AND SUPPLIES REQUIRED

The student should provide himself with the following supplies:

One half-gross box best grade glass slides, standard size (25×75 mm.).

One-half ounce, 18 mm. or ¾ in., round cover-glasses, medium thickness, (o. 18 mm.).

Thirty, 25×50 mm. cover-glasses, medium thickness.

Two or three Pillsbury slide boxes (Fig. 1).

One box of labels for slides.

Three to six camel's hair brushes (Fig. 2).

Six pipettes (Fig. 3).

One set of dissecting instruments as follows:

- One large scalpel or cartilage knife (Fig. 4).
- One small scalpel (Fig. 5).
- Two needles (Fig. 6).
- One fine straight scissors (Fig. 7).
- One fine straight dissecting forceps, file-cut points (Fig. 8).
- One blow-pipe (Fig. 9).
- One section lifter (Fig. 10).

To which may well be added:

- One heavy scissors (Fig. 11).
- One curved scissors (Fig. 12).
- One heavy forceps (Fig. 13).
- One fine forceps, curved tips (Fig. 14).

One horn spoon.

One desk memorandum calendar.

Blank cards (about 75×100 mm.) for keeping records of experiments. The kind of card used for library card catalogue will do.

One section razor (Fig. 15).

A piece of moderately heavy copper wire with one end hammered out to a width of 7 to 10 mm.

Towels.

A glass-marking pencil (wax) or writing diamond will be found useful. See, however, Memorandum 21, chap. vi.

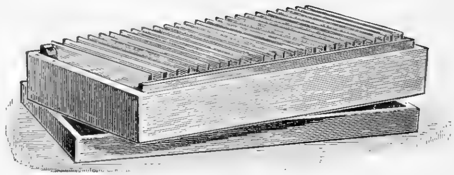


FIG. 1

FIG. 2

FIG. 3

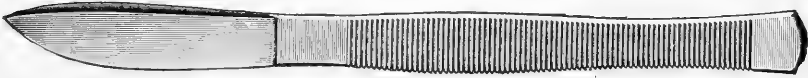


FIG. 4

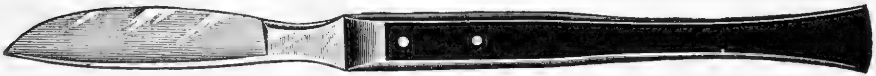


FIG. 5

FIG. 6

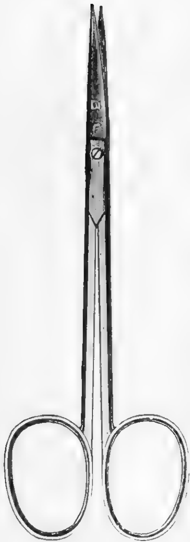


FIG. 7

FIG. 8

FIG. 9

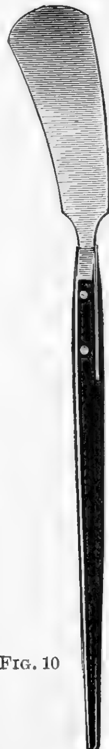


FIG. 10

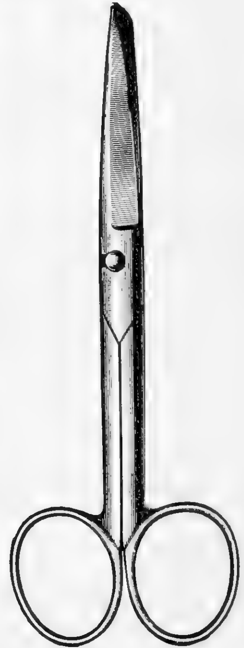


FIG. 11

Fig. 15

Fig. 16

Fig. 17

Fig. 12

Fig. 13

Fig. 14

Fig. 18

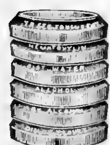


Fig. 19

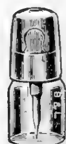


Fig. 20

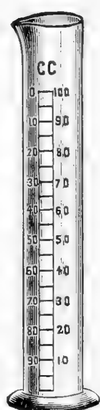


Fig. 21

Fig. 22

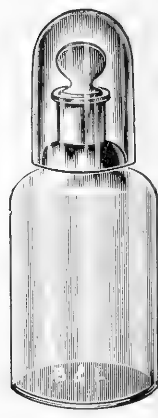


Fig. 23

Apparatus ordinarily supplied by the laboratory:

Desk with drawers.
Locker for microscope.
Compound microscope and accessories (Appendix A).
Dissecting microscope (Fig. 66).
Microtomes (Figs. 27, 28, 29, 32, 33).
Paraffin oven (Figs. 24, 25, 26).
Tall stenders (about 85 mm. deep). Each student should have at least eight (Fig. 16).
Coplin staining jars (Fig. 17). Tall stenders may be used instead. About eight are needed for each student.
Flat stenders (Fig. 18); half a dozen for each student.
Syracuse watch-glasses (Fig. 19); eight to each student
Balsam bottle (Fig. 20).
Graduated cylinders for measuring liquids (Fig. 21).
Wash-bottle (Fig. 22).
Celloidin bottle (Fig. 23).
Turntable (Fig. 36).
Injecting apparatus (Fig. 35).
Reagent bottles and vials.
Other apparatus and supplies such as bone-forceps, bone-saws, glass tubing, glass rods, beakers, burners, filter paper, funnels, evaporating dishes, sand bath, dropping-bottles, balances, mortar and pestle, etc.

For apparatus or supplies not listed in this book the student is referred to the illustrated catalogues of dealers and manufacturers such as: The Bausch and Lomb Optical Co., Rochester, N. Y.; The Ernst Leitz Optical Works, Wetzlar, Germany (American branch, 30 E. 18th St., New York City; or, 32 Clark St., Chicago); The Spencer Lens Co., Buffalo, N. Y.; Carl Zeiss Optical Works, Jena, Germany; R. & J. Beck, 68, Cornhill, London; The Kny-Scherer Co., New York City; Eimer and Amend, New York City; Whitall, Tatum and Co. (especially for glassware), New York City.

IMPORTANT GENERAL RULES

1. Keep everything clean!

2. Have a definite place in your desk for each piece of apparatus and arrange reagents in order on top of it.

3. Use cards for keeping records of materials. Each card should have a number corresponding to that of each special object or piece of tissue, and should show the name of the preparation, date, reagents used, time left in each reagent, in short, all data concerning the manipulation of the material.

4. Jot down in a blank calendar the various things to be done at future dates, such as changing of reagent on tissues, etc., and then go over this memorandum carefully each day when you first come into the laboratory.

5. Use only clean vessels in preparing reagents, and clean up all glassware while it is yet moist.

6. Reserve and mark a separate pipette for each of the chief reagents (absolute alcohol, oils, acids, etc.).

7. In making up solutions, 1 gram of a salt in 100 c.c. of liquid is reckoned ordinarily as a 1 per cent. solution, 3 grams as a 3 per cent. solution, etc. A saturated solution contains all of a given substance that the liquid will take up. When a solution is called for without specifying the solvent an aqueous solution is meant.

8. In weighing salts, always first put paper in the scale pans to protect them.

9. In making solutions or mixtures in which only a small amount of one reagent is used, after mixing, pour back some of the mixture into the small vessel and rinse it thoroughly in order to get all of the original contents out.

10. When pouring liquids from bottles keep the label of the bottle turned toward the palm of the hand. Do not lay down stoppers but hold them by their tops between the knuckles.

11. Before leaving the laboratory put away your instruments and clean and put in its place whatever laboratory apparatus you may have been using.

CHAPTER I

PREPARATION OF REAGENTS

The following reagents should be prepared by each student.

1. **Grades of Alcohol.**—To obtain a given per cent. of alcohol through dilution of a higher per cent. with distilled water, subtract the per cent. required from the per cent. of the alcohol to be diluted; the difference is the proportion of water that must be added. Thus, if 35 is the per cent. required, and 95 the per cent. to be diluted, then 95—35=60; hence, 60 parts of water and 35 parts of 95 per cent. alcohol are the proportions for mixing.

This means that in practice one needs only to fill the graduated measuring cylinder to the same number as the per cent. required (e. g., 35) with the alcohol to be diluted (e. g., 95) and then fill up to the per cent. of the latter with distilled water. In this way one would obtain 95 c.c. of alcohol of the per cent. required, if the measuring cylinder is graduated in cubic centimeters.

Prepare about 250 c.c. of 35, 50, 70, and 83 per cent. alcohols respectively, from 95 per cent. alcohol and distilled water. The commercial alcohol used, though really about 96 per cent., may be figured on the basis of 95 per cent.

Owing to the differences in the specific gravities of the different percentages of alcohol, the above method gives only approximate results; they are sufficiently accurate, however, for most biological work.

2. **Absolute Alcohol.**—It is customary in most laboratories to purchase so-called absolute alcohol specially prepared for laboratory purposes. Squibb's absolute alcohol (99.8 per cent.) is commonly used. Inasmuch as such alcohol is an expensive reagent, economy sometimes necessitates that the student undertake the more tedious process of making his own absolute alcohol. Crystals of copper sulphate are heated until the water of crystallization is driven off and the sulphate is left as a white powder. Such anhydrous sulphate is added to a bottle of commercial

(96 per cent.) alcohol. The water in the alcohol immediately unites with it, turning it blue. Anhydrous sulphate should be added until it no longer turns blue. The alcohol is then filtered into a clean, dry bottle which must have a tight-fitting cork or ground-glass stopper. It is well to smear the glass stopper with vaselin, so that when placed in the bottle, all moisture from the air may be completely excluded.

3. **Acid Alcohol.**—

Alcohol (70 per cent.)	99 c.c.
Hydrochloric acid (pure)	1 c.c.

For sections use the mixture only a few seconds or minutes. For material stained in bulk, add twice as much 70 per cent. alcohol and leave the object in it until sufficiently decolorized (2 to 24 hours).

4. **Ether and Alcohol.**—Absolute alcohol and sulphuric ether equal parts. Quantity, 250 c.c. Keep the ether distant from all flames.

5. **Normal Saline.**—Prepare a 0.7 per cent. solution of sodium chloride in distilled water. This is termed a normal salt solution because it is a solution of about the same density as natural lymph, and is much less harmful to living tissues than is distilled water. Quantity, 500 c.c.

6. **Formalin** (also termed formal, formol, formolose).—Commercial formalin is a 40 per cent. solution of formaldehyde in water. A 4 per cent. solution of *formalin* would be made by taking 4 volumes of commercial formalin and 96 volumes of water. This is, however, only a 1.6 per cent. solution of formaldehyde. Make a 10 per cent. solution of *formalin.* Quantity, 250 c.c.

7. **Gilson's Mercuro-Nitric Fixing Fluid.**—

Bichloride of mercury (corrosive sublimate)	5 grams
Nitric acid (approx. 80 per cent.)	4 c.c.
Glacial acetic acid	1 c.c.
Alcohol (70 per cent.)	25 c.c.
Distilled water	220 c.c.

Quantity, 250 c.c.

Caution.—In handling corrosive sublimate do not use metal instruments because it corrodes metal. Use a glass or horn spatula.

8. **Erlicki's Solution.**—

Bichromate of potash	5 grams
Sulphate of copper	2 grams
Distilled water	220 c.c.

Pulverize the crystals before adding the water.

9. **Gage's Carbol-Xylol Clearer.**—

Carbolic acid crystals (melted)	25 parts
Xylol	75 parts

If the carbolic acid does not dissolve in the xylol, increase the amount of the latter. Handle the acid with great care. Quantity, 250 c.c.

10. **Gage's Formaldehyde Dissociator.**—

Formalin, commercial	0.5 c.c.
Normal saline	250.0 c.c.

11. **Decalcifying Solution.**—

Nitric acid (strong)	10 c.c.
Alcohol (70 per cent.)	90 c.c.

12. **Borax Carmine Stain** (Grenacher's).—

Borax (4 per cent. aqueous solution)	100 c.c.
Carmine	1 gram

Heat until the carmine dissolves, then add 100 c.c. of 70 per cent. alcohol. Filter after 24 hours.

13. **Delafield's Hematoxylin.**—Prepare 100 c.c. of a saturated aqueous solution of ammonia alum. Dissolve 1 gram of hematoxylin crystals in 10 c.c. of absolute alcohol, and add it, drop by drop, to the first solution. Expose this mixture to air and light for several weeks (two months is not too long) to "ripen." (Ripening consists in an oxidation of the hematoxylin to form hematein. This may be accomplished at once with some degree of success through the addition of a few cubic centimeters of a neutralized solution of peroxide of hydrogen.) When ripe, filter the solution and add 25 c.c. of glycerin, and 25 c.c. of methyl alcohol (see memorandum 1). It is well to have a stock solution

of this stain already prepared to be used in case the student's preparation is not ready in time

NOTE.—*At this point the student should begin chapter iii in order that no time may be lost. The present chapter may then be completed while the tissues are becoming fixed and hardened.*

14. **Picro-Carmine.**—This stain is perhaps best procured ready made from dealers. If it is desired to make it, consult Appendix B, reagent 63. Quantity necessary, about 50 c.c.

15. **Bordeaux Red.**—

Bordeaux red	1.0 gram
Distilled water	100.0 c.c.

16. **Lyons Blue.**—

Absolute alcohol	100.0 c.c.
Bleu de Lyon	0.3 gram

17. **Eosin.**—

Eosin	0.5 gram
Alcohol, 95 per cent.	100.0 c.c.

18. **Iron Hematoxylin** (Heidenhain's).—Two solutions are used. They are not to be mixed.

Solution I.

Ferric alum (clear violet crystals)	2.5 gram
Distilled water	100.0 c.c.

Solution II.

Hematoxylin	0.5 gram
Distilled water	100.0 c.c.

The hematoxylin should ripen (see 13) for some three or four weeks.

19. **Safranin.**—

Safranin "O"	1.0 gram
Absolute alcohol	10.0 c.c.
Anilin water	90.0 c.c.

Make the "anilin water" by shaking up 5 c.c. of anilin oil in 90 c.c. of distilled water. Filter through a wet filter. Dissolve the safranin in the anilin water, then add the alcohol.

20. **Canada Balsam.**—Dry 2 grams of Canada balsam on a sand bath, or in a warm chamber until it becomes hard (1 to 2 hours at 65°C.). Do not overheat. When cool add enough xylol to make a thin, syrupy fluid. Roll a sheet of paper into a cone to serve as a funnel, and filter the fluid through absorbent cotton.

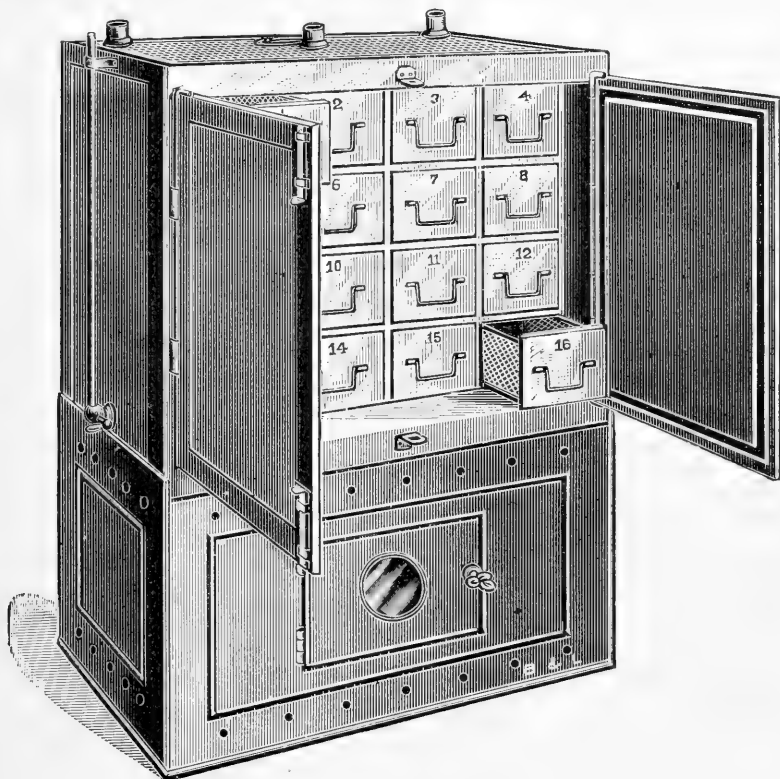


Fig. 24.—The Lillie Water-Bath.

The bath consists of a large chamber containing a series of drawers of equal size, 250 mm. long, 100 mm. wide, 80 mm. deep. Each drawer has copper front and bottom; the sides and back are perforated zinc, thus securing free circulation of warm air. The drawers are separated by perforated cross partitions and run on slides free from the lateral supports, thus permitting sufficient circulation of warm air to secure equal temperature in the top and bottom of the bath. Water gauge and tubulatures for gas regulator and thermometer are provided. This bath is especially adapted to class work, since each student may carry on his work in a separate drawer.

Thicken the solution slightly by leaving the cap off the bottle in a place free from dust, and allowing some of the xylol to evaporate. Or, fill your balsam bottle one-third full of the liquid xylol-balsam now on the market, and dilute to the proper consistency.

21. **Mayer's Albumen Fixative.**—Chop the white of an egg with scissors and filter it through moist filter paper. It filters through very slowly. Add an equal volume of glycerin, and a bit of salycilate of soda (1 gram to 50 c.c.) or thymol to prevent putrefaction.

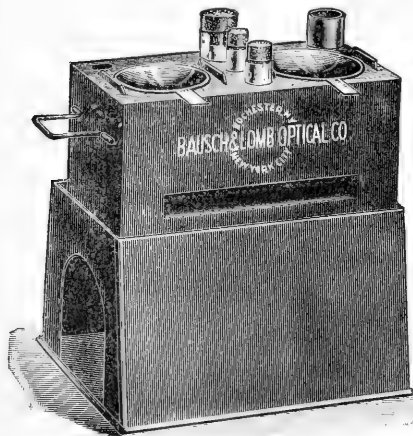


Fig. 25.—Simple Water-Bath.

This is a useful bath for individual workers. It is provided with imbedding-cups, infiltration vials, a shelf for watch-glass imbedding or for warming instruments, and tubulatures for gas regulator and thermometer.

22. **Celloidin.**—Put 5 grams of Schering's shredded or granular celloidin into a celloidin bottle (a bottle with glass stopper and ground glass cap) and dissolve it in equal parts of absolute alcohol and ether (see 4). Add only sufficient fluid (about 100 c.c.) to make a thick, syrupy mass. In a second celloidin bottle make a thin solution by taking about one-third of the original solution and diluting it with its own volume of the ether-alcohol. Label the bottles thick and thin celloidin, respectively.

23. **Paraffin.**—In one of the cups of a warm paraffin oven (Fig. 24, 25, or 26), put 75 grams of paraffin, melting at about 53° C. The bath should be kept at a temperature of some two degrees above the melting-point of the paraffin. A supply of softer and of harder paraffin (e. g., melting at 43° and 60° C.) should also be at hand.

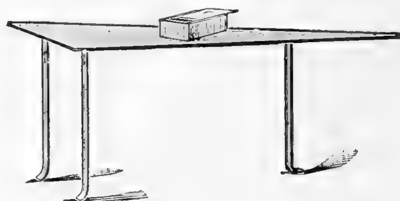


Fig. 26.—Imbedding-Table.

There should be two rectangular boxes (about $3 \times 3 \times 16$ cm.) to contain paraffin. When in use the boxes are so placed on the imbedding-table that the paraffin in one end remains melted; in the other, solid. Regulate the temperature by placing the flame at the proper distance under the acute angle of the table. It is best, when gas is used, always to turn on the gas completely and then regulate the height of the flame by means of a clamp on the rubber tubing which conducts gas to the burner.

Other Reagents.—Provide yourself with 200 c.c. of xylol, 25 c.c. of clove oil, 25 c.c. of glacial acetic acid, 50 c.c. of cedar-wood oil, 75 c.c. of chloroform, 30 c.c. of glycerin and 250 c.c. of absolute

alcohol if it has not already been prepared. Keep the absolute alcohol and the xylol carefully corked to exclude moisture. Before measuring out any of these reagents, see that both the graduate and bottle are perfectly clean and *dry*.

MEMORANDA

1. **Ethyl Alcohol** is the kind commonly used in histological laboratories. Upon presentation of the proper credentials to the internal revenue officers, it may be purchased by the barrel from distillers, tax free, by educational institutions. Such commercial alcohol is of about 96 per cent. strength. When the strength is unknown it should be tested by means of an alcoholometer (see 2, below).

Methyl Alcohol (called also wood alcohol or wood spirits) is cheaper than ethyl alcohol in case the latter cannot be had tax free, and is fairly satisfactory in most cases. It is poisonous and must be carefully handled. It is of about 90 per cent. strength.

Synthol is a manufactured product now on the market which seems to answer the purposes of ordinary absolute alcohol. It is designated as a synthetic alcohol by its manufacturers and is cheaper than absolute alcohol.

Rectified Spirit is a 91 per cent. alcohol (84 per cent. in England).

2. **The Alcoholometer** is a convenient instrument for determining the strength of alcohol, or the percentage of absolute alcohol in a spirituous mixture. It is a kind of hydrometer with a scale marked to indicate the percentages of alcohol. Different strengths of alcohol have different specific gravities, consequently, the instrument will float higher or lower in the liquid depending upon the percentage of alcohol present. The number on the scale just at the surface of the liquid indicates its strength.

3. **Rule for Dilution** of a given strength of a solution with a lower per cent. of the same solution. (For where the diluent is water, i. e., zero per cent., see rule under reagent 1.) Subtract the per cent. required from the per cent. of the solution to be diluted; also subtract the per cent. of the diluent from that of the strength required. The differences are the relative proportions of the diluent and the solution to be diluted that must be used. Thus, to prepare a 35 per cent. solution from 95 and 20 per cent. solutions: $95-35=60$; $35-20=15$; hence, 60 to 15, or 4 to 1 are the proportions desired. That is, 4 parts of the 20 per cent. and 1 part of the 95 per cent. solution must be used to obtain a 35 per cent. solution.

4. "**To Remove Fixed Stoppers**, take the bottle in the left hand with the forefinger applied to one side of the stopper, then tap the other side of

the stopper with some heavy instrument, such as the handle of a pocket-knife, pressing the forefinger against the direction of the tap. Turn the bottle round, gradually tapping until the stopper loosens. Should this device prove of no avail (which is very rarely), hold the neck of the bottle in a spirit flame, and quickly withdraw the stopper as the glass of the neck expands. This is a somewhat risky procedure, but is very effectual if done smartly." (*Journal of Applied Microscopy*, Vol. VI, p. 2116.) The glass of the neck may be more safely heated by looping a heavy cord about it and sawing the cord back and forth until the friction warms the glass.

CHAPTER II

GENERAL STATEMENT OF METHODS

Each of the reagents which has been prepared is used for one or more of the purposes to be discussed in this chapter.

All methods of preparation in microscopy are to enable us to learn more of the structure and functions of objects than would otherwise be apparent. We endeavor to study them in as near their natural condition as possible. While the study of living or of fresh material is desirable it can be carried on only to a very limited extent. Most structures of the animal body, though opaque, must be examined largely by transmitted light, hence, special preparation is necessary to put them into suitable condition. This is accomplished—

1. By cutting them into thin slices (*section method*).
2. By separating them into their elements (*isolation*)—
   a) Mechanically (*teasing*), or
   b) With the aid of fluids which remove the cement substance (*dissociation* or *maceration*).

In most instances, the minute structure of a tissue or of an organism can be studied to the best advantage only after the application of certain agents which serve to emphasize the various structural elements. A tissue so prepared is an artificial product in that it is not exactly the same as it was in the living organism, but recent studies of protoplasm in the living condition by competent investigators strengthen the belief that many reagents preserve very faithfully the actual structure of the cell contents. The liquid albuminoids are apparently the materials which suffer the greatest modifications. Since alterations do occur, however, it is clear that in our interpretations of prepared material we must reckon carefully with both the original nature of the object and with the factors introduced by ourselves.

KILLING, FIXING, AND HARDENING

The first step in the preparation of tissues ordinarily is the employment of some reagent which will kill the tissues and fix their various components in the characteristic stages of their activities. Such material may then be preserved indefinitely for future use.

It is customary to discriminate between killing, fixing, and hardening, although the same reagent may fulfil all three requirements. Killing refers particularly to the destruction of the life of the tissue, a process which may be either slow or instantaneous. In slow killing it is usual to employ narcotics such as ether, chloroform, chloral hydrate, chloretone, carbon dioxide, nicotin, cocain, or weak alcohol. Ice is also used sometimes. Such methods are of particular value with highly contractile animals which are desired in the extended condition. Such forms are narcotized completely or until they are unable to contract and then frequently fixed and hardened in other or stronger fluids. Where practicable, instantaneous killing and fixing is preferable because tissues have then no time to undergo postmortem changes. The same fluid ordinarily is employed for killing and fixing.

The purpose of fixation is—

a) To preserve the actual form of tissue elements.

b) To produce optical differences in structure, or so to affect the tissues that such differences will be brought out through subsequent treatment with stains or other reagents.

To accomplish this the fixing agent must possess the following qualities:

1. It should kill the tissue so quickly that few structural changes can occur.

2. It should neither shrink nor distend the tissue.

3. It should be a good preservative; that is, it must render the tissue elements insoluble and prevent postmortem changes.

4. It should penetrate all parts equally well.

5. It should put the tissue in condition to take stains unless it of itself produces sufficient optical differences in the various parts of the tissue.

No ideal single reagent has been discovered which meets all of these requirements, hence it is customary to combine two or more reagents which individually possess certain of these desirable qualities. All of the best fixing reagents are mixtures. For example, acetic acid is very generally used in fixing mixtures because it penetrates well, produces good optical differentiation, and counteracts the tendency of some reagents (e. g., corrosive sublimate) to shrink tissues. Again, osmic acid, which is an excellent fixing agent for very small pieces of tissue, penetrates very poorly; consequently for most objects it must be mixed with reagents which penetrate rapidly and thoroughly.

Some fixing agents (corrosive sublimate, chromic acid, osmic acid, etc.) enter into chemical combination with certain of the tissue elements, others (alcohol, picric acid, nitric acid, hot water, etc.) act by coagulating or precipitating certain constituents of tissues.

The chief object of *hardening* is to bring tissues to the proper consistency for cutting sections. The process, although begun ordinarily by the fixing agent, is usually completed in alcohol. Some objects are not sufficiently hardened until they have remained in alcohol for many hours, or even days. As a rule, tissues should remain in alcohol of at least 70 per cent. strength for a minimum of 24 hours after the preliminary operations of fixing, washing, etc., before they are subjected to further treatment.

WASHING

Fixing agents ordinarily, with the exception of alcohol, must be washed out thoroughly or they are likely to interfere with subsequent processes. Aqueous solutions are washed out usually in water or a low per cent. of alcohol; alcoholic solutions, with alcohol of about the same strength as that of the fixing agent. Washing usually requires from 10 to 24 hours, with several changes of the liquid. If water is the washing agent it is best where practicable to use running water.

Chromic acid and its compounds should be washed out in running water. This should be done in the dark in order that precipitation may be avoided.

Picric acid, or solutions containing it, must be washed in strong alcohol (70 per cent.), never in water because the latter seems to undo the work of fixation.

Corrosive sublimate and mixtures containing it are washed out in water or alcohol. A little tincture of iodine should be added to the wash from time to time to insure the removal of all corrosive sublimate crystals. Sufficient iodine has been added when it no longer loses its reddish color after being in contact with the preparation for a short time.

Osmic acid and mixtures containing it should be washed in running water.

DEHYDRATING

While under certain circumstances objects may be mounted in aqueous media for examination, in the majority of cases, especially where the preparation is to be a permanent one, it has been found best to remove all water from the tissues, that is, to *dehydrate* them. This renders preservation more certain, and it is a necessity, moreover, if the object is to be imbedded later in paraffin or celloidin, for neither of these substances is miscible with water. Because of its strong affinity for water and the ease with which it may be manipulated, alcohol has come to be used universally for this purpose. It completes the process of hardening at the same time. The dehydration must be gradual. In tissues transferred from water or aqueous solutions directly to strong alcohol (or vice versa) violent diffusion currents are set up which produce serious distortion of the tissue elements. For this reason a series of alcohols of gradually increasing strength (e. g., 35–50–70–83–95 per cent.) is used. The more delicate the object, the closer should be the grades of alcohol.

PRESERVING

After fixing and washing, the process of dehydration is begun ordinarily and tissues are carried as far as 70 per cent. alcohol. It is customary to leave them in alcohol of from 70 to 83 per cent. strength until they are needed. They may remain here indefinitely. If they are to be preserved for a long time (for

months), however, it is better to keep them in a mixture of equal parts of glycerin, distilled water, and strong (commercial) alcohol.

STAINING

A few fixing agents produce sufficient optical differentiation in tissues, but as a rule this must be accomplished through the addition of certain stains. Most of the stains used have more or less of a selective action; that is, they pick out certain elements of the tissue, and thus enable one to see details of structure that would otherwise be invisible. Their action, however, depends largely upon the nature of the fixing agent which has previously been used. The secret of good staining, indeed, lies largely in proper fixation.

There are large numbers of stains of very different chemical constitution (acid, neutral, and alkali), and they may act in very different ways upon the material to be stained. For example, some show affinity only for certain elements of the nucleus, others for the cytoplasm of cells, and some are present in tissues only physically as deposits, while others enter into chemical combination with certain of the cell constituents. A few, such as borax-carmine, are general stains, and affect to a greater or less degree practically all the tissue elements.

It is not the purpose of the present book to enter into a prolonged discussion of the theory of staining or to undertake a description and classification of stains. For this the reader is referred to the excellent compendium of Lee (*The Microtomist's Vade-Mecum*).

The stains of widest application are (1) the **Carmines,** (2) the **Hematoxylins,** (3) the **Anilins,** and (4) **Metallic substances.**

Carmine is a brilliant scarlet or purplish coloring matter made from the bodies of the cochineal and kermes scale insects. The carmine stains, including cochineal, have been largely used in the past for all kinds of work, but at present they are used more particularly for staining objects in bulk before sectioning, or objects which are not to be sectioned. They are easy to use, and will follow almost any fixing agent. In case of over-staining, weak

hydrochloric acid (0.1 to 1 per cent.) is used to decolorize the tissues. For formulae see Appendix B.

Hematoxylin is a compound containing the coloring matter of logwood. The hematoxylins follow well almost any of the fixing agents; they are especially recommended after fluids containing chromic acid or its salts. According to Mayer, the active agent in these stains is a compound of hematein with alumina. The hematein is produced by the oxidation of hematoxylin. The so-called "ripening" is simply this change, which is brought about by exposing the hematoxylin solution to air. If the pure hematein is used in making the stain, therefore, the latter will be ready for use immediately, because it need not undergo the ripening process (see reagent 47, Appendix B). For formulae see Appendix B.

Anilin is a colorless coal-tar derivative, and is the base from which many of the numerous coal-tar dyes are made. The anilins are brilliant stains of all colors. They are used almost exclusively for staining sections or thin membranes, and are of great service to the microscopist, although, as a rule, they fade in time.

The basic anilin stains, such as methyl green, methyl violet, gentian violet, methylen blue, safranin, Bismarck brown, toluidin blue, and thionin are usually nuclear stains. On the other hand, the acid anilin stains, such as acid fuchsin, eosin, erythrosin, light green, orange G, bleu de Lyon, nigrosin, benzopurpurin, and aurantia are ranked as cytoplasmic stains. These stains must be made up fresh every two or three weeks, as they frequently spoil if kept much longer.

The metallic substances used for color differentiation operate principally as *impregnations* rather than as stains. The coloring matter is held physically as a precipitate or reduction product in certain of the tissue elements. The commonest reagents of this class in use are silver nitrate and gold chloride.

The different tissue elements frequently show affinity for different stains, consequently it is a common practice to use more than one stain. Very decided contrasts may thus be produced, such as red and blue, red and green, green and orange, etc. It

is not uncommon, in fact, to have triple and even multiple staining. In such staining, the stains are sometimes applied consecutively; in other cases, at different points in the process of general manipulation. Sometimes all the stains may be mixed together, so that immersion of the sections in one liquid is all that is required for double or multiple staining.

A general rule in staining, especially for entire or bulky objects, is that the specimen should be transferred to the stain from a reagent in which the percentage of water is approximately the same as that of the stain. The same is true when the object is removed from the stain. For example, if the stain to be used is an aqueous solution, the object should enter it from an aqueous solution; if the stain is made up in 95 per cent. alcohol, the object should enter from 95 per cent. alcohol, etc. For reasons see "dehydrating."

CLEARING

In the vast majority of cases tissues are too opaque for satisfactory examination until they have been treated with certain clarifying reagents or *clearers* which render them more transparent.

Such reagents as glycerin, glycerin-jelly, etc., are used when the object is to be cleared, without alcoholic dehydration, directly from water. Usually, for permanent preparations, the alcoholic dehydration method is employed and it then becomes necessary to use a clarifying reagent which will replace the alcohol and facilitate the penetration of the final mounting-medium (balsam or damar).

Perhaps the most useful and rapid clearer is xylol. Xylol, however, is very sensitive to moisture and if the preparation has not been thoroughly dehydrated the final mount will appear milky. For this reason the beginner is recommended to use a carbol-xylol mixture (see reagent 9, chap. i). Carbolic acid has a great affinity for water, and the mixture will therefore clear preparations that are not fully dehydrated. Cedar-wood oil, though somewhat slower than xylol, is one of the best clearers. It is also one of the safest, because tissues may be left in it

indefinitely. Other good clearers after alcohol are oil of origanum, sandal-wood oil, oil of cloves, toluol, oil of bergamot, anilin oil (for watery specimens), carbolic acid (for watery specimens), and beechwood creasote. Clove oil should not be used for celloidin sections because it dissolves celloidin. It is also inapplicable ordinarily after most anilin dyes because of its tendency to extract them. Among the best reagents for celloidin sections are cedar-wood oil, carbol-xylol, oil of origanum, creasote, and Eycleshymer's clearer (memorandum 4, chap. vii).

While "clearing" refers especially to the rendering transparent of tissue elements, and *dealcoholization* to the removal of alcohol previous to imbedding in paraffin, very frequently the same reagent is used for either purpose and the term "clearing" has come to be used in either sense.

MOUNTING

After tissues have been cleared the final step is to mount them in some suitable medium for preservation and inspection.

If tissues are to be mounted directly from water or aqueous media, glycerin, glycerin-jelly, or Farrant's solution is used ordinarily. If the alcoholic dehydration method is employed, balsam or gum damar is the final mounting medium. The balsam or damar is dissolved commonly in xylol, although turpentine, chloroform, or benzol may be used as the solvent. Xylol-balsam is the most satisfactory for ordinary purposes.

IMBEDDING

In order to section tissues or objects satisfactorily it is frequently necessary to imbed them in a suitable matrix. *Simple imbedding* consists in merely surrounding the object by an appropriate medium to hold it in place while it is being cut. In *interstitial imbedding* the object is saturated (*infiltrated*) with the imbedding substance which, when all cavities and intersticies are filled, is caused to set; thus it supports all parts of the tissue and holds the components in place when sections are made. Infiltration imbedding is of great importance to microscopists and much of the space of the present book is given up to

drilling the student in the details of the two chief infiltration methods, viz., the *paraffin method* and the *celloidin method.* Infiltration with gum is also not infrequently resorted to, especially for tissues which would be injured by alcohol, or for sectioning by the freezing method.

Paraffin is a translucent, waxy material derived from various sources, one of the commonest of which is crude petroleum. Paraffins of low and of high melting-points, termed respectively soft and hard paraffin, should be kept on hand so that mixtures of different degrees of hardness may be made up as necessity demands.

Celloidin is a form of pyroxilin (gun cotton or collodion cotton) specially prepared for interstitial imbedding. It is dissolved in a mixture of ether and alcohol (chap. i, reagent 4) and solutions of two or three strengths are used for infiltration. For details see the method, chap. vii. Collodion instead of celloidin is used by some workers (see memorandum 11, chap. vii).

AFFIXING SECTIONS

When mounting sections upon a slide, especially if they are yet to be stained, it is usually necessary to affix them firmly to the slide to prevent later displacement. For paraffin sections Mayer's albumen fixative (reagent 21, chap. i), or a combination of this method with the water method, is most widely used. The water method alone often proves adequate, particularly with thin sections. The slide is flooded with water and the sections are floated upon its surface. As the layer of water evaporates the sections are slowly drawn down into close contact with the slide. When perfectly dry they are usually so firmly affixed that they will not become detached even after the removal of paraffin from them. It is common, however, and safer to use a thin film of albumen fixative as a cementing substance between the water and the surface of the slide.

In the case of celloidin sections, if only one or a few sections are to be mounted on one slide, it is a common practice to stain the sections and transfer them through the various reagents, even

to clearing, before mounting them on the slide. In such cases the sections need not be fixed to the slide. With serial sections, however, the sections must be held in place some way during their transition through the reagents (see memorandum 12, chap. vii). Unlike paraffin, the celloidin is not ordinarily removed from the tissues.

DECOLORIZING

Not infrequently in staining the tissue becomes overstained and requires that some of the color be extracted from certain of the elements to bring about a proper differentiation. The fact that certain tissue elements retain stain more tenaciously than others is sometimes taken advantage of and overstaining followed by decolorization is practiced intentionally. Alcohol slightly acidulated with hydrochloric acid (0.1 to 1 per cent.) is commonly used for the extraction of surplus color. In special cases other decolorizers are used: for example, iron-alum in the iron-hematoxylin method (reagent 18, chap. i).

BLEACHING

In some cases, tissues are obscured because of the presence of natural pigments or on account of blackening caused by the fixing reagent. Such tissues must be bleached. Chlorine, peroxide of hydrogen, or sulphurous acid are commonly employed. A method is given in chap. v, memorandum 12.

CORROSION

To obtain skeletal structures, as for example the spicules of sponges or the hard parts of insects, various methods of corrosion are employed. Nitric acid, caustic potash, caustic soda, eau de Javelle are reagents often used for this purpose. Corrosive preparations of injected vessels and cavities may also be made.

DECALCIFICATION AND DESILICIDATION

Tissues impregnated with lime salts or with silica must have such hard parts removed usually before they can be sectioned. For decalcification, one of several acids may be used. The details are given in the chapter on bone, tooth, etc. (chap. xi). For decalcifying reagents, see Appendix B, v.

Where desilicidation is necessary hydrofluoric acid may be employed, although, because of its property of attacking mucous membranes, its use is attended with more or less danger for the operator. It is added drop by drop to the tissue which has previously been placed in a paraffin-coated vessel (the acid attacks glass). If the tissue is not too heavily impregnated with silica, it is safer to use an old section razor and try to cut sections without previously treating them with hydrofluoric acid.

INJECTION METHODS

The injection of colored masses into the blood vessels and other vessels of the body is frequently practiced to aid in determining their distribution and their relation to the surrounding tissues. The dye is termed the *coloring mass* and the substance to which it is added, the *vehicle.*

ISOLATION OF HISTOLOGICAL ELEMENTS

Isolation is one of the most valuable means of forming a correct conception of cells and fibers. It has the advantage over sections that the elements may be inspected in their entirety and from all sides. The separation is accomplished, as already noted, by (1) reagents which dissolve or soften cell cement and interstitial material without seriously affecting the cells (*maceration* or *dissociation*), or (2) mechanically by means of dissecting needles (*teasing*), or both. Hardening and fixing reagents in general if diluted to about one-tenth are efficient for dissociation. Gage recommends normal saline as preferable to water for dilution. The dissecting microscope or some kind of lens-holder and lens are valuable aids in isolating tissue elements. For practical methods consult chap. x; for reagents, Appendix B, iv.

NORMAL OR INDIFFERENT FLUIDS FOR EXAMINING FRESH TISSUES

It is desirable frequently to examine fresh material in as near a natural condition as possible, hence recourse is had to the so-called indifferent fluids. While not wholly indifferent, they ordinarily produce but slight changes in tissues and their elements from the view-point of the microscopist. The liquids most commonly used for this purpose are discussed in Appendix B, iii.

GENERAL SCHEME FOR MOUNTING WHOLE OBJECTS (*IN TOTO PREPARATIONS*) OR SECTIONS

Whole Objects (for balsam mounts)

Killing and fixing
|
Washing
|
Staining
|
(Decolorizing if necessary)
|
Dehydrating
|
Clearing
|
Mounting

Section Methods (paraffin and celloidin)

Killing and fixing
|
Washing
|
(Staining, if to be stained in bulk)
|
Hardening and dehydrating
|
Absolute alcohol

Paraffin Method

Dealcoholization (xylol)
|
Melted paraffin
|
Imbedding
|
Sectioning
|
Affixing sections
|
Removal of paraffin
|
Absolute alcohol
|
Clearing
|
Mounting

If not stained in bulk (from Sectioning to Mounting):
Through alcohols to stain
|
Staining
|
Washing
|
Dehydrating (and decolorizing if necessary)

Celloidin Method

Ether-alcohol
|
Thin celloidin
|
Thick celloidin
|
Imbedding
|
Sectioning *
|
Dehydrating to 95 per cent. alcohol
|
Clearing
|
Mounting

If not stained in bulk (from Sectioning to Dehydrating):
Staining
|
Washing (and decolorizing if necessary)

* If sections are to be arranged serially they must be affixed to the slide as soon as cut.

CHAPTER III

KILLING AND FIXING

CAUTIONS.—1. *Use only fresh tissues and work rapidly so that the tissue elements will not have time to undergo postmortem changes.*

2. *Remove organs carefully, and avoid crushing or pressing the parts to be prepared.*

3. *Tissues should never be allowed to dry from the time they leave the animal until they are finally mounted for microscopical examination except at one point in the paraffin method.*

4. *Use only small pieces (2 to 6 mm. cube) of tissue whenever possible, or penetration of the reagent will be insufficient. Embryos and small objects up to 4 cm. in size may be placed entire in certain of the fixing fluids.*

5. *For fixing and hardening, the bulk of the fluid should be from 10 to 50 times that of the object. Too many pieces should not be placed in the same vial.*

6. *Use only clean reagents. It is well to let the object rest on a bit of cotton in the bottom of the vial or have it suspended from the vial mouth so that the reagent may penetrate equally from all sides. Penetration is aided by heat.*

7. *When necessary to wash fresh tissue, it is usually best to use normal saline, and not water. Let it flow gently over the surface of the object or slowly twirl the latter in the fluid. Do not scrape off foreign matter.*

8. *In many cases the killing and fixing reagent does not harden the tissue sufficiently and the hardening process must be completed in alcohol.*

9. *Keep the reagents and preparations from direct sunlight.*

10. *Carefully label each vessel containing tissue. State the contents, the fluid used, and the date. Label on the side.*

11. *Keep a careful record on cards of the reagents used, and the time when changed, for each separate piece of tissue.*

PRACTICAL EXERCISE

Kill a frog by placing it under a bell jar which contains a bit of cotton saturated with chloroform. Open the body as soon as possible after death and secure the tissues specified below.

1. **Alcohol Fixation.**—Remove the dorsal aorta and small pieces of the liver and harden in absolute alcohol (at least, not less than 95 per cent.) in a vial or small bottle. The tissue will be ready for further treatment in two days.

Larger pieces of tissue require longer time. The pieces should be thin. Change the alcohol every day for the first three days.

Alcohol is in many instances an unsatisfactory fixing reagent, but it is frequently employed because it is usually at hand and is easily manipulated. Hot absolute alcohol is very often used for insects. If absolute alcohol is used, the fixation may be fairly good, but because of the expense attached to the best absolute alcohol, the lower percentages are more frequently used. They shrink protoplasm, however, and are not to be recommended for the finer histological work. Ninety-five per cent. alcohol is as low as should be used for fixing, although 70 per cent. is sufficient to preserve specimens for other than microscopical work. Acetic acid (Appendix B, 2) is used with alcohol sometimes to increase penetration and to counteract its tendency to shrink tissues. The mixture is usually preferable to alcohol alone.

2. **Fixing with Gilson's Mercuro-Nitric Mixture.**—Place small pieces of liver, kidney, pancreas, esophagus, cardiac and pyloric ends of the stomach, apex of the heart, bladder, testis or ovary, and tongue in Gilson for from two to six hours. Remove a piece of intestine about 12 mm. long, and after washing it thoroughly in normal saline place it in a small vial containing about fifty times its bulk of fixing mixture and leave it for two hours. After fixation wash the objects thoroughly in water followed by 35 and 50 per cent. alcohol (15 minutes each), and preserve them in 70 per cent. alcohol. Read remarks on washing out corrosive sublimate, Appendix B, reagent 13, caution 1.

Gilson's is an excellent general reagent and gives a very delicate fixation. It is perhaps the most satisfactory killing and fix-

ing reagent that the beginner can use. The time which objects should be left in the fluid varies from ten or fifteen minutes for very delicate objects to six hours for larger or denser tissues, although many objects may be left for thirty-six hours without injury. When an object becomes opaque throughout it is sufficiently fixed. This holds true of other corrosive sublimate fixing fluids. Corrosive sublimate alone is also widely used as a general reagent. See caution 2 under reagent 13 in Appendix B.

3. **Fixing with Erlicki's Fluid.**—Remove a small piece of the spinal cord 1 cm. in length and place it in about one hundred times its volume of Erlicki's fluid. Likewise place the brain in this fluid. The spinal cord must remain about five days and the brain a week or ten days in the liquid. At the end of this time transfer the object to 35 per cent. alcohol, keeping it in the dark for two hours to avoid precipitation. The alcohol should be changed occasionally during this time. Repeat the process using 50 per cent. alcohol, and finally preserve the material in 70 per cent. alcohol.

Erlicki's fluid is an excellent reagent for general use, and is especially valuable for voluminous objects such as advanced embryos. Its principal drawback is the length of time required properly to harden objects (ten days to three weeks for objects larger than the above tissues). The process may be hastened by keeping the fluid containing the tissue at the temperature of an incubator (39° C.).

4. **Formalin as a Fixing Reagent.**—Place a piece of spinal cord, liver, and fragments of muscle in which nerves terminate in 10 per cent. formalin and leave until needed for work later. Formalin in varying percentages is widely used for the preservation and fixation of specimens for dissection. It is especially serviceable for the central nervous system. Most specimens may remain in it indefinitely without injury. For simple preservation, solutions ranging from 2 to 5 per cent. are adequate, but for fixation, it should be stronger (10 per cent.). Entire human brains may be fixed and hardened in a 10 per cent. solution with fairly good results.

MEMORANDA

1. **Tissues Are Preserved in Alcohol** of from 70 to 85 per cent. strength, but if they are to remain several months it is better to preserve them in a mixture of equal parts of glycerin, distilled water, and 95 per cent. alcohol.

2. **Hardening.**—Read carefully the remarks on hardening in chap. ii.

3. **Tissues Should Not Be Left in the Fixing Agent** longer, ordinarily, than is necessary to get results. Some, however, require a long time to bring out the optical differences of their elements. Experience alone can teach the time required in a given case. Such a reagent as formalin kills, fixes, hardens, and preserves, all at the same time.

4. **For Transferring Small Objects** through reagents the method of Walton is an excellent one. For the several reagents, he uses shell vials which measure about 10 cm. in height by 3 cm. in diameter. Through the center of a flat cork which fits the vials, a hole is made and a glass tube (about 9 cm. by 1.5 cm.) is inserted so that its lower end dips well into the reagents in the vials. The lower end of the tube is closed with fine-meshed cloth and the objects are placed within the tube. To transfer the objects one simply removes the cork bearing the tube, and inserts it in the vial containing the desired reagent. The upper end of the tube may be closed with a cork of the proper size. To avoid disturbance from changes in air pressure a small hole should be bored in the side of the tube just below the lower level of the larger cork. The vials are supported as indicated in memorandum 5.

5. **Shell Vials, Small Bottles,** etc., when in use are best supported in shallow auger holes of proper size in thick blocks of wood.

6. **Material Which Is To Be Kept Indefinitely** should be put in tightly stoppered vials in a place away from strong light. It is best to pack the vials in a museum jar on cotton and then seal the jar securely to prevent evaporation. Material is even more secure if the museum jar is partly filled with alcohol; in such a case each small vial should have a label of the contents placed within it.

Another way to prevent evaporation from vials or bottles is to "cap" them with a suitable varnish (see 7).

7. **To Seal Bottles and Preparation Jars** ("bottle-capping") dip the stopper and part of the neck in collodion varnish made as follows:

Pyroxylin	1 oz.
Ether	6 oz.
Alcohol	8 oz.

When the pyroxylin has completely dissolved add 2.5 drams of camphor. (From *Pharmaceutical Era*, Vol. XXX, p. 528.)

8. **For the Preservation of Anatomical Specimens** for other than histological purposes, Galt (*The Lancet*, Nov. 16, 1901, p. 1334) recommends the following fluid as superior to the well-known Kaiserling's fluid.

Sodium chloride	5 parts
Potassium nitrate	1 part
Chloral hydrate	1 part
Water	100 parts

Wash fresh tissues for several hours in running water, then "set" in an excess of methyl alcohol to which 0.5 per cent. formalin has been added (time required: six hours to a week according to nature and size of specimen). Next transfer the specimen directly to the preserving fluid, changing the latter after two or three weeks if necessary. In case the preparation is not sealed, sufficient water to make up loss by evaporation must be added occasionally. Specimens are said to retain their natural colors.

The following mixture, recommended to the author by Professor Kincaid of the Washington State University, has given most excellent results. To a mixture of equal parts of glycerin and strong alcohol sufficient formalin is added to make the whole about a 2 per cent. formalin. Specimens remain perfectly flexible in this mixture, and, indeed, after they have become thoroughly saturated, many forms (crustacea, insects, etc.) may be removed and kept as dry specimens which still retain their flexibility.

CHAPTER IV

SIMPLE SECTION METHODS

FREE HAND SECTION CUTTING

This method is important because it requires no costly appliances; although the sections are not as accurately cut as when mechanical aids are used, the method is simple, rapid, and adequate for the more general histological and pathological work.

1. The section razor is flat on one side (the lower), and hollow ground on the other (Fig. 15). *It must be sharp.*

2. A shallow glass dish or watch-glass partly filled with water is also necessary. Before making a section dip the razor flatwise into the liquid, or use a camel's hair brush; see that the upper surface is well flooded.

3. Sit in such a way that the fore-arm may be steadied against the edge of the table.

4. Use a piece of liver which was fixed in formalin, first rinsing it in water. Take the tissue between the thumb and forefinger of the left hand, and hold it in such a way that a thin slice may be cut by drawing the knife along the surface of the forefinger.

5. Rest the flat surface of the knife upon the forefinger, and, beginning at the heel of the knife, carefully draw the blade toward you diagonally through the tissue, slicing off a thin section of as uniform thickness as possible.

6. As each section is cut, float it off into the water; if it adheres to the blade, remove it by means of a wet camel's hair brush.

7. Practice until very thin sections are obtained, then place the dish upon a black surface, and with a needle or section lifter transfer the thinnest and best sections, if only fragments, to a watch-glass containing water.

NOTE.—In case the tissue has been preserved in alcohol, cut the sections under 70 per cent. alcohol instead of water, then transfer them to 50 and 35 per cent. alcohol successively and finally to water, leaving them in each liquid from 3 to 5 minutes.

8. Next, place the sections in about 3 c.c. of Delafield's hematoxylin diluted with an equal volume of water, and leave them for various lengths of time (3, 7, 12 minutes) to determine the time for successful staining.

9. Transfer the sections from the stain to tap water, and gently move them about for from 5 to 10 minutes to wash out the excess of the stain. If the sections are still overstained, place them in 5 c.c. of distilled water to which 3 drops of acetic acid have been added. Leave for 5 minutes, or until they become lighter in color, then wash in several changes of tap water until they have again become blue.

10. Remove the sections from the water and transfer them through 35, 50, 70, 85, and 95 per cent. alcohol successively, leaving them from 3 to 5 minutes in each, and lastly transfer them to absolute alcohol for 10 minutes, and finally to carbol-xylol for 10 minutes, or until clear.

11. Select one or two of the best sections and transfer them to the center of a clean glass slide. After straightening them out properly, drain off the excess of the carbol-xylol, and before the sections can become dry, add a drop of Canada balsam. Carefully lower a *clean* cover-glass (for cleaning see memorandum 14, chap. vi) on to the balsam. There should be just sufficient balsam to spread evenly under the cover without exuding around the edges.

12. Label, stating card number, name of the preparation, and other data that it is desired to add (see chap. vi, i, step 10).

13. Carry one of the pieces of stomach prepared in Gilson through the same treatment. The sections should be transverse sections of the stomach wall.

14. Clean up all dirty glassware *immediately*.

MEMORANDA

1. **The Thinnest Sections** are not always the best. For a general view of an organ, large, comparatively thick sections are usually better; for details of structure, thin sections.

2. **Small Pieces of Tissue** may be cemented to a cork if too small to hold conveniently between thumb and forefinger. A piece of stout copper wire is heated for a moment in the flame and touched to a bit

of paraffin. As the paraffin melts transfer drops of it to the edge of the tissue, which has been previously placed on the cork. The paraffin cools and holds the tissue fast.

Another and better method of handling a small object is to imbed it in a piece of hardened liver. In sectioning, the liver as well as the object is sliced, but they readily separate when placed in alcohol. Beef liver or dog liver is prepared for such purposes by hardening pieces about 5×2×2 cm. in size in 95 per cent. alcohol for 24 hours, and then transferring to fresh 95 per cent. alcohol until needed. When much hand sectioning is to be done, a supply of hardened liver should be kept on hand. Many small objects may be held between pieces of pith, and successfully sectioned.

3. **Well Microtomes** (Fig. 27) are inexpensive instruments which are used for simple sectioning. Such a microtome consists of a tube in which the object is placed, and at one end of which is a plate to guide the razor. The other end is provided with a screw, which, when turned, pushes the contents of the tube above the plate, thus making it possible to cut sections of a uniform thickness. The object to be cut must be firmly fixed in the well. Such tissues as kidney, liver, spleen, hard tumors, cartilage, etc., may be held sufficiently rigid by wedging small slabs of carrot, turnip, pith, or hardened liver in about them. These supporting substances must, of course, rest squarely against the bottom of the well. Soft tissues, such as soft tumors or brain, must be imbedded. Three parts of paraffin and one part of vaselin melted together and thoroughly mixed makes a very good imbedding-mass for a well microtome. To imbed, warm the microtome slightly and fill the well with the imbedding mixture. Remove all liquid from the surface of the tissue, and pass it below the surface of the mixture just as it begins to harden around the edges. When the imbedding mass has become cold the sections are cut in the ordinary way.

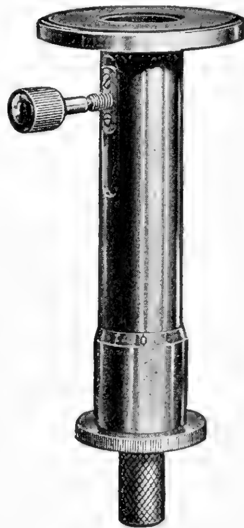


Fig. 27.—Well Microtome.

4. **Temporary Mounts** may be made directly from water after staining by using glycerin as a mounting-medium. Transfer the section to the slide, add a drop or two of glycerin, and a clean cover-glass.

CHAPTER V

THE PARAFFIN METHOD: IMBEDDING AND SECTIONING

1. From 70 per cent. alcohol take a small piece of intestine (6 mm. long) fixed in Gilson, and also pieces of kidney and tongue, and proceed according to the following schedule. Keep accurate records on your cards.

2. Ninety-five per cent. alcohol, 30 to 45 minutes. A longer time will do no harm.

3. Absolute alcohol, 45 minutes. Before transferring to absolute, remove the excess of 95 per cent. alcohol from the object by touching it with a piece of blotting paper or a clean cloth.

4. Xylol, 2 hours or until the object looks clear. It may be left several hours. Rapidly remove all excess of xylol before proceeding with step 5, but do not allow the tissue to become dry or dull looking.

5. Melted paraffin (melting-point about 53° C.), 2 hours. The object may be left an hour or two longer, but it is best to avoid as much as possible subjecting tissues to an elevated temperature. Shift its position in the paraffin once or twice to facilitate penetration of the latter.

Cautions.—a) Do not have the bath too hot. Cooked tissues are worse than useless.

b) To keep material clean, it is well to have a false bottom of paper in the vessel containing paraffin. Make this by swinging a strip of white paper into the cup so that the loop of the paper is submerged in paraffin and the ends attached on either side to the mouth of the cup.

6. Prepare paper boxes according to the following instructions:

A small rectangular block of wood or a stick with a flat end measuring approximately 15×20 mm. is used. Cut a strip of stiff paper so that it measures about 4×7 cm. Place the flat end of the block in the center of the paper with its long diameter coinciding with the long diameter of the

paper. Fold the narrow side margins of the paper up along the sides of the block first, then do likewise with the ends of the paper. Turn the ears which have been formed at each corner back over what is to be the end of the box, and then fold the long end of the paper back to hold the ears in place, and also to make the end of the box of the same height as the sides. Manifestly, any size of box may be made by varying the size of the block. With a little practice, the same kind of box may be folded without the use of a wooden block.

7. With a *warm*, wide-mouthed pipette transfer sufficient melted paraffin to a paper box to cover the bottom, then, with warm forceps, remove the tissue to the box. Next, fill the box with melted paraffin. Orient the object with heated needles if necessary. As soon as the paraffin has congealed sufficiently for the surface to become opaque, cool it rapidly by plunging it into cold water; otherwise, the paraffin will crystallize and become unsuited for sectioning.

Cautions.—*a*) Tissues must be oriented (i. e., placed in proper position for cutting) while the paraffin is still in liquid condition. Arrange the tissue so that it will be cut at right angles (transverse) or parallel to the surface of the organ. Avoid oblique sections as they are very puzzling. For present purposes of practice cut transverse sections.

b) If whitish-looking patches are present in the block after imbedding they are due to xylol which has been carried over into the paraffin. If they occur in the immediate vicinity of the object, the block should be placed in the bath again until melted, and the object be reimbedded.

c) Be sure that every piece of tissue is marked after it is imbedded. Tissues are sometimes kept in paraffin for months or even years before they are finally sectioned. To mark, scratch the number of the record card in the paraffin, or better, write it on the paper box and leave the box in place.

CUTTING SECTIONS

8. Study the paraffin microtome (e. g., Fig. 28); identify the parts and learn how the thickness of sections is controlled.

9. Proceed with the block of paraffin containing the intestine. Make it fast to the carrying disk of the microtome in the follow-

ing manner: Remove the disk from the machine and by means of a heated steel spatula or copper wire flattened at one end, melt a small chip of paraffin on to it. Likewise warm the end of the paraffin block and quickly press it into the melted paraffin on

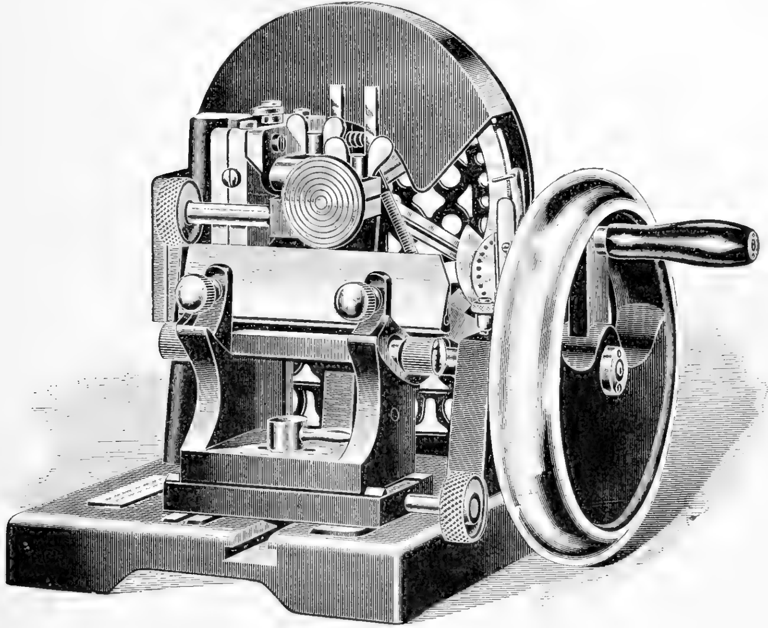


FIG. 28.—Minot Automatic Rotary Microtome.

The object carrier is adjustable in three planes and is perfectly rigid. The knife carrier is also adjustable and extra heavy and solid. The feed is controlled by an adjustable cam, giving cuts of any number of microns in thickness from 1 to 25. By means of an automatically closing split-nut the carriage is returned to the beginning position after the screw is fed out the entire length.

the disk. Cement it firmly in place by means of the heated wire or spatula and cool in water.

10. With a sharp scalpel trim the free end of the block so that it presents a perfectly rectangular outline (however, see caution *c*). The length should exceed the breadth by at least one-fourth.

Cautions.—*a*) In trimming do not cut farther back than the base of the object. This leaves a wide shoulder for support.

b) Leave a margin of about 2 mm. around the object.

c) To avoid reversing sections in mounting, it is frequently advantageous to have the imbedding mass trimmed unsymmetrically. The edge which first comes in contact with the knife is left longer than the opposite edge. One may thus readily discover when a section or part of a series has been turned over.

11. Mount the object firmly in the microtome. It should just clear the knife. The flat end-surface of the paraffin block should

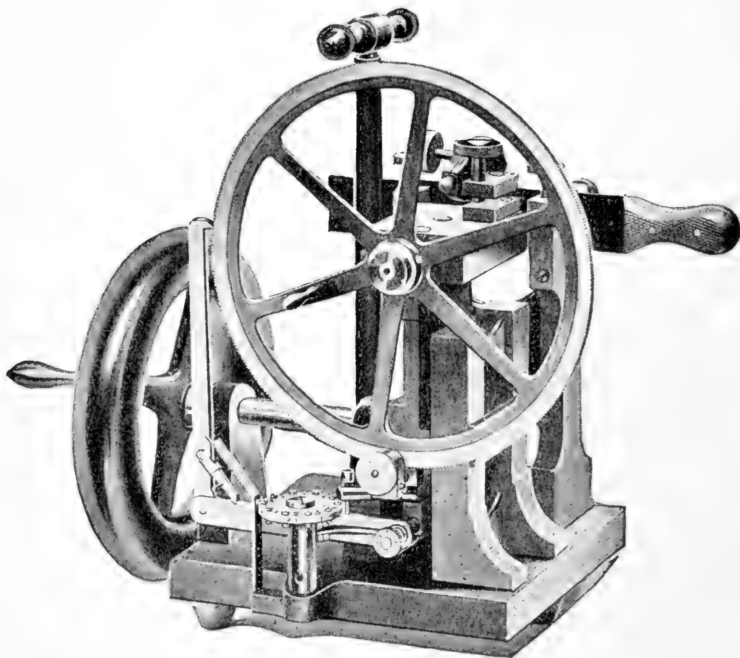


Fig. 29.—Minot-Blake Microtome, designed especially for cutting thin sections. Manufactured by *Buff & Buff M'f'g Co.*, of Boston, Mass.

be parallel to the edge of the knife, and the block so oriented that in cutting, the long edge will meet the edge of the knife squarely.

12. Place the knife in position with the handle to the side away from the wheel (if a rotary microtome is used). By means of the adjusting screws tilt the cutting edge slightly toward the object so that the side of the knife will not remain in contact with

the paraffin block after a section has been cut. If the knife has a flat under surface it requires more tilt than if the surface is hollow ground. For a flat under surface the tilt should be about 9 degrees from the perpendicular. See that the knife is held firmly in place.

Caution.—The knife should be kept in its case when not in the machine. The edge is very easily injured.

13. Set the regulator so that the microtome will cut sections about 10 microns thick. A micron is one-thousandth of a millimeter.

14. Unloose the catch which locks the wheel and revolve the wheel with the right hand. A few revolutions should bring the block of paraffin into contact with the knife. As each new section is cut, it displaces the last one and if the paraffin is of the proper consistency unites by one edge with the displaced section. Thus a ribbon or chain is formed. When the ribbon becomes of sufficient length support the free end by means of a hair brush held in the left hand. To prevent breaking the ribbon avoid pulling it taut. A silk carrier for it may be attached to the machine but there is little need for such after one has acquired a little skill in supporting it.

Caution.—Never bring a needle or other hard object near the edge of the knife. If the paraffin does not ribbon properly consult the table at the end of this chapter.

15. When a sufficient number of sections have been cut, carefully place the ribbon on a piece of paper. Protect it from draughts of air which will carry away or disarrange the sections.

16. Cut the ribbon into strips of such length that they may be placed in successive rows one above the other under the cover-glass that is to be used. Mark out on a sheet of paper the exact size of the cover-glass so that there can be no mistake in cutting strips of the proper length. A margin of 2 or 3 mm. should be allowed for the cover.

17. Place a small drop of albumen fixative on a clean glass slide (for cleaning see memorandum 14, chap. vi), and spread it evenly over the surface, except the end which is to bear the label

(see step 10, chap. vi). With a clean finger, rub off all of the fixative that can be easily removed so that only a very thin film remains.

18. Flood the slide with a few drops of distilled water until the entire surface bearing the fixative is covered by a thin layer of water, but do not put on sufficient to overflow the edge.

19. Take up the first strip of paraffin ribbon with a brush or needle and float it onto the surface of the water. The first section of the series should be in the upper left-hand corner, but back at least 5 mm. from the end of the slide. In case the label is to be placed on the left end of the slide, allowance must be made for it, of course. Add the successive strips of the ribbon in the order of the lines of a printed page until as many rows are in place as will conveniently lie under the cover, allowing for the proper margins. See that each section presents the same aspect to the observer as its predecessor (see 10, *c*).

20. Warm the slide gently by holding it well above a small flame until the paraffin flattens out and becomes free from wrinkles. Be careful not to melt the paraffin, for heat sufficient to do so will render the albumen useless. It is safer to heat the slide by placing it upon the warm paraffin oven for a few minutes, instead of holding it above a flame.

21. Drain off the excess of water and set the slide away to dry after properly numbering it with your glass-marking pencil. As the water evaporates the sections are drawn down tightly into the film of fixative. The slide is seldom sufficiently dried under six hours. It is well to leave it twelve hours; it may be left indefinitely. The time may be shortened by placing a few thicknesses of blotting paper under the slide and drying it on the paraffin oven. Unless the slide is perfectly dry the sections will float off during subsequent treatment. Take precautions to prevent particles of dirt from settling upon the surface of the sections. This is usually accomplished by placing the slides upon some kind of a rack and covering them with a bell-jar. Prepare several other slides in the same manner as the above if sufficient of the ribbon remains.

NOTE.—As time permits, cut the other sections which are imbedded in paraffin. When, as in the present case, it is not necessary to have a complete series of sections, you may place fewer sections on a slide and use smaller covers.

When a small cover is to be used, place the sections at the center of the slide. The center may readily be determined by drawing the outline of a slide on a card and connecting the opposite corners of the figure by means of diagonal lines. When mounting, place a slide over the diagram; the intersection of the diagonals shows the center.

At this point the student should make a careful study of Appendix A if he is not already thoroughly acquainted with the optical principles involved in microscopy.

MEMORANDA

1. **If Paraffin Becomes Dirty** it should be melted and filtered.

2. **Oil of Cedar,** if used for dealcoholization before imbedding, should be followed by at least two changes of paraffin or the paraffin does not thoroughly replace the oil and the object is likely to drop out of the sections as they are cut. In my experience this is the commonest difficulty which beginners encounter if they use cedar oil for dealcoholization. For this reason xylol is recommended as preferable for general work.

3. **Objects Imbedded in Paraffin** may be preserved in that form indefinitely. It is one of the most convenient ways, in fact, of preserving material which is to be sectioned in paraffin.

4. **Small White Objects,** if not stained before imbedding, should be tinged with a dilute solution of Bordeaux red to facilitate orientation. For orientation in general see chap. xvi, memorandum 12.

5. **With Delicate Tissues** it is necessary that the transition from alcohol to clearer be gradual, hence it is best to add the clearer, a little at a time, to the last alcohol, transferring it with a pipette *to the bottom* of the alcohol.

6. **The Temperature of the Laboratory** must be taken into account when sectioning in paraffin. In summer use a harder, in winter a softer, paraffin.

7. **For Thin Sections** use a hard paraffin, for thick sections, a softer paraffin.

8. **For Valuable Tissues Which Crumble in Paraffin Alone** the following somewhat tedious process (Mark, *American Naturalist* [1885], p. 628) may be resorted to. Prepare a very fluid collodion in ether-alcohol and

coat the exposed surface of the object immediately before cutting each section. If the collodion leaves a shiny surface or produces a membrane when applied to the paraffin, it is not thin enough and must be further diluted with ether-alcohol. Apply the collodion with a brush with all excess of the fluid wiped away so that the brush is just moist. The fluid should touch only the face of the block in which the object is exposed. After applying, wait a few seconds for the solution to dry before cutting. See also memorandum 9.

9. **Johnson's Paraffin-Asphalt-Rubber Method** for brittle objects is a very useful one. One part of crude India rubber cut into very small pieces is mixed with ninety-nine parts of hard paraffin which has previously been melted and tinged to a light amber color with a small amount of asphalt ("mineral rubber"). The mixture is then subjected to a temperature of 100° C. (not higher) for 24 to 48 hours, or left in a paraffin oven at 60° C. for several days. Use only the supernatant fluid. It is allowed to cool and remain cold until needed, because the rubber separates out after a time if the mixture continues melted. Johnson (*Journal of Applied Microscopy*, Vol. VI, p. 2662) recommends it as even better than paraffin for all kinds of work for which paraffin is commonly employed. Proceed as in the ordinary method, using xylol (*not* cedar oil) for dealcoholization and also for clearing sections.

10. **Keep All Parts of the Microtome** clean and well oiled with watch oil or pure paraffin oil of 25 degrees. The instrument should be covered when not in use.

11. **Keep the Microtome Knife Sharp.** It should receive frequent stroppings. For sharpening the knife two hones are commonly used.

Honing.—If the knife is very dull it is first honed on a Belgian yellow hone, an open-grained stone which cuts the metal of the knife rapidly. The surface of the stone is kept moist with filtered kerosene oil or lathered with palm-oil soap. After the nicks and other inequalities of the edge of the knife have been removed, the honing is best finished on a good fine-grained blue-water stone.

In honing the stone is laid flat on the table with its end toward the operator and its surface properly lubricated. A very dull knife is ground at first on the concave side only until it developes a fine "wire edge" along the full length of the blade. It is then ground on each side alternately until the wire edge has disappeared completely. In grinding, the knife must remain flat on the hone and pass lightly over the full length of the surface, edge foremost in a diagonal direction from point to heel, although itself remaining at right angles to the long axis of the hone. The honing has been sufficient when all nicks and wire edges have disappeared and the knife, instead of catching and hanging when the edge is

drawn lightly across the ball of the thumb, freely enters the moist epidermis. Finally the blade is wiped clean with a soft cloth, great care being taken not to injure the edge.

Stropping.—A broad firm strop of finest calfskin is best. It should be affixed to a solid back so that it will not spring and thus round off the delicate edge of the knife.

In stropping, the motions are the same as in honing (both sides of blade), only the knife passes *back* foremost and from heel to point. The blade must move lightly over the surface of the strop with very slight pressure on the part of the operator. The stropping is ordinarily considered sufficient when the blade will cut a loose hair freely along every part of the edge. An examination under a low power of the microscope should reveal no nicks in the edge.

12. **To Remove Pigments and to Bleach Osmic and Chromic Acid Materials** a 3 per cent. solution of peroxide of hydrogen frequently is sufficient. Tissues left too long in this liquid macerate.

Mayer's chlorine method is one of the best for bleaching. To several crystals of chlorate of potash in a glass tube a few drops of hydrochloric acid is added. When the greenish fumes of chlorine appear, add from 5 to 10 c.c. of 50 per cent. alcohol. The object, which in the meantime has been standing in 70 per cent. alcohol, is transferred to the tube. From 15 minutes to 24 hours are required for bleaching, depending upon the nature of the material. It is well to suspend the object from the mouth of the bottle. Sections on the slide may be bleached in a few minutes. This method is especially recommended for removing natural pigments and for bleaching osmic material.

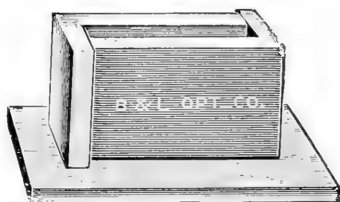


Fig. 30.—Metal Ls for molding imbedding masses.

13. **Large Objects May Be Cut in Paraffin** better with a slanting knife than with a square-set one. The block of paraffin must be trimmed to a three-sided prism with its most acute angle farthest from the object. A sliding microtome is used ordinarily and the block of paraffin is so oriented that the knife enters at the sharpest angle of the prism. Each section as cut is removed with a brush.

14. **Metal "Ls" Are Frequently Used Instead of Paper Boxes** for molding paraffin blocks. The two Ls (Fig. 30) may be placed together on a small glass or metal plate in such a way as to mold blocks of any desired size. Before pouring the melted paraffin in, the inner walls of the metal pieces should be lightly smeared with glycerin so that the block of paraffin will easily separate from them when cool.

DIFFICULTIES LIKELY TO BE ENCOUNTERED IN SECTIONING IN PARAFFIN, AND THE PROBABLE REMEDY

1. **Crooked Ribbons.**—*a*) Usually caused by wedge-shaped sections. Correct by trimming the block of paraffin so that the edge which strikes the knife first and the edge on the opposite side are strictly parallel. See that the block strikes the knife exactly at right angles.

b) The paraffin may be softer at one end of the block than at the other. This can only be corrected by imbedding the object over again in a homogeneous paraffin.

2. **The Object Makes a Scratching Noise on the Knife or Cuts with a Gritty Feeling** and the sections perhaps crumble and tear out from the paraffin.

a) This is generally caused by too high heating of the object while in the paraffin oven. Not only is such an object worthless but it endangers the edge of the microtome knife. Correct by limiting the bath in paraffin to the minimum time necessary for a proper penetration of the object, and keeping the temperature barely above the melting-point of the paraffin.

b) The fixing reagent has formed crystals (e. g., corrosive sublimate) which have not been thoroughly washed out.

See also 5.

3. **The Sections Wrinkle or Jam Together**; the object itself may be compressed before the knife. This is a serious fault because the arrangement of the parts of a tissue are greatly deranged. It may be due to various causes.

a) The microtome knife may be dull. Examine the knife and sharpen it if necessary.

b) The paraffin may be too soft. To remedy this defect employ one or more of the following means: (1) cool the paraffin block in water; (2) cut the sections in a cooler room; (3) cut the sections thicker; (4) reimbed in harder paraffin. If sections are not too badly wrinkled they may be flattened out by warming on water as directed in steps 18–20.

c) A possible reason is that the tilt of the knife is insufficient (see step 12).

d) The edge of the knife may be smeared with a layer of paraffin. Clean the edge with a cloth moistened in xylol.

4. **The Sections Roll and Refuse to Ribbon.**—This is one of the most exasperating of all defects. If the sections are not tightly curled they frequently unroll when placed on warm water (step 18). Various mechanical devices have been constructed to prevent this evil, but most of them are impractical. Sometimes when a section begins to roll, if the edge is held down by means of a flat-pointed hair brush, the curling can be overcome. If a ribbon can once be started the difficulty is frequently corrected. The sections should be cut rapidly.

a) The commonest cause of rolling is the hardness of the paraffin. This may sometimes be remedied by one or more of the following means: (1) warming the knife with the breath; (2) cutting in a warmer room; (3) placing a lamp or burner near the imbedded object; (4) warming the knife *very carefully* by holding the back on a warm paraffin bath; (5) cutting the sections thinner; (6) reimbedding the object in softer paraffin.

b) The tilt of the knife may be too great (step 12).

c) The knife may be dull.

5. **The Sections Split Longitudinally or Are Crossed by Parallel Scratches.**—*a*) Look for a nick in the edge of the knife. Cut in a new place on the knife or sharpen it.

b) A bit of grit may have gotten into the object or the paraffin. Reimbed after carefully cleaning the object in the clearing fluid.

c) Tissues may contain hard substances (lime salts, silica, crystals precipitated from fixing reagents) which have been imperfectly washed out. It is best to take an entirely new piece of tissue in which these defects do not exist.

d) The tilt of the knife may be too great (step 12).

e) The object may be too large to cut in paraffin. Try smaller pieces of tissue or use the celloidin method.

6. **The Knife Scrapes or Rings as It Passes Back** over the object after having cut a section.

a) This is sometimes caused by a knife with either too great or too little tilt (step 12).

b) The object may be too tough or hard to cut in paraffin without springing the edge of the knife (see 7 *b*).

7. **The Sections Vary in Thickness**; the machine cuts one thick and one thin or misses a section.

a) This may be caused by the imperfect mechanical construction of the machine. Old machines in which the parts are worn are especially liable to this defect. It may be remedied to some extent by tightening up the parts of the machine.

b) The object may be too hard for the knife to cut and, as a consequence, the edge of the knife springs. When tough or hard objects must be cut, use an old microtome knife or a sectioning razor. See if there is not some means of softening such a tissue without obscuring the microscopical structures sought.

c) Either too great or too little tilt may cause the defect (step 12).

d) See that the disk bearing the object is securely clamped in the machine.

8. **The Object Crumbles or Drops out of the Paraffin as Cut.**—It has probably been insufficiently penetrated by paraffin. Some of the

following precautions may prevent the defect: (1) Leave the object in the paraffin bath longer. (2) See that it is entirely free from the dealcoholizing fluid before placing it into the melted paraffin. Objects which have been immersed in cedar oil are particularly subject to this defect. For this reason xylol is better than cedar oil for dealcoholization in general work. (3) If the object is impervious to paraffin or very friable, as are many ova, some other method must be tried. Consult memoranda 8 and 9; see also the celloidin method (chap. vii) or the combination celloidin-paraffin method (chap. vii, memorandum 8).

9. **The Ribbon Twists or Curls about or Clings Closely to the Side of the Knife.**—This is due to the electrification of sections. If the fault is excessive it is best to postpone the cutting until the atmospheric conditions have changed.

CHAPTER VI

THE PARAFFIN METHOD: STAINING AND MOUNTING

I. STAINING WITH HEMATOXYLIN

Place enough of the following reagents in tall stender dishes or Coplin staining-jars to cover the slides lengthwise, up beyond the sections affixed to them: xylol, carbol-xylol, absolute, 95, 70, 50, 35 per cent. alcohols respectively, clear water, acid alcohol, and for washing out the acid alcohol in the case of hematoxylin preparations, a separate jar of 70 per cent. alcohol to which a few drops of a 0.1 per cent. aqueous solution of bicarbonate of soda has been added. Arrange these reagents in a row in the order named with the exception of the acid alcohol and its accompanying alkaline alcohol wash of 70 per cent. alcohol, which may be placed immediately back of the ordinary 70 per cent. alcohol. Put a little vaselin along the upper edges of the jars containing absolute alcohol, xylol, and carbol-xylol and press the cover down tightly to prevent evaporation or the entrance of moisture.

In like manner place in Coplin staining-jars (tall stenders will answer) a supply of Delafield's hematoxylin diluted one-half with distilled water, eosin, Lyons blue, borax-carmine, Bordeaux red, and solutions A and B for the iron-hematoxylin method. Arrange these stains in a row back of the alcohol series.

1. Remove the paraffin from the sections of intestine (see last lesson) by placing the slides in xylol (turpentine will answer) for 10 or 15 minutes. The process may be hastened by first gently warming the slide until the paraffin begins to melt.

2. Remove the xylol from the sections by transferring the slides to absolute alcohol for 1 minute.

3. Pass the slides through the alcohols (95, 70, 50, and 35 per cent.) leaving them for a half-minute in each.

4. Remove to Delafield's hematoxylin for 10 to 30 minutes or until stained a pronounced blue.

5. Wash in water for 5 minutes.

6. Pass the slides up through the series of alcohols to 70 per cent., leaving them about half a minute in each alcohol.

7. Dip each slide for from thirty seconds to five minutes into the acid alcohol until the sections are of a reddish hue, then rinse them in 70 per cent. alkaline alcohol until the blue color is restored. This last alcohol must be kept very slightly alkaline through the occasional addition of a few drops of a 0.1 per cent. solution of bicarbonate of soda (see memorandum 10). The alkaline alcohol may be omitted when other than hematoxylin stains are used as its purpose is merely to restore the blue color of the latter.

8. Pass the slides through 95 per cent. alcohol (1 minute), absolute alcohol (3 minutes), into carbol-xylol for 5 minutes or until clear.

9. Carefully drain off all excess of the clearer, wipe the under side of a slide and lay it down flat with the sections uppermost. Put a few drops of thin balsam on the sections near one end. Take up a clean cover-glass and holding it by the edges between the thumb and first finger of one hand, lower it upon the balsam by bringing one end into contact with the slide near the balsam, and supporting the other end by means of a needle held in the free hand. Lower the cover slowly so that as the balsam spreads no air bubbles will be inclosed under the glass. If a slide is tilted a little and allowed to remain in that position small bubbles will frequently work out unaided. They may sometimes be removed by pressing gently above them with the handle of a needle and gradually working them to the edge of the cover-glass. Keep the slide in a horizontal position until the balsam hardens.

Caution.—Do not allow the sections to become dry before adding the balsam and cover.

10. Attach the permanent label. It should contain at least the following data: the number of the record card; the name of the tissue; the kind of section (plane of section, thickness, etc.), if one of a series the number of the slide in the series and the number of the first and last section on the slide; the date, and if desired the name or the initials of the preparator.

If the operator is accustomed to manipulate the fine adjustment of his microscope with the left hand and the slide on the stage with the right, it is best to have the label on the right end of the slide; if not, then on the left.

NOTE.—Prepare four slides each of the other objects which have been imbedded. Stain and mount one of each kind as you did the intestine, and also one of each kind in the same way, only substitute borax-carmine for the hematoxylin. The borax-carmine may require 6 to 12 hours for staining. Preserve the others for double staining.

As time permits prepare and section the other tissues which were fixed in alcohol and Gilson. After you have had the preliminary practice in double staining, stain and mount these as you prefer.

II. DOUBLE STAINING IN HEMATOXYLIN AND EOSIN

1. Proceed according to the regular schedule with one each of the slides reserved above, and stain in Delafield's hematoxylin.

2. Wash the sections in water, and proceed farther according to the regular schedule to 95 per cent. alcohol.

3. Transfer the slide to the eosin stain for 10 to 30 seconds, and after rinsing again in 95 per cent. alcohol, place it in absolute alcohol.

4. Clear in carbol-xylol and mount in balsam.

NOTE.—The sections should show both the blue stain (in nuclei) and the red stain (in cytoplasm) when examined under the microscope. If either is too dense or too light, make a note of the fact and vary the time accordingly when staining other sections by this method.

III. DOUBLE STAINING IN CARMINE AND LYONS BLUE

1. Pass the remaining reserved slides through xylol and the alcohols, descending to 35 per cent. alcohol.

2. Stain in borax-carmine for from 30 minutes to several hours, until the sections are well colored.

3. Rinse in water or 35 per cent. alcohol, and pass the sections up through the alcohols to 95 per cent. If the sections are deeply stained, however, remove the excess of stain with acid

alcohol (a few seconds) when the sections are in 70 per cent. alcohol.

4. Stain for 10 to 20 seconds in Lyons blue. It is very easy to overstain with this dye.

5. Rinse in 95 per cent. alcohol, and transfer the sections to absolute alcohol (3 minutes), clear in carbol-xylol, and mount in balsam.

IV. STAINING WITH HEIDENHAIN'S IRON-ALUM HEMATOXYLIN

This stain is very valuable in the study of cell division and in determining the finer structure of the nucleus. The iron-alum acts as a mordant, preparing the tissue for the action of the hematoxylin.

1. Prepare two sets of sections of intestine, testis or ovary, bladder, pancreas, and stomach. The sections should not be over 6 or 7 microns in thickness. Preserve one set for double staining.

2. Pass the slides bearing the sections through xylol, absolute alcohol, 95 per cent. alcohol, and thence directly into water.

3. Transfer from water to the iron-alum, and allow this solution to act for from 6 to 8 hours.

4. Rinse in water 5 minutes.

5. Stain in the 0.5 per cent. hematoxylin 24 to 36 hours. If a trace of the iron-alum remains in the sections the hematoxylin will turn black. This, however, does not impair its power of staining.

6. Rinse in water 5 minutes.

7. Place the sections into iron-alum again, which will now extract the excess of stain. The time required for proper differentiation varies with the kind of tissue and the fixing agent that has been used. From 10 to 30 minutes is usually sufficient, though no definite time limit can be set. Remove the slide from the iron-alum from time to time and inspect it. When the sections become of a dull-grayish hue the decolorization is usually sufficient. If very accurate results are necessary, the slide should be removed from the iron-alum frequently and examined under

the microscope. When in a dividing cell the chromosomes become sharply defined, the decolorization should be stopped.

8. Wash in several changes of water for 2 to 3 hours. If any of the iron-alum is left in the sections the color will fade later.

9. Wipe off the excess of water, transfer the slide to 95 per cent. alcohol, followed by absolute alcohol and carbol-xylol.

10. Mount in balsam.

NOTE.—Iron hematoxylin is perhaps the one most important stain in use today. The student should practice the method until he has mastered it.

It is better though not absolutely essential that the stain be "ripe." If the stain is to be simply for general histological instead of cytological work, the baths may be curtailed considerably. For example, immersion for 30 minutes in the iron solution, then for 45 minutes in the stain followed by very brief differentiation in the iron solution, yields a good general preparation but the finer details of cell structure (centrosome, etc.) are not brought out.

V. BORDEAUX RED AND IRON-ALUM HEMATOXYLIN

Use the sections which were reserved for this method. The method is identical with the one just outlined, except that between step 2 and step 3 the following directions should be inserted: 2*a*, transfer the sections from water to Bordeaux red for 2 hours (12 hours will do no harm), then wash them in water and proceed to step 3.

NOTE.—*Before proceeding further, kill a female cat or rabbit to secure tissues for the celloidin method and to correct failures in the paraffin method. In addition to the tissues specified before, prepare (fix in Gilson) pieces of tendon, cartilage, spleen, lymph gland, pancreas, and salivary glands. (If the reagents are at hand and time permits, the student, indeed, might advantageously prepare a number of tissues according to the methods indicated in Appendix C.) Fix the ovary in Gilson, and reserve it for the paraffin method for delicate objects. Fix parts of the brain and cord in Erlicki and in formalin as previously indicated, and place bits of muscle in which nerves terminate plentifully (e.g., intercostals) in formalin. Larger pieces (up to 2 cm.) may be used of such tissues as are to be imbedded in celloidin. Bear in mind that the* **larger** *the tissue the* **longer** *must it be left in the different reagents. Select the necessary parts of the digestive tract to prepare longitudinal sections in celloidin from esophagus to stomach and from stomach to intestine. As soon as possible begin the preliminary steps in the celloidin method (chap. vii) so that there may be no loss of time.*

Prepare a piece of intestine for staining in bulk (see vi). It should be placed in the stain after thoroughly washing out the fixing reagent. Preserve parts of it to cut in celloidin. Remove the lower jaw, and prepare it for decalcification of teeth as indicated in chap. xi. Likewise prepare pieces of femur and of tarsal bone for sectioning (chap. xi).

VI. STAINING IN BULK BEFORE SECTIONING

It is sometimes desirable to stain objects before sectioning. The method is a slow one, and requires stains which penetrate evenly and thoroughly. Various preparations of carmine and cochineal give the best satisfaction, although several hematoxylin stains are also frequently used in this way. It is best to stain immediately after fixing and washing out, before the object has been carried into higher alcohols. In general, it is advisable to section tissues and stain on the slide, because the staining can be controlled more effectually. Use the piece of intestine already prepared (see note above).

1. After fixing in Gilson and thoroughly washing out in water, place the tissue in borax-carmine for 24 hours.
2. Wash in 35 per cent. alcohol for 5 minutes.
3. Fifty, 70, 95 per cent. alcohols, 30 minutes each.
4. From this point proceed through absolute alcohol, xylol, and imbedding, sectioning, and mounting precisely as in the general paraffin method, except that after the sections have been freed from paraffin in xylol, do *not* mount immediately in balsam, but first transfer the slide back into absolute alcohol, and thoroughly wash it in order to remove the glycerin from the fixative and so prevent cloudiness of the final mount. From alcohol the slide is passed through xylol, or carbol-xylol, and mounted in the usual way.

Note.—When borax-carmine or Delafield's hematoxylin is used as the stain for an entire object, the preparation usually needs to be decolorized with acid alcohol. This may be deferred, however, until after the object is sectioned.

VII. PARAFFIN METHOD FOR DELICATE OBJECTS

To prevent the distortion of delicate objects which are to be sectioned in paraffin the transition of the material from one reagent to the other must be very gradual and the heat be mini-

mized. Observe the following modifications of the general method and prepare pieces of ovary which have been fixed in Gilson.

1. Pass the object in the usual manner up through the series of alcohols to absolute. It is sometimes necessary to use a more closely graded series of alcohols if the object be very delicate.

2. From the absolute alcohol, pass to a mixture of absolute alcohol two-thirds and chloroform one-third; gradually add more chloroform until at the end of an hour the mixture is at least two-thirds chloroform.

3. Transfer to pure chloroform for 30 minutes.

4. Add melted paraffin little by little during the course of an hour or two (24 hours will do no harm), until the chloroform will hold no more in solution.

5. Transfer the object to pure melted paraffin in a small vessel on the paraffin oven for 10 to 20 minutes, changing the paraffin once. Imbed in the usual way.

6. Cut the sections about 7 microns thick. Mount and stain some in Delafield's hematoxylin and eosin, and others in iron-hematoxylin and Bordeaux red, according to the directions already given for these methods.

NOTE.—For very sensitive objects Schultz's dehydrating apparatus (to be obtained from dealers) may be used. It consists of a tube within a tube, each having the lower end covered by an animal membrane. The tubes are suspended in the neck of a much larger bottle which contains 95 per cent. alcohol. The object is placed in the inner tube and both tubes filled with water. When suspended in the alcohol, a very gradual hardening or dehydration of the object takes place as the alcohol slowly diffuses through the membrane. Sometimes it is necessary to use only one tube, and in such a case the hardening proceeds more rapidly.

MEMORANDA

1. **In Passing from One Liquid to Another,** one corner of the slide-bearing sections should first be touched by blotting paper to remove any excess of the liquid last used. This is especially necessary in transferring from absolute alcohol to xylol, or from 95 per cent. to absolute alcohol.

2. **Sections Once Placed in Turpentine or Xylol** for the removal of paraffin must never in any subsequent step be allowed to become dry. Particular care must be taken to prevent sections from drying out after removing them from xylol to mount in balsam because the xylol evaporates rapidly. If the sections become dry the preparation is usually rendered valueless.

3. **Xylol Used for Removing Paraffin** should be kept in a jar separate from that which contains xylol for clearing before mounting and it should be changed occasionally because it tends to become saturated with paraffin.

4. **Sections Not over 10 Microns Thick** may be plunged directly from 95 per cent. alcohol into an aqueous medium and vice versa. If sections are over 10 microns thick it is better to put them through the complete series of alcohols. With thick sections diffusion is less rapid, and too abrupt a change from one fluid to another may produce distortions or wrench the sections loose from the slide.

5. **To Avoid Rubbing Sections off the Slide,** hold the slide with one end toward the light before wiping it and glance obliquely along the surface. The shiny side is the one to wipe.

6. **The Series of Alcohols and Stains** ordinarily may be used a number of times without replenishing. When the alcohols become very much discolored or the stains cloudy they should be renewed. Alcohols should not be used too often, however, as they soon accumulate particles of dirt which settle upon the sections and render preparations unsightly.

7. **Absolute Alcohol** must be kept free from water. It may be tested from time to time by mixing a few drops with a little turpentine. If the mixture appears milky the alcohol contains a harmful amount of water and should be renewed.

8. **Two Slides Placed Back to Back** can be handled as readily as a single slide in passing through the various liquids.

9. **Gentle Agitation of a Slide** in any liquid facilitates the action of the liquid. Observe this precaution especially with absolute alcohol.

10. **For Washing Sections after Staining in Hematoxylin** tap water is preferable to distilled water because it is usually slightly alkaline. When acid alcohol is used to decolorize sections stained in hematoxylin, the sections should be washed in 70 per cent. alcohol rendered alkaline by the addition of a few drops of 0.1 per cent. solution of bicarbonate of soda. The alkali neutralizes the acid and restores the bluish-purple color to the section; it also renders the blue color more permanent. If too much of the soda is added the color will be a hazy disagreeable blue.

11. **To Obtain a More Precise Stain** with Delafield's hematoxylin it is well to dilute it with three or four times its bulk of distilled water. The sections must be left in this solution a correspondingly longer time. Sections stained in this way may not require treatment with acid alcohol. Most workers, however, prefer to overstain and decolorize.

12. **The Length of Time Required for Staining Different Tissues** is exceedingly variable. Upon removal from the stain after rinsing, if the sections are insufficiently colored, put them back into the stain and examine from time to time until they are properly stained (30 minutes to 24 hours).

13. **If Objects Refuse to Stain** it is usually due to one of the following causes: (*a*) The fixing agent has not been sufficiently washed out.

This is a frequent cause of poor staining. (*b*) The fixation has been poor. The success of a preparation depends largely upon proper fixation in most cases. (*c*) The stain is at fault. Hematoxylin will not stain properly until ripe (see *Hematoxylin*, page 20). Many stains, especially the anilins, deteriorate and must be replaced. (*d*) Certain stains will not follow some fixing agents. This can be remedied only by using a different stain or by fixing tissues in a different fluid. The hematoxylins and carmines are applicable after a very large variety of fixing agents. (*e*) The paraffin has been insufficiently removed from the sections. This may be corrected by dissolving off the cover-glass in xylol and after thoroughly removing all paraffin, restaining and mounting the sections again in the ordinary way.

14. **Use Only Clean Slides and Covers.**—Always grasp a slide or a cover by its edges to avoid soiling its surface. All cloudiness (seen by looking through the glass toward some dark object) must be removed. For wiping slides and covers, a piece of cloth which does not readily form lint should be used. Slides may often be cleaned after simply dipping them into alcohol or into alcohol followed by water. If this treatment is insufficient, place them for several hours into equal parts of hydrochloric acid and 95 per cent. alcohol, keeping them well separated so that the liquid may act on the entire surface of each. Then rinse them in water and place them in ether-alcohol. It is well to keep a stock supply of such slides and cover-glasses in ether-alcohol.

To clean a cover-glass grasp it by the edges in one hand, cover the thumb and first finger of the other hand with the cleaning cloth and rub both surfaces of the glass at the same time. To avoid breaking the cover, keep the thumb and finger each directly opposite the other. A large cover-glass may be cleaned by rubbing it between two flat blocks which have been wrapped with cleaning cloths.

To clean slides which have been used, if balsam mounts, warm and place in xylol or turpentine to dissolve off the covers. Put the slides and covers into separate vessels and leave them for a few days in the following cleaning mixture:

Potassium bichromate	10 parts
Hot water	50 parts
Sulphuric acid	50 parts

Add the acid very cautiously after the bichromate solution cools. When the slides are freed from balsam, wash them in water, rinse in a dilute solution of caustic soda, again in water, and finally place them in ether-alcohol until needed.

15. **If Sections Appear Milky or Hazy** under a medium power of the microscope, when finally mounted, the effect is probably due to one of the following causes: (*a*) The clearer is poor and needs replenishing or correcting. (*b*) The absolute alcohol contains water (see 7). (*c*) The

cover bore moisture. Passing a cover-glass quickly through a flame before putting it onto the object will remove moisture. (*d*) The acid has not been entirely removed from the sections. (*e*) Too much albumen fixative has been used. (*f*) The glycerin of the albumen fixative has not been removed by passing sections of objects stained in bulk (see vi, 4) back into absolute alcohol after removing paraffin from them.

Fig. 31. Dropping-Bottle.

The defect may be remedied frequently by dissolving off the cover in xylol or turpentine, descending through the series of reagents to the point where the fault lies, correcting and ascending again according to the regular method. To remove water, for example, it is only necessary to go back as far as absolute alcohol which has a great affinity for water.

16. **Dry or Dull-Looking Areas under the Cover-Glass** indicate that the sections were allowed to get dry after the removal from the clearer, or that insufficient balsam was applied.

17. **Balsam Which Exudes from under the Cover** may be scraped off with an old knife after it hardens. Remove the last traces by means of a brush or a cloth dipped in turpentine or xylol. Balsam may be removed from the surface of a cover by means of a brush dipped in xylol.

18. **If Sections Wash off the Slide** the defect is probably due to one of the following causes: (*a*) The slide was soiled or oily. Remedy by cleaning slides thoroughly (see 14). (*b*) The albumen fixative is too old. (*c*) The transitions in the alcohol have been too great. This is true sometimes of thick sections.

19. **Flooding Sections with the Dye** by means of a pipette, especially in case of stains which act rapidly (e. g., eosin, acid fuchsin, Lyons blue, picric acid, etc.), is sometimes more convenient than immersing the sections in a jar of the staining fluid. Small bottles with combination rubber stopper and pipette (Fig. 31) are now provided for this purpose by dealers.

20. **Balsam Mounts in Which the Stain Has Faded** may frequently be restained, either with the original or with other stains. All that is necessary is to dissolve off the cover in xylol (2 to 3 days) and pass the preparation down through the alcohols to the stain in the usual manner.

21. **Ink for Writing on Glass** (Hubbert, *Journal of Applied Microscopy*, Vol. V, p. 1680).—Mix drop by drop 3 parts of a 13 per cent. alcoholic solution of shellac with 5 parts of a 13 per cent. aqueous solution of borax. If a precipitate forms, heat the solution until it clears. Add enough methylen blue to color the mass deep blue.

CHAPTER VII

THE CELLOIDIN METHOD

Use the tissues which were prepared for this method including pieces of the brain and spinal cord which were fixed in Erlicki's fluid. Reserve a piece of spleen for the freezing method, chap. viii.

1. Fixing, washing, and dehydrating are the same as usual (chap. iii). If the object is in 70 per cent. alcohol, complete the dehydration by using successively 95 per cent. and absolute alcohol. It should remain in the absolute alcohol for from 12 to 24 hours.

2. From absolute alcohol transfer the object to equal parts of absolute alcohol and ether 12 to 24 hours.

3. Next, to thin celloidin for from 36 hours to several days.

4. Thence to thick celloidin for from 24 hours to several days.

5. Prepare a wooden block in such a manner that it will have surface enough to accommodate the object, leaving a small margin, and length enough to be readily clamped into the carrier on the microtome (Fig. 32). Dip the end of the block to which the object is to be attached into ether-alcohol for a minute and then into thick celloidin. Let it dry so that later air bubbles will not work up out of the wood into the imbedding mass.

6. Oil one side of a strip of stiff paper by rubbing on a very little vaselin, and wrap it, oiled surface in, about the prepared end of the block in such a way that it will project beyond the end of the block, forming a collar high enough to extend a little beyond the object which is to be placed within it. Tie the paper in place by means of a thread.

7. Pour a small amount of thick celloidin into the paper cup thus formed and with forceps remove the piece of tissue and place it in celloidin. Add more thick celloidin until the cup is full. By means of needles which have been moistened in ether-alcohol arrange the object so that it will be cut in the desired plane.

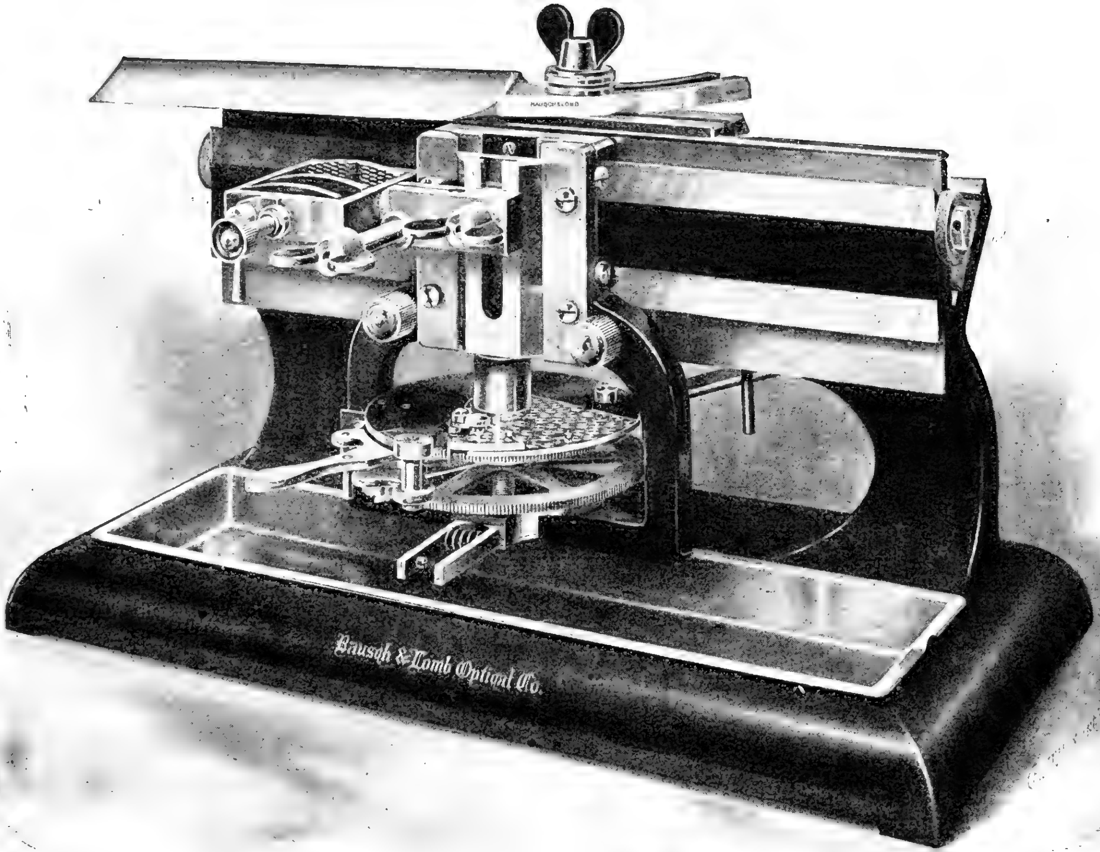


Fig. 32.—Automatic Celloidin Microtome.

Adapted for celloid or paraffin cutting. The object remains stationary during cutting; it is held by a rigid clamp which is adjustable in two planes and vertically. The knife is carried on a block which slides in ground ways. The motion of the knife operates the feed screw, leaving the hand free for the manipulation of the sections. The feed is from 2 to 60 microns, in two microns. A split-nut permits the object-holder to be lowered again when the screw has been fed out.

8. Into a small stender dish put chloroform to the depth of 3 mm. When a film has formed over the exposed surface of the celloidin place it in the chloroform to harden. It need not be submerged. Keep the vessel tightly covered. The object may be left for a day or two, but 1 to 3 hours usually suffices.

9. Transfer the block to 70–83 per cent. alcohol, where it may remain indefinitely.

10. Make a careful study of the microtome used for cutting celloidin (Fig. 32).

11. Place the block in the object carrier of the microtome at the proper level and arrange the microtome knife obliquely, so that it will slice through the object with a long drawing cut for at least half the length of the blade. If the object is oblong it is advantageous to have the long diameter parallel to the edge of the knife.

12. Keep both the knife and the object flooded with 70 per cent. alcohol.

13. Draw the knife through the object with a straight steady pull; avoid pulling down on or lifting the knife carrier.

14. If the feed is not automatic push the knife back to position always before turning the screw which raises the object. Cut the sections about 15 or 20 microns thick.

15. As the sections are cut, transfer them by means of a small soft brush or a paper spatula to a flat stender or a watch-glass containing 70 per cent. alcohol.

16. Transfer some of the sections through 50 and 35 per cent. alcohol, 2 minutes each, into borax-carmine for from 20 to 30 minutes, or until stained (12 to 24 hours).

17. Wash successively in 35, 50, and 70 per cent. alcohols, leaving the sections from 2 to 3 minutes in each.

18. Transfer the sections to 95 per cent. alcohol for 3 to 5 minutes. Absolute alcohol is not to be used with celloidin because it dissolves the celloidin.

19. Clear in carbol-xylol for from 10 to 20 minutes.

20. Mount in balsam (see chap. vi, **I**, step 9).

STAINING CELLOIDIN SECTIONS IN HEMATOXYLIN AND EOSIN

The objects are killed, fixed, and preserved as usual in 70 per cent. alcohol, and sectioned as in the above method.

1. Fifty and 35 per cent. alcohol each 3 to 5 minutes.
2. Delafield's hematoxylin, 10 to 30 minutes.
3. Water 5 minutes.
4. Thirty-five, 50, and 70 per cent. alcohol each 3 to 5 minutes.
5. Acid alcohol until the celloidin which surrounds the object shows but little of the stain.
6. Seventy per cent. alcohol, barely alkaline (see chap. vi, memorandum 10), until the red color caused by the acid is replaced by bluish purple.
7. Alcoholic eosin, 30 seconds to 1 minute.
8. Ninety-five per cent. alcohol, 2 to 5 minutes. Clear in carbol-xylol and mount in balsam.

NOTE.—As time permits section other tissues by the celloidin method and stain as above.

MEMORANDA

1. **If Chloroform is Not at Hand,** 80 per cent. alcohol will harden the celloidin, although more slowly.

2. **The Length of Time** that objects should be left in ether-alcohol and the celloidin mixtures depends upon the size and density of the objects. When time permits it is always best to leave them several days, or even weeks in the mixtures of celloidin. For large objects such as the medulla of a large brain this is a necessity. For an embryo of large size months may be required.

3. **Blocks for Celloidin Mounting** may be of white pine, glass, vulcanized fiber or even a very hard paraffin. Cork should not be used because it is liable to give or bend. The vulcanized fiber is the most satisfactory. It may be purchased from dealers in the form of strips which may easily be sawn to the necessary dimension. It is well to saw several parallel cuts into the upper edge of the block to provide points of attachment for the celloidin.

4. **Other Clearers** may be substituted for carbol-xylol. One which clears from 95 per cent. and which does not dissolve celloidin must be chosen. Cedar oil is an excellent clearer as is also beechwood creasote. Other good clearers are (1) origanum oil, (2) a mixture of oil of thyme (3 parts) and castor oil (1 part), and (3) Eycleshymer's clearing fluid

which is a mixture of equal parts of bergamot oil, cedar oil, and anhydrous carbolic acid.

5. **Imbedding a Number of Objects** in one mass is frequently convenient. Fold a stiff paper into a box of the proper size (chap. v, step 6) or use metal **L**s (Fig. 30). Pour in thick celloidin, put the objects in place and orient them properly for cutting. Leave a space of about 8 mm. between adjacent objects. Fill the box with thick celloidin and set it in a dish containing a little chloroform, or leave it in 80 per cent. alcohol to harden. When ready to proceed, cut the large blocks into smaller ones each containing a piece of tissue. To fasten it to the wood, trim the small celloidin block to the proper dimensions, soften for a few minutes in ether-alcohol, the side to be attached, then dip it into thick celloidin and apply to the end of a wooden block which likewise has been dipped into the ether-alcohol and the thick celloidin. Press the two together and place them in chloroform or 80 per cent. alcohol to harden.

6. **Anilin Dyes** are usually avoided in the celloidin method because they stain the celloidin intensely and are not removed in subsequent treatment. When necessary, however, some (e. g., eosin) may be used. Saffranin, for example, may be removed satisfactorily from the celloidin by means of acid alcohol without extracting all the stain from the tissue. If anilin dyes have been used it is sometimes better to remove the celloidin by treating the sections with absolute alcohol or with ether before the final clearing and mounting.

7. **Relative Merits of the Paraffin and the Celloidin Methods.**—Celloidin is good for large objects, for brittle or friable objects, and for delicate objects which heat would injure. It does not require removal from the tissues ordinarily, hence it holds delicate structures together permanently. Very thin sections cannot be cut, consequently it is of little value in cytological work. It is usually impractical to attempt to cut sections under 10 microns in thickness. The method, moreover, is extremely slow. The paraffin method is comparatively rapid, serial sections may be cut and mounted with ease, and very thin sections may be obtained. Large objects do not section satisfactorily, although up to 10 mm. or even greater diameter they cut readily. The rule is to use the paraffin method when you can.

8. **For Brittle Objects, a Combination of Celloidin and Paraffin Infiltration** sometimes proves successful. The method is too tedious for ordinary use although it must sometimes be resorted to with friable or delicate objects such as eggs. Infiltrate with celloidin in the usual manner and imbed in a paper box, but do not mount on a block. Harden in 80 per cent. alcohol, transfer to 95 per cent. alcohol for 12 hours, immerse in pure origanum oil and 95 per cent. alcohol equal parts for 12 hours, then in pure origanum oil for 2 to 3 hours; next transfer to a mix-

ture of equal parts of origanum oil and xylol for a few hours, and finally to pure xylol. Proceed from this point as in ordinary paraffin infiltration and sectioning, although the length of time in the paraffin bath should be curtailed as much as possible to avoid making the celloidin brittle.

9. **To Transfer Celloidin Sections from the Knife** it is an excellent plan to use a paper spatula; a bit of postal card held in the cleft end of a small stick answers very well. Press the paper down evenly on the section and then slide it off the edge of the knife. The section adheres to the paper. In carrying loose sections from one fluid to another an ordinary section lifter may be used or a glass rod around which the section is allowed to curl answers very well.

10. **Objects Stained in Bulk May Be Cleared while Yet in the Block,** then sectioned, and mounted without passing back into the alcohols. After the block of celloidin has hardened sufficiently in chloroform it is transferred directly to the clearer (cedar oil, or a mixture of oil of thyme 3 parts and castor oil 1 part). In cutting objects thus cleared the knife must be flooded with the clearer instead of alcohol. Do not allow the sections to become dry. If it is desired to use this method for a celloidin block which has already been preserved in 70 to 83 per cent. alcohol, the block must pass through 95 per cent. alcohol (1 to 2 hours) before it is placed in the clearer.

11. **Collodion instead of Celloidin** is used by some workers. Celloidin, in fact, is only a patent preparation of collodion, which is a solution of gun cotton in ether and strong alcohol. Thin and thick solutions are employed and the method is in every respect similar to the celloidin method. Collodion is cheaper than celloidin.

12. **Fixing Serial Celloidin Sections to the Slide** is accomplished, (1) by covering the sections, when mounted in proper order, with a strip of tissue paper which is then bound fast by wrapping thread around it. Lee (*Microtomist's Vade-Mecum*, 6th ed., p. 144) recommends (2) the albumen method for celloidin sections as well as for paraffin. (3) If the sections on the slide are carefully flooded with 95 per cent. alcohol two or three times, this drained off and followed by a small amount of ether-alcohol or ether fumes until the edges of the sections begin to soften perceptibly (10 to 20 seconds), the sections will generally adhere to the slide sufficiently when the celloidin becomes hard again upon exposure to the air (30 seconds) after the ether-alcohol has been drained off; they must then be immersed in 95 per cent. alcohol before any further steps are taken.

13. **Gilson's Rapid Celloidin Process** (Lee, *The Microtomist's Vade-Mecum*, 6th ed., p. 131) is a very valuable one because of the great saving of time. After dehydration the object is saturated with ether and finally placed into a test-tube containing thin celloidin. The lower end

of the tube is then dipped into melted paraffin and allowed to remain there until the celloidin solution has boiled down to about one-third of its original volume. The mass is then mounted in the ordinary way, hardened for an hour or more in chloroform, and cleared in cedar oil. Sections are cut as directed under memorandum 10.

CHAPTER VIII

THE FREEZING METHOD

1. Use a piece of spleen which has been properly fixed and later preserved in 70 per cent. alcohol. Transfer it through 50 and 35 per cent. alcohol successively to water, and wash it for 12 hours in running water.

2. Place it into a gum and syrup mass for 24 hours (a saturated solution of loaf sugar in 30 c.c. of distilled water, added to 50 c.c. of gum mucilage. Prepare a supply of gum mucilage by dissolving 60 grams of best gum acacia in 90 c.c. of distilled water).

3. Examine the freezing microtome carefully (Fig. 33).

4. Remove the gum and syrup mixture from the outside of the tissue with a cloth, put a little gum mucilage (not gum and syrup) on the freezing disk of the microtome, and place the tissue in it in such a way that longitudinal sections through the hilum may be cut. Surround the object with gum mucilage and set the freezing apparatus to going. If carbon dioxide is used, open the valve very cautiously, and let only a small quantity of the gas escape.

Note.—Carbon dioxide is commonly used for charging soda water and beer. It may be purchased in iron cylinders containing about 20 pounds of the liquified gas. The cylinder, when empty, is exchanged for a charged one, so that the purchaser pays only for the contents. The Bardeen microtome (Fig. 33) may be screwed directly upon the carbon-dioxide cylinder when the latter is in a horizontal position, or, if desired, the cylinder may be placed vertically and the microtome attached by means of an L-shaped piece of heavy tubing. This microtome has the advantage over the common forms of freezing microtomes of wasting less gas and of greater freedom from clogging. It is advantageous to have an extra long handle to the key which is used for opening the escape valve of the carbon-dioxide cylinder.

5. As soon as the gum is frozen, continue to add more until the tissue is completely covered and frozen.

6. Work the microtome screw with one hand and plane off sections (15 to 20 microns thick) with the other. The well-

sharpened blade of a carpenter's plane is the best instrument for cutting. It must be frequently stropped.

The blade should be mounted in a short, broad handle, which may be grasped easily and firmly with one hand. In cutting, the bevel edge of the knife should set squarely on the glass ways of the microtome so that the handle of the knife is inclined toward the operator at about an angle of 45 degrees from the perpendicular. The hand guiding the knife should be firmly supported against the chest while pressing the cutting edge steadily against the glass ways of the microtome. The cutting stroke is made by bending the body forward from the waist and thus forcing the blade squarely across the surface of the tissue.

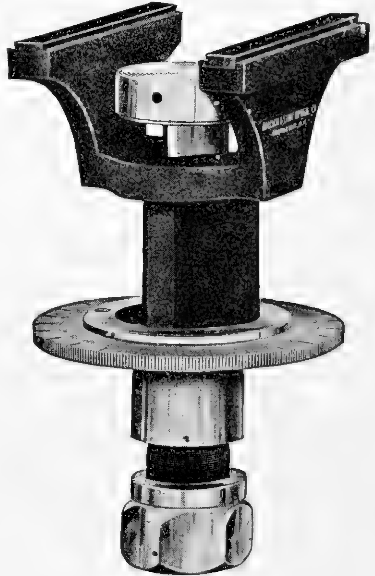


FIG. 33.—Bardeen Carbon-Dioxide Freezing Microtome.

The freezing chamber contains a spiral passage through which the expanding carbon-dioxide passes, securing the maximum freezing power. The knife slides on glass guides. The finest feed is twenty microns.

The blade must be kept cold to prevent sections from sticking to it. If the sections fly off or roll, the tissue is probably frozen too hard. The same defect may arise if there is insufficient syrup in the gum with which the tissue has been saturated. To correct, let the tissue thaw a little, and if still at fault, soak it again in a mixture which contains a greater proportion of syrup. Work rapidly, so as to cut sections in quick succession. Several sections may be allowed to collect on the blade before they need be removed.

7. Transfer the sections to distilled water. The water should be changed several times to dissolve out the gum. Reserve a few sections in water for later use (step 11).

8. Immerse a few of the sections for 10 to 30 minutes in Delafield's hematoxylin, then wash them in several changes of tap water.

9. Transfer the sections through the successive grades of alcohol (decolorizing with acid alcohol if necessary) up to absolute alcohol, leaving them two minutes in each, after which remove them to carbol-xylol for 5 minutes or until clear. If desired, stain with eosin (30 to 60 seconds) after 70 per cent. alcohol.

10. Remove one or two of the best to a slide, drain off the excess of carbol-xylol, add a few drops of balsam and a cover-glass of suitable size, and label.

11. Remove the sections reserved in step 7 to a test-tube containing a small amount of water, and shake the test-tube vigorously

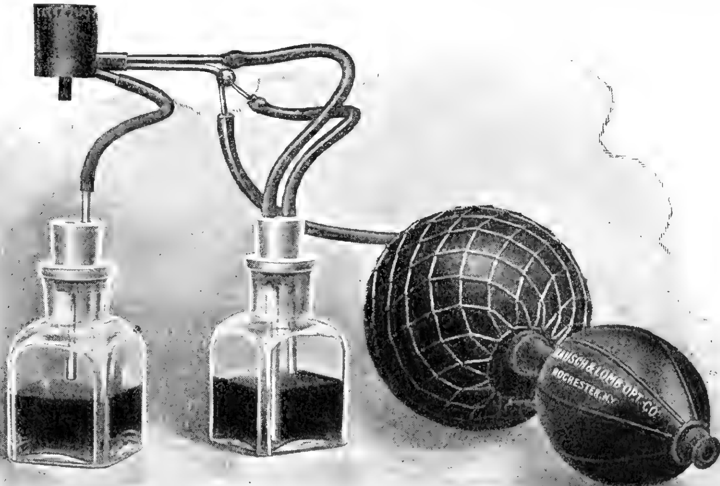


Fig. 34.—Ether or Rhigolene Freezing Attachment.

for a minute or two. This removes the lymphocytes from the sections, and exposes the reticular connective tissue so that it may be examined. Dehydrate the sections and mount in balsam.

MEMORANDA

1. **Fresh Tissues Are Frequently Sectioned** by the freezing method. The tissue may be transferred directly to the disk of a microtome without previous imbedding, and sectioned after freezing. This affords a ready

means of rapidly determining the nature of a given tissue, and is very serviceable, especially to the pathologist. The principal objection is that crystals of ice form in the cells and distort them badly. This is avoided when syrup and gum is used for imbedding.

2. **Sections May Be Preserved** in alcohol in the usual way after being cut by the freezing method. All trace of gum should be washed out and the sections passed through the grades of alcohol to 83 per cent., where they may remain indefinitely.

3. **Sections of Fresh Tissue May Be Fixed** and washed out after cutting if desired. This requires but little time, and the sections will take stain much more satisfactorily after having been subjected to a fixing reagent.

4. **Objects Which Alcohol Would Injure** may be sectioned by the freezing method and mounted in aqueous media.

5. **Ether or Rhigolene is Sometimes Used for Freezing,** although the method is more expensive and less satisfactory on the whole than the carbon dioxide method. Fig. 34 shows a common form of freezing attachment used for either of these liquids.

CHAPTER IX

METALLIC SUBSTANCES FOR COLOR DIFFERENTIATION

I. A GOLGI METHOD FOR NERVE CELLS AND THEIR RAMIFICATIONS

The Golgi chrom-silver method is one widely used for the demonstration of nerve cells together with their various processes. There are many modifications of the method all of which are more or less inconstant in their results. In a successful preparation, the various cells and nerve processes are not equally blackened, a fact which allows of discrimination between the different elements. Sometimes the ganglion cells and fibers remain unstained while the neuroglia cells are impregnated, or occasionally other elements than nervous tissue (e. g., blood-vessels) are affected.

The following method is applied to material preserved in 10 per cent. formalin and is a so-called "rapid method."

1. From the brain and spinal cord which have previously (see note, p. 38) been subdivided and placed in at least 10 times their volume of 10 per cent. formalin (3 days to an indefinite time), cut out small pieces 4 to 5 mm. thick from the region desired for study and transfer them to a vessel containing from 15 to 20 times their volume of a 3.5 per cent. aqueous solution of potassium bichromate. They should remain in this solution for from 2 to 5 days. Renew the fluid at the end of 12 hours. Keep the different pieces of tissue in separate vessels so as to avoid confusion.

2. For impregnation, transfer the tissues to a silver nitrate solution made as follows:

Silver nitrate (crystals)	1.5 grams
Distilled water	200.0 c.c.
Concentrated formic acid	1.0 drop

3. Rock the tissues gently in a small amount of this fluid until the brown precipitate of silver chromate ceases to appear, then transfer them into from 20 to 40 times their bulk of fresh silver nitrate solution and leave them *in the dark* for from 3 to 6 days. Change the fluid after the first 12 hours.

4. Transfer a few of the brown pieces of tissue to 95 per cent. alcohol for half an hour, renewing it once or twice during this time. Leave the rest of the tissue in the silver-nitrate solution for future use in case the first attempt proves unsuccessful.

5. Remove the pieces from 95 per cent. to absolute alcohol for 20 minutes, changing the latter once. Then transfer them to ether-alcohol for 20 minutes.

6. Imbed in celloidin without waiting for infiltration to occur (thin celloidin 30 minutes, thick celloidin 10 minutes). Mount directly on a block and harden in chloroform for 20 minutes.

7. From chloroform transfer directly to the clearing fluid (e. g., cedar oil), and as soon as clear (30 to 60 minutes) cut sections 50 to 100 microns thick, only keep the knife flooded with the clearing fluid instead of alcohol. Cut sections of cortex so that they will be perpendicular to the surface of the brain.

8. When the sections are thoroughly cleared, transfer them to a slide flooded with the clearing fluid, select such as prove desirable upon microscopic inspection, and discard the remainder.

9. Replace the oil with xylol, then remove the xylol by pressing upon the sections with blotting paper. Add enough *thick* Canada balsam to cover the sections.

Caution.—Do not put on a cover-glass; moisture must evaporate from the section. If this is prevented the metal deposits break up and the sections become worthless.

10. Keep the preparations level and put them away in a dry place free from dust. If the balsam runs off the sections more balsam must be added at once. Do not attempt to examine under a high power until the balsam is thoroughly hardened.

MEMORANDA

1. **A Fuller Account of the Golgi Methods** will be found in Hardesty's *Neurological Technique* (pp. 55-61), or in Lee's *Microtomist's Vade-Mecum* (pp. 411-27).

2. **An Osmium-Bichromate Mixture** is frequently used instead of formalin for fixing fresh tissues. To 85 parts of a 3.5 per cent. solution of potassium bichromate add 15 parts of a 1 per cent. solution of osmic acid. Small pieces (4 to 6 mm. thick) of fresh tissue are placed in 40 times their volume of this mixture and kept in the dark for from 12 to 24

hours. This fixing fluid is then replaced by a 3.5 per cent. solution of potassium bichromate as in the case of material fixed in formalin (see above). From this point the method is identical with the one given above.

3. **The Determination of the Elements That Will Be Impregnated** appears to depend upon the length of time the tissue is left in the 3.5 per cent. solution of potassium bichromate. Hardesty gives the following lengths of time for different structures: neuroglia, 2 to 3 days; cortical cells, 3 to 4 days; Purkinje cells, spinal cord, peripheral ganglion cells, 4 to 5 days; nerve fibers of the spinal cord, 5 to 7 days.

4. **Mounting the Sections upon a Cover-Glass** is preferred by some workers. The cover-slip is then fastened over the opening of a perforated slide with the section downward.

5. **For Permanently Mounting Golgi Preparations under a Cover-Glass,** Huber recommends the following method: the sections are removed from xylol to the slide and the xylol then removed by pressing blotting paper ovor the sections. A large drop of xylol balsam is then quickly applied and the slide is carefully heated over a flame from 3 to 5 minutes. A large cover-glass is warmed and put in place before the balsam cools.

II. SILVER NITRATE METHOD FOR NERVE. (*After Hardesty*)

1. The fresh nerve, or better a spinal nerve root, may be obtained from a frog which has just been killed. Without stretching the nerve, carefully insert beneath it the end of a strip of postal card or similar card which has been trimmed to the width of 50 mm. The nerve when cut off at each side of the card will adhere to it and remain straight and at approximately normal tension.

2. Clip off the end of the card bearing the nerve into a clean vial which contains 0.75 per cent. aqueous solution of silver nitrate. Place the vial in the dark for from 12 to 24 hours.

3. Transfer the nerve to pure glycerin on a slide and tease the fibers apart thoroughly under the dissecting microscope.

4. Add a cover-glass and expose the fibers to sunlight until they become brown (30 minutes).

5. To make the preparation permanent, take off the cover and remove the glycerin by means of filter paper, add a few drops of warm glycerin-jelly, put on a clean cover-glass, and press it down. Wipe away the exuded jelly and when the preparation has cooled,

seal the cover with gold-size, followed by Bell's cement. (See chap. xiii, **iii**, A.)

The preparation should show the "cross of Ranvier" and the "lines of Fromman."

III. GOLD CHLORIDE METHOD FOR NERVE ENDINGS

1. Trace some of the motor nerves of a reptile or mammal to where they enter the muscles (intercostals are best) and clip out small pieces of the muscle. Use material that has been preserved in 10 per cent. formalin.

2. Place the bits of muscle in 10 or 12 times their volume of a 10 per cent. solution of formic acid in distilled water and leave them for from 30 to 40 minutes.

3. Transfer the tissue into from 8 to 10 times its volume of a 1 per cent. solution of gold chloride in distilled water for from 30 to 40 minutes. Avoid direct sunlight. The muscle should become yellow in color.

4. Remove the tissue without washing it to about 25 volumes of a 2 per cent. formic acid solution and keep it in the dark until it assumes a purple color (24 to 48 hours). When the fibers appear reddish violet in color the reduction has gone far enough; if they show a decidedly bluish tinge the process has gone too far.

5. Wash the tissue in several changes of distilled water for an hour and transfer a small piece to a slide. Tease the fibers apart very carefully under a dissecting lens. Great care must be exercised to avoid tearing the nerve fiber from its endings. Examine from time to time under a low power of the compound microscope, and when a nerve fiber and its termination is found, carefully separate it as much as possible from the other fibers.

6. Add glycerin-jelly and a cover-glass. Seal in the ordinary way (chap. xiii).

NOTE.—Tissues may be dehydrated in the ordinary way and mounted in balsam or imbedded in paraffin or celloidin and sectioned.

CHAPTER X

ISOLATION OF HISTOLOGICAL ELEMENTS. MINUTE DISSECTIONS

I. ISOLATION

A. Dissociation by Means of Formaldehyde; ciliated and columnar epithelium.—1. Kill a frog and secure the hinder part of the roof of the mouth and the stomach. Slit open the latter. Place the objects in the dissociating fluid (see reagent 10, chap. i) for a day or two.

2. Scrape the roof of the mouth after removal from the fluid and mount the ciliated cells thus obtained on a slide. Similarly remove some columnar epithelium from the internal surface of the stomach and mount on another slide.

3. Add a cover-glass and examine. If the cells cling together in clumps, separate them by drumming gently upon the cover-glass with the handle of a needle.

4. Stain by placing a drop of picro-carmine (reagent 14, chap. i) on the slide just at the edge of the cover and applying a bit of filter paper to the opposite edge of the cover. The filter paper absorbs the fluid from under the cover and the stain replaces it.

5. After 15 or 20 minutes replace the stain by glycerin in a similar manner.

6. If a permanent preparation is desired the cover-glass must be sealed (see chap. xiii), or, after staining, the tissue must be dehydrated and mounted in balsam in the usual manner.

B. Isolation of Muscle Fibers by Maceration and Teasing.—1. Place small fragments of voluntary muscle, of the root of the tongue, and of heart muscle of the frog into separate vials containing MacCallum's macerating fluid (reagent 80, Appendix B). After 2 days pour off the fluid, fill the vials about half full of water and separate the fascicles by shaking the vial. Further isolate the fibers by teasing.

Teasing.—In teasing the important thing to remember is that the elements of the tissue are to be separated, not broken up. Both patience and sharp clean needles are indispensable. The process is best carried on under the lens of a dissecting microscope, although it may be done without such aid. A background which enables the tissue to be seen distinctly should be selected, black for colorless or white for colored objects. Black-and-white porcelain slabs are made for this purpose and are very convenient. A good dissecting microscope has attached beneath the stage a reversible plate one side of which is black, the other white. Use a small piece of tissue and begin teasing at one end of it.

2. With the aid of a dissecting microscope carefully tease out in water a number of fibers. Use a small piece and, beginning at one end, with both needles separate the piece along its entire length into two; likewise further subdivide these until the ultimate fibers are isolated.

3. Transfer some of the fibers through the alcohols and xylol and mount in balsam. Stain others in picro-carmine for 5 to 10 minutes and mount in glycerin as above.

C. Maceration by Means of Hertwig's Fluid (Hydra. Testis).—1. The solution consists of:

0.05 per cent. aqueous solution of osmic acid .	1 part
0.2 per cent. acetic acid	1 part

Prepare the ingredients for this mixture by diluting the stock solution (1 per cent.) in each case with distilled water. Make a separate 0.1 per cent. solution of acetic acid also.

2. Treat a hydra with the osmic and acetic acid mixture for 3 minutes and then transfer it to the 0.1 per cent. solution of acetic acid. Wash in several changes of this fluid to remove all osmic acid and let the hydra remain in the acetic acid for 12 hours.

3. Wash in water, stain in carmalum (reagent 34, Appendix B) or better in Acid carmine (reagent 37), and mount in glycerin as above. If the cells are not sufficiently separated, gently tap on the cover glass.

4. Submit small bits of the testis of some animal to the same treatment. Stain with methyl green (reagent 56) or acid carmine (reagent 37).

II. MINUTE DISSECTIONS

A. Alimentary Canal and Nervous System of Insects.—1. Carefully dissect out the alimentary canal of a cockroach and the central nervous system of a grasshopper with the aid of the dissecting microscope or lens. Wash each by gently flooding it with distilled water from a pipette, and then cover it with Gilson's fluid (reagent 15, Appendix B) or corrosive sublimate (reagent 13) for 5 minutes.

2. Wash in several changes of water during the course of half an hour and stain for 20 minutes in borax-carmine.

3. Wash in 50 per cent. alcohol and decolorize in 70 per cent. acid alcohol until the objects become bright scarlet in color.

4. Wash in 95 per cent. alcohol for 5 minutes and then transfer to absolute alcohol for 5 minutes, xylol or turpentine 10 minutes and mount in balsam. Apply cover and label.

B. Gizzard of Cricket or Katydid.—Pull off the head of a cricket or katydid. The gizzard usually remains attached to the head part. Cut it open lengthwise, wash out the contents and mount as above; only, omit the staining. The inside should be turned uppermost.

C. Sting of Wasp or Bee.—1. Place a wasp or bee in water, cover to keep out dust and let it stand for two or three days until the smell becomes unpleasant.

2. Wash in clear water and squeeze the abdomen gently until the sting protrudes. With forceps pull it out carefully. The poison gland and duct should come away with it.

3. Place the parts removed on a slide and under a lens draw the sting out of its sheath by means of a small needle which should be drawn over the outer surface of the sheath from the base to the apex of the sting.

4. Stain and follow out the same subsequent treatment as for **II, A** or mount without staining. It is advisable to compress the object between two slides as soon as the acid alcohol is washed out. The slides should be tied together and left in 95 per cent. alcohol several hours. Then proceed in the ordinary way.

D. Salivary Gland of Cockroach or Cricket.—Let the animal soak in water as for preparation of sting. When sufficiently decayed pull off the head carefully with forceps. The esophagus, the salivary glands, and crop usually come along with it. Stain and mount as for sting. For preparation of fresh salivary gland see Appendix C, II, "Salivary gland of Chironomous larva."

E. Mouth Parts of Insect.—1. Place the head of a bee or cockroach in 95 per cent. alcohol for 2 or 3 hours. Transfer to absolute alcohol for 30 minutes, and then to cedar oil for 30 minutes to an hour.

2. Remove the head to a slide and in a drop of the oil dissect out the mouth parts. Transfer them to a clean slide, remove the excess of oil and arrange them in their relative positions in sufficient balsam to hold them in place, then set the slide aside in a place free from dust until the balsam hardens enough to keep the parts from shifting. Make any necessary rearrangement. Add more balsam and a cover.

MEMORANDA

1. **The Cover-Glass May Be Supported** by means of small wax feet or bits of broken cover-glass when the tissue is too bulky to allow the cover-glass to fit down closely to the slide.

2. **A General Rule for Dissociating Tissues** is to use small pieces of the tissue and not a very great amount of the fluid.

3. **For Minute Dissections** clove oil is often a convenient medium. It tends to form very convex drops, clears well, and renders the object brittle; any or all of which properties may be useful in such dissections.

4. **The Fixation of Pieces of Macerated Tissue** (e. g., macerated epithelium) in 0.5 to 1 per cent. osmic acid for an hour or so, often proves advantageous.

CHAPTER XI

TOOTH, BONE, AND OTHER HARD OBJECTS

Sectioning Decalcified Tooth.—1. Kill a cat and remove the lower jaw (p. 53). With a fine saw cut out about a quarter of an inch of the bone bearing a tooth (e. g., canine), remove as much of the surrounding tissue as possible and place the object in Erlicki's fluid for several days. Transfer to nitric acid decalcifying fluid (reagent 11, chap. i). Use a relatively large quantity of the fluid and change it each day until the tooth is decalcified (2 to 6 days). It is sufficiently soft to cut when a needle can be thrust into it easily.

2. Wash it in repeated changes of 70 per cent. alcohol until all trace of the acid is removed (about 2 days) as shown by litmus paper.

3. Transfer the object through 50 and 35 per cent. alcohol successively to running water and wash for 24 hours.

4. Cut sections by means of the freezing microtome as directed under that method (chap. viii). If a freezing microtome is not available use the celloidin method.

5. After dissolving out all of the gum from the sections in distilled water, immerse them for half an hour in picro-carmine, then remove one or two of the best (through the center of the tooth) to a slide, drain off the excess of stain and add a few drops of melted glycerin-jelly. Cover with a circular cover-glass.

6. When the jelly has hardened, seal the cover with gold size and when this is dry, add a thin coat of Bell's cement (see chap. xiii, **ii**, A, 6).

7. Stain other sections in 1 per cent. osmic acid for 24 hours and mount in glycerin-jelly as above or dehydrate and mount in balsam.

Sectioning Decalcified Bone.—Saw out a short piece from the femur of a cat (p. 53). Prepare transverse sections by decalcify-

ing and sectioning in the same manner as for teeth. Do not destroy the periosteum. Prepare likewise longitudinal sections of a tarsal bone.

Sectioning Bone by Grinding.—1. With a fine saw cut a thin transverse section of the femur of a cat. Let it macerate in water until quite clean, then dry it carefully.

2. Grind the disk of bone between two hones, keeping the hones parallel in order to avoid wedge-shaped sections. The section is not thin enough until fine print can readily be distinguished through it.

3. Wash the section thoroughly in water, transfer it to absolute alcohol for 10 minutes, then to pure ether for half an hour

4. After removal from the ether, clamp it between two slides by means of a string or a rubber band and let it dry thoroughly.

5. Place some xylol-balsam in the center of a slide and heat it for a few minutes to drive off the xylol, then press the section of bone down firmly into it and put on the cover-glass. The balsam should not be thin enough to penetrate the tissue of the bone.

MEMORANDA

1. **Proper Fixing Before Decalcification** is necessary for the best results. Müller's fluid (reagent 8, Appendix B) is perhaps the most common reagent used in the fixation of bones or teeth. It requires 2 to 4 weeks to fix properly.

2. **Failure to Stain Properly** is due ordinarily to insufficient washing-out of the acid.

3. **Teeth and Other Hard Objects** may be prepared by grinding in the same way as bone.

4. **For Other Decalcifying Fluids** than nitric acid, see Appendix B, v.

CHAPTER XII

INJECTION OF BLOOD AND LYMPH VESSELS

Red Injection Mass.—1. Rub up 4 grams of carmine thoroughly with 8 c.c. of distilled water in a mortar and add ammonium hydrate drop by drop until a transparent red color results.

2. Soak 50 grams of best French gelatin in distilled water until it is swollen and soft (18 hours), then remove it to a porcelain evaporating dish and melt it at a temperature of about 45° C.

3. While the gelatin is yet fluid, slowly add the coloring matter, stirring constantly until a homogeneous mixture is obtained.

4. Before the mass cools add also some 25 per cent. acetic acid solution drop by drop, stirring thoroughly until the mass becomes slightly opaque and the odor of ammonia gives place to a faint acid smell. Watch for this change closely, for a few drops too much of the acid will spoil the entire mass by precipitating the carmine. If the ammonia is not completely neutralized, on the other hand, the coloring matter will diffuse through the walls of the injected vessels and stain the surrounding tissues. Just before using, the mass should be heated and strained through clean flannel.

With a large animal it is advisable to keep animal and apparatus submerged in warm normal saline during the operation of injection, but with a small animal this is unnecessary if the operator works rapidly.

Blue Injection Mass.—Prepare a gelatin mass as directed above. To the warm mass add sufficient quantity of saturated aqueous solution of Berlin blue to give the desired blue color. If the blue does not dissolve, add a little oxalic acid to the mixture. The blue mass need not be made for the present practical exercise unless the student wishes to undertake a double injection as indicated in memorandum 2.

INJECTING WITH A SYRINGE; SINGLE INJECTION

A common method of injection and one which proves satisfactory in many instances, is by means of a metal or glass syringe.

Although not as desirable in the main as the method of continuous air pressure, many good injections may be made by means of the syringe. The apparatus (Fig. 35) consists of a syringe fitted with a stop-cock in the nozzle, and a separate tube, known as the cannula, which fits on to the end of the nozzle. The syringes are made in different sizes and each is provided with an assortment of cannulae to fit vessels of different caliber.

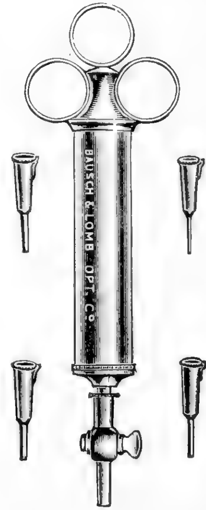


Fig. 35.—Injecting Syringe.

1. Provide yourself with several strong threads about four inches in length for ligating blood vessels. Have the red injection mass melted and heated to about 50° C. Also have ready some hot water to warm the syringe.

2. Kill a cat or a rabbit by means of chloroform. In death from chloroform the blood vessels are left dilated. Work rapidly so that the entire animal may be injected while yet warm. Stretch it out in a dissecting pan or tie it out onto a board.

3. Slit the skin along the ventral surface of the body to the middle of the neck and reflect it to the right and left sides. Pin it back out of the way.

4. Snip a small hole through the body wall just posterior to the ensiform cartilage. Insert the index finger of the left hand to guide the scissors and prevent injury to the underlying organs, and cut the costosternal cartilages of the right side up to the first rib. In like manner cut the cartilages of the left side up to the first rib.

5. Ligate the sternum tightly as close to the first ribs as possible to prevent leakage from cut blood vessels.

6. Cut off the apex of the heart and expose the ventricles. The left ventricle is seen as a round opening, the right as a slit.

7. With a sponge wrung out of warm water, rapidly absorb the blood from the thorax.

8. Choose the largest cannula that the aorta will admit and thrust it through the left ventricle into the aorta.

9. With a pair of fine-pointed forceps (preferably with curved points) pick up one end of a thread for ligating and carefully work it through under the aorta (do not mistake the vena cava superior for the aorta). Tie the thread around the aorta over the cannula, making a double or surgeon's knot. Draw it tightly on the cannula so that the latter will be held firmly in place. Run another thread through under the aorta and have it in readiness to ligate the aorta when the cannula is withdrawn.

10. Warm the syringe by sucking hot water into it repeatedly, then fill it with warm normal salt solution. Force out a little of the salt solution into the open end of the cannula, then connect cannula and syringe and force the warm solution through the blood vessels to cleanse them thoroughly of blood.

11. Empty the syringe completely and rapidly fill it and the cannula with the warm injecting fluid.

12. Force out a little of the fluid from the syringe to expel all air, and connect it carefully with the cannula.

13. Force the injecting mass into the blood vessels by a slow steady pressure. Begin with a very low pressure, so that the large vessels will be thoroughly filled before the mass enters the capillaries. The pressure should be gradually increased. Avoid sudden increase of pressure or too strong pressure, for either may cause a rupture of the blood vessels and consequent extravasation. From 8 to 10 minutes is about the time required to make a good injection of the cat.

14. Examine the intestines and the gums from time to time and also the inside of the thigh (from which the skin has been reflected); they should be deeply colored by the mass before the injection is complete. If the mass begins early to flow from the right ventricle, the ventricle should be ligated. In any event, it is well to tie the ventricle a few minutes before completion of the injection, to insure filling of all blood vessels.

Note.—If the gums remain uncolored, the cannula has probably been forced past the arteries which lead to the head. In such a case, complete the injection of the trunk and then, if injected tissue from the head region is desired, cut obliquely into one side of the innominate artery, tie a cannula in place and inject toward the head as in the case of the aorta.

15. When the injection is complete, shut the stop-cock, ligate the aorta, or clamp it with pressure forceps beyond the end of the cannula and then remove the latter.

16. Place the animal in cold water or cold alcohol for half an hour, then remove pieces of liver, spleen, pancreas, stomach, intestine, salivary glands, kidneys, and voluntary muscle and harden in absolute alcohol, or in 10 per cent. formalin.

17. When sufficiently hardened transfer the objects to ether-alcohol and proceed to imbed and cut in celloidin according to the method already given. Make longitudinal sections of the kidney parallel to its flat surface. Cut transverse sections of liver, stomach and intestine, longitudinal of the muscle, and sections passing longitudinally through the hilum of the salivary glands and spleen. The sections should not be under 30 microns thick. Mount some unstained; stain others in diluted Delafield's hematoxylin or in hemalum.

MEMORANDA

1. **Apparatus for Continuous Air Pressure Injections** is now provided in many laboratories. If a regular cylinder for air pressure is not present, however, an assistant with a little ingenuity can readily fit up a suitable apparatus. A carboy or large-mouthed bottle which can be tightly corked will answer as a chamber for compressed air, a water tap, or a tank of water elevated to the height of 7 or 8 feet will provide sufficient pressure. By making the proper connections by means of rubber and glass tubing a steady stream of compressed air may finally be conducted to a flask containing the injection mass; the flask works in the same way as an ordinary wash bottle. All corks and fittings must be tightly secured with wire or strong cord. If desired, by adding an extra perforation to the cork in the air chamber, a mercury manometer may be added to register the amount of air pressure. If a metal cannula is not at hand a glass one may be made as indicated under memorandum 8. In lieu of a stop-cock, use a pinch-cock on the rubber delivery tube.

2. **A Double Injection of the Vascular System** may be made by first injecting the blue mass until it is seen to flow from the right ventricle, then detaching the tube which conveys the blue mass, and slipping over the end of the cannula a tube conveying a red mass. This second mass should be in a bottle or flask connected with the pressure bottle by means of an additional tube through the cork of the latter, or the two flasks containing the colored masses may be connected with the tube

from the pressure bottle by means of a Y-tube. Each must be provided with a pinch-cock or clamp to hold back its contents while the other is in operation. If a syringe is used, it is better to have a second syringe for the second mass, although one will answer if it is rinsed out with hot water before being filled with the second mass. The second mass should have a quantity of very finely pulverized starch mixed with it, so that when it reaches the capillaries they will become completely plugged.

It should be borne in mind that the larger veins cannot be injected in a direction contrary to their flow, because of the valves they contain.

3. **The Lungs, Liver and Kidney** are readily injected through their larger blood vessels with two masses, and afford very instructive material when thus prepared. A triple injection of the liver may be made by injecting the hepatic artery and the hepatic and portal veins. The third mass may be colored with China ink. Whitman (*Methods in Microscopical Anatomy and Embryology*) recommends first injecting the hepatic artery and afterward the two veins. The blood should be washed out of the organ to be injected with warm salt solution.

4. **To Inject Lymphatics** the puncture method is commonly employed. For example, an aqueous solution of Berlin blue is drawn into a hypodermic syringe, the sharp point of the cannula is thrust into the tissue, and the syringe emptied by slight, steady pressure. For practice, thrust the cannula into the pad of a cat's foot, and force in some of the injection mass. If the leg is rubbed upward, the fluid will flow along the lymph channels and into the glands of the groin.

5. **To Keep Gelatin Injection Masses** let them congeal, then cover the surface with 95 per cent. alcohol, and leave in a well-stoppered vessel until needed.

6. **Injection through the Femoral Artery** is frequently practiced, and is preferred to injection through the aorta by some workers. An oblique cut is made in one side of the artery and the cannula inserted pointing toward the heart. Others prefer to cut into the dorsal aorta and inject both anteriorly and posteriorly.

7. **The Injecting Syringe** must work without jerking or catching along the wall of the barrel. It should always be carefully cleaned after using. If the piston does not fit the barrel tightly enough it should be wrapped with gauze.

8. **Glass Cannulae** may be made by grasping the ends of a short piece of soft glass tubing and heating the middle in a flame until the glass becomes soft, which is indicated by the yellow color of the flame. The tubing should be constantly rotated, so that all sides heat equally. When the glass becomes soft, draw the tube out steadily until the diameter of the soft portion becomes as small as desired. When the glass

has cooled, the tube should be cut with a file at the proper place to make two cannulae of it.

9. **If the Blue Color Fades** in the gelatin mass in the tissues, it may frequently be restored by treating the tissue or section with oil of cloves or turpentine.

10. **A Cold Fluid Gelatin Mass** has been used very successfully by Tandler (see abstract by A. M. C. in *Journal of Applied Microscopy*, Vol. V, p. 1625). To prepare the mass, dissolve 5 grams of finest gelatin in 100 c.c. of tepid distilled water. Color to the desired shade with Berlin blue, and then add slowly 5 to 6 grams of potassium iodide. The mass remains fluid at ordinary temperatures, but when injected objects are placed in 5 per cent. formalin, it sets completely and is thereafter unaffected by reagents. The minutest vessels are injected, and sections may be stained in the usual ways. Subjection to strong acids, such as sulphuric or hydrochloric, does not affect the mass, hence it may be used for injecting specimens that are to be decalcified afterward. To preserve the fresh mass, add a few crystals of thymol and keep in a stoppered bottle.

11. **Corrosion of Injected Vessels or Cavities** is sometimes practiced. A mass must be employed which will not be attacked by the reagent used for destroying the surrounding tissues. One of the best masses consists of white wax 5 parts and colophonium 6 parts, melted together at a temperature of about 75° C. For fine vessels increase the proportions of wax, for larger ones add more colophonium. Vermilion, Prussian blue, or chromate of lead may be used for coloring. The part to be injected should be placed in warm water and the mass injected at a temperature of from 50° to 60° C. The injected part is left in cold water for from 1 to 2 hours, and is then corroded in pure hydrochloric acid for from 6 to 48 hours, according to the resistance of the tissue. Finally, wash the preparation thoroughly in running water. For bibliography and more detailed directions see *Technique des Injections*, par Hermann Joris, Université Libre de Bruxelles, 1903.

CHAPTER XIII

OBJECTS OF GENERAL INTEREST: CELL-MAKING, FLUID MOUNTS, "IN TOTO" PREPARATIONS, DRY MOUNTS, OPAQUE MOUNTS

When objects of considerable thickness are to be mounted it is sometimes necessary to resort to cells which will contain the object and support the cover-glass. Fluid mounts and aqueous media must occasionally be resorted to for delicate objects which would be injuriously affected by alcohol, or which are unsuitable for mounting in balsam. When such mounts are used, whether in a cell or not, the cover-glass must ordinarily be sealed with a cement if the preparation is to be permanent. In all cases where it is at all practicable, balsam mounts are to be preferred for permanent preparations. Glycerin is a convenient mounting medium for many objects, especially for temporary mounts. It is often used where such media as balsam would render the preparation too transparent; it is much more favorable, moreover, to the preservation of color than are resinous media. For making cells and sealing circular covers, a turntable (Fig. 36) is desirable, although the work may be done by following a guide ring drawn on paper and placed under the slide.

I. TURNING CELLS

Prepare 12 or 15 slides as follows: 1. Place a slide on a turntable and adjust it so that its center lies over the center of the turntable.

2. Dip a small camel's hair pencil into gold size, but do not take up enough of the fluid to drop.

3. Choose a guide ring on the turntable which is of slightly smaller diameter than the cover-glass to be used, whirl the table and hold the pencil lightly over the guide ring. The ring which has been spun should be even. If it is not, practice turning rings until satisfactory ones are made. If the gold size is old it is probably too thick to make suitable rings. Pure linseed oil may

be used to dilute it, but it is advisable to use only fresh gold size if it is obtainable.

4. The slide must be set aside to dry before it can be used for mounting. A gentle heat will aid in drying.

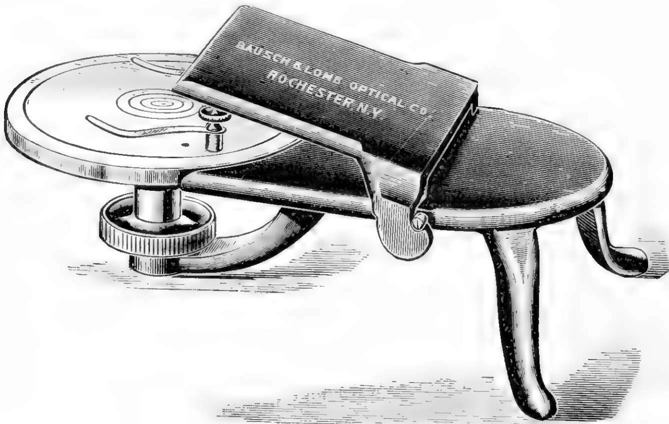


Fig. 36.—Turntable.

5. To some of the cells add successive coats of gold size as the previous one dries, so that you will have cells of varying depth.

II. MOUNTING IN GLYCERIN

A. Water Mites and Transparent Larvae.—1. Kill several small, colored water mites or transparent larvae of insects by means of chloroform (a few drops in water) and place them for half an hour (two or three hours for larger objects) into a mixture of water and glycerin equal parts, after which transfer them to pure glycerin,

2. Apply a thin coat of gold size to the upper edge of a cell which is of sufficient depth to accommodate the object.

3. Breathe into the cell to moisten it so that the glycerin will adhere throughout and prevent the formation of air-bubbles.

4. Fill the cell flush with glycerin and put the object into it, carefully spreading out all parts.

5. Breathe on the lower surface of a clean cover-glass, put one edge down on the edge of the cell and then gradually lower the

cover so as to avoid bubbles of air. When in place, press the cover down gently with the handle of a needle and see that it adheres all around. Wash off the exuded glycerin and carefully wipe the slide with a cloth.

6. Turn a comparatively broad ring around the edge of the cover to seal it, and when this is dry add a very thin coat of Bell's cement. Label and put away in a horizontal position until dry.

Caution.—It is *indispensable* that the edges of the cover-glass be perfectly dry before attempting to seal the preparation; otherwise the cement will not adhere.

B. Killing and Mounting Hydra.—1. With a dipping-tube remove a hydra to a warm watch-glass and leave it in only a few drops of water. Have ready some hot Gilson's fluid or corrosive acetic, and when the hydra sends out its tentacles and expands its body, apply the reagent by suddenly squirting it into the watch-glass so that it sweeps over the hydra from aboral to oral extremity and carries the tentacles out straight. Then fill the watch-glass with the hot fluid.

2. After 5 minutes pour off the fixing fluid and wash the animal thoroughly in 50 followed by 70 per cent. alcohol to which a little tincture of iodine has been added.

3. Replace the alcohol with borax-carmine or dilute hematoxylin and stain for from 30 minutes to several hours.

4. Remove the stain with a pipette and replace it with a mixture of equal parts of glycerin and water for half an hour, followed by pure glycerin. Proceed farther as in the preceding exercise.

Note.—After removal from the stain, if necessary, decolorize in acidulated water or alcohol (0.5 per cent. hydrochloric acid), then wash out the acid thoroughly in tap water.

Hydra may also be dehydrated, cleared and mounted in balsam. See also Appendix D, "Hydra."

III. MOUNTING IN GLYCERIN-JELLY

Glycerin-jelly is frequently preferable to pure glycerin for mounting because it is a solid at ordinary temperatures. One formula for making it is as follows.

Water	42 c.c.
Gelatin	6 grams
Glycerin	50 c.c.
Carbolic acid crystals	2 grams

Dissolve the gelatin in the water and add the glycerin and the carbolic acid. Warm for 10 or 15 minutes stirring continually until the mixture is homogeneous. Do not heat above 75° C. or the gelatin may be transformed into metagelatin which will not harden at ordinary temperatures. Filter through fine hot flannel. Use only clean gelatin of the best quality.

A. Small Crustacea.—1. By means of a dipping-tube isolate such small creatures as Cyclops, Daphnia, or Cypris.

2. Kill by warming slowly in a drop of water on a slide.

3. Place them in a cell of proper depth, draw off all water with a pipette, and gently warm the slide.

4. Place the bottle of glycerin-jelly into a vessel containing warm water until the jelly becomes liquid, but do not let it get any warmer.

5. Fill the cell flush with the warm jelly and arrange the objects in suitable positions.

6. Breathe upon the lower surface of a clean cover-glass and put it in place in the usual way.

7. Wash away any trace of the jelly from the outside of the cell and when the slide is dry run a ring of gold-size cement around the edge of the cover. After this dries varnish with Bell's cement. It is not an absolute necessity to seal glycerin-jelly mounts, but the writer has always found it a wise precaution.

B. Muscle of Insect.—1. Cut off the head of an insect and bisect the trunk so as to expose the interior. Observe two kinds of muscular tissue, that of grayish color belonging to the legs, the yellowish to the wings.

2. Take a shred of muscle and on a dry slide carefully separate pieces of muscle fiber and stretch them out, while keeping them moist by breathing on them.

3. Mount in glycerin-jelly as directed in the previous exercise. See also Appendix C, IX.

IV. MOUNTING IN BALSAM

A. Flat Worms.—1. Obtain specimens of Planaria from the under surface of flat rocks in the edge of streams (see Appendix D, "Planaria").

2. Place the animal in a little tepid water. Watch until it is extended full length, then flood it quickly with hot corrosive sublimate, or hot Gilson's fluid. The animal may be removed after 10 or 15 minutes and washed thoroughly in 50 per cent. alcohol to which a little tincture of iodine has been added.

3. Stain for 24 hours in borax-carmine, or in Delafield's hematoxylin diluted one-half with water.

4. Wash in water followed by 35 and 50 per cent. alcohol each 15 minutes.

5. Decolorize in acid alcohol until the color ceases to come away freely (10 to 30 minutes).

6. Wash out the acid in 70 per cent. alcohol, using the alkaline alcohol if hematoxylin was used in staining.

7. Flatten the animal by compressing it between two slides by means of a rubber band, and place it for 24 hours in 95 per cent. alcohol.

8. Transfer to absolute alcohol for 1 hour, and to xylol until clear.

9. Mount in balsam in a thin cell or without a cell at pleasure. If on examination the separate organs of the animal are not seen distinctly, the reason is probably that the object has not been compressed sufficiently. This difficulty may also sometimes be overcome in a measure by letting a cover-glass rest upon the live Planarian to flatten it out slightly, and then running the fixing fluid under the cover. Specimens which have been in the laboratory for some weeks or months make better preparations than those fresh from the stream.

B. Mosquito, Gnat, or Aphid.—1. Kill a mosquito with cyanide or chloroform and place it in cedar oil or turpentine for an hour.

2. Remove, and place it on its back on filter paper. Carefully spread the legs of the insect, put a drop of thick balsam on a slide, invert the slide, and bring the balsam in contact with the thorax of the mosquito. Spread the wings and the legs of the insect and gently press it down into the balsam.

3. Add thinner balsam, see that the proboscis and antennae

are floated out properly, then add more balsam, and put on a cover-glass.

V. OPAQUE MOUNTS

Some objects are mounted to be viewed by reflected instead of transmitted light. They may be mounted in the ordinary way, and when examined as opaque objects, the light from the mirror should be turned away and, if necessary, a strip of dark paper placed under the slide to shut off all light from below.

A. Beetles.—Choose a shallow cell for mounting the wing cases and legs of one of the Curculionidae, preferably *Curculio imperalis*, the South American diamond beetle.

1. Soak the part in cedar oil or turpentine for half an hour, then place it in the cell in the proper position, the outer side of the case toward the observer.

2. Fill up the cell with balsam and add the cover.

B. Wings of Moths or Butterflies.—Prepare parts of the wings of moths or butterflies as in **A.** The wing of the clothes moth makes a good opaque mount.

C. Head of a Fly.—1. Secure the specimen (preferably one having colored eyes, as one of the gad-flies) and choose a cell of the proper size for it. The cell should be of such a depth that the cover will rest lightly upon the object and retain it in the center of the cell. The head should present the front view when mounted.

2. Spin a very thin coat of gold size on to the dry edge of the cell so that the cover will adhere.

3. Soak the head of the fly for a couple of hours in equal parts of glycerin and water.

4. Moisten the cell by breathing into it, fill it with glycerin and transfer the object to it.

5. Breathe on the cover-glass and apply it very carefully to avoid air-bubbles. When the cover settles into place, press it down gently to make it adhere to the cement.

6. Set it aside to harden. When hard, seal on the turntable with gold-size followed by Bell's cement when the gold-size is dry.

D. Foreleg of Dytiscus, the Great Water Beetle.—1. Detach the foreleg of a male, and soak it in 10 per cent. potash solution (see Appendix B, reagent 76) for a day or two.

2. Wash it in water, run it up to 95 per cent. alcohol, and leave it there for 24 hours.

3. Pass it through absolute alcohol and clear in cedar oil, turpentine or xylol.

4. Lay the leg, disk side uppermost, in a drop of balsam on a slide, add another drop of balsam and carefully cover with a clean cover-glass. Place a small weight (e. g., half of a bullet) on top of the cover to hold it down until the balsam hardens.

VI. DRY MOUNTS

A. Scales.—Prepare a very shallow cell and let it dry. Thoroughly dry the scales from a moth's wing by gently heating them on a slide over a flame. Place the scales in a cell, warm the slide until the cell wall becomes sticky, put on the cover and press it down until it adheres all around, and finally seal as in previous exercises.

B. Eggs of Butterflies, Small Feathers, Antennae of Insects, etc., may be mounted as dry objects. Care must be taken to have them *perfectly* dry, or they will in time cloud the cover with moisture from within.

MEMORANDA

1. **Small or Soft Insects or Their Larvae** may frequently be mounted directly in glycerin, or they may be dehydrated and mounted in balsam. A method often used is to kill them in strong carbolic acid and mount them directly in balsam. The carbolic acid both dehydrates and clears. It is better, however, to clear the preparation further by immersion in cedar oil or xylol before adding the balsam.

2. **Insects Having Hard Shells** must first be soaked in 10 per cent. potash to soften them and render them transparent if they are to be examined by transmitted light. The softer parts of insects so treated are destroyed and only the external parts remain. Such insects may be mounted in glycerin or glycerin-jelly, or they may be dehydrated, cleared and mounted in balsam.

3. **Delicate Insects,** which are too frail to withstand much handling, may be placed at once in cedar oil or turpentine and after an hour mounted in balsam.

4. **Wings, Legs, Antennae, Mouth-Parts, etc., of Such Forms as Flies and Bees** which have been preserved in alcohol, should be completely dehydrated, cleared and mounted in balsam in cells of the proper depth.

5. **Transparent and Soft Insects** may be stained in borax-carmine or hematoxylin in the ordinary way and mounted as whole objects, if desired. They will stain better if they have been fixed previously in some corrosive sublimate mixture and then washed properly (see Appendix B, reagent 13). To stain, follow the method outlined in **IV**, A.

6. **To Center an Object in a Cell** (the head of an insect, for example), thread a fine needle with a hair and run it through the object. Remove the needle and imbed the ends of the hair in the cement on opposite sides of the cell. When the cover-glass is put in place the object may be

adjusted by pulling the hair. After the slide is finished and dry, the ends of the hair should be cut off at the edge of the cell.

Another method which will frequently answer for an object to be mounted in balsam is to place the object (after clearing) in the center of the cell, coat it with balsam, adjust it properly, and then set the slide away in a place free from dust till the balsam thickens. Finally fill the cell with balsam and add the cover.

7. **The Radula or Lingual Ribbon of the Snail or Slug** should be dissected out and soaked for a day or two in a 10 per cent. solution of potash. If the animal is a small one, cut off the head including the buccal mass and soak it in a solution of potash until the soft tissues are destroyed and only the radula remains. From the potash the radula is transferred to water and washed for some hours. With a strip of paper on each side to prevent crushing it, it should be placed between two slides, and the slides bound together by means of string or rubber bands. While held in this position, dehydrate and clear it. Finally remove one slide and the paper and mount the object in balsam on the other slide. A shallow cell may be used if desired.

8. **Flukes and Tapeworms** are prepared in the same manner as planaria (see IV, A). The time of immersion in the various fluids should be lengthened in proportion as the object is larger than the planarian. See also Appendix D.

9. **Spirogyra, Protococcus, Volvox, Desmids, etc.,** may be mounted in a cell in the following copper solution:

Acetate of copper	1. gram
Camphor water	240. c.c.
Glycerin	240. c.c.
Glacial acetic acid	0.3 c.c.
Corrosive sublimate, saturated aqueous solution	0.1 c.c.

Mix thoroughly, filter and keep in a glass-stoppered bottle. The green color of the plant may frequently be preserved for some time in this medium. The specimen is washed in water, transferred to the cell, then the solution is added. The cell is covered and sealed in the usual way.

10. **A Dipping-Tube** is a simple glass tube. To operate it, hold the tip of the forefinger over the upper end and dip the lower end into the water until it comes just above the object desired; lift the finger and let the air out of the tube, and the water will rush in at the lower end carrying the object with it. Replace the finger over the top of the tube and remove it; the water will remain in it as long as the finger is held firmly over the upper end. When the finger is removed the water and the object pass out. The object may sometimes be more readily discharged if the tube is rotated.

11. **To Keep Water from Evaporating from a Cell too Freely** use a round cell and cover it with a square cover-glass. Apply a brush wet with water to the slide beneath one of the projecting corners of the cover from time to time. Capillary attraction will draw in the water and will keep the cell full. If a continuous supply of fresh water is necessary, one end of a loosely twisted cotton thread may be laid along one side of the cover and the other end of the thread immersed in a small vessel of water which stands within half or three-quarters of an inch of the cell. A reservoir made from the bottom of a shell vial or homeopathic vial answers very well; it may be cemented to the slide.

Protozoa and other small forms may be kept alive on a slide for a number of hours by simply mounting them in water under a cover in a cell of blotting paper which has been saturated with water.

12. **Deep Cells** are made frequently by cutting out rings of paper, lead, or block-tin with gun punches and cementing them to the slide. Glass and hard rubber rings of various sizes may be purchased from dealers.

CHAPTER XIV

BLOOD

I. EXAMINATION OF FRESH BLOOD

a) *General.*—1. Thoroughly clean a slide and cover, bathe a finger in ether-alcohol (reagent 16, Appendix B), sterilize a sharp needle by heating it in a flame and then prick the finger with the needle.

2. Place a small drop of the resulting blood on a slide and quickly put on a cover-glass. To prevent evaporation, the edges of the cover may be surrounded by olive oil or vaselin.

Living corpuscles may also be studied in a drop of normal saline.

b) *Effects of reagents.*—When it is desired to study the effects of reagents on fresh blood (e. g., distilled water, 1 per cent. tannic acid, etc.) a drop of fresh blood is placed on a slide, the cover is put on and then the blood is "irrigated" with the reagent. That is, a drop of the reagent is placed at the edge of the cover to be drawn under by capillary action. The process may be hastened by applying the edge of a bit of blotting paper to the opposite edge of the cover.

c) *To demonstrate blood-platelets.*—Place a small drop of a 1 per cent. solution of methyl violet (reagent 57, Appendix B) in normal salt solution, on a finger which has been cleaned by washing it in ether-alcohol. With a sterilized needle prick the finger through the stain and mount a drop of the blood which exudes. Examine it under a high power. Both platelets and white corpuscles are stained.

d) *Stained preparation of fibrin.*—Mount a drop of blood on a slide as in *a*. Place it in a moist chamber for from 20 to 30 minutes to coagulate. Loosen the cover with a few drops of water and then thoroughly irrigate the preparation with water. Drain off the water, blot the preparation with blotting paper and add immediately a drop of a 1 per cent. aqueous solution of eosin (reagent 40, Appendix B). Remove this after 3 minutes, rinse the preparation in water, then treat it 3 minutes with a 1 per cent. aqueous solution of methyl violet (reagent 57, Appendix B). Rinse the preparation in water, let it dry, and finally mount in balsam.

e) *Crystals of the blood.*—

1) **Hemoglobin Crystals.**—Allow a drop of blood to dry on the slide without covering it. Long rhombic prisms of a red color crystallize out. The blood of a rat is best for demonstration. A more certain method is as follows: To 5 c.c. of blood in a test-tube add a few drops of ether

and shake the mixture vigorously until the blood becomes laky. Place a drop or two of the laked blood on a slide and allow it to dry in the cold.

2) **Hematoidin Crystals**; reddish-yellow crystals (rhombic plates). They can be obtained from old blood extravasations (e. g., cerebral hemorrhage, corpora lutea, etc.) by teasing. Mount in Canada balsam.

3) **Hemin or Teichmann's Crystals.**—To a small drop of blood on a slide or a bit of cloth which has been previously saturated with blood, add a few crystals of common salt. Heat over a flame until the mixture has become dry, leaving a reddish-brown residue. Apply a cover-glass and flood the preparation with as much acetic acid as will remain in place under the cover. Heat the preparation until the acetic acid boils. After the acid has evaporated the preparation may be made permanent by adding Canada balsam. The crystals are very small, narrow rhombic plates of dark brown color. They vary in size and may lie singly, across one another, or in stellate groups.

The presence of these crystals is positive evidence of the presence of blood, hence their demonstration is of great importance in stains or fluid suspected of containing blood.

II. COVER-GLASS PREPARATIONS

a) *Dry preparations* (Ehrlich's method).—1. In this method the preparation is "fixed" by means of heat. Under one end of a copper bar or copper triangle (Fig. 26) place a flame. After 15 or 20 minutes a given point on the bar will have a practically constant temperature. Thoroughly clean the bar, run a stream of water along the top of it toward the flame, and locate the point farthest from the flame at which the water boils. The blood smears when prepared are to be placed film side up in a row across the bar about three-fourths of an inch nearer the flame than the point at which the water just boiled. This will subject them to a temperature of about 120° C.

2. Thoroughly clean and dry two cover-glasses, touch one to a drop of perfectly fresh blood as it comes from the finger or lobe of the ear and instantly drop it onto the second cover. The blood should spread in a thin film between the covers; if it does not, it has begun to coagulate and the preparation will be inferior. Rapidly separate the covers by sliding them apart, wave them in the air a minute to dry the films, then place them down with the smear side uppermost. *Do not press the covers together* to spread the blood because this ruins the corpuscles. If the red corpuscles are to retain their shape the film of blood must be *extremely* and *uniformly* thin. Practice until you have prepared such a film.

NOTE.—In the clinical examination of blood great care must be exercised to have it absolutely fresh; furthermore, the cover-glasses should be handled with forceps instead of by means of the fingers. It is recommended that one pair of the forceps be Coronet or spring forceps of some kind (Fig. 39). The lobe of the ear is perhaps the best region from which to obtain the blood. The needle with which the puncture is made should always be sterilized. Wipe away the first drop of blood that appears. The drop finally chosen should be one that has appeared *immediately* after the spot has been wiped and it should be but little larger than a pin-head. The whole operation cannot be performed too rapidly. To shorten the time it is well to have an assistant to prick and manipulate the ear while the operator attends to the preparation of the film.

3. When several satisfactory films have been prepared, place them on the heated bar as indicated in step 1. Cover them to keep out dust and leave them for from 30 to 60 minutes.

4. Remove the covers and stain the preparations 15 or 20 minutes with Ehrlich's triple stain (reagent 39, Appendix B) by flooding the film with the stain by means of a pipette. Rinse off the surplus stain with water, blot the film with blotting paper and dry it by holding it with the edge downward high above the flame. When dry, mount in balsam on a slide.

NOTE.—Instead of heating the preparation, much the same results may be obtained by subjecting films (prepared as in step 2) to ether-alcohol (reagent 16, Appendix B) for from 1 to 12 hours, drying them again in the air and then staining as above.

b) *Rapid method.*—1. Prepare a film as above (*a* 2), only, before it has dried treat it with concentrated aqueous solution of corrosive sublimate (reagent 13, Appendix B).

2. Wash the preparation thoroughly in water or in 50 per cent. alcohol.

3. Stain for 10 minutes in Delafield's or Ehrlich's hematoxylin (reagent 49 or 50, Appendix B), rinse in 70 per cent. alcohol and stain for 20 seconds in eosin (0.5 per cent. solution in 70 or 95 per cent. alcohol).

4. Rinse in 95 per cent. and in absolute alcohol each for 2 minutes, pass through xylol and mount in balsam.

After rinsing following staining, some workers simply blot the preparation with blotting paper, dry it in the air and mount it in balsam.

III. ENUMERATION OF BLOOD CORPUSCLES

"The instrument used is the hemocytometer (Fig. 37). Obtain a drop of blood from the lobe of the ear or from the finger. Fill the clean pipette of the hemocytometer to the mark 1 by careful suction. (If the blood is drawn beyond the 1 mark, blow it out immediately, clean the

tube and repeat the operation.) Wipe the blood from the outside of the pipette and draw in sufficient Toisson's solution to make the level of the combined liquids stand precisely at the mark 101.

Toisson's solution:

Sodium sulphate	8.0	grams
Sodium chloride	1.0	gram
Neutral glycerin	30.0	c.c.
Methyl violet, 5b	0.025	gram
Distilled water	160.0	c.c.

"Mix the blood thoroughly with the solution by shaking the tube for a few minutes. The blood is thus diluted 100 times.

"Blow out a drop of the liquid to remove the unmixed solution remaining in the capillary tube. Have the counting disk and cover-glass per-

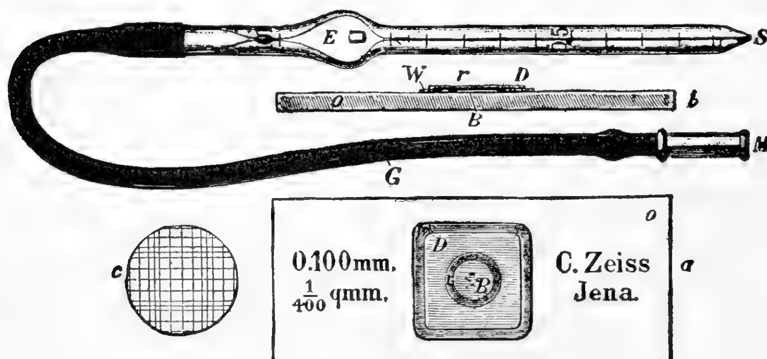


Fig. 37.—Hemocytometer.

a, view of slide from above; *b*, view of slide from one side; *c*, counting-disk which lies at the center of *B*; *E*, bead for mixing; *M*, mouthpiece.

fectly clean. Allow a drop of the diluted blood to flow onto the disk and place the cover-glass over the drop. The cell of the disk must be entirely filled by the drop of blood.

"Examine the preparation under a high power of the microscope, and count the number of red corpuscles in 20 to 40 small squares; of those corpuscles which happen to lie on the boundary line, count the ones that lie only in the upper and on the left sides of each square. Take the average number in a square and calculate the number of corpuscles in a cubic millimeter of blood.

"The depth of the entire cell is 0.1 mm., the area of each small square is $\frac{1}{400}$ sq. mm., consequently the volume of blood in each square column is $\frac{1}{4000}$ c. mm., or 1 cubic millimeter of diluted blood would contain 4,000 times the average number in a square. One cubic milli-

meter of undiluted blood contains 100 times as many, or 400,000 times the number in one square. What result do you obtain?

"After finishing the count, clean the pipette by successively drawing into and expelling from it water, alcohol, and finally ether. Do not blow through it, but cause the ether to evaporate by sucking air through the tube. For counting the white corpuscles use the large pipette and dilute the blood 10 times with one-third of 1 per cent. glacial acetic acid. The acid destroys the red corpuscles and thus the white corpuscles are more readily seen. Proceed in the same manner as for red corpuscles." (From *Laboratory Outlines for Physiology*, by Guyer and Pauli.)

IV. OBSERVATION OF THE BLOOD CURRENT

a) *Circulation in the web of a frog's foot.*—Wind a long strip of cheese cloth around a frog stretched out upon a narrow piece of thin board, leaving one hind foot exposed. Soak the cloth in water in order to keep the animal's skin moist. Pin the extended foot onto a ring of cork in such a way that the web between the toes is stretched over the opening in the cork. Examine under the microscope. If the preparation is favorable leucocytes may perhaps be seen penetrating the walls of the vessel (*diapedesis*) and passing into the surrounding tissues.

b) *Circulation in the mesentery. Inflammation.*—Immobilize a frog (the male is better) by injecting a few drops of a 1 per cent. solution of curare into one of the dorsal lymph sacs. Curare paralyses the nerve endings. After waiting 20 minutes for the curare to be absorbed into the circulation, cut open the abdominal wall for a short distance along the left side and draw out several loops of the intestine. Pin out a favorable area of mesentery over a cork ring, and, after covering it with a cover-glass, examine under the microscope. Keep the parts moistened with normal salt solution. Such a preparation is especially favorable for studying the migrations of leucocytes through the walls of the vessels. Do not have the mesentery stretched too tightly or the circulation will cease. After a time the phenomena of inflammation may readily be observed. It is hastened if some irritant (e. g., a drop of creosote) is applied to the mesentery.

MEMORANDA

1. **For Demonstration of the Different Granules of Leucocytes,** etc., see Appendix C, I, under the general topic of blood.

2. **To Study Blood in Sections,** ligate a small vessel in two places to keep in the corpuscles, then remove the piece so prepared and fix it in Gilson's fluid (reagent 15, Appendix B) or Hermann's fluid (reagent 26, Appendix B). Imbed in paraffin and cut thin sections. Stain material fixed in Gilson or other corrosive sublimate reagents by the hematoxylin

eosin method (reagents 49, 40, Appendix B) or with the Ehrlich-Biondi stain (38, Appendix B). The blood fixed in Hermann's fluid may be stained by the saffranin-gentian violet method (66, Appendix B).

3. **Amoeboid Movements in Leucocytes** may readily be observed in blood (preferably amphibian) which has been mounted on a slide in very slightly warmed normal saline. Place a hair under the cover-glass and seal the edges of the latter with vaselin or melted paraffin. For continuous study of the white corpuscles of warm-blooded animals a warm stage of some kind is necessary, to keep the temperature of the blood near the temperature of the body.

4. **Feeding Leucocytes.**—Rub up sufficient India ink in a few drops of normal saline to make a grayish fluid. With fine scissors make an incision into one of the dorsal lymph sacs of a frog (parallel to and close beside the urostyle). Introduce a capillary pipette into the wound and obtain a small drop of lymph. Mix it on a slide with a drop or two of the prepared ink. After placing a hair across the field put on a cover-glass and seal the edges with vaselin or melted paraffin. Under a high power of the microscope the cells may be seen engulfing the colored particles.

5. **Wright's Stain for Blood.**—To prepare the stain make a 0.5 per cent. solution of sodium bicarbonate in distilled water and add to it 1 per cent. of methylen blue (Grübler). Subject the mixture to steam in an ordinary steam sterilizer (e. g., Arnold; not a pressure sterilizer or a water-bath) for one hour. When the mixture is cool, without filtering, pour it into a large dish. Prepare a 1 per cent. aqueous solution of Grübler's yellowish eosin (soluble in water) and, with constant stirring, add it to the methylen-blue solution until the blue color is replaced by purple, and a yellowish scum with metallic luster forms on the surface of the mixture, while a finely granular black precipitate appears in suspension. The proportions required will be about 500 parts of the eosin to 100 parts of the methylen blue solution. Collect the precipitate on a filter, dry it thoroughly, make a 5 per cent. solution in pure methylic alcohol. To prevent the alcohol from evaporating keep the bottle containing the solution tightly stoppered. Should precipitation occur, filter the stain and add a small quantity of methyl alcohol.

Mallory and Wright in their *Pathological Technique*, p. 374, give the following summary of the method for staining blood films:

"1. Make films of the blood, spread thinly, and allow them to dry in the air.

"2. Cover the preparation with the staining fluid for one minute.

"3. Add to the staining fluid on the preparation sufficient water, drop by drop, until a delicate, iridescent, metallic scum forms on the surface. Allow this mixture to remain on the preparation for two or three minutes.

"4. Wash in water, preferably in distilled water, until the film has a pinkish tint in its thinner or better-spread portions and the red corpuscles acquire a yellow or pink color.

"5. Dry between filter-paper and mount in balsam. The preparations retain their colors as long as any preparations stained with anilin dyes.

"Unstained blood films may be kept for some weeks without impairment of their staining properties. Films months old will probably not give good results."

6. **For Malarial Parasites** Wright's stain (memorandum 5) is excellent. It yields the so-called Romanowsky stain; the color of the chromatin varies from lilac to very dark red, while the body of the parasite stains blue. A full account of the method will be found in Mallory and Wright's *Pathological Technique*, p. 421.

CHAPTER XV

BACTERIA

No attempt is made here to give even an elementary account of bacteriological technique. Only such phases of the work as are concerned with the immediate microscopical examination of bacteria are touched upon, and these chiefly to afford some practice in this kind of manipulation. For special technique, identification, or descriptions of apparatus and accessories, the student is referred to standard textbooks.

BACTERIAL EXAMINATION

Bacteria when prepared for microscopical examination are in the form of

A. Cover-glass preparations,

B. Bacteria in tissues (section method), or

C. Hanging-drop preparations.

A. Cover-Glass Preparations

I. *Killing and fixing.*

1. **From Fluid Media** (e.g., bouillon, milk, water, saliva, blood, pus, etc.).—Sterilize a platinum wire loop by heating it red hot in a flame. When cool, touch the loop to the culture and spread the adherent bacteria in a thin film over the surface of a cover-glass which has been sterilized in a flame. After the film has dried in the air, kill and fix the bacteria to the cover by passing it three times, film side uppermost, through the apex of a flame. Each time should not exceed half a second. Prepare several films from a given material. Coronet or similar forceps (Figs. 38, 39) should be used for handling such films, because the cover-glass can be left in them through the entire operation of fixing and staining.

If a platinum loop is not at hand a second cover-glass may be used to spread the smear. The first cover-glass is held in a pair of cover-glass forceps and the second cover-glass is dropped on to it. The glasses are then rapidly drawn apart with a sliding motion by means of forceps. The glasses should not be pressed tightly together. Proficiency in making such preparations is gained only after considerable practice. The chief secret in making a good preparation is to get the films extremely thin and evenly distributed.

2. **From Solid Media** (gelatin, agar, meat, potato, animal tissues and organs, etc.).—The procedure is the same as for 1, except that a drop of

sterilized water or bouillon is put on the cover-glass to facilitate the spreading of the bacteria in a film over the cover.

II. *Staining and Mounting.*

1. Gentian violet (memorandum 3*a*) 5 minutes. The cover-glass is left in the forceps, film side up, and the film flooded with the staining fluid.

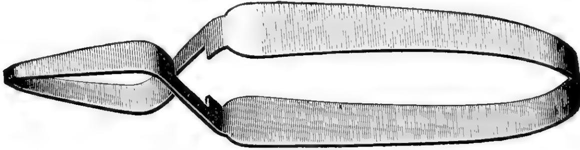


Fig. 38.—Cornet's Cover-Class Forceps.

2. Rinse in water.

3. Gram's solution (memorandum 3*f*) until the color becomes black (2 to 3 minutes).

4. Ninety-five per cent. alcohol until the violet color has almost completely disappeared.

5. Rinse in water and examine by placing the cover-glass film side downward on a slide. Only a thin film of water should remain between the slide and the cover. Remove surplus water by means of blotting paper. If a prolonged examination is to be made, water lost by evaporation must be replaced by occasionally placing a small drop of water at

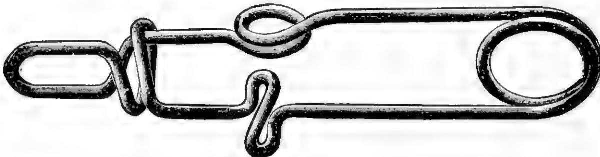


Fig. 39.—Stewart's Steel Wire Cover-Glass Forceps.

the edge of the cover. In ordinary work the final inspection is frequently made at this stage. If a permanent preparation is desired, however, proceed with the following steps:

6. If the bacteria are well stained, a counterstain of Bismarck brown (memorandum 3*d*, sol. 2) may be added (5 to 10 seconds). This step may be omitted.

7. Absolute alcohol, 10 to 15 seconds.

8. Xylol.

9. Xylol-balsam.

Note.—In staining, if the cover-glass is warmed over a flame some 15 or 20 seconds until the stain steams, the action of the stain is usually more intense and more rapid. Boiling, however, must be avoided.

B. Bacteria in Tissues

Tissues may be fixed and hardened (e.g., Gilson's fluid, Appendix B, reagent 15; or Zenker's, reagent 6; or formalin, reagent 17) in the ordinary way, and sections made by the usual methods. Where practicable paraffin sections are preferable to celloidin sections, because the celloidin tends to hold the stain and thus obscure the bacteria. Sections should be fixed to the slide (paraffin by albumen fixative, celloidin by ether vapor).

Bacteria which do not stain by the Gram method (memorandum 3*f*) or the tubercle bacillus method (memorandum 3*e*) are difficult to demonstrate, because it is hard to stain them so as to differentiate them from the tissues in which they lie; furthermore, most of them easily lose whatever stain they may have taken up. Löffler's alkaline methylen blue (memorandum 3*b*) is, perhaps, the most useful stain for these organisms.

Methylen Blue Stain for Bacteria in Tissues.—1. Stain sections (paraffin) 30 minutes to 24 hours.

2. Acetic acid (1 to 1,000 of water) 10 to 20 seconds.
3. Rinse in absolute alcohol 20 to 30 seconds.
4. Xylol.
5. Xylol-balsam.

With celloidin sections substitute 95 per cent. alcohol for absolute (step 3), then treat with xylol or, better, carbol-xylol until sections are clear. Mount in xylol-balsam.

Anilin gentian violet, methyl blue, methyl violet, or fuchsin (memorandum 3*a*), also carbol-fuchsin (memorandum 3*c*) may be used in the same way.

Gram's Method for Bacteria in Tissues (Weigert's modification).—
1. Stain sections (any kind) in lithium carmine 2 to 5 minutes.

Lithium Carmine (Orth's):

Carmine	2.5 to 5 grams.
Carbonate of lithium, saturated aqueous solution	100 c.c.
Thymol	a crystal or two.
Filter.	

2. Anilin gentian violet 5 to 20 minutes (celloidin sections should first be dehydrated in 95 per cent. alcohol and affixed to the slide with ether vapor).
3. Rinse in normal saline.
4. Gram's solution (memorandum 3*f*) 1 to 2 minutes.
5. Rinse in water.
6. Blot sections with filter paper to remove as much water as possible.
7. Anilin oil, several changes. The oil dehydrates, and at the same time decolorizes the celloidin.
8. Xylol, several changes.
9. Xylol-balsam.

C. Hanging-Drop Preparations

1. A slide with a concave center is used (Fig. 40). With a fine-pointed brush paint a narrow strip of vaselin around the margin of the concavity. The vaselin makes the cover-glass stick to the slide and also prevents evaporation.

2. Place a small drop of the fluid containing bacteria in the center of the cover-glass. If the bacteria to be examined are on a solid medium the "drop" should be made by mixing a small portion of the growth with a drop of bouillon, normal saline, or serum. Place the cover-glass, drop downward, over the depression in the slide and press it down well into the vaselin.

Fig. 40.—Culture Slide.

3. Use only a small opening in the diaphragm when examining the bacteria, in order to get as much contrast by refraction as possible. Focus first with a medium-power dry objective on the edge of the drop, then employ the oil immersion. Such unstained organisms are frequently difficult to find and there is great danger of breaking the cover-glass with the objective.

Hanging-drop preparations are used mainly in determining the motility of bacteria, or in the study of spore formation. For the latter purpose, the slide and cover-glass must be carefully sterilized and the sealing with vaselin complete. The preparation may then be placed on a warm stage or in an incubator and examined from time to time.

MEMORANDA

1. **The Main Points to Be Observed in the Microscopical Examination of Bacteria** are as follows: (1) form of the individual, whether spherical (*coccus*), spiral (*spirillum*), or rodlike (*bacillus*) with end square, pointed, or rounded; (2) uniformity in size; (3) the arrangements of individuals whether single (*micrococci*, *etc.*), in pairs (e. g., *diplococci*), in chains (e. g., *streptococci*), groups of four (e. g., *tetracocci*), cubical groups of eight or more (*sarcinae*), or small grape-like bunches of various-sized cocci (*staphylococci*); (4) presence or absence of cell-wall, gelatinous capsule, etc.; (5) motility in living forms (do not confuse with Brownian movement); (6) reaction to stains; (7) presence of spores which are recognizable as bright, highly refractive rounded bodies.

2. **Material for the Demonstration of Bacteria** (coccus, bacillus, spirillum, and beggiatoa forms) will be found in abundance in foul water, especially when contaminated with sewage. By scraping the inside of the cheek such forms as Leptothrix may often be found. Make a cover-glass preparation; kill and fix in the flame in the ordinary way; stain in

methyl violet, gentian violet, or fuchsin (basic) and, if desired, counterstain lightly with Bismarck brown; examine in water or dehydrate in absolute alcohol, clear in xylol and mount in balsam.

To demonstrate bacteria in tissues, a mouse may be inoculated with anthrax, and paraffin sections of the spleen prepared. Stain by the gentian violet method.

3. **Some of the Most Important Stains for Bacteria** are as follows:

a) *Anilin water solution of gentian violet* (Koch-Ehrlich's).—

Gentian violet, saturated alcoholic solution . . .	10 c.c.
Anilin water (see Appendix B, reagent 29) . . .	100 c.c.

After shaking it, the mixture should be set aside for 24 hours because of the precipitation which takes place soon after making. Solutions of fuchsin (basic) and methyl blue are made in the same way. These solutions begin to decompose after about 10 days and must then be freshly prepared. They yield good results with many species of bacteria. The gentian violet, particularly, is widely used in connection with Gram's method (see *f*).

b) *Alkaline methylen blue* (Loeffler's).—

Methylen blue, saturated alcoholic solution . .	30 c.c.
Caustic potash, aqueous solution (1: 10,000). . .	100 c.c.

This stain keeps well and is one of the most widely used of the general stains. It is especially serviceable in staining the bacillus of diphtheria or of glanders.

c) *Carbol-fuchsin* (Ziehl-Neelson's).—

Fuchsin, saturated alcoholic solution	10 c.c.
Carbolic acid, 5 per cent. aqueous solution . . .	90 c.c.

This stain keeps well, stains powerfully, and can be used on many forms of bacteria.

d) *Neisser's method for the diagnosis of Diphtheria.*—

Solution I.

Methylen blue (Grübler's)	1 gram
Alcohol, 96 per cent.	20 c.c.
Distilled water (add after the methylen blue has dissolved in the alcohol)	950 c.c.
Glacial acetic acid	50 c.c.

Solution II.

Bismarck brown	1 gram
Distilled water (should be boiling when the Bismarck brown is added)	500 c.c.

Cover-glass preparations are stained for from 2 to 3 seconds in Solution I, rinsed in distilled water, placed in Solution II for from 3 to 5 seconds,

rinsed again in water, and examined in the ordinary way. The bacteria of virulent diphtheria should appear as pale-brown rods, some of which show at one or both ends bluish-black oval bodies of greater diameter than the rod. Such dark bodies will not be seen in the pseudo-diphtheria bacilli.

The bacilli must have been grown for from 12 to 18 hours on Löffler's blood-serum which is a mixture of glucose bouillon 1 part and beef-blood serum 3 parts. The mixture is run into test-tubes and coagulated at 100° C.; the tube should be tilted to one side to give a slanting surface for culture purposes. The formula for glucose bouillon is as follows: dry glucose, 10 grams; Liebig's extract of beef, 3 grams; peptone, 10 grams; sodium chloride, 5 grams; water 1,000 c.c.

e) *Gabbet's solution for demonstrating tubercle bacilli.*—

Methylen blue	1 to 2 grams
Distilled water	75 c.c.
Concentrated sulphuric acid	25 c.c.

The acid decolorizes, while the methylen blue serves as a contrast stain. The solution acts rapidly. A modification of the method to be commended is first to stain the preparation with carbol-fuchsin (see *c*) by warming the stain on the slide until it steams, rinsing in water and then proceeding with the methylen-blue solution. Smegma, leprosy, and syphilis bacilli are also stained by this method. Tubercle bacilli are also stained by Gram's method (see *f*). To examine sputum for tubercle bacilli, the sputum is carefully inspected for small yellowish-white cheesy masses varying in size from the diameter of a pin-head to that of a small pea. Very thin smear preparations (see A) are made from such masses.

f) *Gram's method.*—

Gram's solution.

Iodine crystals	1 gram
Iodide of potassium	2 grams
Distilled water	300 c.c.

The preparations are first stained in anilin gentian violet (memorandum 3*a*), and then immersed in Gram's solution for from 1 to 2 minutes. They are then rinsed in alcohol until the violet color is no longer visible to the naked eye. To decolorize them sufficiently, it may be necessary to treat them again with the iodine solution. Finally rinse in water and examine, or if a permanent preparation is desired, rinse in absolute alcohol, transfer to xylol, and mount in balsam. If the preparations are from cultures, it should be borne in mind that the method works well only when applied to bacteria from actively growing cultures; old cultures seldom yield satisfactory results.

Pathogenic Bacteria Stained by Gram's Method	Pathogenic Bacteria Decolorized by Gram's Method
Bacillus aerogenes capsulatus	Bacillus of bubonic plague
Bacillus of anthrax	Bacillus of chancroid
Bacillus diphtheriae	Bacillus coli communis
Bacillus of malignant edema	Bacillus of dysentery
Bacillus of tetanus	Bacillus of glanders
Bacillus of tuberculosis	Bacillus of influenza
Micrococcus tetragenus	Bacillus mucosus capsulatus
Pneumococcus	Bacillus proteus
Staphylococcus pyogenes aureus	Bacillus pyocyaneus
Staphylococcus pyogenes albus	Bacillus of typhoid
Streptococcus pyogenes	Diplococcus intra cellularis meningitidis
Streptococcus capsulatus	Gonococcus
	Spirillum of Asiatic cholera

4. **Staining Spores** (Abbott's method).—Prepare a cover-glass smear in the usual way. Apply the stain (e. g., methylen blue) and hold the cover-glass over a flame until the liquid steams. Repeat the heating several times, but do not boil continuously. Rinse the cover-glass in water and then decolorize the preparation in a 0.3 per cent. solution of hydrochloric acid in 95 per cent. alcohol, until all color visible to the naked eye has disappeared. Wash in water. If a counterstain is desired, stain for from 8 to 10 seconds in anilin-fuchsin solution. Rinse in water and mount in the usual way. The spores are stained blue.

5. **Staining Flagella** (Bunge's modification of Loeffler's method).—The locomotor organs of motile bacteria are long, hairlike prolongations (1 to many) termed flagella. Special methods of staining are necessary for their demonstration.

Make thin cover-glass smears of an 18-hour culture which contains motile forms. Dry and fix in the ordinary way.

The mordant.—

Ferric chloride, aqueous solution (1:20) . . . . 25 c.c.
Alum, saturated aqueous solution . . . . 75 c.c.
Shake well and add,
Fuchsin (basic), saturated aqueous solution . . . 10 c.c.

Filter and allow to stand for some time before using. Treat the smear for 5 minutes with this preparation, gently warming by holding it high above a flame. The fluid must not boil. Rinse in water, then stain faintly with carbol-fuchsin. Repeat the process until a successful result is obtained. Mount in the usual way.

CHAPTER XVI

SOME EMBRYOLOGICAL METHODS: THE CHICK, SECTIONS AND "IN TOTO" MOUNTS; AMPHIBIA; FISH; MAMMALS; OTHER FORMS

THE CHICK

The hen or an artificial incubator is necessary. In many ways the latter is more convenient as it may be kept in the laboratory and is ready at all seasons of the year. There are many kinds of good incubators on the market at present which may be had for a small sum.

Whatever method of incubation is employed the eggs must be fresh and must not have been subjected to rough handling. The date and hour at which incubation is to begin should be written on the shell of each egg in ink. If late stages of development are desired the egg must be turned every few days. All products of combustion from the lamp or burner should be kept from the eggs and the supply of fresh air and moisture carefully maintained. The temperature should be maintained at 38° C. (100.4° F.). Should it rise above 40° C. embryos will be destroyed.

Prepare at least 5 embryos as directed in the practical exercise; 2 for *in toto* preparations and 3 for sections.

1. Place an egg which has been incubated for between 46 and 54 hours, while it is yet warm, in a vessel which contains sufficient normal saline warmed to 38° C. to cover the egg. In the chick the embryo always makes its appearance as a germinal disc or *cicatricula*, as it is termed, situated on one side of the yolk, which is the real egg of the hen, the white being simply a nutritive mass added in the oviduct. This disc or *blastoderm* in the early stages of incubation always turns uppermost no matter in what position the egg may be placed. Moreover, it has been found that the embryo in nearly every instance lies in such a position that when the blunt end of the egg is toward the left,

the head of the chick is directed away from the operator. This fact affords a very reliable means of orienting the embryo, especially in the very young stages when the anterior and posterior ends are not easily recognized by the observer.

2. Break through the shell at the broad end over the air chamber by tapping it sharply and let out the air, or the broad end will tilt up.

3. Begin at the hole made in the end and with blunt forceps remove the shell and shell membrane bit by bit from the upper surface of the egg until the embryo comes plainly into view. Remove with a pipette the thin layer of albumen which lies above the blastoderm.

4. With as little agitation of the liquid in the vessel as possible, by means of fine scissors cut rapidly around the blastoderm just outside the vascular area.

5. Carefully float the blastoderm into a thin watch-glass, keeping it as flat as possible. Shake it gently to remove the piece of vitelline membrane covering it, or any yolk which may adhere. The aid of a needle may be necessary to remove the vitelline covering.

6. With a pipette remove all fluid from the watch-glass, leaving the blastoderm to become dry enough to adhere to the glass, but take great pains that the embryo itself does not become dry. If the edges are not thus kept down they curl up and obscure the embryo when the fixing fluid is added. (Some workers employ a ring of paper to hold down the edges of the blastoderm.)

7. Carefully add Gilson's fluid (reagent 15, Appendix B) until the embryo is completely immersed. The fluid should be allowed to act for from 2 to 3 hours.

8. Wash in repeated changes of 50 per cent. alcohol to which tincture of iodine has been added (see caution 1 under reagent 13, Appendix B), and stain in Borax-carmine (reagent 32, chap. i) for 24 hours (Conklin's hematoxylin may be used if preferred; see reagent 48, Appendix B).

9. Wash the object in water and transfer it through 35 and 50 per cent. alcohol to acid alcohol, leaving it 30 minutes in each.

Decolorize until the embryo becomes bright scarlet in color, then wash in 70 per cent. alcohol and leave it there until ready to proceed.

10. Transfer the object through 95 per cent. (1 hour) absolute alcohol (2 hours) to xylol, where it should remain about 2 hours or until it ceases to appear opaque.

Moant two embryos entire, one with the ventral, the other with the dorsal side uppermost. Put bits of broken cover-glass under the edges of the cover to avoid crushing them.

The three remaining embryos are to be so sectioned (step 11 ff.), that the student will have a complete series of sections in each of the three different planes of the body with reference to the axis of the spinal cord: viz., transverse, sagittal, and frontal. Read carefully memorandum 12 on orientating serial sections.

CAUTION.—*Before sectioning any embryo always make an outline drawing of the entire embryo, then rule lines across the drawing parallel to the plane of section. Unless this is done great difficulty will be experienced frequently in understanding the sections.*

11. Infiltrate the embryo with paraffin in the usual manner by leaving it in melted paraffin for 2 or 3 hours. A softer paraffin (melting at 43° C.) than has been used heretofore may be employed and the sections cut thicker (15 to 20 microns).

12. Imbed and cut in the usual way (chap. v). Mount the entire series.

MEMORANDA

1. **For the Average Course in Embryology of the Chick** the following mounted stages are the most useful:

I. *Mounted in toto.*—

Approximately,

48	hours	viewed	from	above	and	below.
36	"	"	"	"	"	"
30	"	"	"	"		
24	"	"	"	"	"	"
18	"	"	"	"		
12	"	"	"	"		
64–72	"	"	"	"		

96 hours (studied in alcohol under the dissecting microscope).

II. *Sections.*—

48	hours,	transverse,	sagittal,	and frontal.
36	"	"	"	
30	"	"	"	
24	"	"	"	
18	"	"		
10	"	"		
72	"	"	'	"
96	"	"	"	

The number of embryos needed for the above preparations is as follows:

5	embryos	of	48	hours	(27–29	somites).
4	"	"	36	"	(15–18	").
3	"	"	30	"	(10–14	").
4	"	"	24	"	(4–6	").
2	"	"	18	"		
1	"	"	12	"		
1	"	"	60	"		
3	"	"	64–72 hours (cervical flexure formed).			
3	"	"	96	hours.		

2. **In Measuring the Length of Embryos** some embryologists (e. g., Minot) measure the greatest length of the embryo along a straight line (limbs not included) when the embryo is in its normal attitude; consequently in some early stages where the embryo is greatly flexed the neck-bend would be the point to which to measure instead of the tip of the head, because it is the most anterior region; in stages where the embryo is straight, the head would be included. Other embryologists (e. g., His and German authors in general) make use exclusively of the so-called "neck-length;" that is, the distance in a straight line between the neck-bend and the caudal-bend. In this volume the full length measurements are employed unless otherwise specified.

3. **To Mark Anterior and Posterior Ends of Young Chick Embryos** in blastoderms which still have a homogeneous aspect, Duval's osmic acid method is very useful. With a strip of paper 5 mm. wide by 50 mm. long a triangular bottomless box with narrow base is constructed. This is placed on the yolk inclosing the blastoderm in such a position that the base of the triangle corresponds to what will be the anterior region of the embryo (for orientation of embryo in the egg see step 1 of the practical exercise). Press the box down against the yolk and fill it with a 0.3 per cent. aqueous solution of osmic acid. In a short time the preparation begins to darken and the osmic acid should be removed. The blastoderm may then be removed in the ordinary manner and fixed as desired (Duval used chromic acid for fixing). However, it is very diffi-

cult to separate the blastoderm from the egg during the first 24 hours of incubation and it is advisable therefore, to fix and harden both together and to remove the blastoderm later.* The blackened area affords a convenient means of orienting the preparation for sectioning.

4. **For the Embryology of Teleosts** the following are the most useful mounted stages:

I. *Whole mounts.*—Two-, 4-, 8-, 16-, 32-, and 64-cell stages (only the blastodisc segments); early periblast; late periblast; early germ-ring; embryonic shield; various stages of early embryos, such as embryos of 45, 50 and 60 hours.

II. *Sections* (paraffin).—Four, 16, and 32 cells (vertical sections parallel to the first plane of cleavage); late cleavage (vertical sections); early, mid, and late periblast (vertical sections); transverse and sagittal sections of early germ-ring, embryonic shield, early embryo, late germ-ring, and closing of blastopore, respectively.

All stages may be fixed in picro-acetic (reagent 23, Appendix B) for 30 to 40 minutes. Later stages may also be fixed in Hermann's fluid (reagent 26) 2 to 4 minutes. Eggs fixed in Hermann's fluid should be washed for an hour in frequent changes of water, and the membrane of each egg should then be pricked with a needle before passing it through the series of alcohol. The eggs are finally preserved in 83 per cent. alcohol. (Child finds that fixation for about a minute in 10 per cent. acetic acid saturated with corrosive sublimate, followed by 10 per cent. formalin, gives good results without the yolk becoming hard.)

Before the preserved material can be mounted *in toto* or sectioned, the essential part (the blastoderm) must be dissected off under a dissecting lens by means of sharp needles. If the blastoderms are to be mounted entire they may be passed down through the alcohols (see Walton's device, memorandum 4, chap. iii), stained in Conklin's hematoxylin (reagent 48, Appendix B), then dehydrated and mounted in the usual way. To avoid crushing the objects, the cover-glass should be supported by means of bits of broken cover. Material which is to be sectioned may be stained *in toto* or the sections may be stained on the slide. In the latter event, to facilitate orientation, it is necessary to tinge the blastoderms slightly with Bordeaux red or some other cytoplasmic stain unless the fixing reagent has already done so. For the same reason it is best to imbed the material in a watch glass, arranging it near the bottom of the paraffin mass so that one can see with a microscope how to shape the

*Andrews (*Zeitschrift für wissenschaftliche Mikroskopie*, Vol. XXI, 1904, p. 177) injects picro-sulphuric acid (1) between the vitelline membrane and the blastoderm and (2) between the blastoderm and the yolk, by means of a pipette which has a fine upcurved point. The blastoderm may then be readily freed from the yolk. This operation should be performed before the egg has been subjected to the action of any reagents.

paraffin block in order to cut sections in the proper plane. The immersion in the melted paraffin should not be longer than 5 or 10 minutes. The paraffin is best hardened under 95 per cent. alcohol. The sections may be stained by any of the hematoxylin methods; iron-hematoxylin (reagent 51, Appendix B) yields excellent results.

5. **For the Embryology of the Frog and other Amphibia** the following are the most useful stages:

I. *Surface views* (from side and from animal pole).—Unsegmented egg; 2-, 4-, and 16-cell stages; 32 becoming 64 cells; later cleavage; blastopore forming; yolk-plug stage; early medullary folds; late medullary folds; later embryo; embryo just before hatching; tadpole shortly after hatching; later stages of tadpole for gross dissection.

II. *Sections.*—Late cleavage; blastopore forming (sagittal); yolk-plug stage (sagittal); late medullary fold (transverse and sagittal); later embryo (transverse and sagittal); embryo just before hatching (transverse and sagittal); tadpole shortly after hatching (sagittal); head and anterior part of the tadpole about the time the hind legs appear (sagittal).

To study amphibian eggs entire use a hand lens or dissecting microscope. Place the eggs on a bit of absorbent cotton under 70 per cent. alcohol in salt cellars. The eggs are fragile, consequently to manipulate them use a soft hair pencil or a current from a pipette. Use the same egg for surface view and for sectioning when possible.

Amphibian eggs may be fixed (in masses of 15 or 20) in Gilson's mercuro-nitric (reagent 15, Appendix B) or in Worcester's aceto-formol-sublimate mixture (reagent 19*b*, Appendix B). Chromic acid (reagent 10) brings out surface views well but the material becomes very brittle and does not take stains readily. If surface views alone are desired formalin-preserved material will answer.

Before the egg can be prepared for sectioning the thick albuminous coat which surrounds it must be removed. The fixed eggs may be shelled out by means of needles. Whitman (*American Naturalist*, Vol. XXII, p. 857) recommends putting the fixed eggs into a 10 per cent. solution of sodium hypochlorite diluted with 5 or 6 volumes of water and leaving them until they can be shaken free. This requires only a few minutes. Rinse the eggs in 35 per cent. alcohol. It is advisable to remove the albuminous coats before hardening in alcohol.

Child (*Zeitschrift für wissenschaftliche Mikroskopie*, Vol. XVII, 1900, p. 205) states that the albumen which surrounds many ova becomes transparent and dissolves if after fixation (in any way except with chromic acid) the ova are passed up through the grades of alcohol to 80 per cent., hardened, and then passed down again through the alcohols into water which has been slightly acidified with any acid except chromic.

Amphibian eggs are so friable that they must be sectioned in celloidin (see, however, Johnson's asphalt-rubber method, chap. v, memorandum 9).

Stain the eggs in borax-carmine (reagent 32, Appendix B), then pass them through the alcohols (35, 50, 70, 95, 100), leaving them half an hour in each (if necessary decolorize in acidulated 70 per cent. alcohol); thence into ether-alcohol for an hour or more, followed by thin celloidin (12 hours) and thick celloidin (6 hours). Imbed in the usual manner, but clear in the block and section as directed in memorandum 10, chap. vii. Older embryos may be sectioned in paraffin.

6. **For Early Stages of the Mammalian Embryo** rabbits are commonly employed because they breed readily, especially in the spring of the year, and the observer can note the exact time when the female is covered if she has been kept separate from the buck until she comes into heat. The period of gestation is 4 weeks and impregnation takes place again immediately after littering. The two uteri of the rabbit diverge as two anterior horns from the single median vagina and each terminates in front in a narrow, coiled tube, the oviduct or Fallopian tube. To obtain the early stages the abdomen is slit open from pubis to sternum, the intestinal tract is cut away or pushed to one side, and each uterus and oviduct carefully removed and stretched out along a glass plate. The segmenting ova are found in the oviduct up to nearly 70 hours from the time of copulation. After that period of time they must be looked for in the uterus. Fecundation takes place about 9 hours after coition. While in the oviduct, with the aid of a lens they may sometimes be seen through its walls. A segmenting ovum once located, a transverse cut is made to one side of it through the wall of the oviduct, and the ovum which is very small is gently squeezed out by compressing the oviduct behind it. With a spear-headed needle or the point of a scalpel the ovum is conveyed to the fixing fluid. In case segmenting ova are not visible from the exterior of the oviduct, the latter must be slit open carefully with a pair of fine-pointed scissors, and the eggs sought for by means of a lens. In case no red corpora lutea are visible on the surface of the ovary, indicating a recent discharge of ova from the Graafian follicles, further search is useless.

Rabbit ova of 18 hours show 4 blastomeres; 36 to 48 hours, advanced segmentation; 72 hours (about 0.6 mm. in diameter; in anterior end of uterus) show the fully segmented ovum—an outer layer of clear, cubical cells, an inner mass of irregular granular cells; 72 to 90 hours show enlarged blastodermic vesicle and establishment of embryonic area; fifth and sixth days (0.8 to 4 mm.) show germinal layers; seventh day, primitive streak; 8th day, medullary folds.

The earlier stages (up to 70 hours) may be fixed for from 5 to 8 min

utes in a 0.3 per cent. aqueous solution of osmic acid, stained in picro-carmine, and transferred to a mixture of glycerin and water, equal parts. They should remain in this fluid for a week under a bell-jar so that the water gradually evaporates. The object may then be mounted in formic-glycerin (formic acid 1 part, glycerin 99 parts). To avoid pressure of the cover-glass, the object should be mounted in a cell or between two slips of paper or pieces of cover-glass. If the preparation is to be permanent the cover-glass should be sealed (see chap. xiii, II, A, 6).

To render the cell outlines distinct stages of from 70 to 80 hours are best treated, after rinsing in distilled water, with a 1 per cent. aqueous solution of silver nitrate for 3 minutes and then exposed to light in a dish of distilled water until they become brown. They are then treated with water and glycerin and mounted in formic-glycerin as in the case of younger stages.

For sections, the embryos should be placed in Hermann's (reagent 26, Appendix B) or Zenker's (reagent 6) fluid for about an hour, then washed in the customary way for these methods, stained in borax-carmine or alum-cochineal, and sectioned in paraffin.

In opening the uterus, the incision should always be made along the middle of the free side, opposite the insertion of the peritoneal fold, because this line of insertion marks the region of attachment of the embryo within the oviduct. By the seventh or eighth day the developing ova have taken up positions at intervals along the inner walls of the uterus and have become so firmly attached to the mucous membrane that they can no longer be detached unmutilated. For further particulars regarding the embryology of the rabbit, the reader is referred to E. Van Beneden and Charles Julin's "Recherches sur la formation des annexes foetalis chez les mammifères," *Archives de Biologie*, Vol. V (1884), p. 378.

7. **For Older Stages of the Mammalian Embryo,** pig embryos are commonly employed. They may often be procured in large numbers and with little trouble at the larger pork-packing establishments. The most valuable stage for study is an embryo of from 10 to 13 mm. in length. In most laboratories it is customary to make a detailed study of an embryo of about this stage and then a more general survey of both smaller and larger sizes.

Early stages are much more difficult to obtain than advanced stages. Embryos of 6 mm. length and over may usually be readily located by the enlargements which they cause in the uterine walls. The uterus should be handled carefully and opened as soon as possible. The embryo is best removed by means of fine forceps and a horn spoon. It is very delicate and should not be handled roughly. The chances are that

in removing the embryo the membranes will be ruptured and the amniotic and allantoic fluids will escape.

Submerge the embryo without removing the membranes in a bountiful supply of Kleinenberg's picro-sulphuric acid (reagent 25, Appendix B), moving it about gently to rinse off any coagulum that may form on the surface.

Leave embryos of 6 to 9 mm., 2½ hours; 12 to 15 mm., 4 hours; 20 to 25 mm., 6 to 8 hours.

For washing and subsequent treatment see reagent 25, Appendix B. Embryos may be stained *in toto* in alum-cochineal (reagent 27) or borax-carmine (reagent 32).

For studying the uterus, placentation (diffuse in the pig), and embryonic membranes in place, formalin-hardened material may be used after first thoroughly washing it in water.

For gross dissection of embryos, the specimen should be studied in alcohol under the dissecting microscope.

Because of the asymmetry of young embryos it is impossible to secure strictly *transverse*, *sagittal*, and *frontal* sections. Minot recommends, therefore, that for practical purposes the plane of section be taken with regard to the head alone irrespective of how it may cut the other parts of the body and suggests the floor of the fourth ventricle of the brain as the guide for orientation. In his *Laboratory Text-Book of Embryology* he especially recommends that each student prepare sections of the following stages of pig embryos: 9 mm., transverse and sagittal, frontal of the head; 6 mm., transverse, frontal of the head; 17 mm., transverse and sagittal, frontal of the head; 20 mm., transverse and sagittal, frontal of the head; 24 mm., frontal of the head.

8. **For the Stages of Maturation, Fertilization, and Segmentation in Mammals** white mice will prove most useful because these processes are better known in them than in other mammals; furthermore, an abundance of material may be procured. The ovum, however, is extremely small, measuring only 59 microns in diameter. It is surrounded by a very thin zona pellucida (1.2 microns). In the majority of ova (80 to 90 per cent.) only a single polar body is formed; it corresponds in every way apparently to the second polar body when two are formed. It has been inferred, therefore, that probably in the mouse the first polar body ordinarily forms at an early stage in the history of the germ cells and is thus overlooked by observers.

When two polar bodies have been observed, the first always appears while the egg is yet in the Graafian follicle of the ovary; the second, after the egg has entered the Fallopian tube. When only one polar body is present it, likewise, appears after the egg has entered the tube. The

spermatozoon reaches the egg in the upper end of the Fallopian tube some time after coitus. Formation of the single (or the second) polar body and the entrance of the spermatozoon may be found in the same egg.

The female comes into heat 21 days after littering and coitus can take place only when she is in heat because at other times the vagina is closed. During heat the periovarial space and the beginning of the Fallopian tube are distended with a clear fluid. Ovulation occurs before or at the time of coitus. (See "Die Befruchtung und Furchung des Eies der Maus" by Sobotta: *Archiv für mikroskopische Anatomie*, Vol. XLV [1895], pp. 15–93, Plates II–IV.)

9. **Artificial Fecundation** when it can be practiced is the most convenient means of securing early stages of development. This is possible with many worms, coelenterates, echinoderms, cyclostomes, teleosts, and anuran amphibia.

a) *In echinoderms* (e. g., sea urchin) the female is cut open and a number of the living eggs transferred to a watch-glass which contains fresh sea water. The testes of a male are teased out in sea water and a drop of the mixture is conveyed by means of a pipette into the dish containing eggs. Immediately upon fertilization a membrane forms around each fertilized egg. In about 40 to 50 minutes after fertilization the signs of the first cleavage should appear. The blastula forms in about 6 hours, and the gastrula in about 12 hours. For the study of fertilization, etc., the following stages should be fixed in picro-acetic (reagent 23, Appendix B) for 30 minutes and stained in Conklin's hematoxylin (reagent 48); 5 minutes after fertilization, nucleus giving off polar bodies; 30 minutes after fertilization, approaching pronuclei; 50 to 55 minutes after fertilization, division of nucleus (mitotic figure) in the first cleavage.

b) *In amphibia* (e. g., frog) both male and female are cut open, the vasa deferentia or testes are teased out in a watch-glass full of water and the ova are then removed from the lower ends of the oviducts and placed in this water. After fertilization the eggs should be placed in glass dishes in not over 4 inches of water. Many eggs should not be placed in one dish. See also memorandum 5.

c) *In teleosts* the eggs are obtained by stripping the female when she is in spawning condition. At such times the eggs are loose in the body cavity and may be pressed out by gently manipulating the belly of the fish. The head of the fish should be held in one hand, the tail in the other and the thumb or the thumb and forefinger used to press out the ova; the vent of the fish should remain submerged in water. The milt of the male is obtained in the same manner and in the same dish. Eggs and sperm are then gently stirred about by means of a feather to insure thorough mixing. However, in some teleosts (e. g., stickleback) it is necessary to kill the male and tease out the testes. In the cunner (Cten-

olabrus) 10 minutes after fertilization the formation of blastodisc and polar bodies may be observed; 30 to 33 minutes after fertilization the two pronuclei may be found in close approximation.

If other than the very early stages are required, the fertilized eggs must be transferred to a hatching-box. This is best done by means of a horn spoon and a feather. The hatching-box must be provided with a very gentle (drop by drop) stream of running water. According to Exner the eggs are best placed in the box on a layer of glass rods which are from one-twelfth to one-sixth of an inch apart. Three-eighths of an inch below the rods there should be a layer of pebbles covering the floor of the box. Dead eggs, recognizable by their opacity, should be removed at least once a day. See also memorandum 4.

10. **For the Study of Early Cleavage in Living Material** the eggs of some of the water snails afford an abundance of excellent material. By watching aquaria which contain snails the fresh material can easily be obtained during the spring and summer. Twigs and bits of board to which the egg-masses may be attached should be placed in the aquaria.

11. **For the Study of the Formation of Polar Bodies, Fertilization, and Early Cleavage in Sections** nothing surpasses the eggs of Ascaris. The Ascaris (*A. megalocephala*) from the horse is preferable although *A. lumbricoides* from the pig will answer.

The ovisacs, two in number, are very long convoluted tubes. Different regions contain eggs in different stages of development. The thicker tubes toward the anterior end of the animal contain cleavage stages; back of these are cells showing extrusion of the polar bodies and fertilization stages. The material must be fresh; either bring the live Ascaris to the laboratory or take the fixing fluid to the place for obtaining the material. Slit open the abdominal wall of the worm and remove the ovisacs and after separating the numerous convolutions somewhat, fix them entire for 24 hours in picro-acetic acid (reagent 23, Appendix B), or for 15 to 25 minutes in acetic-alcohol-chloroform (reagent 2*b*) saturated with corrosive sublimate. Preserve in 80 per cent. alcohol. To locate eggs of the desired stage tease out eggs at intervals along the ovisacs, stain with acid carmine (reagent 37) and examine. The proper region once located, cut out small lengths of the tube, imbed it in paraffin and make thin transverse sections. In order to keep the eggs from shriveling, the bath in hot paraffin must be curtailed. Use the method for delicate objects (chap. vi, vii). Stain by the iron-hematoxylin method (reagent 51, Appendix B).

12. **Directions for Orienting Serial Sections.**—*a*) In mounting *transverse sections* (sections across the main axis of the object), the sections, beginning at the anterior end of the object, are laid on the slide in the same sequence as the reading on the page of a book. In order to have right

and left sides and dorsal and ventral surfaces in proper relation to the observer, mount the object in such a way that in cutting the knife will enter it on the right side and at the anterior end. In mounting, each section (or strip of ribbon) is turned over and mounted with its posterior face toward the observer and its ventral edge toward the upper edge of the slide. Leave room at one end of the slide (see chap. vi, I, 10) for a label and also a small margin at the opposite side.

b) To get proper orientation of *frontal sections* (sections lengthwise of the object in a plane including right and left sides) arrange the object so that the knife will enter it on the right side and slice off the dorsal surface first. Mount sections, with their posterior ends toward the upper edge of the slide placing the first section of the series to the left end of the upper row. This throws left and right, dorsal and ventral into their proper position as viewed through the compound microscope and the observer looks from the dorsal toward the ventral aspect of the object.

c) To mount *sagittal sections* (sections lengthwise of the object in a plane including ventral and dorsal sides) arrange the object in such a position that the knife enters the ventral surface and slices off the left side first. Turn the section (or ribbon) over and mount with the posterior end toward the upper edge of the slide, placing the first section of the series at the left end of the upper row. Through the compound microscope, the observer views the object from the right toward the left. The head will appear to be toward the upper end of the slide, the dorsal surface toward the left.

It is frequently advantageous to have the imbedding mass trimmed unsymmetrically by leaving the edge which first comes in contact with the knife longer than the opposite edge. One may thus readily discover if a section or part of a series has been accidentally turned over.

13. **Orientation of Objects in the Imbedding Mass** so that sections can be cut accurately in definite planes is frequently difficult to accomplish. The following methods are useful in many instances:

I. *For paraffin sections.*—With a soft pencil rule the strip of paper which is to be used for making the imbedding-box into small squares or rectangles. After imbedding, upon removal of the paper a copy of the pencil marks will be found upon the block of paraffin. If the object has been arranged in the melted paraffin with reference to these lines, it is easy so to arrange the block in the microtome as to cut the object along any desired plane. It is frequently an aid to orientation by this method to have one of the central ruled lines broader than the others, or double.

Small objects which cannot conveniently be oriented in melted paraffin may be properly oriented and fixed to a small strip of paper ruled

as above, before they are placed in the paraffin bath, by a mixture of clove oil and collodion of about the consistency of thick molasses, as in Patton's method (*Zeitschrift für wissenschaftliche Mikroskopie*, Vol. XI [1894], p. 13). One or a number of small objects which have previously been cleared in oil of bergamot or cloves are mounted in small separate droplets of the reagent and oriented under a dissecting lens with reference to the ruled lines. The paper is then placed in turpentine which washes out the clove oil and fixes the object in place. The paper with objects attached is then passed through melted paraffin and imbedded in the ordinary way. Upon removal of the paper from the hardened block a sufficient number of pencil marks remain to be used as a guide in sectioning. Instead of pencil marks Patton employed ribbed paper.

II. *For celloidin sections.—*

Eycleshymer's Methods.—*a*. For imbedding, metal boxes made of two Ls (Fig. 30) are used. The Ls are held together by overlapping strips. The ends and sides of the box are perforated at regular intervals by small holes which have been drilled opposite one another in such a way that threads drawn through them are parallel. Threads of silk are run through the holes from side to side, drawn taut, and cemented to the outside of the box with a drop of celloidin. Each piece of thread should have an end two or three inches long hanging outside the box. A piece of heavy blotting paper is used as a bottom for the box. The object is oriented on the parallel threads and the imbedding mass poured in and hardened. The loose ends of the threads are then soaked in a solution of thin celloidin which contains lamp-black, the celloidin drops holding the threads taut are dissolved by a drop of ether-alcohol, and the blackened ends are drawn through the block of celloidin. The lamp-black leaves distinct black lines through the mass which will serve for properly orienting the celloidin block on the microtome.

This method is valuable also in reconstructions from sections (see chap. xvii). In such work it is very desirable to establish "reconstruction points" to guide in fitting the wax plates together properly. The black rings of lamp-black left in the sections answer admirably for this purpose.

b. For small objects in which reconstruction points are not required Eycleshymer uses fine insect pins from which the heads have been clipped and the headless ends loosely inserted in handles. The objects are mounted on the points of the pins and oriented in the desired position. Each pin is then removed from its handle, and the free end is inserted from below into a small perforation which has been made by passing a somewhat larger pin lengthwise through a cork. A number of pins may be mounted on the same cork. To prevent the objects from becoming dry the cork must frequently be inserted into the mouth of a

vial full of alcohol in such a way that the objects are immersed. If desired, the objects may be sketched *in situ* under alcohol by weighting the cork with lead and placing it in a beaker of alcohol. To pass the objects through the various grades of alcohol, etc., simply transfer the cork bearing them to successive vials of proper size containing the different fluids. For imbedding in celloidin use the method given on p. 59, steps 2 ff. When the celloidin mass has hardened the paper is removed and the pins are drawn out through the cork, thus leaving the objects in place ready for sectioning.

14. **Human Embryos** of all ages are very valuable material for scientific purposes. Physicians and surgeons are urged to preserve such material properly and turn it over to some competent embryologist. Very young human embryos are exceedingly desirable.

An excellent fixing reagent, the ingredients of which a physician can usually readily procure, is the acetic-alcohol-chloroform mixture described in Appendix B, reagent 2*b*. The embryo should remain in this fluid from 6 to 24 hours according to size and then be preserved in 80 per cent. alcohol (or commercial alcohol to which has been added about one-fifth its volume of distilled water). Use a wide-mouthed bottle with tightly fitting stopper.

Zenker's fluid (reagent 6, Appendix B) is better for larger-sized embryos. Material should be left in it from 18 hours to several days. For washing and preserving follow the directions given under the description of the fluid. For fetuses use a fruit jar of such a size that the embryo can be kept in about 10 times its volume of fluid.

In case the above fluids are not available, the material may be placed in 10 per cent. formalin (1 part of commercial formalin to 9 parts of distilled water) and left indefinitely. As a last resort, if no other fixing reagent is available, the embryo may be placed in the strongest alcohol which can be secured and later transferred to 80 per cent. alcohol for preservation.

The specimen should not be handled nor allowed to lie in water. When the proper reagents are not at hand, carefully wrap the object in cloth and keep it on ice if possible until they can be secured. Very small embryos may be fixed and preserved with membranes intact; older ones (6 weeks to 3 months) should have the membranes ruptured. To secure the best fixation of fetuses (2 months and beyond), the specimen should be divided, or at least the body cavity should be opened.

CHAPTER XVII

RECONSTRUCTION OF OBJECTS FROM SECTIONS

In investigating objects which possess complex internal cavities or complicated structures, it is frequently very difficult to gain an adequate idea from the direct study of serial sections, or by means of macerated or teased preparations, consequently, various methods of plastic or geometrical reconstructions from the sections are resorted to. For such reconstruction, sections must be of uniform thickness, serial, and they must possess similar orientation.

RECONSTRUCTION IN WAX

Born's method of constructing wax models of objects from serial sections is widely used for both embryological and anatomical subjects. The thickness of the sections, the magnification of the microscope, and the plane of section must be known.

Wax plates are prepared as many times thicker than the actual sections as the latter will be magnified in diameters. For example, if the serial sections are $\frac{1}{30}$ of a millimeter thick ($33\frac{1}{3}$ microns), and they are to be magnified 60 diameters, then the wax plates must be made 60 times as thick as the sections, or 2 millimeters thick. This is the thickness commonly used. Count the number of sections to be reconstructed, and prepare an equal number of plates.

Preparation of the Wax Plates

(*a*) *The original method.*—1. To prepare wax plates of the proper thickness (2 mm.) use several straight-walled rectangular tin pans 25 mm. deep and measuring 270×230 mm. in area.

2. In order to make the beeswax which is to be used flexible, add a little turpentine to it. The specific gravity of the mixture will be about 0.95, and the weight necessary to make a plate of wax 2 mm. thick in one of the pans will be about 118 grams.

3. Fill one of the pans with boiling water to the depth of about 1.5 cm., melt 118 grams of the prepared wax and pour it upon the water. The wax should spread evenly over the surface of the water if both wax and water are sufficiently hot. If gaps remain, close them by drawing a

glass slide over the surface of the wax. To prevent the plate from splitting while cooling, after it has stiffened somewhat, cut the edges free from the walls of the pan. When the water has become tepid, remove the wax plate to a flat support and leave it to harden.

(*b*) *Huber's method.*—Several instruments have been devised for making the plates more rapidly and more accurately than by the original method. Huber's apparatus, for instance, consists of a heavy cast-iron plate with moveable side pieces which can be adjusted to a height corresponding to the desired thickness of the wax plates. The whole instrument is supported upon three adjustable legs, by means of which it can be made exactly level. Melted beeswax slightly in excess of the quantity necessary for a wax plate is poured on to the iron plate in an even layer, and rolled out with a hot roller until the roller comes to run directly on the side pieces of the instrument. When the wax plate is cool enough to handle it may be placed in a pan of cold water to harden.

Practical Exercise.—When possible an outline drawing of the part to be reconstructed should be made before it is sectioned.

1. Reconstruct the heart of a chick at the end of the third day of incubation, under a magnification of 60 diameters. For this magnification, if it is desired to use a wax plate 2 mm. thick, the original sections should have been 33.3 microns thick.

2. Place a sheet of blue tracing-paper on the wax plate with the colored side toward it. Over the tracing-paper place a sheet of ordinary drawing-paper. With the aid of a camera lucida or other projection apparatus, outline on the drawing-paper the part to be reconstructed. In doing this the outline is also traced in blue on the wax. Number each drawing, and also indicate the number of the section on the slide to which it corresponds; also number the wax plates with reference to the drawings.

3. Lay the wax plate on a suitable flat surface, and cut out the outlined parts with a sharp, narrow-bladed knife. Leave bridges of wax to hold in place the parts that would otherwise be separate pieces. Pile up the successive sections in proper sequence as they are cut out.

4. In finally putting the model together, accurately adjust the parts (for reconstruction points see chap. xvi, memorandum 13, II*a*), and build up the model in blocks of five sections each (Bardeen's suggestion). If necessary, unite the essential parts

by means of pins or fine nails. Remove all temporary wax bridges (see 3) by means of a hot knife.

When all blocks are properly adjusted and united, smoothe over the surface by means of a hot spatula.

MEMORANDA

1. **Geometrical Reconstructions,** first described by Professor His, are often all that is necessary to give one the desired information about internal organs. Before sectioning, an outline drawing of the object is made in a plane at right angles to the intended plane of section, and under the same magnification that will be used for the reconstructed drawing. For example, if the sections are to be transverse, the outline drawing of the object would be a profile view from the side. After sectioning the object, each section is drawn under the same magnification as was used for the outline drawing.

To reconstruct any special part of the object, draw a median line on the outline drawing corresponding to the long axis of the object. At right angles to this line, draw a series of equidistant parallel lines corresponding in positions to the sections that have been made. For example, if the magnification is 100 diameters and the sections 10 microns thick, then the parallel lines must be 1 mm. apart. Then, beginning with the first section, indicate by dots in the proper plane in the profile drawing the relative distances of the part in the sections above or below the median line along the proper one of the parallel lines. All of the sections having thus been plotted, connect the dots of corresponding parts in the successive zones. It is frequently sufficient to reconstruct only every fifth or even every tenth section. When the plane of section is not quite at right angles to the axis of the object, an equal alteration of angle must be made between the median line of the outline drawing and the parallel lines.

Such a reconstruction as the above would give lateral views of the various internal parts. To get their aspects as seen from above or below, the original outline drawing of the specimen as a whole should have been made from this point of view instead of from the side. In actual work one should make reconstructions in both planes.

2. **A Special Drawing-Table** for rapid and convenient drawing of sections for reconstruction has been devised by Bardeen. For details, see *Johns Hopkins Bulletin*, XII, p. 148.

APPENDICES

APPENDIX A

THE MICROSCOPE AND ITS OPTICAL PRINCIPLES

For an understanding of the optical principles involved in microscopy, four things must be borne in mind with regard to a ray of ordinary daylight:

1. It has an appreciable breadth.
2. It travels in a straight line in a homogeneous medium.
3. It is bent (*refracted*) in passing obliquely from one medium into another of different density.
4. It is in reality a composite of a number of different colored rays, ranging from violet to red, and each of these has a different refrangibility.

The amount of refraction undergone by light in a given case depends upon the difference in density of the two media which the light traverses. Thus, glass is denser than air, hence, in passing from air obliquely through a glass plate (Fig. 41), a ray of light *AB* would be bent out of its original course. On reaching the air again, however, it would resume its original direction, although it would be displaced to an amount equal to the distance between *A* and *A′*. It is on account of such displacement that an object in water, for example, appears to be at a different point from where it really is.

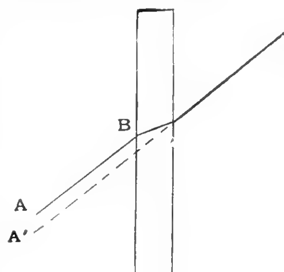


Fig. 41.

On the other hand, after traversing a prism, a ray does not resume its former direction, but takes a new course upon leaving as well as upon entering the prism (Fig. 42). This new direction is always toward the base of the prism, and the amount of deviation depends upon the shape and density of the prism. If the base is down, then the ray is bent downward; if the apex is down, the ray still deviates towards the base, that is, it is bent upward

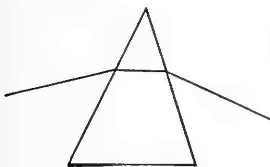


Fig. 42.

Lenses.—Each of the two principal forms of lenses is in effect practically two prisms, (1) with the bases placed together (Fig. 43*a*, *convex lens*), or, (2) with the apices together (Fig. 43*b*, *concave lens*).

In the convex lens, since rays of light are refracted toward the bases of the respective prisms, they will converge; in the concave lens, for the same reason, they will diverge. The terms *converging lens* and *diverging lens*, therefore, are used frequently as synonymous with the terms convex lens and concave lens. All lenses are modifications or combinations of these two types.

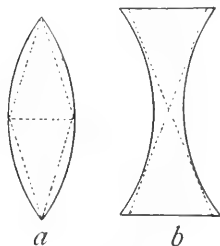


Fig. 43.

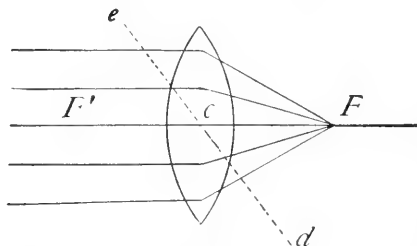


Fig. 44.

If parallel rays of light pass through a convex lens (Fig. 44) they are so refracted as to meet in one point *F*, which is termed, in consequence, the focal point or principal focus. If, on the other hand, the source of light be placed at the focal point, then, after traversing the lens, the rays of light will emerge parallel. If parallel rays of light came from the opposite side of the lens, manifestly there would be a second focal point at *F'*. The two principal foci are termed *conjugate foci*, and will be equidistant from the center of the lens when both sides of the lens have equal curvature.

The ray which passes through the center of the lens (Fig. 44*c*) and the focal point, traverses what is termed the *principal axis* of the lens. The *optical center* of the lens is a point on the principal axis at or near the actual center of the lens, through which rays pass without angular deviation. Any line (*c d*), other than the principal axis, which passes through the optical center of the lens is termed a *secondary axis*.

In the case of a concave lens, parallel rays will be caused to diverge (Fig. 45) and the principal focus, *F*, of the lens is determined by the extension of the divergent rays till they meet at a point which lies on the same side of the lens as the source of light. Such a point has no actual existence, and is known, consequently, as a *virtual focus*. The focus of a convex lens, on the other hand, is *real*, and may be determined readily by allowing the sun's rays, which are practically parallel, to pass through it on to a screen. By moving the lens backward and forward, the spot of projected light varies in size and brightness. When smallest and brightest the spot is at the focal point of the lens.

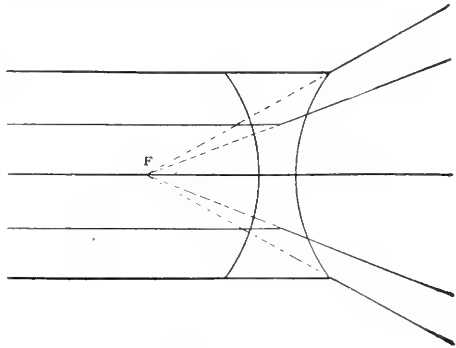


FIG. 45.

Images.—In Fig. 46 the object, represented by an arrow, lies beyond the principal focus of a convex lens as in a photographic camera, for example, or the objective of a compound microscope. Light rays pass out in all directions from any luminous point. Hence, one ray from any point on the arrow, the tip, for instance, will pass through the focal point, *F*, and one will pass through the optical center of the lens. From what was determined above, manifestly the ray through *F* will emerge as one of the parallel rays upon leaving the lens, and the one through the optical center of the lens, since it traverses a secondary axis, will not be refracted, hence the two rays must cross. Their point of intersection is the point at which the image of the arrow-tip will be formed. The same fact may be determined, likewise, for any other point of the arrow, for example, the opposite end. Thus the distance from the lens

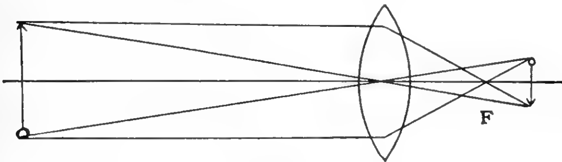


FIG. 46.

at which the image is formed may readily be determined. In focusing a photographic camera, for example, the image comes sharply into view on the ground-glass plate at the back of the camera when the plate is brought into the plane in which these rays through the focus and the optical center intersect beyond the lens. It will be observed from the figure that the image is reversed. The size of the image diminishes as the object lies farther beyond *F*.

In case the object lies between the lens and the principal focus, as in Fig. 47, parallel rays from the object would converge to meet at the conjugate focus *F′*, and an eye at this point would see the image projected and enlarged without being reversed. The plane in which the image is formed is determined by finding the points of intersection of the secondary axis through points of the object with the imaginary elongation of the refracted rays as shown in the figure. The image is magnified because the observer judges of the size of an object by the visual angle which it subtends. The greater the convexity of the lens, the shorter the focus, and also, since the rays are bent more, the greater the magnification.

The Simple Microscope.—The simple microscope (the ordinary so-called magnifiers, etc.) operates upon this principle; the image of an object is projected and enlarged but not inverted (Fig. 47).

The question arises as to why there is a *best distance* to hold the simple microscope from an object. Why will not any point answer so long as it is within the focal point? As a matter of fact, the object may be placed at any point within the focus, and it will be found that the nearer it is brought to the lens the less it is magnified. There is one most favorable point for observation, however, which is neither at the point of highest nor of lowest magnification, but an intermediate point, where the lens is freest from chromatic and spherical aberrations.

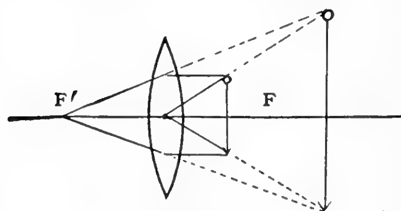


Fig. 47.

The Compound Microscope.—The general principle of the compound microscope is represented in Fig. 48. The object *ab* lies beyond the principal focus of the first lens or *objective* (really a system of lenses), hence the image *AB* is reversed. This image, in turn, is viewed through a lens, the *eyepiece* or *ocular* situated nearer the eye of the observer. The ocular acts as a simple magnifier, projecting and enlarging the image but not

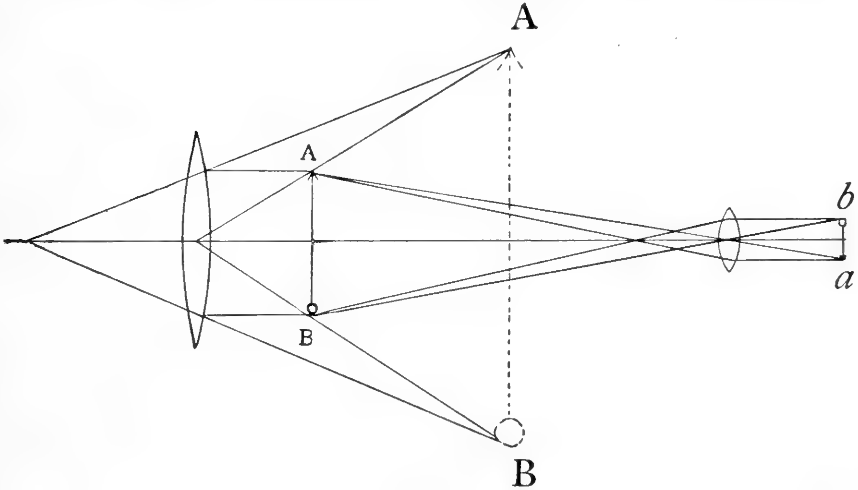
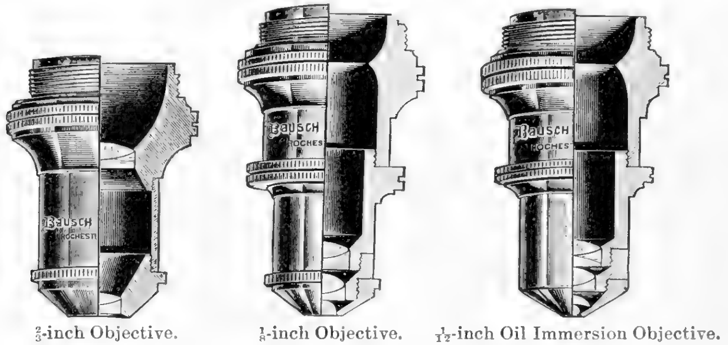


Fig. 48.

reversing it again. As a matter of fact, the ordinary ocular of a compound microscope cannot be taken from the instrument and used as a simple magnifier because it is made of two planoconvex lenses which are so adjusted that the image from the objective of the compound microscope is not brought to focus until it has traversed the larger or field lens of the eyepiece (Fig. 52). The image is really examined, therefore, at a point between the two lenses of the eyepiece. Such an eyepiece is termed a *negative* eyepiece or ocular and is widely used today for microscopical work. *Positive* eyepieces are made, however, and they may be used as simple magnifiers when removed from the compound microscope.

A good objective is made up of from two to five systems of lenses as shown in Fig. 49. A single system in turn may be a

doublet (Fig. 54) or a *triplet*, each made of different kinds and shapes of glass. A good objective is a very delicate piece of apparatus and must be handled with great care. Each component is very accurately ground and the systems distanced with extreme precision in order to get a clear image. If not already familiar



$\frac{2}{3}$-inch Objective. $\frac{1}{8}$-inch Objective. $\frac{1}{12}$-inch Oil Immersion Objective.

FIG. 49.—Lens Systems of Various Objectives.
Bausch and Lomb $\frac{2}{3}$-inch, $\frac{1}{8}$-inch, and $\frac{1}{12}$-inch oil-immersion objectives respectively.

with the parts of the compound microscope, the student should study Figs. 51 and 52 with a microscope before him.

DEFECTS IN THE IMAGE

Spherical Aberration.—A simple convex lens, unless corrected, will not give a sharply defined image because it does not refract to the same degree all rays passing through it. Those which traverse its edges are brought to a focus nearer the lens (Fig. 50). This results not only in an indistinct image but in a distortion of shape as well. Straight lines, for example, appear curved and when the parts of the object are in focus in the center of the field, those nearer the margin are hazy and indistinct. This defect is greatest in strongly curved lenses, that is, in high powers, since magnification increases with increased curvature. Spherical aberration is corrected by one or more of the following processes:

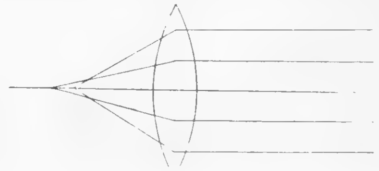


FIG. 50.

1. Cutting off the marginal rays.
2. Changing the shape of the surface of the lens.
3. Combining several lenses equivalent to a single lens.

Chromatic Aberration.—As with a prism, ordinary light in passing through a lens is broken up into its component colors. This

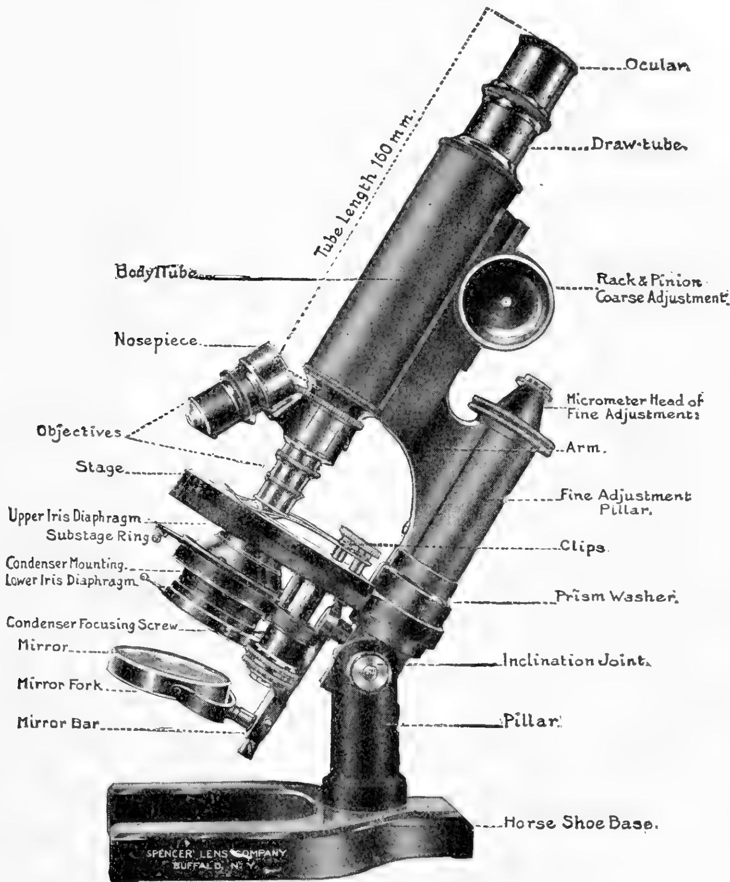


FIG. 51.—A Compound Microscope with Parts Named.

process is technically termed *dispersion.* Since the colors are not all bent to the same extent, the result is that each color has a different focus; the ones which are bent most (violet rays) come to a focus nearest the lens and those which are least affected (red

rays) meet at a point farther away (Fig. 53). This failure of the color rays to meet in one focal point is termed *chromatic aberration*, and if uncorrected causes the image of an object viewed through such a lens to be bordered by a colored halo.

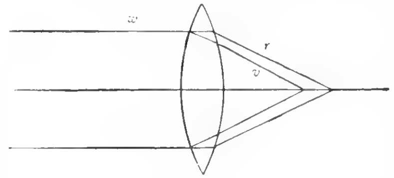


Fig. 53.

The defect is corrected by properly combining glasses of different dispersive powers but of kindred refractive powers. Flint glass (silicate of potassium and lead), for example, has a dispersive power equal to about twice that of crown glass (silicate of potassium and lime), although their refractive powers are nearly the same. By combining a biconvex lens of crown glass with a concave lens of flint glass so constructed that its dispersive power will just equal that of the crown glass (Fig. 54), the error may in large measure be corrected. Such an arrangement does not interfere seriously with the refractive powers of the lens so constructed. Unfortunately no two kinds of glass have been found which have proportional dispersive powers for all colors, so that in the ordinary achromatic objective only *two* of the different colors of the spectrum have been accurately corrected and brought to one focus. The colors

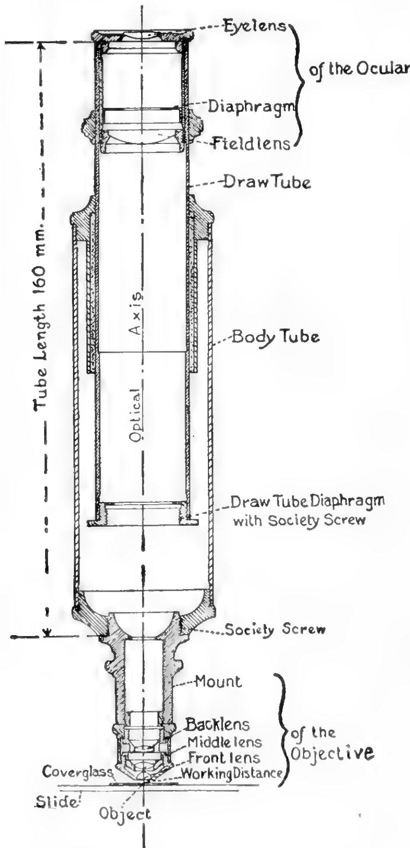


Fig. 52.—Sectional View of Microscope Tube including Ocular and Objective.

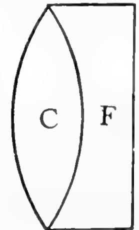


Fig 54.

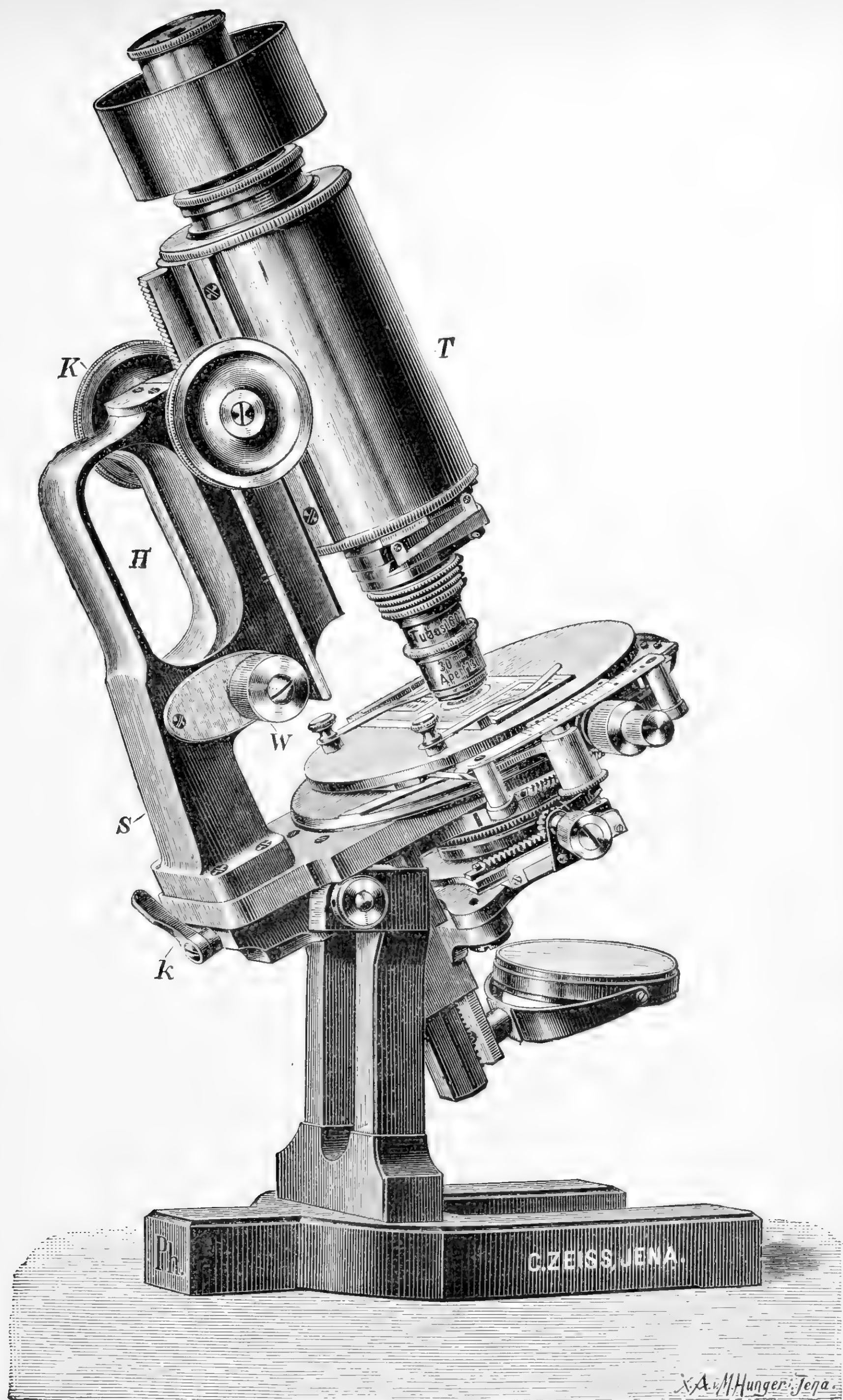


Fig. 55.—The Zeiss Stand, IC.
Equipped with Accessories for Micro-Photography.

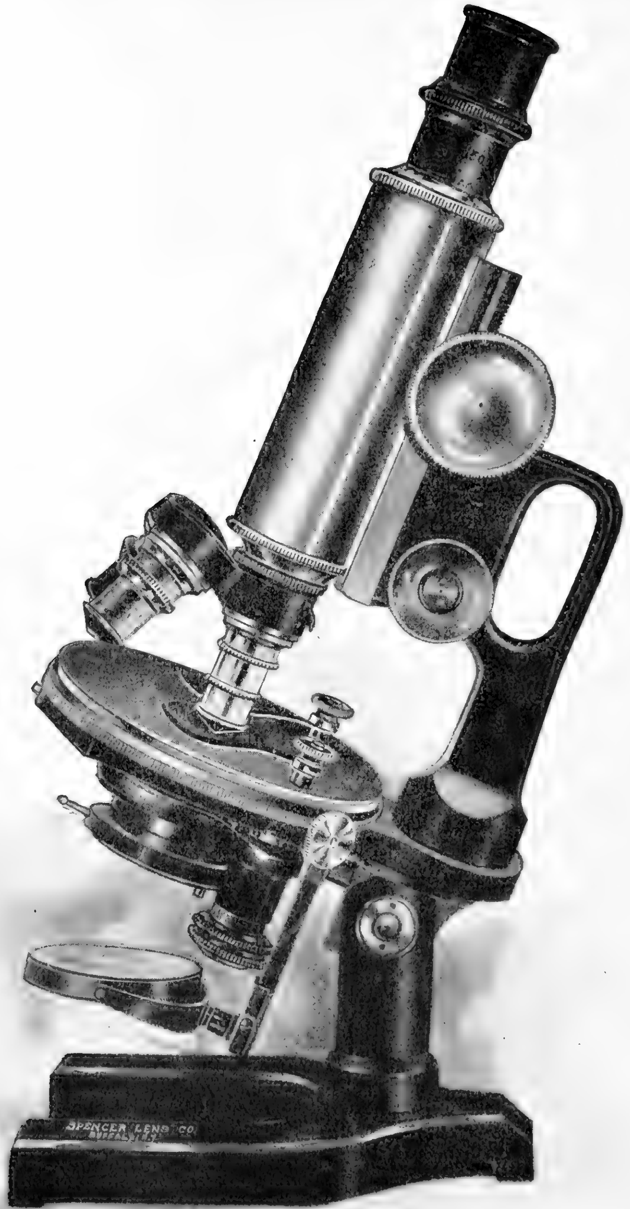


Fig. 53.—The Spencer No. 25 Stand.

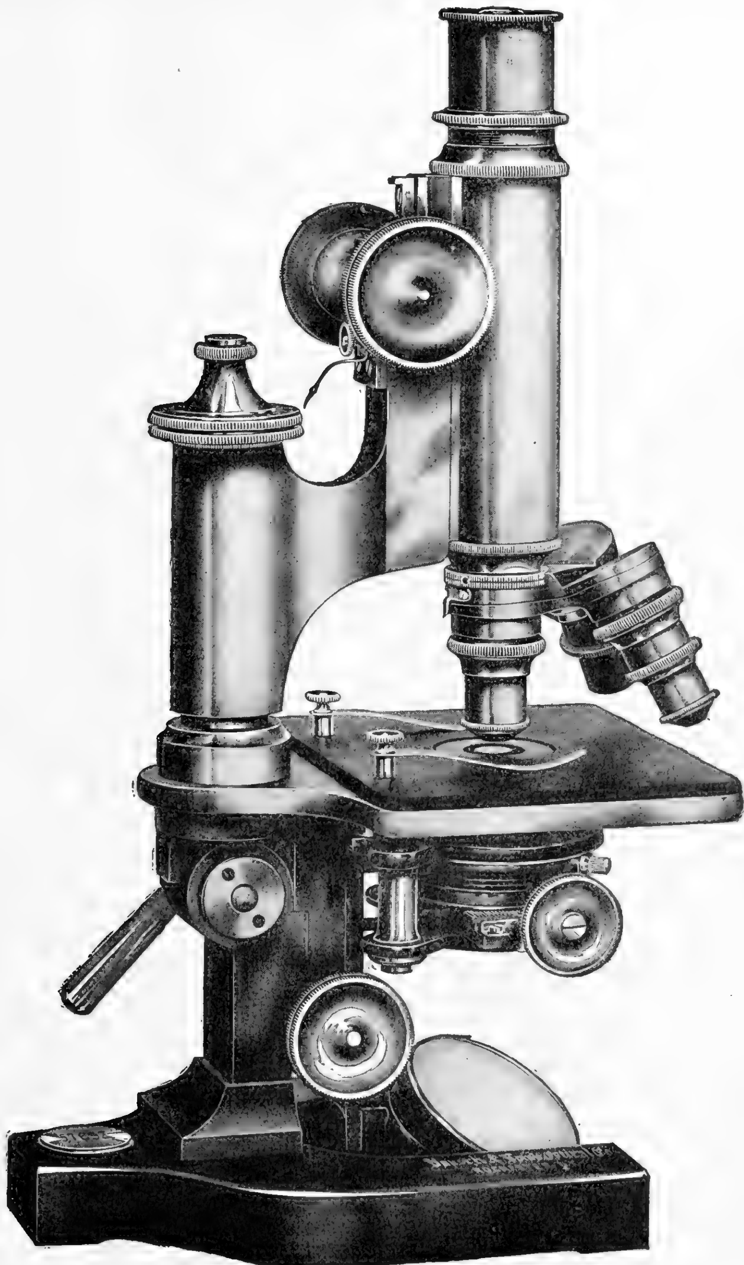


FIG. 57.—The Bausch and Lomb CA Stand.

left outstanding form the defect known as a *secondary spectrum*. In the apochromatic objectives (p. 146) *three* rays are brought to one focus, leaving only a slight *tertiary spectrum*.

NOMENCLATURE OR RATING OF OBJECTIVES AND OCULARS

Oculars.—Different makers, unfortunately, use different systems in marking their lenses to indicate relative powers of magnification. In the case of lettering the system is wholly arbitrary; the only rule is that the nearer to *A* the letter is, the lower the magnification. When the objective bears a figure it is usually indicative of the magnifying power of the part marked. Thus a $\frac{1}{12}$ inch objective magnifies approximately 120 diameters; a $\frac{1}{8}$ inch, 80 diameters; a $\frac{1}{2}$ inch, 20 diameters; a 1 inch, 10 diameters: a 2 inch, 5 diameters; and so on. This means that an objective which forms an image 10 times the real diameter of the object itself, on a screen placed 10 inches (the conventional distance of vision) from its back lens, is rated as a 1-inch objective. If it formed an image only 5 times the real diameter of the object it would be a 2-inch objective, if 30 times, a $\frac{1}{3}$-inch objective, and so on. Such magnification is termed the *initial magnifying power* of the objective.

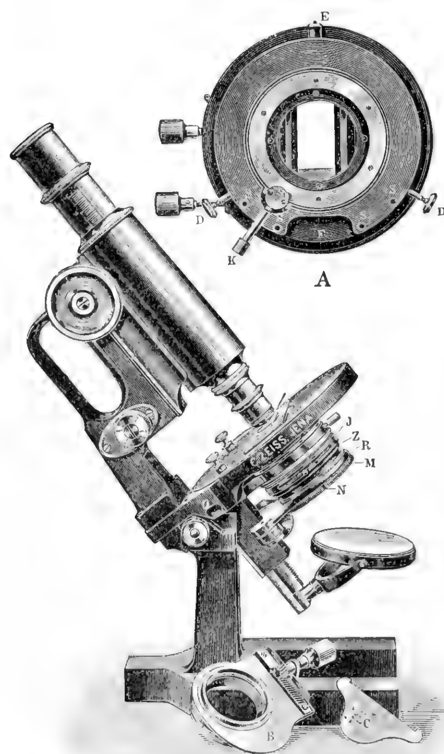


FIG. 58.—The *Zeiss* IIIB Stand with Diaphragm-Carrier (B) and its Key (C).

Zeiss is represented in America by *The Scientific Shop*, 324 Dearborn street, Chicago, Ill.

The objectives of French and German instruments are rated in millimeters and the conventional distance of vision taken as 250 millimeters. An objective of 3 millimeters focus, therefore, yields an initial magnification of 83.3 diameters ($\frac{1}{3} \times 250 = 83.3$). Compensating oculars (see below) bear numbers which indicate the number of times the eyepiece, when used at a given tube-length, increases the initial magnification. Ocular 12, for example, with a 3-millimeter objective would yield a magnification of $83.3 \times 12 = 1{,}000$ diameters, with a standard length of tube. Unfortunately this simple system does not apply to most ordinary oculars which are more or less arbitrarily lettered or numbered.

SOME COMMON MICROSCOPICAL TERMS AND APPLIANCES
(Alphabetically Arranged)

Achromatic Objective.—An objective corrected for chromatic aberration. The correction is not absolute.

Achromatism.—Freedom from chromatic aberration.

Angular Aperture.—The angle (measured in degrees) formed at the point of focus (*F*, Fig. 59) by the outermost rays (*a F*, *b F*) which traverse the objective to form an image. This angle is an important consideration because on it depends in large measure the defining or resolving power of the objective. It is evident that the larger the angle is, the greater the number of rays of light that will be admitted from an object. Thus the object will be better defined to the eye. In low powers the angle may be very wide, in high powers it must necessarily be small. Two objectives, even though they may possess different powers of magnification, will have the same brilliancy if they are of the same angular aperture; on the other hand, if they have the same magnifying power but differ in angular aperture, the brilliancy is reduced in the one of smaller angle. In immersion lenses the liquid used between the lens and the object, by reducing refraction has the effect of increasing the angle of aperture. See *immersion objective*, also *numerical aperture*.

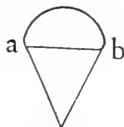


Fig. 59.

Apertometer.—An instrument for measuring both the angular and the numerical aperture of objectives. It is fitted to the stage of the microscope.

Aplanatism.—Freedom from spherical aberration. The result is a flat field as viewed through the microscope. Aplanatic lenses are usually also achromatic.

Apochromatic Objective.—An improved form of objective which is more exactly achromatic than the ordinary objective because it is corrected for rays of three colors instead of two, and this correction is equally good in all parts of the field. In the ordinary achromatic objective after correction there is a residue of color which is known as the secondary spectrum. In the apochromatic lenses correction is made for a third color, and usually only a slight tertiary spectrum is left uncorrected. Spherical aberration is also more fully corrected. Furthermore, in these objectives the foci of the optical and the chemical rays are identical, hence the lenses are well adapted to photography In the glasses of the apochromatics, silicon is replaced by boron in the flint series, and by phosphorus in the crown series. Fluorite was used in conjunction with the glasses in the earlier forms of apochromatic lenses, with the result that the lenses frequently deteriorated in warm, moist climates. Several makers are now able to construct apochromatic objectives without the use of fluorite. Both dry and immersion apochromatics are made.

Binocular Microscope.—A microscope adapted to vision with both eyes at once. By means of a prism part of the light from the object is diverted into a second tube which like the main tube is provided with an eyepiece. Binocular eyepieces for attachment to an ordinary microscope are now made. Binocular microscopes yield a stereoscopic view so that objects which have any amount of depth stand out in relief exhibiting their natural contour. The instruments can be used successfully only with objectives of comparatively low power.

Brownian Movement or Pedesis.—An oscillating or dancing motion observable in small particles in a liquid when seen under the microscope.

Camera Lucida.—An apparatus containing a glass prism or thin glass plate so arranged that when placed over the eyepiece of the microscope the observer may see the image of the object under the microscope projected on to his drawing-paper on the table. The point of the pencil is also visible, consequently the outline of the object may be readily traced on the paper. In the simpler camera lucidas a thin neutral tint glass slip is so arranged that it is in alignment with the eye-lens of the ocular, except that it sets at an angle of 45° to it. When the microscope is tilted into a horizontal position the observer sees the image of the object reflected from the upper side of the glass slip, but, since the latter is somewhat transparent, he also sees the white paper spread below on the table (Fig. 60).

Another form of simple camera lucida is the *Wollaston.* To use it the microscope must be inclined. The essential part of the camera consists of a quadrangular prism. The eye of the observer is so placed over the edge of the prism as to receive rays of light from the object

with one portion of the pupil, and from the drawing-paper with the remainder.

Some form of the *Abbe camera lucida*, however, is used by most workers. It consists of a cap which is fitted immediately above the eyepiece and which contains two right-angle prisms cemented together to

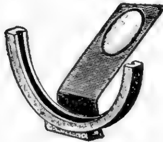


FIG. 60.—Simple Camera, Lucida.

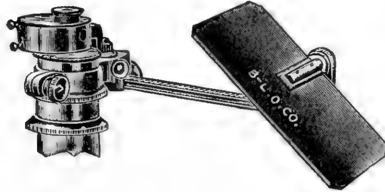


FIG. 61.—Camera Lucida, Abbe.

form a cube (Fig. 61). The lower one of the prisms is silvered along its cemented surface although a small central opening is left through which the object under the microscope may be viewed; connected with the cap is an arm which bears a mirror and this mirror may be so adjusted as to reflect the image of the drawing-paper on the table on to the prisms from one side. The prisms are so set that the silvered surface of the lower one reflects this image upward to the eye of the observer which also, coincidently, is viewing the magnified image of the object through the hole in the silvering. When proper adjustment of the light received from object and paper respectively is made, a pencil point may be distinctly seen when brought into the field of vision over the paper; consequently, the outline of the object may be accurately traced. The secret of success in working with a camera lucida is to have the illumination in the two fields properly balanced. Small screens of tinted glass are provided with the instrument for such regulation. Low-power eyepieces should be used. With the Abbe camera lucida the microscope may be used in a vertical or in an inclined position. If the microscope stand is inclined, the drawing-board upon which the paper rests must have the same inclination, or the outline when drawn will be distorted. Likewise, if the mirror of the camera is at any other angle than 45 degrees, an adjustment of the drawing-surface must be made; in short, the axial ray of the image and the drawing-surface *must always be at right angles* to prevent distortion. This means that if the mirror is depressed below 45 degrees the drawing-surface must be tilted toward the microscope *twice as much* as the mirror is depressed. For example, if the mirror is depressed to 37 degrees (8 below 45 degrees), the drawing-board must be tilted (raised) 16 degrees. When the camera is in proper position the field of the microscope should appear at about the same size as without

the camera. If the field is reduced or unevenly lighted, the camera is too near or too far from the ocular, or it is tilted, or the prism is not properly centered.

Fig. 62 represents a simpler form of camera lucida with Abbe prism; the mirror is fixed and close to the prism.

Compensating Ocular.—A specially designed eyepiece for use with apochromatic lenses. It was found advantageous to undercorrect the objective and then to rectify the aberration by over-correcting the ocular. The so-called *searching ocular* is a low-power compensating ocular used for the first finding of objects. The object once located in the field, the higher *working oculars* are used in observation.

Fig. 62.—Abbe Prism.

Condenser.—A lens or a series of lenses mounted in a substage attachment for the purpose of concentrating light upon the object to be examined. They are made in various grades of excellence non-achromatic, achromatic, and apochromatic. Some wide-angle condensers are used as immersion condensers; the immersion fluid is placed between the upper surface of the condenser and the lower surface of the object slide. Condensers are especially valuable with high-power objectives and oil-immersion lenses. They are constructed to receive parallel rays of light, hence the *plane mirror* only should be used with them if the illumination is from daylight. See *illumination.*

Correction Collar.—A device for adjusting the distance between the lens systems of objectives so that the proper corrections may be made for different thicknesses of cover-glass. Low-power objectives are not so sensitive as those of high power to the influence of the cover-glass. Ordinary objectives, however, are mounted in a rigid setting and corrected for a specific tube-length and a standard cover-glass (about 0.18 mm. thick). With a cover-glass of different thickness correction should be made by altering the tube-length of the microscope, lengthening it for a thinner cover and shortening it for a thicker one. With homogeneous immersion lenses the defect caused by different thicknesses of cover-glass disappears (see *immersion objective*). See also *tube-length.*

Cover-Glass Correction.—See *correction collar.*

Definition.—The power of a lens to give a clear, distinct image and make visible minute details. See *resolving power.*

Diaphragm.—Opaque plates with openings of various sizes for regulating the illumination of the object to be examined. The iris diaphragm (Fig. 63) is the best type. It consists of a series of overlapping plates

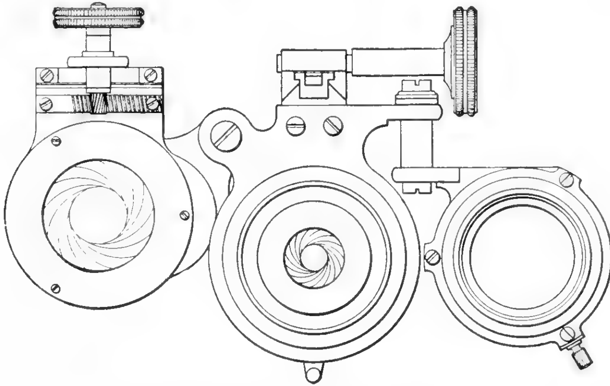


FIG. 63.—Top View of a Substage Attachment with Condenser and Lower Iris Diaphragm thrown out of Optical Axis.

placed around a central opening the size of which may be varied by means of a lever. Revolving diaphragms are commonly used on the cheap grades of microscopes. They consist of round disks perforated by openings of various sizes which may be rotated between the mirror and the object. The nearer to the object the diaphragm is placed, the better the intensity of the illumination can be regulated. Most of the better class of microscopes are provided with two iris diaphragms, one beneath the condenser to be employed when the latter is in use, the other flush with the stage to be used only when the condenser is out. If this second iris diaphragm is lacking, its place is taken by means of a cap-diaphragm which may be fitted into the substage in the place of the condenser.

Dissecting Microscope.—An instrument so constructed as to enable an operator to carry on minute dissections under magnification. Ordinarily they are simple microscopes mounted on a stand of some kind. The best instruments (Fig. 64) are provided with well-corrected lenses, with glass stage, mirror, black and white substage plate, and rests for the hands. See also Figs. 65 and 66 for modified forms.

Embryograph.—A form of camera lucida for drawing at slight magnification small objects, such as embryos. A camera lucida, attached to a simple microscope is frequently used for this purpose.

Eye-Point.—The point above an ocular or lens at which the largest number of rays from the instrument enter the eye. The largest field of the microscope is visible from this point.

Flatness of Field.—See *aplanatism.*

Homogeneous Immersion Objective.—See *immersion objective.*

Illumination.—Any means employed to direct light upon the object under observation. Light which traverses the object is said to be *transmitted* light. Most microscopical work in biology is done by means of transmitted light, hence the object must be rendered more or less transparent if not naturally so. If the object is symmetrically lighted, the

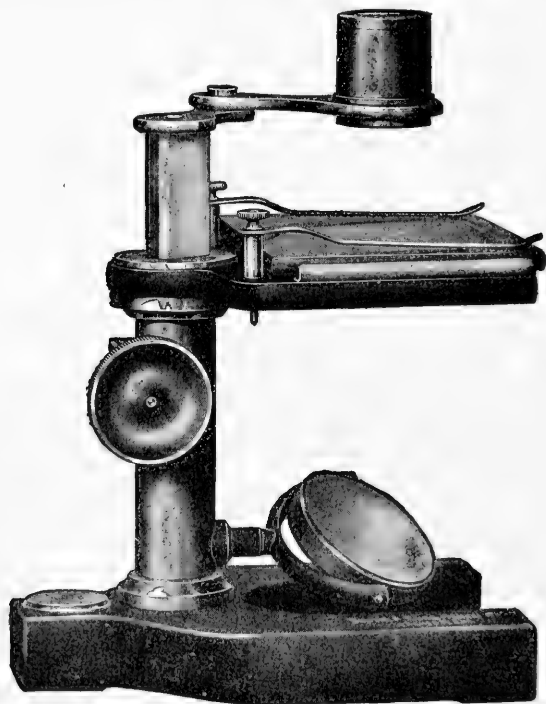


Fig. 64.—Dissecting Microscope.

lighting is designated as *axial* or *central* illumination. If one side is lighted more than another, the term *oblique* illumination is employed. In the case of transmitted light, the light which traverses the object is usually light reflected from a mirror because it is generally inconvenient or impossible to hold the instrument directly toward the source of light.

Light which falls upon the object and is reflected from it to the eye, either directly or through a microscope, is termed *reflected light.* Such illumination is employed but little in ordinary histological work, but it is useful in the examination of opaque objects such as metals, insects,

etc. The illumination may be increased by means of a bull's eye condenser or a mirror. In some microscopes the mirror can be swung above the stage for the purpose of illumining an object which is to be studied by reflected light.

The best light for microscopical work is light reflected from white clouds. Direct sunlight is never used. The light should come from in front of the observer or from one side. Various kinds of artificial light are used for microscopical work, such as an ordinary lamp with flat wick, the Welsbach, or the ordinary electric light. The Welsbach is perhaps the best. Whatever the source, the rays must be steady and brilliant. If a lamp with flat wick is used greater brilliancy is secured when the edge of the flame is turned toward the microscope; the object should be lighted directly by the image of the flame. To do this with low powers, the lamp may have to be turned so that the flame is oblique to the microscope.

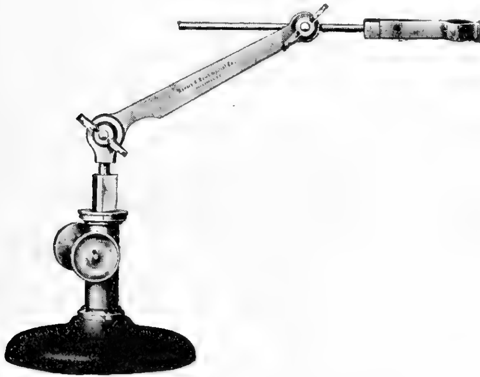


Fig. 65.—Stand for Dissecting Lens.

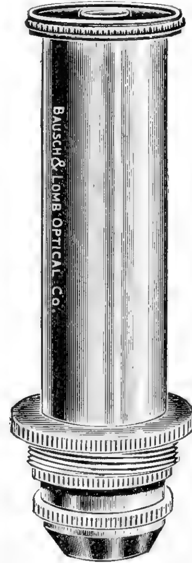


Fig. 66.—High-Power Dissecting Lens, Bruecke Type.

It may be used on the stand of a dissecting microscope or in a lens holder.

In artificial light the rays are divergent, not parallel as in the case of sunlight, hence they will not come to focus at the same point when reflected from the mirror as the latter do. This should be corrected by using a large bull's eye condenser between the source of light and the mirror, or by sliding the mirror along the mirror-bar farther away from the stage so that the concave mirror will have a longer distance in which to bring the rays to focus. If a substage condenser is used the same results may be obtained by depressing the condenser somewhat below the level of the stage. Lamps made for the microscope often have a metal chimney with a bull's eye in one side.

The objectionable yellowness of most artificial light may be eliminated by interposing a piece of green signal glass between the lamp and the microscope. With most microscopes, round slips of blue glass which fit into the substage mechanism are supplied for this purpose. Many workers still employ as a screen an ammonia sulphate of copper solution in a globular flask. To make the solution dissolve a small amount of copper sulphate in water, and add ammonia. At first a precipitate appears, but if an excess of ammonia is added this is dissolved and a transparent deep-blue liquid results. This should be diluted with water sufficiently to get a blue of just the proper depth to render the transmitted light white as seen through the microscope. The globular flask also acts as a condenser.

Immersion Objective.—A kind of objective in which a liquid is used between the front lens and the cover-glass. Cedar oil is the most widely used medium. In as much as the optical properties of cedar oil (refraction and dispersion) are almost the same as crown glass it is often termed a *homogeneous immersion fluid.* A homogeneous immersion lens, therefore, would be one intended for use with such a fluid. The advantage of an immersion over a dry lens lies in the fact that, other things being equal, after leaving the cover-glass rays which would be so refracted in a rarer medium like air as to miss the front end of the objective, reach this lens in the case of immersions and traverse the objective. With homogeneous immersions the rays of light are carried without deflection through cover-glass and fluid and into the glass of the front lens. Water has a greater density than air and less than glass, hence, with a *water immersion* more rays of light reach the front lens than with a dry lens, and less than with a homogeneous immersion lens (Fig. 67). The effect of an immersion is practically to widen the angle of the lens (see *angular aperture*).

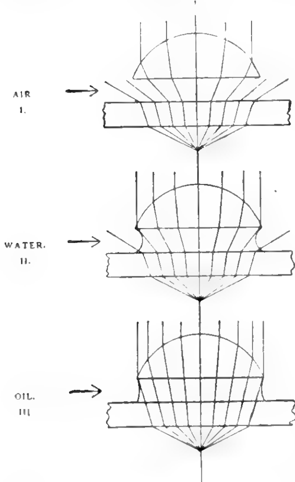


Fig. 67.—(From Bausch, "Manipulation of the Microscope.")

Magnifying Power.—The power of a lens to multiply the apparent dimensions of an object viewed through it. It should be expressed in *diameters* not in areas. While magnifying power is very important it is only so in connection with resolving power. If high power were the only essential, a series of single lenses might be used. The impossibility of using such a series for high magnification is due to the fact that proper

correction of aberrations cannot be made, and consequently, a distinct image cannot be obtained. For determination of magnification see *micrometer*.

Mechanical Stage.—A stage attachment (Fig. 68) for the more accurate manipulation of an object or a series of objects which must be moved about under the objective. The best mechanical stages are provided

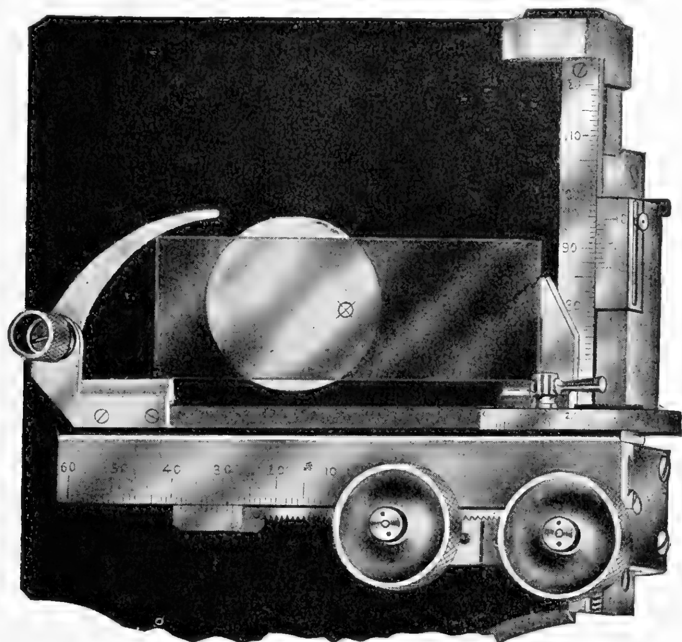


FIG. 68.—Attachable Mechanical Stage.

with scales and verniers so that an object once recorded may be easily found again. They are often very serviceable, especially with high powers.

Micrometer.—A scale for measuring objects under the microscope. The *stage micrometer* consists of a finely divided scale ($\frac{1}{10}$ and $\frac{1}{100}$ mm.) ruled on glass or metal. It is commonly mounted on a glass slide of standard size. To determine the actual size of an object with the stage micrometer, it is most convenient to use a camera lucida. The outline of the object to be measured is projected on to a sheet of drawing-paper and marked off. The object is then replaced under the microscope by the micrometer and the micrometer scale is projected on to the paper. Knowing the actual distance between the lines on the microm-

eter scale, the magnification as well as the real size of the object is readily calculated.

The size of the image projected onto a piece of drawing paper at the level of the table, however, does not represent the true magnifying power of the microscope. The latter is really considerably smaller if the microscope is in a vertical position because the magnification of a lens or a system of lenses is calculated in terms of the conventional distance of vision (250 mm., see page 144) while the distance from the ocular to the table is considerably more than 250 mm. Since the rays of light diverge

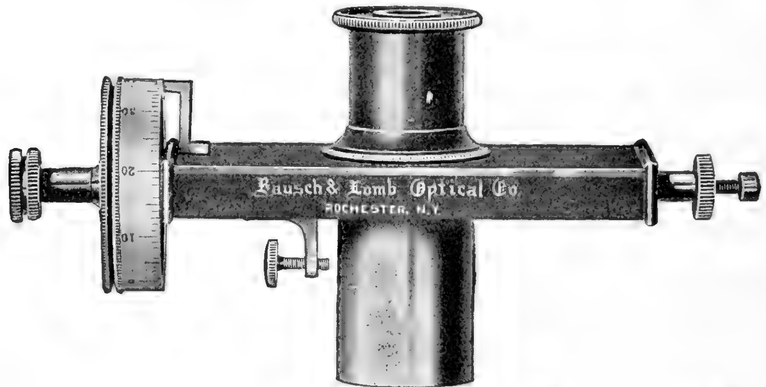


FIG. 69.—Filar Micrometer.

after leaving the ocular, manifestly, the projected image will be larger at the level of the table than at a level just 250 mm. from the point of emergence of the rays from the ocular. To determine the actual magnification of the microscope, therefore, one would have to bring the drawing surface to within 250 mm. of this point of emergence, sketch the projected scale of the stage micrometer on the paper, and then, by means of an ordinary metric rule, compute the number of times the divisions of the micrometer scale have been magnified. The standard distance of 250 mm., if the Abbe camera lucida is used (with camera mirror at 45°), includes the distance along the mirror-bar from the optical axis of the ocular to the mirror, plus the distance from the mirror to the drawing surface.

In practical work it is not necessary to make drawings or measurements exactly at this standard distance; one needs only to have a scale made out for the distance from the camera lucida at which the drawings are actually to be made, although it must be carefully borne in mind that any variation in the elevation of the drawing surface will alter the size of the projected image. A series of carefully prepared scales for

various combinations of objectives and oculars should be made and kept for future use. On each should be recorded the tube-length used, the number of the objective and of the ocular, the length of the camera mirror-bar, and the angle of the mirror, for if any one of these is changed the scale is no longer accurate.

When much measuring is to be done an ocular micrometer is used. It consists of a circular glass disk with a scale ruled on it and is inserted in the ocular between the eye-lens and the field-lens. By means of a stage micrometer the value of the divisions of the ocular micrometer is determined for a known tube-length and every combination of lenses it is desired to use in the work of measurement. Suppose that it takes four divisions of the ocular micrometer to correspond to one of the finer divisions of the stage micrometer, then since the divisions of the latter are equal to $\frac{1}{100}$ mm., each space in the ocular micrometer must be equal to $\frac{1}{400}$ mm., that is 0.0025 mm. A *filar* or *screw micrometer* is a more convenient form of ocular micrometer which is provided with delicate movable spider lines that can be adjusted to the space to be measured by means of a fine screw with very accurately cut threads (Fig. 69). At the end of the screw is a graduated disk which gives the value of the distance between the spider lines. The pitch of the screw is either $\frac{1}{50}$ inch or 0.5 mm.

Micron.—The one-thousandth part of a millimeter; expressed briefly by the Greek letter μ. It is the unit of measurement in microscopy.

Mirror.—The compound microscope is usually provided with both concave and plane mirrors, which may be rotated or swung in any direction. The plane mirror is used with the condenser, the concave, whenever it is of advantage to have light concentrated upon the object with the condenser out. The mirror should be capable of being moved up or down the mirror-bar so that it can be accurately focused upon the object. See also *illumination.*

Muscae Volitantes.—Small filaments or specks which float across the field of vision. They are really small opacities in the vitreous humor of the eye.

Numerical Aperture.—A system which expresses the efficiency of an objective by indicating the relative proportion of light rays which traverse it to form an image. With the introduction of immersion objectives, it became evident that angular aperture alone is not sufficient to indicate the real capacity of an objective. For instance, an immersion and a dry lens may be of precisely the same angular aperture and yet the immersion lens is more efficient because it sends more rays of light through the objective (see *immersion lens*). It was found necessary to take cognizance of the medium which intervenes between the cover-glass and the front lens of the objective.

Professor Abbe, in 1873, proposed the name *numerical aperture* and introduced the formula N. A.=$n \sin u$ in which n signifies the refractive index of the medium between cover-glass and objective, and u equals half the angle of aperture. That is, by multiplying the refractive index of the medium by the sine of half the angle of aperture, the numerical aperture is obtained. For example, suppose that one had an oil-immersion lens of 90 degrees angular aperture, then half the angle of aperture is 45 degrees, and by turning to a table of natural sines, the sine of 45 degrees is found to be 0.707. The refractive index of cedar oil is 1.52. Then N. A.=$1.52 \times 0.707 = 1.075$. Suppose that the lens were a dry instead of an immersion lens; then since the refractive index of air is 1, the formula would read N. A.=$1 \times 0.707 = 0.707$. Thus the two products 1.075 and 0.707 respectively, represent the relative capacities of an oil immersion and a dry objective of 90 degrees angular aperture.

Parfocal.—A term ordinarily applied to eyepieces of different powers that may be exchanged in the microscope without very materially affecting the focus of the instrument. The term is also applied to objectives attached to a revolving nosepiece if each is approximately in focus when turned into place.

Pedesis.—Same as *Brownian movement.*

Penetration.—The quality of an objective that permits of "looking into" an object having sensible thickness. It is greatest with low powers and narrow angles and is antagonistic to resolving power. It is the natural consequence of certain conditions in the making of lenses and is reckoned of secondary importance, because practically the same results are obtained by manipulating the fine adjustment.

Polariscope.—As used in microscopy the polariscope consists of two parts, each composed of a Nicol prism of Iceland spar; one, the *polarizer*, fits into the substage, and the other, the analyzer, is inserted between the objective and the tube of the microscope or, in some forms, just above the ocular. The polariscope is used more in chemical and in geological than in histological work. Some of the uses are as follows: determining whether an object is singly or doubly refractive; detecting the presence of minute crystals; determining the composition of rocks; examining sections of bone, hoof and horn, hairs and fibers of animals and plants, starch, etc., for certain characteristic and striking effects.

Resolving Power.—The quality of an objective which enables the observer to make out fine details of structure. It is the most essential property for precision in observation, and determines largely the excellence of an objective. Resolving power depends upon careful correction of aberrations, general accuracy in the mechanical construction of the microscope, and upon the aperture of the objective (see *angular aperture*,

numerical aperture). Resolving power is tested by the resolution of fine parallel lines ruled on glass or the striae on the surface of diatoms. The test is to determine how many lines to the inch or centimeter may be distinguished, and whether the objective simply glimpses the markings or whether it resolves them clearly. The wider the angle of aperture, the better the resolving power, provided the width is not so great as to interfere with the correction of the lenses. The increased resolution of immersion lenses is due to the fact that the immersion fluid practically widens the angle of aperture (see *immersion objective*).

Tube-Length.—The distance between the places of insertion of ocular and objective into the tube of the microscope. There are two standard tube-lengths; the short standard is 160 mm. ($6\frac{3}{10}$ inches), the long standard, 216 mm. ($8\frac{5}{10}$ inches). Many makers, however, do not adhere to the standards. The optical efficiency of the instrument is the same in either case. The short length is more advantageous in that it is more compact. The lenses must be corrected for the length of tube with which they are to be used. The short standard is in use in most American laboratories.

Overcorrection and Undercorrection.—In correcting for chromatic aberration, if the concave lens is stronger than is necessary to neutralize the aberration of the convex lens, the blue rays are brought to focus beyond the true principal focus of the objective, and the latter is said to be overcorrected; if the concave lens is not strong enough, the result is what is known as undercorrection. In case of overcorrection, the object takes on an orange tint if, after focusing, the distance between object and objective is slightly increased; or it becomes of bluish color if the distance is decreased. In case of undercorrection just the reverse is true. In some instances the objective is purposely undercorrected, and the eyepiece (e. g., compensating ocular) is equally overcorrected.

Working-Distance.—The distance between the front lens of the objective and the object when the latter is in focus. With high powers it is very small, so that with some oil-immersion objectives if a thick cover is used it is impossible to focus upon the object. For this reason thin cover-glasses (No. 1) should be used on preparations which are to be used with high-power immersion lenses.

MANIPULATION OF THE COMPOUND MICROSCOPE

1. Always handle the instrument cautiously; it is a delicate mechanism. Lift it by the base, not by the tube or the arm.

2. The work-table should be of such a height that the observer can sit at it comfortably without compressing the chest or tiring

the neck. Sit as upright as possible. If the instrument is inclined it should set farther in on the table than if it is in the upright position.

3. With a piece of old linen, a chamois skin, or a bit of lens paper, carefully clean the eyepiece to be used and put it in place. Always use the low-power eyepiece first.

4. Likewise clean and attach the objective (low-power first) after elevating the tube far enough above the stage for this purpose. Guard particularly against screwing the objective in crooked, as this will injure the threads. It is best to swing the objective between the first and second fingers of one hand and bring the screw squarely into contact with the screw of the tube (or nosepiece); with the thumb and forefinger of the other hand it is then screwed into place.

5. Bring the draw-tube to the standard length (see *tube-length*) for which the lenses are corrected. If a nosepiece is used, allowance must be made for its height.

6. Place the slide which bears the object on the stage with the object over the central opening of the latter, and clamp it in place by means of the spring clips. While looking at the object from one side, turn the mirror until a flood of light shines up through the center of the stage.

7. Lower the tube until the objective nearly touches the cover-glass, then look through the eyepiece and slowly raise the tube by means of the coarse adjustment until the specimen to be examined is plainly visible. Focus accurately by means of the fine adjustment. If a high-power objective is being used, since it must come very near the cover, the operator should lower his head to the level of the stage, and look toward the light between objective and cover-glass in order to prevent actual contact. This is of great importance, for otherwise the objective or the object is liable to injury. Remember that in focussing *up* the lowest part of the object comes into view first, the highest part last.

8. The higher the power, the more difficult it is to find an object or a particular part of it. For this reason the finding is usually done by means of a low-power objective, or a low-power

ocular, or both, and after accurately centering the object in the field, the high power is attached. In case a revolving nosepiece is used, great care should be used in turning in the high power not to strike the slide with the objective. This is very likely to happen if the objectives are not parfocal.

9. After the object is in focus give any further attention to the illumination that is necessary (see *illumination* and *mirror*). If intensified illumination is desired, use the concave mirror, or use the substage condenser and the plane-mirror. For ordinary purposes the field should be evenly illuminated, although oblique light is frequently useful. Manipulate the diaphragm until the structure to be studied shows with the greatest distinctness. Too much light "drowns" the object, and is hard on the eyes. (To determine the proper distance at which the concave mirror should stand below the stage, let direct sunlight shine upon the mirror, and then adjust the latter so that the apex of the cone of light comes just at the top of the stage where the object will rest.)

10. In using oil-immersion objectives, a small drop of cedar oil (specially prepared by the maker of the lens) is applied to the front lens by means of a small rod or brush. It is very important to keep the oil free from dust, and to see that it does not contain air bubbles when applied to the lens. Carefully lower the tube until the oil on the objective comes in contact with the cover-glass. The operator should lower his head to the level of the stage to observe this properly. Focus up as with a dry objective. With a piece of lens paper or a soft cloth, clean the immersion lens immediately after you have finished using it. Likewise remove the oil from the cover-glass.

11. The range of the fine adjustment is limited. Keep it as near the middle point as possible. If the tube does not respond to the movement of the screw you have probably gone beyond the range of the fine adjustment.

12. In working with the microscope *keep both eyes open.* The eye which is not in use soon becomes accustomed to ignoring objects in the field of vision. To avoid fatigue it is well to use first one eye and then the other for observation. The eye should be placed

at the eyepoint (see above) of the lens. This is some distance from the eye-lens in low-power eyepieces, close to it in high-power eyepieces.

13. Put the microscope in its case when you have finished using it, or at least cover it with a cloth or cone of paper. For further details regarding the use or care of the microscope consult one of the following books: *The Microscope*, by Gage; *Manipulation of the Microscope*, by Bausch; *The Microscope and its Revelations* (1,200 pages), by Carpenter and Dallinger.

14. Do not apply alcohol to any part of the instrument. The lenses may be cleaned ordinarily by breathing upon them and wiping them with a rotary motion on lens paper or a piece of soft old linen. In case a solvent must be used for balsam or oil, benzene is the one commonly recommended. It must be quickly wiped away so that it will not affect the setting of the lens. Bits of dust may be flecked off the surface of a lens by means of a camel's hair brush.

The beginner in microscopy should acquaint himself with various common objects that are liable to get into his preparations in the form of dust, etc., so that he may not mistake them for essential parts of his specimen. Such objects are hairs, fibers of silk, wool, linen, cotton, and the like, and particularly air-bubbles. Air-bubbles are usually circular with black borders and bright centers; they may show tinges of color. Examine a drop of saliva for examples.

APPENDIX B

SOME STANDARD REAGENTS AND THEIR USES

I. FIXING AND HARDENING AGENTS

1. **Acetic Acid.**—Acetic acid is more commonly used in mixtures or in diluted form than pure. It is valuable because it tends to produce good optical differentiation and facilitates penetration. When employed alone it causes some tissues to swell and disintegrate. Inasmuch as most fixing agents give the best results when they have an acid reaction, from 1 to 5 per cent. of acetic acid is generally added to acidify them in case they are not naturally acid. Acetic acid is also of great value in mixtures because it counteracts the shrinking action of certain reagents. Ordinary acetic acid is of about 36 per cent. strength; glacial acetic, of about 99.5 per cent. strength.

A strength of from 0.2 to 1 per cent. is recommended by Flemming for work on cell nuclei. Strong glacial acetic acid is sometimes used for highly contractile animals, such as Coelenterata, Mollusca, and Vermes. The animal is rapidly flooded with the acid and remains immersed until it is thoroughly penetrated (6 to 10 minutes). It is then washed in repeated changes of 50 or 70 per cent. alcohol and left to harden in 70 to 83 per cent. alcohol. The pure acid, if allowed to act for more than a few minutes, swells and softens the tissues. Acetic acid should not be used when connective tissue or delicate calcareous structures are to be preserved.

2. **Acetic Alcohol.**—Carnoy recommends each of the following formulae:

a) Glacial acetic acid . . . . 1 part
Absolute alcohol . . . . 3 parts

b) Glacial acetic acid . . . . 1 part
Absolute alcohol . . . . 6 parts
Chloroform . . . . 3 parts

The chloroform is said to hasten the action of the mixture. Either of these reagents penetrates well and acts rapidly. Almost

any stain will follow them. Even such difficult objects as the eggs of Ascaris may be fixed by the second mixture. The reagent should be washed out in absolute or at least in strong alcohol.

A mixture of absolute alcohol, glacial acetic acid and chloroform, equal parts, saturated with corrosive sublimate (formula of Carnoy and Lebrun) becomes even more valuable for the fixation of difficult objects. According to Lee, isolated ova of Ascaris are fixed in 30 seconds, entire oviducts in 10 minutes, in this liquid.

3. **Alcohol.**—Alcohol is used especially for gland cells and for preserving the brain and spinal cord for Nissl's method of staining nerve cells. See chap. iii, *alcohol fixation;* also chap. i, reagents 1 and 2.

Alcohol and Chloroform.—See 2*b*.

Bichloride of Mercury.—See *corrosive sublimate.*

4. **Bichromate of Potassium.**—Bichromate of potash is one of the oldest and best-known fixing reagents. At present it is more commonly used in mixtures than alone. It is widely used in hardening nervous tissue. Its fixation of nuclei is unsatisfactory unless it is properly corrected through the addition of acetic acid. It acts very slowly, about three weeks being necessary to harden properly a sheep's eye, and from three to six months for a good-sized brain. A weak solution (2 per cent.) should be used at first, to be replaced gradually by stronger solutions up to 5 per cent. When hardening is completed the object should be thoroughly washed in running water and then put into alcohol; begin with low percentages of alcohol and gradually increase the strength up to 70 or 80 per cent. Change the alcohol as often as it becomes yellow. After the object has been placed in alcohol, keep it in the dark in order to prevent a precipitate forming on the surface. Either carmine or hematoxylin may be used as a stain after bichromate of potash. In case carmine is used, the staining is best done before the object is placed in alcohol. Tissues which do not stain well should be placed for 3 hours in acid alcohol and then washed in alcohol before staining.

5. **Bichromate of Potassium and Acetic Acid** (Tellyesnicky's fluid).—

Bichromate of potassium	3 grams
Glacial acetic acid	5 c.c.
Water	100 c.c.

It is best not to add the acetic acid until just before using. This is a good general reagent. It is valuable for embryos. Objects should remain in some 20 volumes of the fluid from 24 to 48 hours, according to size. It is well to change the fluid once, after a few hours. After fixation, tissues should be washed thoroughly in running water (6 to 12 hours) and passed through alcohols of increasing strength beginning with 15 per cent.

6. **Bichromate of Potassium and Corrosive Sublimate** (Zenker's fluid).—

Corrosive sublimate	5 grams
Potassium bichromate	2 grams
Sodium sulphate	1 gram
Glacial acetic acid	5 c.c.
Water	100 c.c.

It is best to add the acetic acid immediately before using. Zenker's is a valuable reagent for both histological and embryological material (embryos up to 25 mm.). Several hours are required for fixation: 2 to 4 hours for a 2 day chick; 8 to 10 hours for objects or embryos of 6 to 8 mm.; 24 hours for embryos of 12 to 14 mm., etc. For washing, running water is employed for from 12 to 24 hours. The object is then transferred to gradually increasing strengths of alcohol up to 70 per cent., leaving it according to size from 1 to 3 hours in each alcohol. To remove the excess of corrosive sublimate, see **13**, caution 1. Almost any stain follows this reagent well. Both nuclear and cytoplasmic structures are properly fixed.

7. **Bichromate of Potassium and Cupric Sulphate** (Erlicki's fluid).—See chap. i, reagent **8** and chap. iii, 3.

8. **Bichromate of Potassium and Sodium Sulphate** (Müller's fluid).—

Bichromate of potassium	20 to 25 grams
Sodium sulphate	10 grams
Water	1,000 c.c.

Müller's fluid is an old and widely used reagent. It is especially valuable for the nervous system. It acts very slowly.

Specimens require immersion in a large quantity of the fluid from 3 to 10 weeks according to size. The solution should be changed every two days for the first ten days, and later, about once a week. If a scum appears at any time the fluid should be changed. In washing, the tissues are placed in running water for a number of hours and are then treated with gradually increasing strengths of alcohol in the usual manner. For some purposes, however, the tissue is transferred directly from the fluid to 70 per cent. alcohol. In any event, the material should always be kept in the dark to prevent precipitation.

Carnoy's Acetic Alcohol, see **2.**

9. **Chloride and Acetate of Copper** (Liquid of Ripart and Petit).—

Camphor water	75.0	grams
Crystallized acetic acid	1.0	gram
Distilled water	75.0	c.c.
Acetate of copper	0.30	gram
Chloride of copper	0.39	gram

This is a good reagent for cytological work where objects are to be studied in as fresh a condition as possible. Methyl green (**56**) should be used for staining. Only aqueous media are employed with such material.

10. **Chromic Acid.**—Aqueous solutions of from 0.2 to 1 per cent. are used. The acid is best kept in the form of a 1 per cent. stock solution. Tissues are left in at least fifty times their volume of the acid for from 24 hours for small pieces to one or more weeks for larger ones. The objects are then washed in running water for several hours, after which they are treated with gradually increasing strengths of alcohol. Do the washing and dehydrating in the dark. If sections of chromic acid material do not stain readily, they should be treated for three hours with acid alcohol, washed out with ordinary alcohol, and then stained. Hematoxylin or some of the anilins are the best stains for chromic material. Chromic acid hardens much more rapidly than bichromate of potash. It makes tissues extremely brittle.

11. **Chromo-Aceto-Osmic Acid** (Flemming's solution).—

Chromic acid, 1 per cent. aqueous solution .	15 parts
Osmic acid, 2 per cent. aqueous solution . .	4 parts
Glacial acetic acid	1 part

This is the so-called "strong" solution of Flemming. The mixture should not be made until immediately before using, because it deteriorates if allowed to stand for any considerable length of time. The fluid is valuable for cytological work, especially for the study of karyokinetic figures. Only small pieces of tissue should be used, as the reagent penetrates poorly. They should remain in the fluid for from 24 to 48 hours and then be washed in running water for from 6 to 24 hours. From water they are transferred to gradually increasing strengths of alcohol. Particles of fat are blackened by the mixture. Sections stain well with safranin or hematoxylin. Read the remarks on osmic acid, **20**.

12. **Chromo-Platinic Mixture** (Merkel's fluid).—

Chromic acid, 1 per cent. aqueous solution	25 c.c.
Platinic chloride ($PtCl_4$), 1 per cent.	25 c.c.
Distilled water	150 c.c.

This is an excellent reagent for delicate objects such as the retina. Objects are left in it for from a few hours to several days. Washing is done with alcohol of 50 per cent. strength followed by 70 per cent. alcohol. Tissues so prepared stain well.

13. **Corrosive Sublimate** (Mercuric Chloride, Bichloride of Mercury).—

Corrosive sublimate is ordinarily used as a saturated solution in distilled water (about a 7 per cent. sol.) or in normal saline. The latter keeps better and contains a greater percentage of the sublimate. Corrosive sublimate is an excellent and rapid fixing fluid for many objects (glands, epithelia, etc.). Objects should remain in the fluid only long enough to become thoroughly fixed; this has been accomplished when they have become opaque throughout. Only a few minutes or even seconds are required to fix very delicate objects, but denser tissues may require from 4 to 24 hours. The value of the fluid is usually enhanced by the addition of 5 per cent. of glacial acetic acid. Small pieces of tissue (not over 0.6 cm. in diameter) should be used where practicable. Washing may be done in running water (several hours) or in 50 to 70 per cent. alcohol.

Cautions.—(1) With corrosive sublimate or mixtures containing it, the mercuric salt is often not wholly removed in washing.

If the tissues are to remain several days or weeks in alcohol, the alcohol will gradually extract it. If they are to be used within a few days, however, it is necessary to remove the excess of sublimate by adding tincture of iodine to the 70 per cent. alcohol. Sufficient of the tincture is added to give the alcohol a port-wine color; as often as the color disappears the iodine must be renewed. After from 12 to 48 hours of this treatment, the iodine color persists and the object should then be transferred to fresh 70 or 80 per cent. which must be renewed until it no longer extracts iodine from the specimen.

(2) In handling corrosive sublimate, a glass or horn spoon should be used instead of a metal instrument, because it corrodes metal.

(3) Use *distilled* water, not tap water, in making an aqueous solution.

14. **Corrosive Sublimate and Acetic Acid.**—

Corrosive sublimate, saturated aqueous solution	100 parts
Glacial acetic acid	5 to 10 parts

This is an excellent reagent for embryonic tissues and for organs which do not contain a very great amount of connective tissue. See remarks under **13.**

15. **Corrosive Sublimate, Nitric Acid Mixture** (Gilson's mercuro-nitric mixture). See chap. i, reagent **7** and chap. iii, 2.

Erlicki's Fluid, see **7.**

16. **Ether Alcohol.**—Equal parts of sulphuric ether and absolute alcohol.

Flemming's Solution, see **11.**

17. **Formalin.**—See chapter i, reagent **6** and chap. iii, 4. It should be borne in mind that formalin is a reducing agent and will rapidly decompose such reagents as osmic acid or chromic acid if mixed with them.

18. **Formalin, Alcohol, and Acetic Acid** (Lavdowsky's mixture).—

Formalin, commercial	10 parts
Alcohol, 95 per cent.	50 parts
Glacial acetic acid	2 parts
Distilled water	40 parts

This mixture is recommended in some cases for the treatment of embryos, especially when the nervous system is to be studied. It penetrates well and preserves faithfully; the alcohol counteracts the swelling effects of the acetic acid and the formalin. Material may remain in it without injury for several days. The fluid should sooner or later be replaced by 70 per cent. alcohol. No preliminary washing is necessary.

19. **Formol Sublimate** (Worcester's fluid).—*a*) Make a saturated solution of corrosive sublimate in 10 per cent. formalin. This reagent is recommended by Raymond Pearl (*Journal of Applied Microscopy*, Vol. VI, p. 2451) as "extremely satisfactory" for killing and fixing protozoa. Washing may be done in water or 4 per cent. formalin. The material may be preserved in 4 per cent. formalin or carried up the grades of alcohol to 70 per cent. alcohol.

b) If to 9 parts of this formol-sublimate mixture, 1 part of glacial acetic acid is added, Worcester's formol-sublimate-acetic mixture is obtained. Pearl recommends this highly for teleost eggs and for embryological material in general. It will not produce coagulations and cloudiness in the gelatinous envelopes of amphibian eggs, if thoroughly washed out after fixing. Preservation is the same as for *a*). Johnson (*Journal of Applied Microscopy*, Vol. VI, p. 2652) also recommends this reagent very highly for general work except in the case of nervous tissue.

Personally, I have found it advisable not to prepare either of the above mixtures until needed because the formalin, which is a reducing agent, causes much of the mercuric salt to pass over into the insoluble mercurous salt.

Gilson's Mercuro-Nitric Mixture, see **15.**

Hermann's Fluid, see **26.**

Kleinberg's Picro-Sulphuric, see **25.**

Lavdowsky's Mixture, see **18.**

Merkel's Fluid, see **12.**

Müller's Fluid, 8.

20. **Osmic Acid** (really the tetroxide of osmium OsO_4).—Osmic acid kills quickly and fixes well. It is exceedingly vola-

tile. The chief objections to it, aside from its extremely poisonous nature, are its poor powers of penetration, and the fact that it becomes reduced in the presence of the least amount of dust containing organic particles. The substance must be handled with the greatest care, as even the vapors are dangerous. It is usually put up in small quantities (0.1 to 1 gram) in hermetically sealed glass tubes. In making up solutions, the wrappings are removed from such a tube and the tube is dropped into a reagent bottle where it may then be broken by means of a glass rod. Aside from its use in mixtures (see **11** and **26**), the vapor or a 0.05 to a 1 per cent. aqueous solution are commonly used. A stock solution of 1 per cent. is usually kept on hand. It *must* be kept free from *dust*. As the most practical way of preventing reduction, Lee recommends that the osmic acid for ordinary work be kept as a solution in chromic acid (a 2 per cent. sol. of osmic acid in a 1 per cent. aqueous sol. of chromic acid). This solution may be employed in making up Flemming's solution or for the purpose of fixation by means of osmium vapor. For vapor fixation, however, many workers prefer the vapor from the solid crystals.

To fix by means of the vapor, the tissue is pinned to the lower end of a cork which fits tightly into the bottle containing the osmic acid, or it is suspended by a thread. Objects which will adhere to a slide are fixed by simply inverting the slide over the mouth of the bottle. The time required for such fixation varies from thirty seconds or a few minutes for isolated cells, to several hours for thicker objects, such as the retina. For fixing in the solution, 24 hours are required ordinarily. Objects are then washed in running water for the same length of time. Only small or thin pieces can be fixed by means of either the solution or the vapor. The stains which follow osmic acid best are hematoxylin, methyl green (for study in aqueous media), alum-carmine, picro-carmine, and safranin.

21. **Picric Acid.**—A cold saturated aqueous solution (about 1.2 per cent.) of picric acid is commonly used. Small objects are fixed in from a few minutes (infusoria) to 6 hours; objects

up to 1 cm. in size, in from 24 to 36 hours. They may be left a much longer time, however, without injury. Large objects may require weeks for proper fixation. After fixing, tissues should be washed in 70 per cent. alcohol until the alcohol is no longer colored by the picric acid. The tissue should not pass, during subsequent treatment (with a few exceptions in case of staining), into an aqueous medium or into an alcohol of less than 70 per cent. strength, because such media seem to undo the work of fixation.

22. **Picric Alcohol.**—Gage recommends a 0.2 per cent. solution of picric acid in 50 per cent. alcohol as an excellent fixer and hardener for almost any tissue or organ. Time required, 1 to 3 days. Entire objects which have been fixed in picric acid or in picric alcohol stain readily in borax-carmine or paracarmine.

23. **Picro-Acetic.**—Saturate a 1 per cent. aqueous solution of acetic acid with picric acid. This liquid is widely used as a general reagent, and is to be preferred for most purposes to picric acid alone. For washing, etc., see remarks under **21.** The author has found that his preparations are improved if about 25 parts of formalin are added to each 100 parts of the picro-acetic.

24. **Picro-Sublimate.**—

Rabl's—

Picric acid, saturated aqueous solution . . .	1 vol.
Corrosive sublimate, saturated aqueous solution	1 vol.
Distilled water	2 vols.

This mixture has been especially recommended for embryos. They are left in the fluid for 12 hours, then washed in weak alcohol and transferred to gradually increasing strengths of alcohol.

O. vom Rath's—

Picric acid, cold saturated solution	1 vol.
Corrosive sublimate, hot saturated solution . .	1 vol.
Glacial acetic acid	0.5 to 1 vol.

After fixing for several hours, transfer the material directly into alcohol.

25. **Picro-Sulphuric** (Kleinenberg's).—

Picric acid, saturated aqueous solution . .	98 vols.
Sulphuric acid	2 vols.
Water	200 vols.

This is an excellent reagent for embryos, either for entire mounts or for sectioning. Chick embryos of 24 to 48 hours should remain in the liquid for from 2 to 4 hours; older embryos, for from 3 to 6 hours. For washing, 70 per cent. alcohol is used. It should be changed (frequently at first) until the color ceases to come out of the embryos. Preserve in about 80 per cent. alcohol.

Lillie recommends the addition of glacial acetic acid sufficient to make a 5 per cent. solution of acetic acid. The reagent, as thus modified, certainly gives beautiful results. For staining, use Conklin's picro-hematoxylin (**48**).

26. **Platino-Aceto-Osmic Mixture** (Hermann's fluid).—

Platinum chloride, 1 per cent. aqueous solution	60 c.c.
Osmic acid, 2 per cent. aqueous solution . .	8 c.c.
Glacial acetic acid	4 c.c.

Hermann's fluid is one of the most valuable cytological reagents. Only small pieces of tissue should be used. The washing and subsequent treatment are the same as for Flemming's solution (**11**). For subsequent treatment with pyrogallol, see **64**. Read, also, remarks on osmic acid (**20**).

Rabl's Picro-Sublimate, see **24**.

Rath's (O. vom) Picro-Sublimate, see **24**.

Ripart and Petit, Liquid of, see **9**.

Tellyesnicky's Fluid, see **5**.

Worcester's Fluid, see **19**.

Zenker's Fluid, see **6**.

II. STAINS

Read the general statement about stains in chap ii.

27. **Alum Cochineal.**—

Potassic alum	6 grams
Powdered cochineal	6 grams
Distilled water	90 c.c.

Boil for half an hour; after the fluid has settled, decant the supernatant liquid, add more water to it, and boil it down until only 90 c.c. of the decoction remains. Filter when cool, and add a bit of thymol or a little salicylic acid to prevent the growth of

mold. Alum cochineal is one of the best stains for entire objects. It is easy to work with, and does not overstain. The time required for staining is from 24 to 36 hours ordinarily. After staining, the object should be washed in water for 15 or 20 minutes to extract the alum, which would otherwise crystallize when the object is placed in alcohol. Too long an immersion in water may extract the stain to too great an extent. From water the object should be passed upward through the grades of alcohol, remaining about an hour in each. The writer has found alum cochineal especially valuable for flatworms (tapeworms, flukes, etc.) and embryos. If it is desired to use a counterstain with it, Lyon's blue, picric acid, orange G, or light green will answer.

28. **Alum Carmine.**

Powdered carmine	1 gram
Ammonia alum (2.5 per cent. aqueous solution)	100 c.c.

Boil for 20 minutes, and filter when cool. The uses and manipulation are the same as for **27**. These stains affect calcareous structures injuriously.

29. **Anilin Stains.**—Read the general remarks about anilin stains in chap. ii. The formulae for some of the most important are given separately in this list in their proper alphabetical position.

The dyes are dissolved in water, in alcohol of any desired strength, or in anilin water, according as they are soluble in these media, or as they meet the needs of the operator. Some workers even use some of them as counterstains dissolved in the clearing fluid. For the study of nuclei, after Hermann's or Flemming's fluid has been used for fixing, the writer has found a weakly alcoholic anilin water solution to be the most satisfactory. As cytoplasmic contrast stains, alcoholic solutions (in 70 to 95 per cent. alcohol) have given the best results. *Anilin water* is made by shaking up 4 c.c. of anilin oil in 90 c.c. of distilled water and filtering the mixture through a wet filter. Enough alcohol may be added to make it a 20 per cent. alcohol, if a weakly alcoholic solution is desired.

The length of time which sections should be immersed in the

stain varies from a few seconds or minutes for some of the dyes (especially when used for cytoplasm) to 24 to 36 hours for others (especially nuclear). Sections usually overstain, in which case they are differentiated by means of alcohol, either pure or slightly acidulated with hydrochloric acid. The color is thus extracted rapidly; decolorization should be stopped immediately after the color ceases to come from the tissue in clouds (20 seconds to 3 minutes). If acidulated alcohol is employed, it must be in much weaker solution than that used for extracting carmines or hematoxylins. One part of hydrochloric acid to 1,000 of water or alcohol is about the correct proportion. When one desires to study the karyokinetic figures of nuclei, the acid-alcohol differentiation should be employed, but if resting nuclei are to be studied, only neutral alcohol should be used.

30. **Anilin Blue and Orange G** (Mallory's connective tissue stain).—

a) *Double.*—

Solution I.

Phosphomolybdic acid	1.0 gram
Distilled water	100.0 c.c.

Solution II.

Anilin blue (soluble in water)	0.5 gram
Orange G.	2.0 grams
Oxalic acid	2.0 grams

The tissue should be fixed in mercuric chloride or in Zenker's fluid. Sections are first placed in Solution I for one or two minutes and then washed well in water. They are then transferred to Solution II for from 2 to 20 minutes, washed in water, dehydrated rapidly, and cleared in xylol. This is an especially differential stain for connective tissue fibrils which are colored a deep blue. Keratin, blood corpuscles, and usually muscle and some nuclei, are stained yellow; mucin and amyloid substances, a light blue.

b) *Triple.*—Stain the sections from 1 to 3 minutes in a 0.1 to 0.5 per cent. aqueous solution of acid fuchsin and wash them in water before treating with Solution I as above. Further procedure is the same as for *a*.

31. **Bismarck Brown.**—Boil one gram of the stain in 100 c.c. of water, filter, and add 30 c.c. of strong alcohol. Bismarck brown is a nuclear stain which does not overstain although it acts rapidly. After staining wash in 95 per cent. or absolute alcohol. This stain is also used in aqueous solution for *intra vitam* staining; the nucleus of the living cell may thus be colored. It has been used as an *intra vitam* stain mostly in the study of infusoria. The stain may be fixed by means of a 0.2 per cent. chromic acid solution, but this, of course, destroys the life of the cells.

32. **Borax-Carmine** (Grenacher's).—See chap. i, reagent **12.**

33. **Bordeaux Red.**—See chap. i, reagent **15.**

34. **Carmalum.** (Mayer's).—

Carminic acid	1 gram
Alum	10 grams
Distilled water	200 c.c.

Dissolve with heat and filter the solution when cold. Add a few crystals of thymol or a little salicylic acid to prevent the formation of mold. Carmalum is one of the best stains for staining objects in bulk and will follow almost any fixing reagent, even osmic acid. If the object has an alkaline reaction it does not stain so well. Washing is done in water.

35. **Carmine** (Beale's).—

Powdered carmine	1 gram
Ammonia	3 c.c.
Pure glycerin	96 c.c.
Distilled water	96 c.c.
Alcohol, 95 per cent.	24 c.c.

The ammonia and part of the water are first mixed and the carmine dissolved in the mixture. The remaining water is added and the solution is left in an open dish until the ammonia has almost evaporated. The alcohol and glycerin are then added. For staining, equal parts of the stain and glycerin are used. The staining is carried on for 24 hours under a bell jar in an uncovered dish. A second open dish containing acetic acid is placed under the bell jar. After staining, the sections are washed in water,

then in weak hydrochloric acid (1 to 500 of water) and again in water. Minot recommends this stain and method of treatment especially for the placenta and for the central nervous system of embryos.

36. **Carmine, Picric Acid, and Indigo Carmine** (Calleja's staining fluid).—

Solution I.

Carmine	2 grams
Lithium carbonate, saturated aqueous solution	100 c.c.

Solution II.

Indigo carmine	0.25 gram
Picric acid, saturated aqueous solution .	100.00 c.c.

Place sections in solution I for from 5 to 10 minutes, then into acid alcohol until they become pale red (20 to 30 seconds); wash well in water. Next, place the sections in Solution II for 5 to 10 minutes, then into acetic acid (0.2 to 0.5 per cent.) for a few seconds and wash well in water. Dehydrate rapidly and clear in xylol. The method is useful for epithelial cells and connective tissue.

37. **Carmine, Acid** (Schneider's).—Add carmine to boiling acetic acid of 45 per cent. strength until no more will dissolve. Filter the solution when cool. This is a valuable reagent for the study of the nuclei of fresh cells. It is very penetrating and gives a brilliant stain. The strong acetic acid ultimately destroys the cell.

38. **Ehrlich-Biondi Triple Stain** (Heidenhain).—The ingredients should be obtained from Grübler and Hollborn, *Baiersche Strasse 63, Leipzig*, or from their agents.

Acid fuchsin, saturated aqueous solution . .	4 parts
Orange G " " " . .	7 parts
Methyl green (Methylgrün OO) saturated aqueous solution	8 parts

The solution of orange should be prepared first, and the solutions of fuchsin and methyl green added to it with continual stirring. Each solution must be thoroughly saturated; it takes

several days for this to occur. The above mixture constitutes a stock solution which should be diluted with about 50 or 100 times its volume of water before using. According to Lee (*Microtomist's Vade-Mecum*), "if a drop be placed on blotting paper it should form a spot bluish green in the center, orange at the periphery. If the orange zone is surrounded by a broader red zone, the mixture contains too much fuchsin." For use with this method, tissues should be fixed in pure corrosive sublimate solution. Sections should be thin (3 to 5 microns) and must remain in the stain from 18 to 24 hours. They should then be rapidly washed in 95 per cent. alcohol, placed for a short time in absolute alcohol and cleared in xylol. If the sections remain in the alcohols any considerable length of time, the methyl green will be extracted. The stain is very uncertain in its action but when successfully applied the results are excellent. It is used chiefly in cytological studies, especially in connection with gland cells. Grübler prepares a dry powder for this three-color mixture, but the results are usually not as satisfactory as when the mixture is properly made fresh. To prepare the stain from the powder, a 0.4 per cent. solution of the latter in distilled water is made, and to 100 c.c. of this solution 7 c.c. of a 0.5 per cent. aqueous solution of acid fuchsin are added.

39. **Ehrlich's "Triacid" Mixture.**—For blood films Ehrlich's so-called triacid mixture is a serviceable stain which is widely used.

Orange G, saturated aqueous solution . . .	14.0 c.c.
Acid fuchsin, saturated aqueous solution .	7.0 c.c.
Distilled water	15.0 c.c.
Absolute alcohol	25.0 c.c.
Methyl green, saturated aqueous solution .	12.5 c.c.
Glycerin	10.0 c.c.

Each solution must be thoroughly saturated (several days). Add the ingredients in the order named, shaking the mixture well between each addition. It is best for the stain to stand a week or two before it is used. Neutrophil granules stain violet, oxyphil granules a brownish red. The mixture stains in from 5 to 15 minutes.

40. **Eosin.**—See chap. i, reagent **17**. This anilin dye is often used after hematoxylin as a contrast stain. It is specific for cer-

tain granules of leucocytes and for red blood corpuscles, giving to the latter a very characteristic coppery-red tinge. Some workers prefer to dissolve it in water or in some cases in the clearer.

41. **Erythrosin.**—An eosin; properties and manipulation, much the same as ordinary eosin (see **40**).

42. **Fuchsin, Acid** (Rubin S, Acid Majenta, Majenta S).—

Acid fuchsin	0.5 gram
Distilled water	100.0 c.c.

This is an excellent anilin stain for cytoplasmic structures. It is also used in some instances as a specific stain for nerve tissue. Acid fuchsin should not be confounded with *basic fuchsin* which is a nuclear stain. It too is used in aqueous solution. When fuchsin alone is mentioned by writers, without specifying whether it is acid or basic, the basic fuchsin is ordinarily meant.

43. **Fuchsin (Acid) and Picric Acid** (Van Gieson's stain).—

Acid fuchsin, 1 per cent. aqueous solution	10 c.c.
Picric acid, saturated aqueous solution	90 c.c.

This stain is frequently used in conjunction with hematoxylin in the study of fibrous or of nerve tissue. Small bits of tissue should be fixed in corrosive sublimate or its mixtures. Sections are slightly overstained with hematoxylin, rinsed in water, and then stained 5 minutes in the picro-fuchsin mixture. To avoid extracting too much of the yellow color in dehydrating and clearing, the alcohols and clearer should each have a few crystals of picric acid added to them. The result should be: nuclei and epithelia brown; white fibrous connective tissue red; elastic tissue and muscle yellow.

44. **Gentian Violet.**—This is one of the best of the nuclear anilin stains. It is best made up in anilin water and weak alcohol (see **29**).

Gentian violet	1 gram
Anilin water	80 c.c.
Alcohol, 95 per cent.	20 c.c.

The stain works well with thin sections. It is also widely used in the study of bacteria. For differentiation, Gram's method is used.

Gram's solution.—

Iodine	1 gram
Iodide of potassium	2 grams
Water	300 c.c.

After staining, the sections are placed in this solution until they are black (2 to 3 minutes) and are then decolorized in absolute alcohol until they appear gray. See also **66**.

45. **Gold Chloride.**—The gold chloride method is used chiefly in the study of nerve-fiber terminations, both motor and sensory, although it is sometimes used for the coloration of other tissue elements (capsules of cartilage, etc.). The process is really an impregnation; through the agency of sunlight and of certain reagents (acetic, citric, formic or oxalic acid) the gold is deposited in the tissues in the form of very fine particles. There are numerous modifications of the method, one of which is given in chap. ix.

46. **Golgi's Chrome-Silver Method.**—See chap. ix.

47. **Hemalum** (Mayer's).—

Hematein	1 gram
Alcohol, 95 per cent.	50 c.c.
Alum	50 grams
Distilled water	1000 c.c.

Dissolve the hematein in the alcohol with the aid of heat. Dissolve the alum in the water and slowly add the hematein solution; thoroughly stir the resulting mixture. Filter if there is any residue of solid material, and add a few crystals of thymol to prevent the formation of mold. The stain may be used *immediately after preparation.* It is valuable for staining in bulk, because it does not overstain, especially if diluted one-half with distilled water. Large objects will require at least 24 hours of staining. After staining, tissues are washed in water thoroughly to insure the removal of all alum. If a purely nuclear stain is desired, 2 per cent. of glacial acetic acid may be added to the hemalum solution.

Hematoxylin.—For general statement see chap. ii.

48. **Hematoxylin, Conklin's Picro-.**—

Delafield's hematoxylin	1 part
Water	4 parts

Add one drop of Kleinenberg's picro-sulphuric (**25**) to each

cubic-centimeter of the solution. This is a beautiful stain (1 to 3 hours) for embryos which are to be mounted entire.

49. **Hematoxylin, Delafield's.**—See chap. i, reagent **13**.

50. **Hematoxylin, Ehrlich's.**—

Hematoxylin	2 grams
Absolute alcohol	100 c.c
Glacial acetic acid	10 c.c.
Glycerin	100 c.c.
Distilled water	100 c.c.
Ammonia alum	

Mix the glycerin and the water and thoroughly saturate the resulting fluid with the alum. The solution must be exposed to light and air at least 3 weeks to ripen. It is not ready for use until it acquires a deep red color. This solution is an excellent nuclear stain and will keep for years.

51. **Hematoxylin, Heidenhain's Iron.**—See chap. i, reagent **18**, and chap. vi, **iv**. This stain is used chiefly in the study of cell structures such as centrosomes, chromosomes, etc. Tissues are best fixed in some of the sublimate solutions or in acetic alcohol, although it will follow liquid of Flemming or Hermann. Sections should be not over 6 microns thick. The ferric solution must be renewed occasionally as it soon spoils.

52. **Hematoxylin, Weigert's.**—This method together with its modifications is a very important one for the study of the tracts of medullated nerve fibers.

Solution I.

Neutral acetate of copper, saturated aqueous solution	1 part
Distilled water	1 part

Solution II.

Hematoxylin	1 gram
Absolute alcohol	10 c.c.
Lithium carbonate, cold saturated aqueous solution	1 c.c.
Distilled water	90 c.c.

Solution III.

Ferricyanide of potassium	2.5 grams
Borax	2.0 grams
Distilled water	200.0 c.c.

Harden the nervous tissue in Müller's (8) or Erlicki's (7) fluid and without previous washing continue the hardening in alcohol. The hardening complete, imbed in celloidin and place the celloidin block into Sol. I for 36 to 48 hours. Place it next into 70 to 80 per cent. alcohol for 24 hours; the block may be left in such alcohol indefinitely. Cut sections in the usual way (not over 20 to 25 microns) and stain them in Sol. II for 24 hours at room temperature, and then for a few hours in a warm chamber at 40° C. Wash the sections in water and differentiate (a few minutes) in Sol. III until the gray matter (ganglion cells, etc.) of the tissue becomes yellow. The medullary sheath remains dark. Rinse in water, dehydrate, clear in carbol-xylol, and mount in balsam. This method may be used also for demonstrating degenerated fibers; they remain unstained.

53. **Light Green** (Lichtgrün S. F.).—This is a beautiful cytoplasmic anilin stain which is frequently used after safranin as a counterstain. Not more than 0.5 per cent. solution should be used as it stains very rapidly and very deeply. It may be used either as an aqueous or as an alcoholic solution. The writer has found a 0.5 per cent. solution in 95 per cent. alcohol very satisfactory. Sections should remain in it only a few seconds.

54. **Lyons Blue** (Bleu de Lyon).—This is one of the best of the numerous anilin blues. It is a good contrast stain when used after such nuclear stains as safranin and carmine. See chap. i, reagent 16.

Mallory's Connective Tissue Stain.—See 30.

Magenta, Acid.—See 42.

55. **Methylen Blue.**—This reagent is an extremely useful one; it is of great value in the study of the nervous system, and it can be made to give results with intercellular cement substance, lymph spaces, etc., as satisfactory and with greater certainty than impregnations obtained with gold chloride or silver nitrate. It is also serviceable as an *intra vitam* stain. Furthermore, methylen blue (saturated solution in 70 per cent. alcohol) followed by eosin is sometimes used for the double staining of blood corpuscles. Methylen blue should not be confounded with *methyl blue.*

Ordinary commercial methylen blue usually contains, in addition to the blue dye, a small quantity of a reddish-violet dye. Such methylen blue is termed *polychromatic* and is especially serviceable in staining certain cell granules. Only the pure methylen blue, however, should be used for nerve staining and other *intra vitam* work.

a) **Intra Vitam Stain for Small, Comparatively Transparent Aquatic Organisms.**—Add sufficient methylen blue to the water containing the organisms to tinge it a light blue. Different tissues will take up the color after different intervals of time, and a given tissue after having attained a maximum degree of coloration will rapidly lose its color again. It is necessary, therefore, to watch the organisms closely for the maximum of color in the tissue desired. If the observer wishes, the stain may be fixed for more prolonged study by following the processes indicated under *b*). The order in which various tissues take the stain seems to vary in different organisms. Usually gland cells stain first, then with more or less deviation, other epithelial cells, fat cells, blood and lymph cells, elastic fibers, smooth muscle, and striated muscle. Nerve cells and nerve fibers do not ordinarily take the stain when the entire animal is immersed.

b) **Ehrlich's Method for Nerve-Terminations and the Relations of Nerve Cells and Fibers to the Central Nervous System.**—The stain should be Grübler's methylen blue (rectificiert nach Ehrlich). A 1 per cent. solution in normal saline is used Warm the solution till it steams, stir it thoroughly and when cool, filter. The tissue must be perfectly fresh. Chloroform the animal and immediately inject the stain into the main artery of the part to be investigated. If the animal is small, the entire body may be injected. The vessels should be filled full but care must be taken not to rupture them. The part should become decidedly blue in color. It is well after 10 or 15 minutes to inject more stain. At the expiration of half an hour after the second injection remove small pieces of tissue containing the nerve elements desired, and *expose them freely to the air* on a slide wet with normal saline. Examine every two minutes under the microscope (without cover-glass) until

the particular element to be investigated (cell, axone, termination) has developed a well-marked blue color. It is important to catch the color at the proper stage and fix it because it soon begins to fade.

Fixing the Stain.—When the desired element has developed a satisfactory blue color, the tissue is transferred immediately to a saturated aqueous solution of *Ammonium picrate* (Dogiel's method) and left for from 6 to 24 hours. For final mounting the tissue should be teased out sufficiently to show the proper elements and then mounted in a few drops of a mixture of pure glycerin (free from acid) and ammonium picrate (saturated aqueous solution), equal parts. It is well to let the tissue stand in 20 to 30 volumes of this glycerin-picrate mixture for a day or two before mounting it. If the preparation is to be kept the cover-glass should be sealed (chap. xiii, **II,** A, 6).

Sections.—If it is desired to make paraffin sections and mount them in balsam, after treatment with the ammonium picrate (10 to 15 minutes), the tissue must be placed into 20 or 30 volumes of Bethe's fluid, which renders the color insoluble in alcohol.

Bethe's fluid.—

Molybdate of ammonia	1 gram
Chromic acid, 2 per cent. aqueous solution .	10 c.c.
Hydrochloric acid, concentrated C. P. . . .	1 drop
Distilled water	10 c.c.

The tissue is left in this mixture for from 45 to 60 minutes (for small objects) and then washed 1 to 2 hours in distilled water. Dehydrate directly in absolute alcohol; follow this with xylol, imbed in paraffin, and section in the ordinary manner. Sections may be counterstained in alum-carmine or alum-cochineal.

c) **Immersion Method.**—Material which cannot be readily injected or which has failed to stain may be stained by immersion. A 0.1 per cent. solution of the stain is used (dilute 1 volume of the solution used for injection with 9 volumes of normal saline). To small pieces (2 to 3 mm. thick) of the tissue, add a few drops of the stain at intervals of about three minutes. The tissue should always be moist, but never covered sufficiently by the solution to exclude air. Examine the preparation from time to

time under the microscope and when the nerve elements are well stained, fix in ammonium picrate and proceed as in *b*). In case of the central nervous system, fairly good results may sometimes be obtained by dusting the methylen blue powder over the freshly cut surface of the part to be studied. The development and fixing of the color is the same as in *b*).

d) **Nissl's Method of Staining Basophil (Tigroid) Substance in Nerve Cells.**—

Methylen blue	3.75 grams
Venetian soap (white castile soap) . .	1.75 grams
Water	1,000.00 c.c

It is best to keep the stain for some months before using.

Ganglia should be fixed in alcohol, formalin or corrosive sublimate and sectioned in paraffin. Fix the sections to the slide, dissolve out the paraffin with xylol, and run the preparation down to the aqueous stain in the ordinary way. In a test-tube heat a few cubic centimeters of the stain until it steams, then apply it while still warm to the sections on the slide, which has been placed flat on the desk. It takes about 6 minutes for the stain to act. Pour off the surplus stain and rinse the slide in distilled water. Lay it flat on the desk again and flood the sections with *anilin-alcohol* (95 per cent. alcohol, 9 parts; anilin oil, 1 part). Let the sections decolorize (20 to 30 seconds) until they are a pale blue; then drain off the anilin-alcohol and transfer the preparation to absolute alcohol. Clear in xylol and mount in balsam. The basophil granules should appear deep blue in color. They are arranged for the most part concentrically around the nucleus.

e) **Unna's Method of Staining Unstriated Muscle in Sections.**—Stain in a 1 per cent. aqueous solution of polychromatic methylen blue, rinse in water and then leave for 10 minutes in a 1 per cent. aqueous solution of potassium ferricyanide. Transfer to acid alcohol until sufficiently decolorized, then complete the dehydration and mount in the usual way.

f) **For Ordinary Section Staining** where a nuclear stain is desired, methylen blue answers very well. It is usually used (2 to 24 hours) in aqueous solution. The treatment is the same as for safranin.

g) **Impregnation of Epithelia, etc.**—Place the fresh tissue, preferably a thin membrane, into a 4 per cent. solution of methylen blue in normal saline. To demonstrate the outline of cells, leave the tissue in the stain not longer than 10 minutes. To get a negative image of lymph spaces, canals, etc., in contrast to the ground substance which becomes deeply impregnated, leave the tissue in the stain 20 to 30 minutes. For this purpose it is advisable to remove any membranous covering which invests the organ. In either case, after staining, fix the tissue for 30 to 40 minutes in a saturated aqueous solution of ammonium picrate, changing it once or twice, and examine in dilute glycerin. To preserve the preparation permanently, proceed as in *b*). To do away with the macerating action of the ammonium picrate, add 2 per cent. of a 1 per cent. osmic-acid solution to the fixing bath.

56. **Methyl Green.**—This is one of the best of the nuclear anilin stains. It is particularly valuable because it instantly stains the chromatin of nuclei in fresh tissues. Use in strong aqueous solutions, acidulated to about 1 per cent. with acetic acid. It does not give a satisfactory chromatin stain if the tissue has been fixed in acetic acid or mixtures containing it. It follows pure corrosive sublimate solution admirably.

57. **Methyl Violet.**—This stain is commonly used in 0.5 to 2 per cent. aqueous solutions for staining bacteria, nuclei, and amyloid. It may often be substituted for gentian violet.

58. **Neutral Red.**—Neutral red is used widely as an *intra vitam* stain. It is a good stain for cytoplasmic granules, and in some cases for mucus cells. For *intra vitam* staining it may be used in the same way as methylen blue (with the omission of fixation). For staining fixed material, a 1 per cent. or stronger aqueous solution is employed. Granules are stained orange red (bright red in acid medium, yellow in alkaline medium). Rosin finds that in nerve cells stained in neutral red (followed by water, acid-free alcohols, xylol, and balsam) nucleoli and Nissl's granules are stained red, the rest of the cell yellow.

59. **Orange G.**—This is an excellent cytoplasmic stain and is often used on sections as a contrast to carmine, hematoxylin, and safranin. Grübler's Orange G is the most reliable. It should be

used in saturated aqueous solution. The solution does not keep very well.

60. **Orcein** (Unna's method for elastic fibers).—

Orcein (Grübler's)	1 gram
Hydrochloric acid	1 c.c.
Absolute alcohol	100 c.c.

Sections are stained in a watch-glass or porcelain dish. The dish is warmed over a flame or in an oven until the stain becomes thick through the evaporation of the alcohol. Rinse the stained sections thoroughly in alcohol, clear in xylol and mount in balsam. Elastic fibers should appear dark brown.

61. **Paracarmine** (Mayer's).—

Carminic acid	1.0 gram
Aluminium chloride	0.5 gram
Calcium chloride	4.0 grams
Alcohol, 70 per cent.	100.0 c.c.

Paracarmine is an excellent stain for large objects. It does not overstain ordinarily. The stained tissue is washed in 70 per cent. alcohol. In case overstaining occurs add 2.5 per cent glacial acetic acid or 0.5 per cent. aluminium chloride to the alcohol used for washing. Objects to be stained should not have an alkaline reaction nor contain limy materials.

62. **Picric Acid.**—Picric acid is widely used as a contrast stain with carmine, hematoxylin, etc. It is best manipulated as a stain by adding a little to each of the alcohols used in dehydrating, after application of the nuclear stain. However, if acid alcohol is to be used, the picric acid should be used only in the grades above the acid alcohol. It may be employed in staining entire objects as well as sections. See also remarks on washing under 21.

63. **Picro-Carmine.**—This is a very useful double stain. Tissues stained in it should never be washed in water or a low percentage of alcohol because these extract the yellow color very rapidly. If the tissues are to be mounted in glycerin or glycerin jelly, transfer them to the mounting fluid without washing; if they are to be mounted in balsam, to prevent the extraction of the stain, picric acid should be added to each of the alcohols through which the material will pass. Picro-carmine solutions are likely to dissolve off sections which have been affixed by means of albumen

fixative. There are various formulae for making the stain. One of the oldest and at the same time one of the best is the formula of Ranvier.

Ranvier's formula.—A saturated solution of carmine in ammonia is added to a saturated aqueous solution of picric acid to the point of saturation (that is, until precipitation begins). The mixture is next evaporated down to one-fifth of its volume and filtered after cooling. The solution is then evaporated until only a powder remains. A 1 per cent. solution of this powder in distilled water is used for staining. It is well to allow the stain to act for from 12 to 24 hours. If the material is to be mounted in glycerin, it should first be treated (by irrigation under the cover-glass) with formic-glycerin (formic acid 1 part, glycerin 100 parts) for several days until proper differentiation has taken place. If osmic acid has been used for fixing, nuclei should appear red, muscle tissue straw yellow, elastic fibres canary yellow, connective tissue pink, and keratohyalin red.

64. **Pyrogallol.**—Tissues which have been fixed in Hermann's or in Flemming's fluid for 24 to 36 hours may be treated (without previous washing) with a weak solution of pyrogallol or with crude pyroligneus acid. Lee (*Microtomist's Vade-Mecum*) recommends the pyrogallol as much preferable. Tissues should remain in the fluid from 1 to 24 hours depending upon size. The result is a black stain which colors both nucleus and cytoplasm. If desired, an additional chromatin stain may be employed. Safranin (**65**) for 24 hours is recommended; decolorize slightly with very dilute acid alcohol. The stain is excellent for cytological work (for "sphere," etc.).

65. **Safranin.**—Safranin is one of the most important of the basic anilin dyes. Read carefully the remarks on anilin stains under **29**.

Safranin	1 gram
Anilin water (see **29**)	90 c.c.
Alcohol, 95 per cent.	10 c.c.

Filter before using. Grübler's "Safranin O" is the most reliable dye. Sections of tissues fixed in Hermann's or Flemming's

solution are left in the stains for from 24 to 48 hours. Decolorize as directed under 29.

66. **Safranin and Gentian Violet.**—This is a combination that is almost indispensable in the study of cell problems, especially spermatogenesis. For formulae of stains see 44 and 65. Tissues are best fixed in Flemming's or Hermann's solutions. Stain thin sections for 36 to 48 hours in the safranin; differentiate in alcohol very slightly acidulated (see 29), then stain for 5 to 10 minutes in the gentian solution and transfer the sections to Gram's solution (see under 44) for 1 to 3 hours. Finally differentiate in absolute alcohol. As soon as purple clouds have ceased to come from the sections in absolute alcohol, they should be transferred to clove oil for a few minutes and thence to xylol. The clove oil seems to intensify the safranin in the chromatic granules, but too prolonged an immersion in clove oil extracts the gentian violet.

67. **Silver Nitrate.**—The nitrate-of-silver method is used largely as an impregnation method for work on nerve tissue and for demonstrating intercellular substances and outlining boundaries of cells in the epithelial coverings of membranes, etc. Wash the fresh tissue in distilled water, then place it for 2 to 5 minutes in 0.5 to 1 per cent. aqueous solution of silver nitrate. Rinse in distilled water, then expose the tissue to bright sunlight in water or glycerin (or in 70 per cent. alcohol, if it be mounted in balsam) until a brown coloration appears. Temporary mounts should be made in glycerin. For application to nerve see chap. ix.

68. **Sudan III.**—This is a specific stain for fat. A saturated alcoholic solution is used (5 to 10 minutes). Wash rapidly in alcohol. Since alcohol is a solvent of fat, too long an immersion will destroy the preparation. Mount in glycerin. With this dye large fat drops stain orange, small ones yellow. The tissue should have been fixed previously in Müllers fluid (8) or other medium which does not dissolve fat.

Van Giesen's Stain.—See 43.

69. **Wright's Stain** (for blood and for the malarial parasite).—See chap. xiv, memoranda 5 and 6.

III. NORMAL OR INDIFFERENT FLUIDS

(For fresh tissues)

70. **Aqueous Humor.**—Obtained by puncturing the cornea of a freshly excised beef's eye. A small amount may readily be obtained by means of a capillary pipette from the eye of a freshly killed frog.

71. **Blood Serum.**—Blood is allowed to clot and after 24 hours the serum is poured off. If necessary it may be further freed of blood cells by means of a centrifuge. The serum will keep for only a day or two. *Schultze's iodized serum* made by saturating blood serum with iodine is sometimes classed as an indifferent fluid, but it is really a dissociating fluid.

72. **Fluid of Ripart and Petit.**—

Camphorated water	75.0 c.c.
Acetate of copper	0.3 gram
Chloride of copper	0.3 gram
Distilled water	75.0 c.c.
Glacial acetic acid	1.0 c.c.

After the solution becomes clear (a few hours) it should be filtered. It is especially useful for examining fresh animal cells. Methyl green is an excellent stain to follow this fixing fluid.

73. **Kronecker's Fluid.**—

Distilled water	100.00 c.c.
Sodium chloride	0.60 gram
Sodium carbonate	0.06 gram

74. **Normal Saline.**—A 0.7 per cent. solution of sodium chloride in distilled water.

IV. DISSOCIATING FLUIDS

75. **Bichromate of Potassium.**—A 0.2 per cent. aqueous solution is commonly used. Nerve cells of the spinal cord and also various epithelia dissociate well in it (2 to 3 days).

76. **Caustic Potash.**—A solution of 35 parts in 100 parts of water is often used for isolating fibers of smooth muscle or heart fibers. It acts by rapidly destroying the connective tissue (20 to 30 minutes). Examination of the tissue is made by mounting it in the dissociating fluid. If water is added the tissue will be destroyed. Usually only temporary preparations are made in

this fluid, but tissues may be made permanent by neutralizing the alkali by means of acetic acid.

77. **Gage's Formaldehyde Dissociator.**—See chap. i, reagent **10.** For method of using see chap. x, A.

78. **Hertwig's Macerating Fluid.**—See chap. x, C.

79. **Landois' Solution.**—

Neutral ammonium chromate, saturated solution	5 grams
Potassium phosphate, saturated solution	5 grams
Sodium sulphate, saturated solution	5 grams
Distilled water	100 c.c.

This solution is valuable for the central nervous system. Small pieces of tissue are placed in the fluid for 1 to 5 days. For staining after maceration, it is recommended that the material be placed for 24 hours in ammonia carmine diluted with one volume of the macerating fluid.

80. **MacCallum's Macerating Fluid.**—

Nitric acid	1 part
Glycerin	2 parts
Water	2 parts

This fluid is recommended for heart muscle of adults or embryos. Hearts should remain in it from 8 hours to 3 days, according to size. The method is valuable for showing the arrangement of cardiac muscle fibers.

81. **Ranvier's One-Third Alcohol.**—This is one of the commonest as well as one of the best macerating fluids. It is simply a 30 per cent. alcohol. Epithelia will macerate in it sufficiently in 24 hours. A still weaker alcohol (20 to 25 per cent.) is used for isolating the nerve fibers of the retina.

82. **Schiefferdecker's Fluid.**—

Methyl alcohol	5 c.c.
Glycerin	50 c.c.
Distilled water	100 c.c.

This fluid is used for the retina and central nervous system. It should be prepared fresh before using and tissues must remain in it for several days.

83. **Sodium Chloride.**—A 10 per cent. solution of sodium chloride is excellent for tendon, etc. It dissolves the cement sub-

stance of epithelial cells and of connective tissue. As a stain, a saturated aqueous solution of picric acid (stain for 24 to 36 hours) followed, after thorough washing in water, by a dilute alcoholic solution of acid fuchsin gives excellent results.

V. DECALCIFYING FLUIDS

84. **Chromic Acid.**—Chromic acid diluted to 1 per cent. or in combination with other fluids is frequently used for decalcification. Chromic acid, 1 gram; water, 200 c.c.; nitric acid, 2 c.c., is a mixture widely used. It decalcifies well but acts more slowly than the 10 per cent. nitric acid mixture. Bone should first be hardened in Müller's fluid (8).

85. **Nitric Acid.**—A 10 per cent. solution of nitric acid in 70 per cent. alcohol may be used. If nitric acid is used for young or foetal bones, it is advisable to use only 1 part of the acid to 99 parts of the alcohol. After washing out in 70 per cent. alcohol, the decalcified bone may be kept in 95 per cent. alcohol.

86. **Phloroglucin Method.**—This is a rapid method. Young bones may be decalcified in half an hour and old and hard ones in a few hours. Teeth require a somewhat longer time. Phloroglucin itself does not decalcify, but protects the tissue from the action of the strong nitric acid. One gram of phloroglucin is dissolved in 10 c.c. of pure non-fuming nitric acid with the aid of gentle heat. Ten c.c. of nitric acid in 100 c.c. of water is added to the mixture.

87. **Picric Acid.**—A solution kept fully saturated is useful for delicate bones. It stains and decalcifies the tissue at the same time. Wash in 70 per cent. alcohol.

88. **Von Ebner's Fluid.**—

Alcohol, 95 per cent.	500.0 c.c.
Water	100.0 c.c.
Sodium chloride	2.5 grams
Hydrochloric acid	5.5 c.c.

This is an excellent fluid for bone because in it the ground substance of the bone does not swell up. Sections are best examined in a 10 per cent. solution of sodium chloride.

APPENDIX C

TABLE OF TISSUES AND ORGANS WITH METHODS OF PREPARATION

Numbers refer to reagents described in Appendix B. Unless otherwise directed, preparations are to be dehydrated and mounted in balsam. Abbreviations: do. is used to indicate that the same reagent or process is to be used as the one immediately above; p. c.=per cent.; chap.=chapter; mem.=memorandum; sat.=saturated; sol.=solution; alc.=alcohol; aq =aqueous; g.=gram.

Object or Element	*Animal, Organ, or Tissue Recommended for Demonstration*	*Fixing and Hardening, or Other Preliminary Treatment*	*Section or Isolation Method. P.=Paraffin; C.=Celloidin; F.=Freezing; H.=Free Hand*	*Staining, etc.*	*General Remarks*
I. BLOOD AND BLOOD-FORMING ORGANS.					
Blood, cover-glass preparation........	Frog Pigeon Man	Dry		Wright's stain, chap. xiv, mem. 5	See also chap. xiv, **II**
		Corrosive sublimate (13) for 15 minutes		Hematoxylin, eosin (49, 40), or Ehrlich (39)	The preparation should be washed with water or alcohol before staining
Crystals............					See chap. xiv, **Ie**
Erythrocytes (red corpuscles)........					Technique same as for cover-glass preparation
Fibrin.					See chap. xiv, **I***d*
Fresh blood.........	Frog; pigeon; man	Examine fresh			See chap. xiv, **I***b* for effects of reagents, etc., on blood
Granules, acidophil (eosinophil, oxyphil)...............	Leucocytes of man	Ether-alcohol (16) 1-2 hours		Ehrlich's hematoxylin (50). To every 100 c.c. add 0.5 g. eosin. Time 2-24 hours	
Granules, amphophil (indulinophil)	Leucocytes of guinea-pig, rabbit or pigeon. Not in man	Do.		Equal parts, sat. glycerin sol. of indulin, naphthylamin yellow, and eosin. Stain 4-6 hours	Rinse with water, blot, dry and mount in balsam. Nuclei, red; amph. granules, black; acido. granules, red; hemoglobin, yellow
Granules, basophil.	Mononuclear leucocytes of man	Do.		Sat. aq. sol. of methylen blue (55 f.)	Rinse in water, blot, dry, and mount in balsam. Baso. gran., blue

Granules, mast cells	Small numbers in normal tissues and normal blood. Large numbers in leukemic blood	Do., for blood. For tissues use strong alcohol (3)	P. Thin sections. (Mucous memb. of mouth, intestine, etc.)	Stain for 24 hours in alum-carmine dahlia (dahlia, 1 g.; abs. alc., 25 c.c.; pure glycerin, 12 c.c.; glacial acetic, 5 c.c. Into this mixture pour 25 c.c. of alum-carmine; see 28)	Differentiate in abs. alc. for 24 hours and finally mount in balsam. Nuclei, red; mast granules dark blue
Granules, neutrophil	Polynuclear leucocytes of man, some transitional cells, pus cells, and myelocytes	Ether-alcohol (16) 1–2 hours		Ehrlich's triple (39)	Rinse in water, blot, dry, and mount in balsam. Nuclei, green; neut. granules, violet; acido. granules, brownish-red
Leucocytes (white corpuscles)........					Technique same as for cover-glass preparation. See also chap. xiv, **II**
Lymph glands......	Mesenteric glands of kitten or dog	Acetic alcohol (2*a*)	P. or C.	Hematoxylin, eosin (49, 40)	Longitudinal sections passing through the hilum are the best
Malarial parasite...					See chap. xiv, mem. 6
Marrow, red........	Cover-glass preparation from rib of guinea-pig, rat or young rabbit	Ether-alcohol (16) 1–2 hours		Ehrlich-Biondi (38) 24 hours, or hematoxylin, eosin (49, 40)	After 38 rinse quickly in strong alcohol, clear in clove oil followed by xylol, and mount in balsam
Platelets...........					See chap. xiv, **Ic**
Spleen, elements of	Scrape the cut surface of a fresh spleen				Examine in normal saline (74)
Spleen, section.....	Cat	Gilson (15) or Zenker (6)	P. or C.	Hematoxylin, eosin (49, 40) or better, hematoxylin, and Van Gieson's (49, 43)	Fix the spleen entire and later cut out segments of the proper size for sectioning
Thymus............	New-born infant	Acetic alcohol (2*a*)	P. or C.	Hematoxylin, eosin (49, 40)	
II. CELLS.					
Accessory chromosome............	Testis of cricket or grasshopper	Flemming (11)	P.	Iron-hematoxylin (51)	
Amitosis..........	Connective tissue cells of salamander larva, testis of snail, or ovarian follicular epithelium of cockroach	Gilson (15)	P. Thin sections	Iron-hematoxylin, Bordeaux red (51, 33)	Amitosis is not of very common occurrence

TABLE OF TISSUES AND ORGANS WITH METHODS OF PREPARATION (Continued)

Object or Element	*Animal, Organ, or Tissue Recommended for Demonstration*	*Fixing and Hardening, or Other Preliminary Treatment*	*Section or Isolation Method. P.=Paraffin; C.=Celloidin; F.=Freezing; H.=Free Hand*	*Staining, etc.*	*General Remarks*
Bioblasts (granula) **of Altmann**.........	Various organs	Fix for 24 hours in potassium bichromate (5 p. c. aq. sol.) and osmic acid (2 p. c. aq. sol.), equal parts	P. Very thin (1-2 μ) sections	20 g. fuchsin S (42) in 100 c.c. of anilin water (29); pour on to slide and heat over flame till stain begins to steam. Wash with picric acid (concentrated alc. sol. plus 2 vols. water); add fresh picric and heat for 1 min. over flame	Mount in melted Canada balsam free from xylol. Granula appear red in yellowish field. It is questionable if they should be regarded as vital structures
Foam structure (Bütschli).........	Blood cells or intestinal epithelium of frog Young ovarian egg of teleosts	Picric acid (21)	P. Very thin (1 μ) sections	Iron-hematoxylin (51)	It is best to clear sections in oil of bergamot or other clearer having a low index of refraction
Heterotypical mitosis..................	Testis of salamander	Flemming (11)	P.	Safranin, light green (65, 53)	
Gland cells of Chironomous larva.....	Pull off the larva's head; the salivary glands (two small transparent bodies) remain attached	Examine in the blood of the larva		Methyl green (56)	The cells possess very large nuclei and nucleoli. The glands may also be fixed in Flemming (11), sectioned in paraffin and stained in Ehrlich-Biondi (38)
Living or fresh cells	Protozoa and blood corpuscles. Ovarian ova (teased) Scrapings from roof of frog's mouth	Examine protozoa in water; the others in normal saline (74), or better, in fluid of Ripart and Petit (70)		For *intra vitam* staining of protozoa, etc., see 31, 55*a*, or 58	For making permanent preparations of protozoa, see Appendix D, 1*c*. See also Appendix B, 19*a*

Mitosis; method 1.	Epithelia of amphibian larvae. Ascaris ova (chap. xvi, mem. 11). Blastodisc of teleost ova (e. g., Coregonus)	Picro-acetic (23) or Gilson (15)	P. Thin sections	Iron-hematoxylin, acid fuchsin (51, 42)	
Mitosis; method 2.	Do.	Hermann (26) or Flemming (11)	Do.	Safranin, gentian violet (66)	
Pigment cells......	Pigment layer of the eye	Formalin (17) preserved material		Carmalum (34)	Dehydrate, spread flat and mount in balsam
Protoplasmic currents..............	Plasmodia of Myxomycetes Rhizopoda. Stamens of Tradescantia				Examine in water under a supported cover-glass
Reduction division.	Testis of an animal during the breeding season (e. g., salamander, rat, squash-bug)	Hermann (26)	P. Thin sections	Safranin, gentian violet (66)	Gilson (15) followed by iron-hematoxylin (51) often gives excellent results. See also chap. xvi, mem. 11 for Ascaris
III. CIRCULATORY SYSTEM.					
Aorta..............	Man; dog; cat	Absolute alcohol (3) or Gilson (15)	C.	Hematoxylin, acid fuchsin (49, 42)	Make both transverse and longitudinal sections
Artery (medium size)...............	Do.	Absolute alcohol (3) or Zenker (6)	C.	Do.	Do.
Capillaries and small vessels.......	Pieces of pia mater from base of brain	Zenker (6) for 1 hour	Teased preparation	Carmalum (34)	Mount in glycerin or dehydrate and mount in balsam. For distribution of capillaries see injection method, chap. xii
Elastic elements...	Aorta, artery, etc.	Absolute alcohol (3)	C.	Orcein (60)	
Endothelium of blood vessels.......	Vessels from the mesentery			Silver nitrate (67) 1 p. c. solution injected into the blood vessels	Capillaries may be examined entire; larger vessels should be slit open and spread out
Heart..............	Dog; cat; man	Absolute alcohol (3) or Zenker (6)	C.	Hematoxylin, acid fuchsin (49, 42), or borax-carmine, Lyons blue (32, 54)	For muscle fibers, see under "Muscular Tissues," IX
Lymph capillaries..					Injection by puncture, see chap. xii, mem. 4

TABLE OF TISSUES AND ORGANS WITH METHODS OF PREPARATION (Continued)

Object or Element	*Animal, Organ, or Tissue Recommended for Demonstration*	*Fixing and Hardening, or Other Preliminary Treatment*	*Section or Isolation Method. P.=Paraffin; C.=Celloidin; F.=Freezing; H.=Free Hand*	*Staining, etc.*	*General Remarks*
Purkinje fibers	Heart of a sheep	MacCallum (80) until macerated	Teased preparation	Methylen blue (55f.)	Mount in glycerin
Spleen, blood vessels of		Alcohol (3)	C.	Hemalum (47)	First inject the animal with a carmine mass (chap. xii). For elements of spleen, see "spleen" under I
Valves of heart		Absolute alcohol (3) or Zenker (6)	C.	Hematoxylin, acid fuchsin (49, 42)	Longitudinal sections
Vein	Man; cat; dog	Do.	C.	Do.	
IV. CONNECTIVE OR SUPPORTING TISSUES.					
Adipose tissue	Subcutaneous tissue	Gilson (15) or Zenker (6)	C.	Hematoxylin, eosin (49, 40)	Clusters of empty fat envelopes may be observed, the fat itself having been withdrawn by the alcohol
Areolar tissue	Strips of intermuscular connective tissue. Subcutaneous tissue		Spread out the film with needles	Picro-carmine (63)	Mount in glycerin or examine in normal saline without staining
Bone corpuscles and their processes		Fix in a mixture of Müller (8) 9 parts and formalin (17) 1 part. Decalcify (see next)	C.	Stain for 10-15 min. in: thionin (50 p.c. alc. sol.) 1 part; carbolic acid (1 p.c. aq. sol.) 9 parts. Rinse in water and stain 2-3 min. in sat. aq. sol. of picric acid (62)	Sections are brought from water into the stain. Cells, reddish-violet; ground substance, yellow; bone corpuscles and processes, dark brown
Bone, decalcified		Fix as above and decalcify in Von Ebner's fluid (88) or nitric acid	C. or F.	Picro-carmine (63)	See also chap. **xi**
Bone, development of (endochondral)	Extremities of fetal pigs, cats or human fetuses	Zenker (6) or Gilson (15)	C.	Borax-carmine, Lyons blue (32, 54)	In advanced fetuses the bone should be decalcified (see 85)

Bone, development of (intra-membranous)	Parietal, frontal, or lower jaw of embryos	Do.	C.	Do.	
Bone, fibers of Sharpey	Vault of a fowl's skull	Decalcify in Von Ebner's fluid (88)	H. Thick sections		Tease lamellae apart; fine tapering fibers and apertures from which other fibers have been withdrawn should be visible
Bone, Haversian canals and lamellae					See method for grinding bone, chap. xi
Bone, isolation of corpuscles	Thin fragments of bone	Strong nitric acid for several hours			Cover on a slide and drum upon the cover-glass with the handle of a dissecting needle
Bone, isolation of lamellae		Fix in a mixture of Müller (8) 9 parts and formalin (17) 1 part. Decalcify in Von Ebner's fluid (88)	Teased preparation		Examine in 10 p. c. aq. sol. of sodium chloride
Cartilage (in general)		Zenker (6) or corrosive sublimate (13)	H. or C.	Hematoxylin, picric acid (49, 62)	Thin sections of costal cartilage may be cut and examined readily without any previous preparation. Finer details of structure vanish in xylol-balsam mounts
Cartilage, capsule of	Small pieces	Fresh		Treat with 1 p.c. aq. sol. of gold chloride (45)	
Cartilage, connective tissue and elastic fibers in		Fresh, or corrosive sublimate (13)	H.	Picro-carmine (63)	Examine in glycerin. Elastic fibers, yellow; connective tissue fibers, pink
Cartilage, elastic (yellow fibro-)	Epiglottis; vocal process of Arytenoid cartilage (man, beef); external ear (man, rabbit)	Zenker (6) or corrosive sublimate (13)	H. or C.	Hematoxylin, eosin (49, 40)	Thin sections of fresh cartilage should be examined in normal saline (74)
Cartilage, glycogen in		Fresh	H.	Lugol's iodo-iodide of potassium (see Gram's solution, 44)	Glycogen, if present, is stained a mahogony brown; elastic fibers are stained a different shade of brown

TABLE OF TISSUES AND ORGANS WITH METHODS OF PREPARATION (Continued)

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Cartilage, hyaline	Head of femur; costal cartilage; ensiform process of frog	Zenker (6) or corrosive sublimate (13)	H. or C.	Hematoxylin, picric acid (49, 62)	Thin sections of the fresh cartilage also should be examined in normal saline (74)
Cartilage, white fibro-	Intervertebral disks	Do.	H. or C.	Do.	Do.
Cells of fibrillar connective tissue	Intermuscular connective tissue		Spread out the film with needles	Picro-carmine (63)	
Connective tissue (general)	Sections of any organ	Zenker (6) or corrosive sublimate (13)	C. or P.	Mallory (30), or picro-carmine (63), or Calleja (36)	
Elastic fibers (fine)	Intermuscular or subcutaneous connective tissue	Irrigate with acetic acid	Tease		The white fibrous tissue swells; the elastic fibers remain unaltered and stand out distinctly
Elastic fibers (coarse)	Ligamentum nuchae of a beef	Fresh	Tease	Picro-carmine (63)	Tease in normal saline (74); mount in glycerin
Encircling fibers	See preparation for fine elastic fibers				The constrictions seen at intervals along the swollen connective tissue bundles are caused by encircling fibers
Fat cells	Fatty areas of mesentery	Müller (8)	H.	Sudan III (68)	Do not subject to any treatment with alcohol except as directed under the stain. Osmic acid (0.5 to 1 p. c. sol.) is also used as a test for fat; it turns fat black
Fenestrated membrane	Basilar artery, cut open lengthwise; endocardium of sheep's heart	Strong caustic potash solution, 6 hours	Scrape away outer coats	Acid fuchsin (42)	Wash well in water before staining

Fibrillar (white fibrous) connective tissue	Tendon from tail of rat or mouse; intermuscular connective tissue	Fresh	Tease		Examine in normal saline (74). See also "tendon"
Jelly of Wharton	Umbilical cord of young (2-3 months) human embryo, or of a 3-5 cm. pig embryo	Zenker (6) or Gilson (15)	H. or P.	Hematoxylin, eosin (49, 40)	
Ligament	Ligamentum teres	Zenker (6)	H. or C.	Hematoxylin, acid fuchsin (49, 42)	Cross and longitudinal sections
Ligamentum nuchae	Beef	Zenker (6)	Do.	Do.	Do.
Mucoid connective tissue	See "jelly of Wharton"				
Reticular (adenoid) connective tissue	Spleen or lymph gland	Fresh	F. or H. Thin sections		Shake the sections in a test-tube containing a few c.c. of water in order to remove lymphocytes. Examine unstained or stain with acid fuchsin (42)
Synovial villi	From capsular ligament near border of patella	Fresh			With low power and without a cover-glass, examine inner surface of a strip in normal saline (74)
Tendon		Dry	H.	Methylen blue (55 f.)	Cross-section. See also "fibrillar connective tissue"
Tendon cells	Tendon from tail of rat or mouse	Fresh	Tease	Picro-carmine	Examine in glycerin
Tendon to muscle	Tendo-Achillis of frog	Zenker (6) or Gilson (15)	C.	Stain in bulk in borax-carmine (32) and after sectioning, in picric acid (62)	Longitudinal sections
V. DIGESTIVE ORGANS.					
Blood vessels of	Cat or dog				Carmine injection method, chap. xii
Cells of Paneth	Ileum of man or rodents. Not present in carnivora	A mixture of potassium bichromate (3.5 p. c. aq. sol.) 80 c.c.; strong formalin 20 c.c. After 24 hours transfer to 3.5 p. c. potassium bichromate sol. for 3 days	P. Thin sections	Iron-hematoxylin (51)	

TABLE OF TISSUES AND ORGANS WITH METHODS OF PREPARATION (Continued)

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Crescents of Gianuzzi (demilunes of Heidenhain)	Submaxillary gland (man, dog)	Zenker (6) or Flemming (11)	P.	Hematoxylin, eosin (49, 40)	
Duodenum	Dog	Zenker (6) or Gilson (15)	P. or C.	Do.	
Epithelium of mouth	Man or dog	Do.	P.	Do.	For isolation see 77, 75, or 81
Epithelium of stomach.	Do.	Do.	P.	Do.	Do.
Epithelium of small intestine and villi	Dog or cat	Do.	P.	Do.	Do.
Esophagus	Man or dog	Acetic alcohol (2)	P. or C.	Do.	
Gall bladder	Do.	Zenker (6)	C.	Do.	The tissue must be perfectly fresh, otherwise the alkaline bile renders it unfit for microscopical examination
Gall duct	Do.	Do.	C.	Do.	Do.
Gastric glands (sections)	Stomach of cat or dog, mucous membrane only	Acetic alcohol (2)	P.	Stain in dilute hematoxylin (49) 3-5 min.; wash in water; 0.03 p. c. aq. sol. Congo red, 3-6 minutes	The animal should have fasted for 1 or 2 days. Chief cells pale blue, parietal cells red
Gastric glands (fresh)	Rabbit	Fresh. Dissect off mucous membrane and tease		Picro-carmine (63)	Tease in 0.5 p. c. solution of sodium chloride
Goblet cells	Large intestine	Corrosive sublimate (13)	P.	Mallory (30)	
Granules of salivary glands and pancreas					Technique same as for "cells of Paneth"
Intestinal absorption of fat	Frog; rat	Osmic acid, 1 p. c. (20)	P. or tease in normal saline (74)	Safranin (65)	Feed the animal on fat bacon for a couple of days before killing

Large intestine		Zenker (6) or Gilson (15)	P. or C.	Hematoxylin, eosin (49, 40); or Mallory (30)	
Lip	Lower lip of man or dog	Zenker (6) or Tellyesniczky (5)	C.	Hematoxylin, eosin (49, 40)	Vertical sections including both skin and mucous membrane should be made
Liver, amyloid infiltration of		Alcohol (3)	P.	Mallory (30 *a*)	
Liver, blood vessels of	Dog; cat	Do.	C.	Hemalum (47)	First inject with carmine mass, see chap. xii
Liver, bile capillaries	Guinea-pig	Do.	C. or P.		Inject a concentrated aq. sol. of Berlin blue through the bile duct, after clamping the cystic duct. Avoid too great pressure
Liver cells	Man, pig, or dog	Zenker (6) or Gilson (15)	P. Thin sections	Hematoxylin, acid fuchsin (49, 42)	
Liver, hepatic lobules	Pig	Acetic alcohol (2)	P. or C.	Do.	Make sections both parallel and vertical to the surface
Liver, interlobular connective tissue	Do.	Müller (8) or Zenker (6)	F. or H.	Do.	After staining, shake out cells by shaking sections in a test-tube with some water
Mucin	Glands (sections and thin membranes)	Corrosive sublimate (13), 5 p. c. aq. sol. for 2-8 hours	P.	Thionin (2-3 drops of sat. aq. sol. to 5 c.c. of water), 15 minutes	Tissue blue, mucin reddish. See also "goblet cell"
Nerve plexuses (alimentary canal)	Stomach or duodenum of guinea-pig	Macerate in 0.25 p. c. acetic acid	Tease	Gold chloride (45)	
Pancreas	Man or dog	Zenker (6) or Gilson (15)	P.	Hematoxylin, eosin (49, 40). Also stain pieces in borax-carmine and section (32)	The granules have frequently been extracted by water in mounted specimens. To see granules tease fresh pancreas in normal saline (84)
Parotid gland	Do.	Do.	P.	Do.	
Peyer's patches (agminated nodules)	Small intestine at its point of opening into the large intestine (cat or dog)	Do.	P. or C.	Hematoxylin, eosin (49, 40)	
Small intestine	Kitten or puppy	Do.	Do.	Do. Also stain pieces in borax-carmine before sectioning (32)	To prevent the shrinkage of connective tissue of villi away from the epithelium, allow the intestine to cool before fixing

TABLE OF TISSUES AND ORGANS WITH METHODS OF PREPARATION (Continued)

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Stomach, cardiac end	Dog	Acetic alcohol (2)	P. or C.	Hematoxylin, eosin (49, 40), or borax-carmine, Lyons blue (32, 54)	
Stomach, fundus	Do.	Do.	Do.	Do.	
Stomach, pyloric end	Do.	Do.	Do.	Do.	Cut some longitudinal sections to show transition from stomach to intestine
Sublingual glands					Technique same as for "pancreas"
Submaxillary glands					Do.
Taste-buds	Foliate papilla of rabbit (at sides near base of tongue)	Flemming (11)	P. Thin sections	Safranin, gentian-violet (66)	Orient the buds carefully for sectioning. Both longitudinal and cross-sections are instructive
Tongue (general)		Acetic alcohol (2) or Müller (8)		Hematoxylin, eosin (49, 40)	Cross-sections
Tongue, papillae and folliculi linguales	Mucous membrane of upper surface (rabbit, man)	Do.	P. or C.	Do.	For fungiform papillae, tip of tongue. For filiform papillae, middle of tongue. For circumvallate papillae, root of tongue. For folliculi, root of tongue
Tonsil	Cat or rabbit	Acetic alcohol (2) or Tellyesniczky (5)	Do.	Do.	
Tooth, decalcified	Canine tooth of dog, cat, or man				Technique same as for decalcified bone. See also chap. xi
Tooth, development	Sheep or pig embryo	Fix in Tellyesniczky (5); decalcify in 1 p. c. nitric acid (85)	P. or F. Serial sections	Borax-carmine, picric acid (32, 62)	First stage, 7 cm. embryo. Second stage, 10 cm. embryo. Later stages, use new-born kitten
Tooth, enamel prisms					Tease out bits of enamel from teeth prepared as for "odontoblasts"
Tooth, ground					See chap. xi

Tooth, odontoblasts	New-born child or other young animal	Müller (8), 6-8 days	Withdraw pulp and tease a bit of its surface	Picro-carmine (63)	Seen best in teeth which have not cut the gum. Examine in slightly acidulated glycerin
VI. EAR.					
Ceruminous glands.		Alcohol (3)	C.	Iron-hematoxylin (51)	
Cochlea............	Guinea-pig or rabbit. Cut away the lower jaw and reach the inner ear by dissecting through the bulla	Fix in Flemming (11) and decalcify in 2 p. c. chromic acid (10)	C. or P. Cut from base to apex	Do.	In the guinea-pig decalcification is accomplished in about a week. Before fixing make a hole or two in the turns of the cochlea for the reagent to enter; do this under the liquid so that air will not get in
Eustachian tube..		Zenker (6) or Gilson (15)	C.	Hematoxylin, eosin (49, 40)	Sections should be transverse and should include the cartilage
External ear.......		Do.	P. or C.	Do.	
Middle ear.........		Zenker (6)	Do.	Do.	
Nerve fibers and nerve endings of cochlea............	Young fetuses; new-born mouse	Golgi method (46), or methylen blue (55*b*)			
Otoliths...........					Chisel into the sacculus, remove bits of the macula and examine in dilute glycerin
Semicircular canals	Young dog; skate	Flemming (11). Also decalcify in case of dog, as for "cochlea"	P.	Safranin (65)	
VII. EPITHELIAL TISSUES.					
Ciliated epithelium (living)...........	Roof of frog's mouth; gill plate of mussel, clam, or oyster				Scrape the surface with a scalpel and examine the material thus obtained in normal saline (74)
Ciliated columnar epithelium.........	Trachea: epididymus	Gilson (15) or Zenker (6)	P. or C.	Hematoxylin, eosin (49, 40)	
Columnar and glandular epithelium...	Bronchus; intestine; stomach	Do.	Do.	Do.	
Cubical epithelium.	Smaller bronchioles; uriniferous tubules	Do.	P.	Do.	

TABLE OF TISSUES AND ORGANS WITH METHODS OF PREPARATION (Continued)

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Endothelial cells...	Slit open a medium-sized vein or artery from a freshly killed cat or dog	Subject the inner surface to 1 p. c. silver nitrate (67) until opaque (10–15 min.)	To be mounted flat	Expose to sunlight until a brownish-red color is visible, then stain lightly in hematoxylin (49)	The vessel should be pinned out flat, endothelium uppermost, before applying the nitrate. Another method is to inject the solution into the vein or artery
Intercellular bridges	Epidermis of larval salamander	Flemming (11)	P. Thin	Iron-hematoxylin, acid fuchsin (51, 42)	Also mount bits of epidermis flat without sectioning
Isolation of epithelial tissues.	Fresh epithelia	Formaldehyde dissociator (77) or alcohol (81)	Tease, or shake in a vial	Picro-carmine (63)	Examine in the dissociating fluid or in dilute glycerin
Mesothelial cells...	Central tendon of diaphragm, peritoneum, or pericardium	1 p. c. silver nitrate (67) until opaque (10–15 min.)		Expose to sunlight until a brownish-red color is visible, then stain lightly in hematoxylin (49)	Rinse in distilled water before placing in the silver nitrate and again upon removal from it. Mount in glycerin, or dehydrate and mount in balsam
Pigmented epithelium................	Pigment layer of eye	Formalin preserved material (17)	To be mounted flat	Carmalum (34)	
Stratified epithelium................					See "cornea," "skin," "esophagus," etc.
Squamous or pavement epithelium...	Sloughed off epidermis of amphibian	Gilson (15) for 20 min.	To be mounted flat	Hematoxylin (49)	Examine also scrapings from inside your cheek
Terminal bars......	Small pieces of intestine	Corrosive sublimate (13) in normal saline, or Flemming (11)	P. Thin	Iron-hematoxylin (51)	
Transitional epithelium...........	Bladder of frog, guinea-pig, or rabbit	30 p. c. alcohol (81) for 24–36 hours. Distend the bladder with some of the alcohol		Picro-carmine (63). Stain in toto for 24 hours	Scrape off some of the mucous membrane and examine in glycerin

VIII. EYE.					
Blood vessels of eyeball	Injected eye (chap. xii) of albino rabbit or rat	Alcohol (3) or Müller (8)	Bisect into anterior and posterior halves		The halves of the rat's eyeball may be dehydrated and mounted whole in balsam
Choroid	Fresh		Tease		Examine in glycerin. For sections see "eyeball"
Cornea	Cat	Flemming (11)	C. Thin	Iron-hematoxylin (51)	
Corneal corpuscles and nerves		Use the gold chloride method (chap. xi), or the methylen blue method (55*b*)			
Corneal spaces and canaliculi	Fresh eye	Scrape the epithelium from the cornea and then cauterize the latter with stick silver nitrate	Tangential sections with a razor	Place in water for 2–3 days	Spaces and canaliculi show dark upon a light field. See also methylen blue (55*g*)
Eyeball (general)	Bisect the eyeball into anterior and posterior halves	Müller (8) or Flemming (11)	C.	Borax-carmine, picric acid (32, 62)	After fixing, the part desired may be sectioned and mounted. Small eyes may be fixed entire, but the chances are that the retina will wrinkle
Eyelid	Eyelid of an infant	Tellyesniczky (5)	P. or C.	Hematoxylin, eosin (49, 40)	
Harder's glands	Rabbit (median angle of eye)	Zenker (6) or Gilson (15)	P.	Do.	Do not confuse with lachrymal glands
Iris	See "eyeball"				Meridional sections of the anterior half of the eye will show it
Lachrymal gland	Man or rabbit	Zenker (6) or Gilson (15)	P.	Hematoxylin, eosin (49, 40)	In case of the rabbit do not confuse with Harder's glands
Lens	Beef or sheep	Müller (8)	C.	Borax-carmine, picric acid (32, 62)	Make antero-posterior sections, also other sections, at right angles to these
Lens, capsule and epithelium	Do.	Flemming (11)	P.	Hematoxylin, eosin (49, 40)	Fix the lens entire, then peel off the anterior capsule for sectioning
Lens fibers	Do.	0.2 p. c. sol. potassium bichromate for a week, or 30 p. c. alc. for 2–3 days	Tease		Junctional lines of lens fibers will be seen. Peel off lamina and tease

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Membrane of Descemet	Rabbit; fowl	Draw off aqueous humor and inject 1 p. c. silver nitrate (10–15 min.)	Cut out cornea with a razor	Expose under surface to sunlight until it becomes brownish-red	Wash in distilled water and examine in glycerin, or dehydrate and mount in balsam. See also "cornea"
Retina	Both mammal (human if possible) and amphibian. Cone visual cells are particularly well seen in the eyes of fish	Flemming (11). Bisect the eye into anterior and posterior halves. Merkel (12) is also excellent	P.	Hematoxylin, orange G (49, 59)	Some sections should pass through the fovea centralis. Also tease bits of retina from eyes which have been prepared in Müller (8). Try Golgi (46) or methylen blue method (55*b*)
Sclera	See "eyeball"				
Visual purple in rods	Eye of freshly killed frog	Rapidly cut out a segment consisting of a third of the posterior wall of the eyeball. Quickly peel out the retina			Examine in normal saline (74). If the preparation has been made quickly enough, the retina will appear purplish red. Some rods may be green. The color soon fades. Note also the mosaic formed by the ends of rods and cones
IX. MUSCULAR TISSUE.					
Areas of Cohnheim	Man; lingual muscle of rabbit	Flemming (11)	P. Thin	Hematoxylin (49)	
Branched striated fibers	Tongue of frog	Macerate in 20 p. c. nitric acid (2–3 days)	Tease, or shake in a vial of water	Picro-carmine (63)	Examine in glycerin and mount in glycerin jelly, or dehydrate and mount in water
Cardiac muscle	Man; dog; sheep	Corrosive sublimate (13)	P. or C.	Hemalum (47)	Longitudinal and transverse sections
Cardiac muscle, isolated fibers	Adult or embryo	MacCallum (80)			

Ends of striated fibers	Gastrocnemius of frog	Dissociate in 35 p. c. caustic potash for 15 min. (76)			Tease on a slide and examine in the dissociating fluid
Fibrillae in striated muscle	Frog	Macerate in 0.1 p. c. chromic acid (24-36 hours)	Tease in water		Ends of teased fibers show fibrillae. To make a permanent preparation, wash thoroughly, stain in hematoxylin (49), dehydrate
Muscle to tendon	Small muscle with its tendon (e. g., sartorius of frog)	Dissociate in 35 p. c. caustic potash (76) for 15 min.			Tease on a slide and examine in the dissociating fluid
Non-striated fibers (sections)	Bladder; intestine	Flemming (11)	P.	Iron-hematoxylin, acid fuchsin (51, 42)	
Non-striated fibers (isolated)	Intestine, stomach, or bladder of frog	Macerate in 20 p. c. nitric acid (2-3 days)	Tease, or shake in a vial containing water	Picro-carmine (63)	
Purkinje fibers	See "Purkinje fibers" under III				
Sarcolemma	Fresh striated muscle	Cold saturated solution of ammonium carbonate	Tease		Within 5 or 10 minutes the sarcolemma separates from the muscle substance. Examine in normal saline (74)
Striated fibers (fresh)	Frog; mammal; wing (thoracic) muscle of insect	Isolate by teasing in normal saline (74)			One of the thin muscles from the leg of Hydrophilus (a water beetle) is the classic example of insect muscle. Examine without adding fluid; waves of contraction are observable
Striated fibers (fixed)	Do.	Stretch the muscle by extending an extremity; inject 0.5 to 1 c.c. of 1 p. c. osmic acid hypodermically	Tease	Hematoxylin (49). Need not be stained, however	Cut out small pieces of the muscle from the injected area and wash in distilled water before teasing

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X. NERVOUS SYSTEM.					
Axone (axis cylinder)	Small nerve (lay it along a toothpick or small splinter without stretching, and tie it fast)	0.5 p. c. osmic acid (20) for 4 hours; then wash in water 4 hours followed by 90 p. c. alcohol for 24 hours	P. Very thin sections	Sat. aq. sol. of acid fuchsin (42) for 24 hours, followed by absolute alcohol (3 days)	Make longitudinal and transverse sections. In imbedding use paraffin method for delicate objects, only, substitute toluol for chloroform (chap. vi, vii)
Brain cells	Small pieces of brain	Macerate in Landois fluid (79)	Tease	Beale's carmine (35) diluted with 1 volume of the macerating fluid	See also "cerebral cortex," "cerebellar cortex," etc.
Brain sand	Pineal body		Do.		Tease in normal saline (74) and examine under low power
Central nervous system (general topography)	Brain and cord	Müller (8)	C.	Borax-carmine (32), or Beale's carmine (35)	
Cerebral cortex		Müller (8) or Erlicki (7)	P. or C.	Borax-carmine, picric acid (32, 62)	Also try Golgi method (46)
Cerebellar cortex		Do.	Do.	Do.	Do. Failures with the Golgi method are more frequent than in the case of cerebrum or cord
Choroid plexus	Spread out pieces under the microscope and choose one which shows blood vessels well	Flood with acetic alcohol (2) for 30 min., then wash well in 50 p. c. alcohol	To be mounted flat	Hematoxylin, eosin (49, 40)	
Cylindrical end bulbs	Fresh scleral conjunctiva (e. g., of calf). Oral mucous membrane			Methylen blue (55b or c)	The end bulbs lie close beneath the epithelium of the conjunctiva and may be torn off if the tissue is not handled cautiously. This is a difficult preparation to make

Degenerating fibers	See "Marchi's method"				
Ganglion canaliculi	Spinal ganglion of cat	Picro-sublimate (24)	P. Thin	Iron-hematoxylin (51)	
Ganglion cells......	Gasserian or spinal ganglia; cerebral and cerebellar cortex; spinal cord	Macerate in Landois fluid (79)	Tease	Beale's carmine (35) diluted with 1 vol. of the macerating fluid, or picro-carmine (63)	Most of the cell processes will be torn off. See also sections of brain, cord, and ganglia
Grandry's corpuscles..........	Waxy skin from lateral edges of duck's or goose's upper beak; tongue of woodpecker	Treat thin pieces with 1 p. c. osmic acid (20) for 18-24 hours	H. In hardened liver	Mount unstained or stain in carmalum (34)	In sectioning, cut from corium toward epithelium
Herbst's corpuscles	Do.	Do.	Do.	Do.	Do.
Hypophysis........		Müller (8) or Zenker (6)	P. Thin	Hematoxylin, eosin (49, 40)	
Intra-epithelial nerve fibers........	Skin from freshly amputated toe or finger (volar surface)		C. Very thin sections	Gold chloride (45)	Remove all fat before treating the tissue with any of the solutions. Look for tactile corpuscles near the excretory ducts of the sweat glands
Marchi's method for degenerating fibers...............	Nerve or cord, 2-4 weeks after the lesion has been made	Müller (8) for 8 days; then in a mixture of Müller 2 parts and 1 p. c. osmic acid 1 part, for 6 days	C.		Degenerated parts appear black, others, yellowish or gray. [With Weigert's hematoxylin method (52) degenerated fibers are unstained]
Medulla oblongata.		Müller (8) or Erlicki (7)	P. or C.	Borax-carmine, picric acid (32, 62)	Also try Golgi method (46)
Medullary sheath (myelin)...........	Small fresh nerve	Press an end of the nerve firmly against the slide. To prevent drying, breathe on the preparation occasionally	Tease loose end		Finally examine in a drop of normal saline (74). Look for exuded drops of myelin. See also "medullated fiber" and Weigert's method (52)
Medullated fibers of cord and brain......		Müller (8) or Erlicki (7)		Weigert's hematoxylin (52)	

TABLE OF TISSUES AND ORGANS WITH METHODS OF PREPARATION (Continued)

Object or Element	*Animal, Organ, or Tissue Recommended for Demonstration*	*Fixing and Hardening, or Other Preliminary Treatment*	*Section or Isolation Method. P.=Paraffin; C.=Celloidin; F.=Freezing; H.=Free Hand*	*Staining, etc.*	*General Remarks*
Medullated nerve fiber................	Small nerve	1 p. c. osmic acid (20)	Tease		Dehydrate. On a slide tease and at the same time clear small pieces in clove oil. Mount in balsam
Meissner's corpuscles................	Papillae of corium on volar surface of hand, finger-tip, or foot			Gold chloride (45) or methylen blue (55*b* or *c*)	For simple demonstration, boil the fresh skin for 15 minutes, strip off the epidermis, slice off some papillae from the cutis and examine them in 3 p. c. acetic acid
Neurokeratin.......	Frog; rabbit	Osmic acid (1 p. c. for frog; 2 p. c. for rabbit)		Dehydrate. Clear with bergamot oil (48 hours)	The oil dissolves out the myelin and thus renders visible the neurokeratin
Nerve-endings (in general)...........		The gold chloride (45), the methylen blue (55*b* or *c*), or the chrom-silver (46) method			The methods are enumerated in order of their simplicity. For nerve endings in striated muscle see chap. ix
Nerve-fiber bundles (transverse sections)..............	Sciatic	Zenker (6) or Gilson (15)	C. or P.	Carmalum, Lyons blue (34, 54)	
Neuroglia..........	Spinal cord	Müller (8) or Erlicki (7)	P.	Safranin (65) for 24 hours	Differentiate in absolute alcohol. See also Golgi method (46)
Nissl's granules (tigroid granules).	Lumbar enlargement of spinal cord	Corrosive sublimate (13), formalin (17), or alcohol (3)	P.	Methylen blue (55*d*)	See also neutral red (58)
Nodes of Ranvier...	Small nerve		Tease	Silver nitrate (64)	
Non-medullated (Remak's) **fibers**...	Sympathetic nerve; vagus nerve	0.5 p. c. osmic acid (20)	Do.	Picro-carmine (63) 24 to 48 hours	Medullated fibers (myelin) are stained black; non-medullated show no black

Pacinian corpuscle	Mesentery of cat	Spread out in normal saline and isolate from fat		Treat with 1 p.c. osmic acid (20) until the core appears brownish	To find the corpuscles, look for minute oval bodies between the strands of fat
Purkinje cells	Cerebellum of young kitten or new-born guinea-pig				See "cerebellar cortex," Golgi method (46)
Spinal cord (general; transverse sections)	Cat	Corrosive sublimate (13)	C. or P.	Hematoxylin, eosin (49, 40)	Thin paraffin sections may be stained in Ehrlich-Biondi (38)
Spinal cord; axones and cells (transverse sections)	Cat or dog	Müller (8) for 4 weeks. Transfer to stain without washing	C. Stain before sectioning	1 p.c. aq. sol. sodium carminate (3 days)	Before dehydrating wash the stained pieces for 24 hours in running water. Try also Golgi method (46)
Spinal ganglia (sections)	Do.	Zenker (6)	P.	Hematoxylin, eosin (49, 40)	Spinal ganglia of higher vertebrates are difficult to dissect out. The Gasserian ganglia may usually be substituted for them
Sympathetic ganglia (sections)	Frog, mammal (first thoracic or superior cervical)	Do.	P.	Do.	
Tactile corpuscles	See "intra-epithelial nerve fibers"				
Tactile menisci	Snout of pig or of the mole	0.33 p. c. aq. sol. of chromic acid (10)	P.	Overstain with hematoxylin (49)	Differentiate in alcoholic sol. of potassium ferri-cyanide
XI. NOSE.					
Isolated olfactory cells	Small pieces of olfactory mucous membrane	30 p. c. alcohol (81), 24 hours, followed by 1 p. c. osmic acid for 5 minutes		Picro-carmine (63)	Scrape off a little of the epithelial covering and examine in glycerin
Mucous membrane	Rabbit (divide head longitudinally; nasal mucous membrane is of brownish color)	30 p. c. alcohol 2 hours, followed by 1 p. c. osmic acid for 24 hours. Harden in alcohol	P. Make sections perpendicular to surface of membrane	Iron-hematoxylin (51)	
Nerve processes of olfactory cells	Young animals and fetuses	Golgi method (46), or methylen blue method (55*b*)			

TABLE OF TISSUES AND ORGANS WITH METHODS OF PREPARATION (Continued)

Object or Element	*Animal, Organ, or Tissue Recommended for Demonstration*	*Fixing and Hardening, or Other Preliminary Treatment*	*Section or Isolation Method. P.=Paraffin; C.=Celloidin; F.=Freezing; H.=Free Hand*	*Staining, etc.*	*General Remarks*
XII. REPRODUCTIVE ORGANS.					
Clitoris (transverse sections)	Human; monkey	Gilson (15) or Zenker (6)	P. or C.	Hematoxylin, eosin (49, 40)	
Corpus luteum	Rabbit	Do.	Do.	Iron-hematoxylin (51)	
Epididymis		Do.	P.	Do.	
Graafian follicle (ripe)	Rabbit	Acetic alcohol (2)	C.	Hematoxylin, eosin (49, 40)	
Fallopian tube (transverse section)	Child, dog, cat, or rabbit	Gilson (15)	C. or P.	Do.	
Ova	Ripe Graafian follicle of rabbit; ovary of cow	Normal saline (74)	Tease	Methyl green (56)	
Ovary	Mammal	Acetic alcohol (2)	C.	Hematoxylin, eosin (49, 40)	
Ovogenesis	Ascaris (see chap. xvi, mem. 11); mouse (see chap. xvi, mem. 8)				
Oviduct		Gilson (15)	P.	Iron-hematoxylin (51)	
Penis (cross-section of different regions)	Man; monkey	Acetic alcohol (2)	C.	Hematoxylin, eosin (49, 40)	
Placenta	Human	Technique same as for "uterus"			
Prostate	Man	Acetic alcohol (2)	C. or P.	Hematoxylin, eosin (49, 40)	
Pflüger's egg tubes	New-born kitten	Gilson (15)	Do.	Borax-carmine, picric acid (32, 62)	
Seminal vesicle	Man; dog	Do.	P.	Iron-hematoxylin, acid fuchsin (51, 42)	

Seminiferous tubules	See "spermatogenesis"				
Spermatogenesis	Rat; guinea-pig; salamander	Gilson (15) or Hermann (26)	P. Thin	Iron-hematoxylin, Bordeaux red (51, 33)	After Hermann, the safranin-gentian-violet method (66) gives beautiful results. Tubules should also be macerated in Hertwig's fluid (78), teased, and stained in acid carmine (37)
Spermatozoa	Smear preparations from vas deferens or testis. Also, examine some alive in normal saline (74)	Corrosive sublimate (13) for 5 minutes		Cyanin (1 p. c. sol. in anilin water; see 29), followed by erythrosin (41)	For structure of tail let spermatozoa macerate in normal saline (74), then stain with acid fuchsin (42)
		Osmic acid vapor (20)		Safranin, gentian violet (66)	
Testis (general)	New-born child; cat or dog; bisect the organ longitudinally	Tellyesniczky (5)	C.	Hematoxylin, eosin (49, 40)	In the cat or dog the mediastinum is in the interior instead of at the margin of the testis
Umbilicus	Human or pig embryo	Zenker (6)	C.	Do.	
Urethra	See "urethra" under XV. For male urethra see sections of penis				
terus	Human, if possible (fresh after surgical operation)	Fix in a mixture of formalin (17) 1 part, Müller (8) 9 parts, for 4 days: then to pure Müller (8)	C.	Do. or Beale's carmine (35)	
Vagina	Child; dog	Acetic alcohol (2)	C. or P.	Hematoxylin, eosin (49, 40)	
Vas deferens (transverse section)		Flemming (11) or Gilson (15)	P.	Iron-hematoxylin (51)	
XIII. RESPIRATORY ORGANS.					
Bronchi	Rabbit	Flemming (11)	C. or P.	Safranin (65)	
Epithelium of lung	Kitten	Fill the lung with 0.5 p. c. silver nitrate solution and place it in a similar solution for several hours	H.		Before sectioning wash the preparation in distilled water and transfer it to 70 p.c. alcohol
Fetal lung		Gilson (15) or Zenker (6)	C. or P.	Hematoxylin, eosin (49, 40)	
Larynx		Technique same as for "trachea"			

TABLE OF TISSUES AND ORGANS WITH METHODS OF PREPARATION (Continued)

Object or Element	*Animal, Organ, or Tissue Recommended for Demonstration*	*Fixing and Hardening, or Other Preliminary Treatment*	*Section or Isolation Method, P.=Paraffin; C.=Celloidin; F.=Freezing; H.=Free Hand*	*Staining, etc.*	*General Remarks*
Lung, blood vessels of..................					Carmine or Berlin blue injection through pulmonary artery. See chap. xii
Lung, elastic tissue of alveoli.........		Alcohol (3)	P.	Orcein (60)	Also, treat pieces of fresh lung with about 15 p.c. potassium hydrate
Lung (sections; general topography)..............		Acetic alcohol (2)	P. or C.	Hematoxylin, eosin (49, 40)	Fill the lung with the fixing fluid as well as immersing it in the fluid
Thymus gland.....	New-born child	Acetic alcohol (2)	C. or P.	Hematoxylin, eosin (49, 40)	
Thyroid gland......		Flemming (11)	P.	Ehrlich-Biondi (38)	The stain should differentiate the chief from the colloid cells
Trachea............	Child; cat	Acetic alcohol (2) or Flemming (11)	C. or P.	Hematoxylin, eosin (49, 40)	If very accurate results are desired the mucous membrane should be removed and sectioned alone
XIV. SKIN AND ITS APPENDAGES.					
Epidermis, undersurface view.......	Volar surface finger or toe	Macerate in 0.25 p. c. acetic acid until epidermis may be readily separated from dermis	Mount flat with under surface upward	Hemalum (47)	Many of the sweat glands withdraw from the dermis and remain attached to the epidermis
Hair...............	Examine in water (under cover-glass) or mount dry in balsam				The hair of the mouse and of most bats is peculiar
Hair, elements.....	Warm in sulphuric acid until the hair begins to curl				Tease if necessary and examine under a cover-glass
Hair, development..	Skin from forehead (not scalp) of human fetus of 5 or 6 months	Zenker (6) or Müller (8)	C.	Hematoxylin, eosin (49, 40)	

Hair follicle	Preferably the upper lip of man	Zenker (6)	C.	Do.	The orientation should be precise, so that exactly longitudinal or cross-sections result
Hair, renewal	Eyelid of new-born child. If this is not available, try scalp of adult	Tellyesniczky (5)	C.	Do.	
Mammary gland (general)	Preferably human. Nipple and portion of gland	Zenker (6) or Gilson (15)	C.	Do.	Make vertical sections through nipple and gland
Mammary gland (special)	Mammal during gestation or lactation	Flemming (11)	C. or P.	Safranin (65)	
Milk and colostrum	Obtain colostrum from a pregnant animal shortly before parturition	Examine milk in normal saline (74) without further treatment			Add a drop of picro-carmine (63) and examine colostrum in normal saline (74). Avoid pressure of the cover-glass
Nail (sections)	First-finger joint of little finger	Müller (8); then decalcify (84); harden a second time in alcohol (3)	H. or C.	Alum-carmine (28)	Make longitudinal sections of the entire piece of finger
Nail, elements		Heat in strong caustic potash (76) until the latter boils	Scrape		Transfer to a slide without staining and examine in the dissociating fluid
Prickle cells	Stratum Malpighii	Flemming (11)	P. Very thin sections	Unstained	To section in paraffin, take only small pieces. Examine in glycerin
Sebaceous glands	See sections of hair follicle				
Skin (general)	Volar surface of finger or toe	Gilson (15)	C.	Iron-hematoxylin (51)	
Skin, blood vessels	Hand of child. Forefoot of cat or dog	Müller (8) after injection	C.	Unstained	Inject with Berlin blue or carmine mass through ulnar artery. See chap. xii
Sweat glands	See sections of skin, also in the undersurface view of epidermis				
XV. URINARY ORGANS.					
Bladder		Distend the bladder in Gilson (15), tie it shut and suspend it in more of the fluid	P.	Iron-hematoxylin (51)	For epithelial cells see "transitional epithelium"

TABLE OF TISSUES AND ORGANS WITH METHODS OF PREPARATION (Continued)

Object or Element	*Animal, Organ, or Tissue Recommended for Demonstration*	*Fixing and Hardening, or Other Preliminary Treatment*	*Section or Isolation Method. P.= Paraffin; C.=Celloidin; F. = Freezing; H.=Free Hand*	*Staining, etc.*	*General Remarks*
Kidney, blood vessels	Rabbit or cat	Alcohol (3) or Zenker (6)	C.	Hemalum (47)	First inject the fresh kidney (through renal artery) with carmine mass. See chap. xii
Kidney, cortex and medulla	Small mammal	Acetic alcohol (2) or Gilson (15)	C.	Hematoxylin, eosin (49, 40)	Make radial horizontal sections embracing the whole organ
Epithelial cells of uriniferous tubules		5 p. c. aq. sol. of neutral ammonium chromate	Tease		
Kidney; glomerulus and its capsule		Zenker (6) or Gilson (15)	P. Thin	Iron-hematoxylin (51)	
Kidney; isolation of uriniferous tubules	An animal which has been dead for 24 hours	Pure hydrochloric acid (sp. grav. 1.12) for 18–24 hours. Wash thoroughly in water afterward	Tease		Examine in glycerin
Kidney; medullary rays (vertical sections)	Young animals	Treat as for "cortex and medulla"			
Kidney, nerves of		Golgi method (46), or methylen blue method (55)			
Suprarenal gland		Zenker (6)	P.	Hematoxylin, eosin (49, 40)	
Ureter		Gilson (15)	P.	Iron-hematoxylin (51)	
Urethra (female)	Human; dog	Do.	P. or C.	Do.	For male urethra see "penis" under XII

APPENDIX D

PREPARATION OF MICROSCOPICAL MATERIAL FOR A GENERAL COURSE IN ZOÖLOGY

(In addition to the methods enumerated here, see also chap. x, II and chap. xiii.)

PROTOZOA

a) *Cultures.*—**Amebae,** etc., may usually be obtained in quantities sufficient for class use by the following method recommended by H. S. Jennings:

A number of glass dishes measuring 8 or 9 inches in diameter by 3 inches deep are crowded full of water plants (especially Ceratophyllum and Elodea), filled with water, and the plants allowed to decay. Keep the dishes in warm, light places. In two or three weeks the layers of plants at the surface of the water will be covered with a brown slime which should be examined occasionally under the microscope for the desired forms. The scum that appears on the surface of the water consists mainly of bacteria upon which amebae largely feed. They will be found most frequently in the slime that immediately surrounds the plant tissue. Since they frequently last only two or three days in a culture, to insure material for class work, a number of cultures must be made at different dates and from different localities. Other protozoa such as *Arcella*, *Difflugia*, *Carchesium*, *Stentor*, etc., will also be found in the cultures.

Paramoecium may be kept from dying out by keeping bits of stale bread in cultures.

Euglena will be found in some of the cultures, but usually not in any quantities before the end of four or five weeks. They appear along the side of the dish toward the light.

Carchesium and **Vorticella** are frequently found on decaying duckweed (Lemna) and hornwort (Ceratophyllum). To secure a culture, have a more plentiful supply of water than for ameba. Professor Walton tells me that he always finds a supply of *Epistylis* on the shells of fresh water snails.

Opalina may be obtained readily by killing a frog wich chloroform and slitting open the large intestine. Examine scrapings of the epithelial wall in normal saline (reagent 74, Appendix B).

Sporozoa. Gaegarina may be found in the alimentary canal of the cockroach and Monocystis, in the male reproductive organs of the earthworm. They are best studied in normal saline. If it is desired to stain and mount specimens they may be fixed in corrosive-acetic (reagent 14, Appendix B) for 5 minutes, washed thoroughly in 35 per cent. alcohol to which a little tincture of iodine has been added, and stained with Ehrlich's triple stain (reagent 39), or hematoxylin and acid fuchsin (reagents 49 and 42).

b) *Quieting infusoria.*—1. Let sufficient water evaporate from under the cover to permit the latter to press lightly upon the animals. Guard against too great evaporation of water or the infusoria will be crushed.

2. Entanglement in fibers of cotton, etc., sometimes proves efficacious.

3. A small amount of gelatin or better, cherry-tree gum, dissolved in water makes a viscous mass which is often useful in retarding their motions. A bit of white of egg may be used in the same way.

4. Animals may be narcotized by means of a small drop of very dilute alcohol (preferably methyl alcohol) or chloretone (about one drop of a 1 per cent. solution to 10 drops of water). (Chloretone is manufactured by Park, Davis & Co., of Detroit, Mich. For its use as an anaesthetic in biological work see *Journal of Applied Microscopy*, Vol. V, p, 2051.)

c) *Feeding.*—Place finely pulverized carmine or indigo under the cover-glass. The colored powder rapidly accumulates in the food vacuoles. In such a preparation the action of the cilia of infusoria is also indicated by the rapid movement of the particles in the vicinity of the animal. See also chap. xiv, memorandum 4.

d) *Staining.*—For *intra vitam* staining see Appendix B, reagents 55*a*, 31, and 58.

To see *cilia* of infusoria treat the animal with very dilute iodine solution or a drop of a dilute solution of tannin.

To see the *macronucleus* and the *micronucleus* use a drop of a 2 per cent. solution of acetic acid or, better, methyl green (Appendix B, reagent 56).

e) *Permanent mounted preparations.*—Benedict's method is as follows:

"Smear a glass slide with albumen fixative, as in preparing for the mounting of paraffin sections. Then place on the surface of the film of fixative a drop or two of water containing the forms which it is desired to stain. Let nearly all the water evaporate by exposure to the air of the room until only the film of fixative remains moist. The slide can now be immersed in Gilson or any other fixing reagent, and then passed through the alcohols, stains, etc., in the same way that mounted sections are handled.

"I have had no difficulty in getting preparations of Paramoecium by this method, with very little distortion of the body, and any kind of staining desired. By this method students can prepare in ten minutes very satisfactory preparations of protozoa for demonstration of nuclei, etc."—*Journal of Applied Microscopy*, Vol. VI, p. 2647.

SPONGES

To isolate the spicules of calcareous sponges boil a bit of the sponge in 5 per cent. solution of caustic potash for a few minutes.

Fairly thick transverse, longitudinal, and tangential sections of *Grantia* showing spicules in the tissues are useful. Make these with an old razor or sharp scalpel. To hold the object while sectioning, place it between two pieces of pith or cork. For a careful study of the relations of the two systems of canals in the body-wall, thinner sections are necessary. To prepare these it is best to decalcify (2 per cent. chromic acid, 24 to 36 hours) the sponge and cut celloidin or paraffin sections on the microtome although fairly good sections may be made by hand. They should be dehydrated and mounted in balsam if permanent preparations are desired; if not, they may be examined in glycerin.

To color the collar cells use an aqueous solution of anilin blue.

Spicules of *silicious* sponges are isolated by treating bits of

the sponge with strong nitric acid or a mixture of nitric and hydrochloric acid.

COELENTERATES

Hydra should be sought for in spring-fed pools. In the autumn they are found most frequently on *smooth* dead leaves which are completely submerged. Material should be collected and placed in battery jars or larger glass jars, which are then filled with fresh, clear water and placed in a fairly light place, but not too near a window. Put a small amount of hornwort or Chara in each jar. In a few hours (12–36) the hydra will be found attached to the sides of the vessel and to the plants. They may readily be kept in the laboratory throughout the winter if glass plates are placed over the jars to prevent excessive evaporation and the temperature is not allowed to go below freezing. Fresh water should be added from time to time to make up for evaporation. In case their supply of food (Cyclops, Daphnia, and other small crustacea) is exhausted it should be renewed by skimming out from other aquaria the small forms upon which the animal feeds and putting them in the hydra jars.

For staining and mounting entire see chap. xiii **II,** B. Kill in the same way for sectioning. The most instructive sections are (1) transverse sections, (2) longitudinal sections through the mouth and a bud, and (3) sections showing the sexual organs. Stain in bulk with hematoxylin (reagent 49, Appendix B), imbed in paraffin using the method for delicate objects (chap. vi, **VII**), and after the paraffin has been removed from the sections, stain them for a few seconds in acid fuchsin. Dehydrate and mount in the usual way.

The sections are much more satisfactory if the hydra have been placed in small stender dishes filled with filtered water (not distilled) and kept from food for a week or ten days before killing. This eliminates the metabolic products and oil globules which ordinarily obscure the details of structure.

To Stain the Nematocysts of Living Hydra, place several of the animals in a small stender dish of water which has been tinted a

sky blue through the addition of methylen blue solution made up as follows:

Methylen blue	1.0 gram
Castile soap	0.5 gram
Water	300.0 c.c.

After two hours the hydra may be transferred to fresh water; the nematocyst cells are stained a deep blue. (Method of Little, *Journal of Applied Microscopy*, Vol. VI, p. 2116.)

To Discharge Nematocysts drum on the cover-glass gently with a pencil. By using a very small opening to the diaphragm they are usually sufficiently distinct without staining.

For Other Polypoid Forms, the methods given for hydra will answer in most cases.

For Collecting Free-Swimming Medusoid Forms full directions will be found in Brook's *Invertebrate Zoology.*

Compound Hydrozoa should be placed alive into the cells which they are to occupy when mounted. One per cent. formic acid is then added drop by drop to the sea-water. After the animals have been killed, the fluid is replaced by glycerin-jelly and the cover-glass is put in place. Another method is to kill the animals slowly by adding a few crystals of chloral hydrate, from time to time, to the small vessel of sea-water containing them.

Small Jelly-Fish may be fixed and hardened in 1 per cent. osmic acid and, stained or unstained, mounted in cells.

PLANARIA

Look for planarians on the under sides of stones in small streams of running water. They are usually examined alive. To see them thrust out the proboscis, keep them from food for a few days and then feed them on dead flies. Planaria which have been kept in the laboratory for months display the internal organs much more clearly than freshly captured ones.

If it is desired to study stained specimens, for preparation see chap. xiii, **iv,** A.

To Kill Planaria with Pharynx Protruded Cole (*Journal of Applied Microscopy*, Vol. VI, p. 2125) recommends covering

them in a watch-glass with a 1 per cent. aqueous solution of chloretone until they are immobilized and then rapidly transferring them to 5 per cent. formalin. Other fixing agents than formalin can be used.

DISTOMES

Perhaps the most easily obtained form is the one which is found in the liver of the cat. Search for it in the bile passages. Fix it in hot corrosive sublimate, wash out with alcohol to which tincture of iodine has been added, and stain for 24 hours in alum cochineal (reagent 27, Appendix B), or hemalum (reagent 47). As with Planaria, they should be compressed between two glass slides (see chap. xiii, **iv**, A, 7).

If the large liver fluke of the sheep (Fasciola hepatica) can be obtained, both the alimentary canal and the excretory system may be injected with finely powdered carmine in water. A separate fluke should be used in each case. For injection, a very fine-pointed cannula with rubber cap is used, or the manipulator may operate the cannula by simply blowing through it. The excretory system is injected through an incision made with a sharp-pointed scalpel in the median line near the hinder end of the animal. For the alimentary canal, the incision should be made about 1 mm. to one side of the median line. When the injection is completed, flatten the animal somewhat between two slides (see chap. xiii, **iv**, A, 7), harden in 95 per cent. alcohol for 12 to 24 hours, then dehydrate, clear, and mount in balsam.

CESTODES

Near large cities an unlimited supply of the sheep tapeworm (Monieza) can usually be secured from slaughter houses. Ample supplies can ordinarily be obtained from dogs, or, less frequently, from cats. Tapeworms can be kept alive for considerable length of time in tepid normal saline. The most instructive portions to mount are scolex, and sexually mature proglottids. For fixing and staining use the same methods as for distomes with the exception that cold instead of hot corrosive sublimate should be used. The scolex should not be compressed.

To Find Cysticerci, open the body cavity of a rabbit and look for large whitish bodies imbedded in the peritoneum or liver (the cysticercus of T. serrata). Likewise, the cysticercus of T. cras-

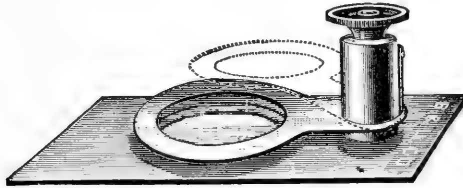

Fig. 70.—Compressor.

sicollis may be found in the liver of the mouse. If a cysticercus is found, its outer wall should be slit open in order to show the reversed scolex.

ASCARIS

See chap. xvi, memorandum 11.

TRICHINA

The simplest way to obtain it is to apply for infected pork to the government inspector whose headquarters are to be found near all large slaughter houses in cities. Bits of the infected

Fig. 71.—Compressor Used by the Government Bureaus for Meat Inspection.

muscle should be teased and flattened out in a compressor (Figs. 70 and 71) until a favorable area has been found. The flattened tissue may then be dehydrated and mounted unstained or it may be stained in hematoxylin (reagent 49, appendix B). Better results will be obtained if the material is fixed for from 4 to 6 hours in

Carnoy's fluid (reagent 2) before dehydrating or staining. If desired, the tissue may be sectioned in celloidin or paraffin.

To Demonstrate Living Trichinae Barnes (*American Monthly Microscopical Journal*, Vol. XIV, p. 104) subjects small bits of trichinized muscle to a mixture of 3 grains of pepsin, 2 drams of water, and 2 minims of hydrochloric acid, for about three hours at body temperature with occasional shaking. When the flesh and cysts are dissolved, the liquid is poured into a narrow glass vessel and allowed to settle. The live trichinae may be withdrawn with a pipette from the bottom of the fluid and examined on a warm stage.

ROTIFERS

Rotifers will usually be found in abundance in some of the laboratory aquaria on the lighted side of the vessel. For ordinary class work they are best studied alive. They are difficult to preserve properly. Full directions for killing and preserving will be found in Jenning's paper, "Rotatoria of the United States," *U. S. Fish Commission Bulletin*, 1902, p. 277.

To Quiet Rotifers, Cole (*Journal of Applied Microscopy*, Vol. VI, p. 2179) anaesthetizes them by adding from time to time a drop of 1 per cent. aqueous solution of chloretone to the water on the slide in which the animals are being examined.

BRYOZOA

They may be treated in the same way as compound hydrozoa. *Plumatella* may frequently be found in shallow fresh-water streams on the under side of flat rocks; *Pectinatella*, in rivers and streams on the upper surface of mussel shells, etc.

EARTHWORM

Earthworms are best collected on warm, rainy nights when they may be found extended on the surface of the ground near their burrows. They are most plentiful in old gardens or rich lawns. A lantern and a pail are the only implements necessary. Earthworms may frequently be found, however, in large numbers on the surface of the ground on cloudy days immediately after prolonged hard rain.

To Prepare Earthworms for Sectioning, secure good-sized specimens, wash them in water and place them in a vessel containing moist filter paper. There must not be sufficient water to drown the worms nor little enough to let the paper dry out. Put only a few worms in each dish and adjust the cover so as to admit a little air. After 12 to 24 hours it is well to remove any dead or injuried specimens and to change the filter paper. The dish should be kept from direct sunlight in a cool place. After two or three days the grit and dirt in the alimentary canal will have been passed out and its place taken by paper which the worms have eaten. They are then ready to kill and preserve or section.

Place the worms in a flat vessel and pour on sufficient water to cover them. During the next two hours add a little alcohol from time to time until the strength of the liquid is increased to about 8 or 10 per cent. Then wash all mucous from the body of the worms and replace them in 10 per cent. alcohol until they no longer respond to pricking or pinching with forceps. Transfer them to 50 per cent. alcohol for several hours, keeping them straightened out as much as possible; then to 70 per cent. alcohol for 12 hours, followed by 95 per cent. alcohol for 24 hours. Preserve finally in 70 per cent. alcohol.

For sectioning the preliminary steps are the same but from 10 per cent. alcohol the worms should be placed into Zenker's fluid (reagent 6, Appendix B) for 4 to 6 hours. For washing, etc., follow the directions given in the discussion of the reagent. To facilitate penetration of the fluid, it is well to slit open the body cavity of the worm in places that are not to be sectioned. The most instructive sections are cross-sections of the middle of the body, and sagittal sections of the anterior end which include the pharynx. The worms may be stained in bulk (24 to 36 hours) in borax-carmine (reagent 32) or hematoxylin (reagent 49) before sectioning.

Entire nephridia together with a small part of the septum which they traverse should be carefully dissected out, stained in borax-carmine (reagent 32), dehydrated, cleared, and mounted in balsam.

An ovary should be removed entire, stained with borax-carmine, dehydrated, cleared, and mounted in balsam.

A testis should be treated in the same way as an ovary. Tease it in the balsam before adding the cover-glass.

To Keep Earthworms Alive in Winter, Jennings (*Journal of Applied Microscopy*, Vol. VI, p. 2412) places them, immediately after collection, into bacteria dishes (9 in. in diameter by 3 in. deep) between folds of muslin which is kept damp but not dripping wet. Not more than a dozen worms should be placed in one dish and the cloth should be changed or washed at least every two weeks. The worms may be fed on leaves, etc., from time to time.

To Immobilize Earthworms for study of circulation of the blood under the microscope or projection lantern, Cole (*Journal of Applied Microscopy*, Vol. VI, p. 2125) places them in a 0.2 per cent. aqueous solution of chloretone for 3 or 4 minutes. Such worms may be slightly compressed between two slides.

To Examine Corpuscles of the Coelomic Fluid, expose the worms for a minute or two, to the vapor of chloroform. The coelomic fluid exudes through dorsal pores. Touch a cover-glass to the fluid and mount.

The Setae Can Be Isolated by boiling a bit of the tissue containing them in a solution of caustic potash. When isolated, dry them and mount in balsam.

LEECH

Leeches are obtained from fresh-water pools, streams and marshes, but to get sufficient numbers for class use it is usually necessary to purchase them from dealers. Live leeches intended for dissection may be killed with chloroform. Cross-sections prepared in the same way as for earthworms are very instructive.

ARTHROPODS

For Mounting Small Crustacea see chap. xiii, **iii,** A.

To Quiet Small Crustacea for Microscopical Examination (Cole: *Journal of Applied Microscopy*, Vol. VI, p. 2180) place them in a watch-glass containing 2 parts of 1 per cent. chloretone and

5 parts of water. The same treatment is useful for the larvae of insects. Some such as the nymph of the dragon-fly, will require more chloretone.

For Various Dissections and Parts of Insects see chap. x, ii.

For Mounting Insects Entire (beetles, mosquitoes, gnats, aphids, larvae, etc.) as microscopic preparations, and for mounting muscle, wings, heads, legs, scales, antennae, etc., see chap. xiii.

Live nymphs of the dragon-fly are especially valuable for study under the compound microscope because they show very clearly the valvular action of the heart, the tracheal gills and tracheae, and the brain and its relation to the eyes. The heart is located well toward the posterior end of the abdomen between the main tracheal trunks. Cole (*Journal of Applied Microscopy*, Vol. VI, p. 2274) recommends that the animals be anaesthetized by subjecting them to a 1 per cent. aqueous solution of chloretone.

MOLLUSKS

Gills of the Fresh-Water Mussel may be fixed in corrosive sublimate (reagent 13, Appendix B) for from 20 to 30 minutes, washed out in water and then in dilute alcohol to which tincture of iodine has been added. Make cross-sections in paraffin, stain in dilute hematoxylin (reagent 49) and mount in the ordinary way.

Cross-Sections of the Entire Mussel are valuable to show the relations of the gills, kidneys, and heart. Wedge the valves apart slightly and immerse the animal for 24 hours in 1 per cent. chromic acid (reagent 10). Wash out thoroughly in running water and transfer the specimens to 70 per cent. alcohol for two or three days or until needed. To section, remove both valves, place the animal on a board and with a razor cut transverse sections. These are to be examined with the naked eye or with a dissecting lens.

To Kill Snails in an Expanded Condition put them into a vessel of cold water, then run a layer of hot water onto the surface of the cold water. See that the vessel is full of water and cover it with a glass plate to exclude the air.

For Lingual Ribbon of the Snail see chap. xiii, memorandum 7.

AMPHIOXUS

Specimens must ordinarily be secured from dealers. The animals should be stained entire in borax-carmine (reagent 32, Appendix B) and sectioned in celloidin. The most instructive sections are cross-sections of a female with well developed gonads, and longitudinal sections of small individuals. Mounts of entire small specimens should also be made.

VERTEBRATA

For any of the tissues of vertebrates which teachers may desire to prepare, ample directions are given in Appendix C.

For Demonstration of Circulation of the Blood in the frog, see chap. xiv.

APPENDIX E

TABLE OF EQUIVALENT WEIGHTS AND MEASURES

WEIGHTS, METRIC AND AVOIRDUPOIS

1 kilo=1,000 grams=1 liter of water at its maximum density=2.2 pounds.
1 gram=1 cubic centimeter of water at its maximum density=15.43 grains=0.035 ounce.
1 pound=453.59 grams.
1 ounce=28.35 grams.
1 grain (Troy)=0.065 gram.
1 dram=1.77 grams.

WEIGHTS, METRIC AND APOTHECARIES'

1 kilo=1,000 grams.
1 gram=15.43 grains=0.032 ounce.
1 pound=373.24 grams.
1 ounce=31.10 grams.
1 dram=3.89 grams.
1 scruple=1.30 grams.
1 grain=0.065 gram.

MEASURES OF LENGTH, METRIC AND ENGLISH

1 meter=1,000 millimeters=39.37 inches.
1 centimeter=0.394 inch.
1 millimeter=0.039 inch.
1 yard=0.914 meter.
1 foot=30.48 centimeters.
1 inch=2.54 centimeters=25.40 millimeters.

LIQUID MEASURES, METRIC AND APOTHECARIES'

1 liter=1,000 cubic centimeters=2.11 pints.
1 cubic centimeter=0.034 fluid ounce=16.23 minims.
1 gallon=128 ounces=3.79 liters.
1 pint=16 ounces=473.18 cubic centimeters.
1 fluid ounce=8 fluid drams=29.57 cubic centimeters.
1 fluid dram=60 minims=3.70 cubic centimeters.

THERMOMETERS

To reduce degrees Fahrenheit to degrees Centigrade use the formula, $C=5/9\,(F-32)$. For example, if the number of degrees Fahrenheit is 77, then $C=5/9\,(77-32)=25$ degrees. Or, for instance, to reduce -31 degrees Fahrenheit to Centigrade, $C=5/9\,(-31-32)=5/9\times-63=-35$ degrees.

To reduce degrees of Centigrade to degrees of Fahrenheit use the formula $F=9/5\,C+32$. For example, if the number of degrees Centigrade is 25, then $F=(9/5\times25)+32=77$ degrees. Or, to reduce -35 degrees Centigrade to Fahrenheit, $F=(9/5\times-35)+32=-31$ degrees.

INDEX

INDEX

Abbe, 156 ; camera lucida, 147.
Abbot's method for staining spores of bacteria, 111.
Aberration, spherical, 138 ; correction of, 139.
Absolute alcohol, testing for water, 56.
Absorption of fat, 198.
Accessory chromosome, 191.
Acetic acid, 161.
Acetic alcohol, 161 ; and chloroform, 123, 125, 161.
Achromatic objective, 145.
Achromatism, 145.
Acid fuchsin, see *fuchsin.*
Acidophil granules, 190.
Action of liquids, to hasten, 56.
Adenoid connective tissue, 197.
Adipose tissue, 194.
Affixing sections, 23, 41, 64.
Air bubbles, 159, 160.
Albumen fixative, 12, 23, 41.
Albumenoids, 15.
Albuminous coats of eggs, to remove, 118.
Alcohol, absolute, 7, 13, 49, 84 ; acid, 8, 24, 50, 162 ; alkaline, 49, 50; and chloroform 162; ether, 8, 23; ethyl, 13; fixation, 28, 162; methyl, 13 ; replenishing, 56.
Alcoholometer, 13.
Alimentary canal, 197, 198; of cockroach, 77.
Alum carmine, 171, 181 ; dahlia, 191.
Alum cochineal, 126, 170, 181.
Aluminium chloride, 184.
Altmann, 192
Ameba, 215.
Ameboid movement in leucocytes, 102.
Amphibia, material for the embryology of, 118; fecundation and early embryonic stages, 122; to study eggs of, 118.
Amphibian eggs, 167.
Amphioxus, 226.
Amphophil granules, 190.
Amitosis, 191.
Ammonia copper sulphate solution, 152.
Ammonium chromate, 214.
Ammonium picrate, 181, 183.
Amyloid, 172, 199.
Anatomical specimens, to preserve, 31.
Andrews, 117.
Angular aperture, 145.
Anilin blue, 217; and orange G, 172.
Anilin, alcohol, 182; dyes, 19, 20, 57, 63, 171; formulae, 171; water, 171.
Antennae of insects, 93.
Anthrax, 109, 111.
Aorta, 193.
Apertometer, 145.
Aphid, 91.
Aplanatism, 145.
Apochromatic objective, 146.
Apparatus, 1 ; dealers in, 5.
Appliances, microscopical, 145.
Aqueous humor, 187.
Arcella, 215.
Areas of Conheim, 204.
Areolar tissue, 194.
Arrangement of apparatus and reagents, 6, 49.
Artery, 193.
Arthropods, 224.
Ascaris, 162; maturation, fertilization, and cleavage, 123.
Asphalt-paraffin-rubber method of imbedding, 44, 119.
Aurantia, 20.
Axial illumination, 150.
Axis cylinder, 206.
Axone, 206.

Bacillus, 108; aerogenes capsulatus, 111; of anthrax, 109, 111; of bubonic plague, 111; of chancroid, 111 ; coli communis, 111 ; diphtheriae, 109, 111 ; of dysentery, 111 ; of glanders, 111; of influenza, 111; of malignant edema, 111; mucosus capsulatus, 111; proteus, 111; pyanocyaneus, 111; of tetanus, 111 ; of tuberculosis, 111 ; of typhoid, 111.
Bacteria, cover-glass preparations of, 105; features to be observed in studying, 108; Gram's method of staining, 107; hanging-drop preparations of, 105, 108; in tissues, 105, 107; material for demonstrating, 108; methylen blue stain for, 107; mounting from fluid media, 105; mounting from solid media, 105; staining and mounting films, 105; spores, 108; staining flagella, 111 ; stains for, 109.
Bacterial examination, 105.
Balsam, 11, 22, 31, 80, 90 ; bottle, 4 ; exudation, to remove, 58.
Bardeen, 128, 129; microtome, 67.
Basophil granules, 190.
Basophil substance in nerve-cells, 182.
Bausch, 152, 160.
Bausch and Lomb microscope, 143.
Beale's carmine, 173.
Bee, 93.
Beetle, 92.
Beggiatoa, 108.
Bell's cement, 79.
Benedict, 217.
Benzopurpurin, 20.
Berlin blue, 81.

Bethe's fluid, 181.
Bichloride of mercury, 165; see also *corrosive sublimate.*
Bichromate of potassium, 162, 187; and acetic acid, 163; and corrosive sublimate, 163; and cupric sulphate, 163; and sodium sulphate, 163.
Binocular microscope, 146.
Bioblasts, 192.
Bismarck brown, 20, 106, 173.
Bladder, 213.
Blastoderm of chick, 113, 114.
Blastoderms, to orient colorless, 117.
Bleaching, 24, 45.
Bleu de Lyon, 20; see also *Lyons blue.*
Blocks for celloidin imbedding, 59, 62.
Blood, 97, 190; cover-glass preparations, 98; clinical examination of, 99; corpuscles, 172, 176; corpuscles, living, 97; crystals, 97, 190; currents, to observe, 101; dry preparations of, 98; effects of reagents on, 97; enumeration of corpuscles, 99; examination of fresh, 97; platelets, 97; rapid method, 99; serum, 187; Loeffler's serum, 110; Schultze's iodized serum, 187; to study in sections, 101; test for, 98; Wright's stain for, 102.
Blood forming organs, 190.
Blow pipe, 1.
Bone, 79; corpuscles and their processes, 194; decalcified, 79, 189, 194; endochondral development of, 194; fibers of Sharpey, 195; grinding, 80; Haversian canals and lamellae, 195; intra-membranous development of, 195; isolation of corpuscles and of lamellae, 195; sectioning, 79, 80; young, to decalcify, 189.
Borax-carmine, 9, 49, 51, 54, 61, 77, 89, 91, 119, 121, 169; formula, 9.
Bordeaux red, 10, 49, 53, 55, 117, 173; formula, 10.
Born, 127.
Bottle-capping, 30.
Bouillon, glucose, 110.
Box, slide, 1.
Brain, 206, 207.
Brain cells, 206.
Brain sand, 206.
Brittle objects, sectioning, 43, 44, 63.
Bronchi, 211.
Brownian movement, 146.
Brushes, camel's hair, 1.
Bryozoa, 222.
Bubonic plague, bacillus of, 111.
Bull's eye, 151.
Bunge-Loeffler, staining flagella of bacteria, 111.
Bütschli, 192.
Butterfly, wings of, 92; eggs of, 93.

Calendar, 1; record, 6.
Calleja's staining fluid, 174.
Camel's hair brushes, 1.
Camera, photographic, 155.
Camera lucida, 146, 147, 148.
Canada balsam, 11.
Cannulae, 82, 85.
Capillaries, blood, 193; lymph, 193.
Carbol-fuchsin for bacteria, 109.
Carbolic acid, 21, 22.
Carbol-xylol clearer, 9, 21, 34.
Carbon dioxide, for freezing, 67; for killing, 16.
Carchesium, 215.
Card records, 1, 6.
Carmalum; 76: formula, 173.
Carmine, 19, 57, 162; acid, 76, 123, 174; alum, 168; and Lyons blue, 51; Beale's, 173; injection mass, 81; picric acid and indigo carmine, 174; picro-, 10, 184.
Carnoy, 161.
Carpenter and Dallinger, 160.
Carrier for paraffin ribbon, 41.
Cartilage, 195; capsule of, 195; connective tissue and elastic fibers in, 195; elastic (yellow-fibro-), 195; glycogen in, 195; hyaline, 196; white fibro-, 196.
Caustic potash, 187.
Cell, animal, 191; living or fresh, 192; of Paneth, 197; pigment, 193; prickle, 213; reduction division in, 193.
Cell-making, 87, 95.
Celloidin, 12, 22; bottle, 4; clearing before sectioning, 64; hardening, 61, 62; imbedding, 59; Gilson's rapid method, 64; method, 26, 59; preparation of material for imbedding in, 53; sections, to transfer from the knife, 64; time required for the method, 62.
Celloidin and paraffin methods compared, 63.
Celloidin-paraffin infiltration, 63.
Cells, to lessen evaporation from, 95.
Cement, Bell's, 79, 89.
Cement substance, 188.
Center of slide, to find, 43.
Centering an object in a cell, 93.
Centigrade to Fahrenheit scale, 228.
Central illumination, 150.
Central nervous system, 188, 206.
Centrosome, 53.
Cerebellar cortex, 206.
Cerebral cortex, 206.
Ceruminous glands, 201.
Cestodes, 220.
Chancroid, bacillus of, 111.
Chick, embryology of, 113; stages necessary for a course in, 115, 116.
Chick embryos, fixing, staining, and mounting, 114, 115; freeing blastoderm from yolk in, 117; orienting, 114, 116; reconstruction of heart, 126; removing blastoderm, 114; sectioning, 116.
Child, 117, 118.
Chironomous larva, gland cells of, 192.
Chloretone, 216.
Chloride and acetate of copper, 164.
Cholera, spirillum of, 111.
Choroid, 203.
Choroid plexus, 206.
Chromatic aberration, 139.

Chromic acid, 118, 164, 189, 225; and its compounds, 17, 164, 165.
Chromic acid material, bleaching, 45.
Chromo-aceto-osmic acid, 164.
Chromo-platinic mixture, 165.
Chromosome, 53.
Chrom-silver method, 71.
Cicatricula, 113.
Cilia of infusoria, 216.
Ciliated epithelium, 75.
Circulation, in foot of frog, 101; in mesentery, 101.
Circulatory system, 193.
Citric acid, 177.
Cleaning lenses, 158, 159, 160.
Cleaning slides and covers, 57.
Cleanliness, 6, 44.
Clearer, 21, 62; carbol-xylol, 9, 21, 30; Eycleshymer's, 22, 62.
Clearing, 21.
Cleavage, in Ascaris, 123; in echinoderms, amphibia, and teleosts, 122; in living material (snails), 123; in mammals, 121.
Clinging of paraffin sections to the knife, 48.
Clitoris, 210.
Clove oil for minute dissections, 78.
Coal-tar dyes, 20.
Coccus, 108.
Cochlea, 201; nerve fibers and nerve endings of, 201.
Cole, 219, 222, 224, 225.
Collar cells of sponge, 217.
Collodion, 23, 44, 64.
Colostrum, 213.
Columnar epithelium, 75.
Compensating ocular, 145, 148.
Compressor, 221.
Concave or diverging lens, 134.
Condenser, 148, 151.
Conheim, areas of, 204.
Conjugate foci, 134.
Conklin, 117, 122, 170; picro-hematoxylin, 177.
Connective tissue, 172, 174, 185, 194, 196.
Contractile animals, 16, 161.
Conventional distance of vision, 144.
Convex or converging lens, 134.
Coplin staining jars, 4, 49.
Copper sulphate method of preparing absolute alcohol, 7.
Cornea, 203.
Corneal corpuscles and nerves, 203; spaces and canaliculi, 203.
Corpora lutea, 119, 210.
Correction collar, 148.
Corrosion, 24, 86.
Corrosive sublimate, 9, 18, 165; handling, 166; and acetic acid, 166; and nitric acid, 166.
Cover-glass, 1; supports for, 78; forceps, 106; correction, 148; to clean, 57.
Creasote, beechwood, 22, 62.
Crescents of Gianuzzi, 198.
Crooked paraffin ribbons, 46.
Crown glass, 140.
Crumbling of tissues in paraffin, 43, 44, 46, 47, 66.
Crustacea, small, 90, 224.
Crystals, blood, 97.
Culture slide, 108.
Curare, 101.
Cyanin, 211.
Cyclops, 90, 218.
Cylindrical end bulbs, 206.
Cypris, 90.
Cysticerci, 221.
Cytological work, reagents for, 162, 164, 170, 175.
Cytoplasmic granules, 183.

Damar, 22.
Daphnia, 90, 218.
Dealcoholization, 22.
Dealers and manufacturers, 5.
Decalcification, 24, 79; fixing before, 80.
Decalcifying fluid, 9, 79; formulae, 189.
Defective mounts, 57, 58.
Definition, 148.
Dehydration, 18.
Delicate tissues, 43; paraffin method for, 54; dehydrating apparatus for, 55; freezing method for, 70.
Demilunes of Heidenhain, 198.
Descemet, membrane of, 204.
Desilicidation, 24.
Desk, 4, 157.
Desmids, 94.
Determination of elements that will be impregnated in Golgi method, 73.
Diamond, writing, 1.
Diapedesis, 101.
Diaphragms, 149, 159.
Difficulties in sectioning paraffin, table of, 46.
Difflugia, 215.
Digestive organs, 197; blood vessels of, 197.
Dilution, rules for, 7, 13.
Diphtheria, 109, 111.
Diplococci, 108.
Diplococcus intra cellularis meningitidis, 111.
Dipping-tube, 94.
Dirty paraffin, 43.
Dirty sections, 42.
Dispersion, 139.
Dissecting instruments, 1.
Dissections, minute, 77.
Dissociation, 15, 25, 75, 78; general rule for, 78.
Dissociators, formaldehyde, 9, 75; formulae for others, 187.
Distomes, 220.
Dogiel, 181.
Double staining, 20, 51, 53, 62.
Doublet lens, 138.
Dragon-fly nymphs, 225; heart beat in, 225.

Drawing-board, with camera lucida, 147.
Drawing-table, Bardeen's 129.
Drawing with camera lucida, 146, 147.
Dropping-bottle, 58.
Dropping out of object from paraffin section, 47.
Dry or dull-looking areas in mounts, 58.
Duodenum, 198.
Duval, orientation of young chick embryos, 116.
Dysentery, bacillus of, 111.
Dityscus, fore-leg of, 92.

Ear, 201.
Earthworm, collecting, 222; coelomic corpuscles of, 224; to immobilize, 224; to keep alive in winter, 224; nephridia of, 223; ovary or testis of, 224; setae of, 224; sectioning, 222.
Eau de Javelle, 24.
Echinoderms, fertilization and early embryonic stages, 122.
Eggs, of butterfly, 93; of chicken, 113; of amphibia, 118; fish, hatching-box for, 123.
Ehrlich-Biondi (Heidenhain) triple stain, 174.
Ehrlich, hematoxylin, 178; method for blood, 198; methylen blue method for nerve tissue, 180; triple stain, 99, 175.
Elastic fibers, 184, 185, 193; fine, 196; coarse, 196.
Embyrograph, 149.
Embryological methods, 113.
Embryology, of amphibia, 118; of the chick, 113; of mammals, 119, 120, 121; of the mouse, 121; of the pig, 120; of the rabbit, 119; of teleosts, 117.
Embyronic membranes, 121.
Embryos, human, 126; measuring, 116; reagents for, 126, 163, 166, 167, 169, 170, 178.
Encircling fibers, 196.
Endothelial cells, 202.
Endothelium of blood vessels, 193.
Eosin, 10, 20, 49, 51, 55, 62, 97, 175; formula, 10.
Epidermis, 212.
Epididymus, 210.
Epistylis, 215.
Epithelia, 165, 174, 176, 186, 187, 188, 201; isolation of, 202.
Epithelium, ciliated, 201; columnar and glandular, 201; cubical, 201; of mouth, 198; of small intestine and villi, 198; of stomach, 198; of lungs, 211; of uriniferous tubules, 214; pigmented, 202; stratified, 202; squamous or pavement, 202; transitional, 202.
Erlicki's solution, 9, 29, 53, 59, 79, 163; formula, 9.
Erythrocytes, 190.
Erythrosin, 20, 176.
Esophagus, 198.
Ether alcohol, 8, 23, 166.
Ether freezing attachment, 68, 69.
Eustachian tube, 201.
Exner, 123.
Eycleshymer's clearer, 22, 62; methods of orientation, 125.
Eye, 203; of sheep, 162.
Eyeball, 203; blood vessels of, 203.
Eyelid, 203.
Eyepiece, 137.
Eyepoint, 149, 160.

Faded preparations, restaining, 58.
Fading of blue color in injection mass, 86.
Fahrenheit to Centigrade scale, 228.
Fallopian tube, 210.
Farrant's solution, 22.
Fasciola, 220.
Fat, 186, 196.
Fatigue of eyes, 169.
Feathers, 93.
Fecundation, see *fertilization.*
Femoral artery, injection through, 85.
Fenestrated membrane, 196.
Fertilization, in Ascaris, 123; artificial, 122; in amphibia, echinoderms, and teleosts, 122; in mammals, 121.
Fetuses, 126.
Fibrillae in striated muscle, 205.
Fibrillar (white fibrous) connective tissue, 197; cells of, 196.
Fibrin, stained preparation of, 97.
Fibrous tissue, 176.
Fine adjustment, 159.
Fixing, 16, 27, 30; purpose of, 16.
Fixing agents, necessary qualities of, 16.
Fixing and hardening agents, formulae, 161.
Fixing sections to slide, 23, 41, 64.
Flagella of bacteria, staining, 111.
Flat worms, 90, 170.
Flatness of field, 150.
Flemming, 161; solution of, 164, 171, 185, 186.
Flint glass, 140.
Fluid mounts, 87, 88, 94.
Flukes, 94.
Foam structure, 192.
Focal point, 134.
Focus, principal, 134; real, 135; virtual, 135.
Formalin, 8, 29, 53, 71, 84, 118, 121, 126; as a reducing agent, 166; with alcohol and acetic acid, 166.
Formic acid, 74, 177.
Formol-sublimate, 167; and acetic acid, 167.
Free-hand sectioning, 33.
Freezing method, 67; carbon dioxide, 67; ether or rhigolene, 70.
Fresh tissues, examination of, 25, 187; fixing and washing after sectioning, 70; sectioning by the freezing method, 69.
Friable objects, sectioning, 43, 44, 63.
Frog, embryology of, 118.
Fromman, lines of, 74.
Fuchsin, acid, 20, 176, 189; and picric acid, 176; basic, 109, 176.

Gabbet's method for tubercle bacilli, 110.
Gage, 9, 160, 169; carbol-xylol clearer, 9; formaldehyde dissociator, 9.
Gall bladder, 198.
Galt, 31.
Ganglia, 182, 207; canaliculi in, 207; peripheral, 73.
Gastric glands, 198.
Gelatin for injection mass, 81.
General rules, 6.
Gentian violet, 20, 106, 109, 176, 186.
Germinal disc of chick, 113.
Gianuzzi, crescents of, 198.
Gilson's mercuro-nitric fixing fluid. 8, 77, 118, 166; formula, 8.
Gilson's rapid celloidin process, 64.
Gizzard of cricket or katydid, 77.
Gland cells, 162, 165, 175; of chironomous larva, 192.
Glanders, 109, 111.
Glomerulus of kidney, 214.
Glycerin, 21, 22, 87, 88.
Glycerin jelly, 21, 22, 79, 89, 90; formula, 90.
Glycerin-picrate mixture, 162.
Gnat, 91.
Goblet cells, 198.
Gold chloride, 20, 177; method for nerve-endings, 74.
Gold size, 87.
Golgi method, 71, 72; mounting Golgi preparations, 72, 73.
Gonococcus, 111.
Graafian follicle, 119, 210.
Grades of alcohol, 7.
Graduated cylinder, 4.
Gram's solution, 110, 177; method, 107, 110, 111.
Grandry's corpuscles, 207.
Grantia, 217.
Great water beetle, foreleg of, 92.
Gregarina, 216.
Grenacher's borax-carmine, 9; see also *borax-carmine.*
Gritty feeling in paraffin sectioning, 46.
Grübler and Hollborn, address, 174.
Gum and syrup mass for freezing, 67.
Gun cotton, 23.
Guyer, 101.

Hair, 212; development of, 212; follicle, 213; renewal of, 213.
Hanging-drop preparations, 108.
Hardening, 15, 17, 27.
Harder's glands, 203.
Hardesty, 72, 73.
Hard objects, to section, 47, 80.
Hatching-box for fish eggs, 123.
Hazy mounts, 57.
Heart, 193.
Heart-beat in nymph of dragonfly, 225.
Heidenhain, 10, 52, 174, 178; demilunes of. 198.
Hemalum, 84, 177.
Hematein, 20, 177.
Hematoidin crystals, 98.
Hematoxylin, 19, 20, 162, 165, 168; ripening of, 9, 20, 57; Delafield's, 9, 34, 49, 54, 55, 56, 57, 68, 84, 89, 91; iron, 10, 52, 53, 55, 123, 178; Ehrlich's, 178; Weigert's, 178.
Hematoxylin and eosin, 51, 62.
Hemin crystals, 98.
Hemocytometer, 99.
Hemoglobin crystals, 97.
Herbst's corpuscles, 12.
Hermann's fluid, 117, 120, 170, 171, 186; formula, 170.
Hertwig's macerating fluid, 76.
Heterotypical mitosis, 192.
His, 116, 129.
Histological elements, isolation of, 75.
Homogeneous immersion lens, 150, 152.
Honing microtome, 44.
Horn spoon, 1.
Huber, 73, 128.
Human embryos, 126.
Hydra, 76, 89, 218; to kill expanded, 89.
Hydrochloric acid, 8, 214.
Hydrofluoric acid, 25.
Hydrozoa, compound, 219.

Illumination, 150, 159.
Images, 135; defects in, 138.
Imbedding, 22; paraffin, 37; celloidin, 59; mass for well microtome, 35; a number of minute objects, 63; Ls, 45.
Immersion objective, 152.
Impregnations, 20.
Incubator, 113.
Indifferent fluids, 25, 187.
Indulin, 190.
Infiltration, 22.
Inflammation, 101.
Influenza, bacillus of, 111.
Infusoria, 168, 173; quieting, 216.
Initial magnifying power, 144.
Injected vessels, corrosion of, 86.
Injecting, with a syringe, 81; blood and lymph vessels, 81; lymphatics, 85; liver fluke, 220; through femoral artery, 85.
Injection, test for complete, 83; double, 84 85; continuous air pressure, 84; syringe, 82, 85.
Injection masses, 81; to keep, 85; fading of blue in, 86; cold fluid gelatin, 86.
Injection methods, 25, 81.
Ink for writing on glass, 58.
Insects, alimentary canal of, 77; antennae of, 93; having hard coverings, 93; delicate, 93; legs of, 93; mounting entire, 225; mouth parts of, 77, 93; muscles of, 90; nervous system of, 77; salivary glands of, 77, 192; scales of, 93; small or soft, 93; stings of, 77; wings of, 93.
Intercellular bridges, 202.
Intercellular substance, 186.

Interstitial imbedding, 22.
Intestinal absorption of fat, 198.
Intestine, large, 199; small, 199.
In toto preparations, 26, 87.
Intra vitam staining, 173, 179, 183, 216.
Iodine, for washing after corrosive sublimate, 166; Gram's solution, 110, 177.
Iris, 203.
Iris diaphragm, 149.
Iron hematoxylin, 10, 49, 178.
Irrigation, 97.
Isolation of histological elements, 15, 25, 75.

Jamming together of paraffin sections, 46.
Jelly fish, 219.
Jelly of Wharton, 197.
Jennings, 215, 222, 224.
Johnson, 44, 119, 167.
Joris, 86.
Julin, 120.

Kaiserling's fluid, 31.
Karyokinesis, 165, 171, 192, 193.
Keratin, 172, 185.
Kidney, blood vessels of, 214; cortex and medulla of, 214; glomeruli of, 214; injection of, 85; isolaticu of tubules, 214; medullary rays of, 214; nerves of, 214.
Killing, 16, 27.
Kincaid, 31.
Kleinenberg's picro-sulphuric, 169.
Koch-Ehrlich gentian violet, 109.
Kronecker's fluid, 187.

Labelling vessels, 27; slides, 50.
Labels, 1.
Lacrymal glands, 293.
Landois' solution, 188.
Large intestine, 199.
Large objects, sectioning in paraffin, 45.
Larvae, transparent, 88; small or soft, 93.
Larynx, 211.
Lavdowsky's mixture, 166.
Lebrun, 161.
Lee, 72, 171, 161, 185.
Leech, 224.
Length of time for staining tissues, 56.
Lens, capsule and epithelium of, 203; fibers, 203.
Lenses, 134; cleaning, 158; systems of, 137.
Leprosy, bacillus of, 110.
Leptothrix, 108.
Leucocytes, 191; feeding, 102; granules of, 190, 191; to demonstrate movement in, 102.
Ligament, 197.
Ligamentum nuchae, 179.
Light, for microscopical work, 151, 152; reflected, 150; transmitted, 150.
Light green, 20, 179.
Lillie, 170.
Lingual ribbon of snail, 84.
Lip, 199.
Lithium carmine, 107.
Liver, amyloid infiltration of, 199; bile capillaries of, 199; blood vessels of, 199; cells, 199; flukes, 220; hepatic lobules of, 199; injection of, 85: interlobular connective tissue of, 199.
Locker, 4.
Loeffler's alkaline methylen blue, 109; blood serum, 110.
Logwood, 20.
Lugol's solution, see *Gram's solution.*
Lung, 212; blood vessels of, 212; elastic tissue of, 212; epithelium of, 211; foetal, 211; injection of, 85.
Lymph, canals, 183; capillaries, 193; glands, 191; spaces, 183.
Lymphatics, injection of, 85.
Lyons blue, 10, 20, 49, 51, 179; formula, 10.

MacCallum's macerating fluid. 188.
Macerated tissue, fixation of, 78.
Maceration, 15, 25, 75, 76.
Magnification, determination of, 153, 154.
Magnifiers, 136.
Magnifying power, 144, 152.
Majenta, 176.
Malarial parasite, 103.
Mallory and Wright, 102.
Mallory's connective tissue stain, 172.
Mammal, maturation, fertilization, and segmentation in, 121.
Mammalian embryos, early stages, 119; older stages, 120.
Mammary gland, 213.
Manufacturers, 4.
Mark, 43.
Marking imbedded specimens, 38.
Marchi, 207.
Marrow, 191.
Mast cells, 191.
Material for a course in zoölogy, 215.
Material, storing, 30.
Maturation, in amphibia, echinoderms, and teleosts, 122; in Ascaris, 123; in mammals, 121.
Mayer, 20, 45, 173, 177.
Mayer's albumen fixative, 12, 23; paracarmine, 184; hemalum, 177; carmalum. 173.
Measurement of microscopic objects, 153.
Mechanical stage, 153.
Medulla oblongata, 207.
Medullary sheath, 270.
Medullated fibers, of cord and tract, 207; tracts of, 178.
Medusoid forms, 217.
Meissner's corpuscles, 208.
Membrane of Descemet, 204.
Mercuro-nitric fixing fluid, 8, 28, 53, 77; formula, 8.
Merkel's fluid, 165.
Mesothelial cells, 202.

Metagelatin, 90.
Metallic substances for color differentiation, 20, 71.
Methods, general statement of, 15.
Methylen blue, 20, 179; for bacteria, 107, 109; for impregnation of epithelia, 183; for nerves and nerve-terminations, 180, 181; for non-striated muscle, 182; for ordinary sections, 182; immersion method, 181; intra vitam stain, 180; Loeffler's alkaline, 109; polychromatic, 180.
Methyl green, 20, 76, 168. 183, 187; formula, 183.
Methyl violet, 20, 57; formula, 183.
Metric weights and measures, 227.
Micrococci, 108.
Micrococcus tetragenus, 111.
Micrometer, stage, 153; ocular, 155; filar, 155.
Micrometry, 153-55.
Micron, 44, 155.
Microscope, 133; simple, 136; compound, 137, 139, 140; makers, 141-44; binocular, 146; dissecting, 4, 149; manipulation of, 157.
Microscopical terms and appliances, 145.
Microtome, well, 35; for paraffin, 39, 40, 60; Minot, 39; Minot-Blake, 40; oil for, 44; celloidin, 60; freezing, 67, 68.
Microtome knife, tilt of, 40; sharpening, 44.
Milk, 213.
Milky looking mounts, 57.
Minot, 116, 121, 174; microtome, 39, 40.
Minute dissection, 77, 78.
Mirror, 155.
Mites, 88.
Mitosis, 165, 171, 192, 193; heterotypical, 192.
Mollusk, 225.
Monieza, 220.
Monocystis, 216.
Moth, wings of, 92.
Mounting, 22.
Mouse, embryology of, 121, 122.
Mouth, epithelium of, 198.
Mucin, 172, 199.
Mucoid connective tissue, 197.
Müller's fluid, 70, 163, 188, 189; formula, 163.
Muscae volitantes, 155.
Muscle, 172, 176, 204; cardiac, 75, 187, 188, 204; to tendon, 205; smooth, 187; voluntary, 75; of insect, 90.
Muscle fiber, branched-striated, 204; non-striated, 182, 205; striated, 75, 204; fibrillae of, 205; end of striated, 205; cardiac, 75.
Mussel, gills of, 225; cross-section of, 225.
Myelin, 207.

Nail, 213.
Naphthylamin yellow, 190.
"Neck length" of embryos, 116.
Needle, 1.
Negative eyepiece, 137.
Neisser, method of diagnosis of diphtheria, 169.
Nematocysts, 218, 219.
Nerve, 73; endings, 74, 177, 180; tissue, 162, 163, 176, 180, 186; degenerated fibers, 179, 207; plexuses in alimentary canal, 199; intra epithelial fibers, 207; medullated fibers, 207, 208; non-medullated fibers, 208; fiber bundles, 208.
Nerve cells and their ramifications, 71, 120, 187.
Nerve cells, Nissl's method for, 162, 182.
Nervous system, of grasshopper, 77; of vertebrates, 206.
Neurokeratin, 208.
Neuroglia, 73, 208.
Neutral red, 183.
Neutrophil granules, 175, 191.
Nitric acid, 9, 79, 189; see also *mercuro-nitric fluid*.
Nissl, method for nerve cells, 162, 182; granules, 182, 183, 208.
Nodes of Ranvier, 208.
Nomenclature of objectives and oculars, 144.
Normal or indifferent fluids, 125, 187.
Normal saline, 8, 187.
Nose, 209; mucous membrane of, 209.
Nosepiece, 139, 158.
Numerical aperture, 155.

Objects which will not stain, 56.
Objects which alcohol would injure, sectioning, 70.
Objects of general interest, mounting, 87.
Objective, 137, 138; immersion, 152, 159; apochromatic, 146; using high power, 158.
Oblique illumination, 150.
Ocular, 137; compensating, 145, 148; searching, 148; working, 148.
Odontoblasts, 201.
Oil, anilin, 22; of bergamot, 22; cedar-wood, 21, 43, 48, 62; of cloves, 22, 78; of origanum, 22, 62; of sandal-wood, 22; of thyme and castor oil, 62.
Oil immersion lens, 152, 159.
Olfactory cells, 209; nerve processes of, 209.
Opalina, 216.
Opaque mounts, 92.
Opaque objects, 150.
Optical center of lens, 134.
Optical principles, 133.
Orange G, 20, 183.
Orcein, 184.
Organs and tissues with methods of preparation, 190.
Orientation, 38.
Orienting chick embryos, 113.
Orienting objects in the imbedding mass, 124; Patton's method, 125; Eycleshymer's methods, 125.
Orienting serial sections, 124.
Orth's lithium carmine, 107.
Osmic acid, 18, 78, 79, 116, 120, 167, 168, 183, 185; discussion of, 167; vapor, 168.
Osmic material, bleaching, 45.
Osmium-bichromate mixture, 72.
Otoliths, 201.

Ova, 210.
Ovary, 210.
Over-correction, 157.
Oviduct, 210.
Ovogenesis, 210.
Oxalic acid, 87, 177.
Oxyphil granules, 175.

Pacinian corpuscles, 209.
Pancreas, 199; granules of, 198.
Paneth, cells of, 198.
Paper box for paraffin imbedding, 37.
Papillae of tongue, 200.
Paracarmine, 169, 184.
Paraffin, 12, 23; oven, 12; dirty, 43.
Paraffin-asphalt-rubber method, 44.
Paraffin block, trimming, 39.
Paraffin method, 26; imbedding and sectioning, 37; staining and mounting, 49; for delicate objects, 54; compared with celloidin method, 63.
Paramoecium, 215, 217.
Parfocal, 156.
Parotid gland, 199.
Pathogenic bacteria, 111.
Patton, 125.
Pearl, 167.
Pectinatella, 222.
Pedesis, 146.
Pencil, glass-marking, 1, 42.
Penetration, 156.
Penis, 210.
Peroxide of hydrogen, 9, 24.
Peyer's patches, 199.
Pflüger's egg tubes, 210.
Phloroglucin method, 189.
Picric acid, 18, 168, 184, 189.
Picric alcohol, 169.
Picro-acetic, 117, 122, 123, 169.
Picro-carmine, 76, 79, 168.
Picro-hematoxylin, 177.
Picro-sublimate, Rabl's formula, 169; vom Rath's formula, 169.
Picro-sulphuric acid, 117, 121, 169.
Pig embryos, to obtain and prepare, 120, 121; stages necessary for study, 120; placentation of, 121; orientation of, 121.
Pigment cells, 193.
Pigments, to remove, 45.
Placenta, 174, 260.
Placentation, 121.
Planaria, 90, 219.
Platino-aceto-osmic mixture, 170.
Plumatella, 222.
Pneumococcus, 111.
Polariscope, 156.
Polypoid forms, 219.
Positive eye-piece, 137.
Potassium bichromate, 162.
Pouring liquids, 6.
Preparation of reagents, 7.
Preservation of anatomical specimens, 31.
Preserving, 18, 30; mixture, 30; objects imbedded in paraffin, 43; sections cut by the freezing method, 70.
Prickle cells, 213.
Principal axis of lens, 134.
Protococcus, 94.
Plotoplasmic currents, 193.
Protozoa, 95, 167; cultures, 215; feeding, 216; permanent mounted preparation, 217; quieting, 216; staining, 216.
Purkinje cells, 73, 209; fibers, 194.
Pyrogallol, 170, 185.
Pyroxilin, 23.

Rabbit, embryology of, 119; dissection of, to obtain embryos, 119, 120; ova, time to obtain various stages, 119.
Rabl, 169.
Radula of snail, 94.
Ranvier, one-third alcohol, 188; picro-carmine, 185; cross of, 74; nodes of, 208.
Rath, O. vom, 169.
Rating of objectives and oculars, 144.
Rays of light, 133.
Razor, section, 1, 33.
Reagent bottles, 4.
Reagents and their preparation, 7; also Appendix B.
Reconstruction of objects from sections, 127; in wax, 127; geometrical, 129.
Reconstruction points, 125.
Records, card, 6, 28; calendar, 6.
Rectified spirit, 13.
Reduction division, 193.
Refraction of light, 133.
Remak's fibers, 208.
Removal of liquid from slide, 55.
Reproductive organs, 210.
Resolving power, 156.
Respiratory organs, 211.
Restaining old mounts, 58.
Reticular connective tissue, 197.
Retina, 165, 168, 188, 204.
Reversing sections, to avoid, 40.
Rhigolene freezing attachment, 68, 70.
Ripart and Petit, 164, 187.
Rolling of paraffin sections, 46.
Romanowsky stain, 103.
Rosin, 183.
Rotifers, 222.
Rubbing sections off the slide, to avoid, 56.
Rubin S, 176.
Rules, general, 6.

Safranin, 10, 20, 165, 185; formula, 185; and gentian violet, 186.
Salivary gland, 199, 200; granules of, 198; of cockroach or cricket, 77.
Salycilic acid, 170.
Sarcinae, 108.
Sarcolemma, 205.

Scales from wings of insects, 93.
Scalpel, 1.
Scissors, 1.
Scheme for mounting whole objects or sections, 26.
Schiefferdecker's fluid, 188.
Schneider's acid carmine, 174.
Schultz's dehydrating apparatus, 55.
Schultze's iodized serum, 187.
Sclera, 204.
Scolex of tapeworm, 220.
Scraping of microtome knife, 47.
Scratches across paraffin sections, 47.
Sealing bottles and preparation jars, 30.
Sealing mounts, 89.
Sebaceous glands, 213.
Secondary axis of lens, 134.
Section lifter, 1.
Section method, 15; simple, 33.
Sectioning in paraffin, 37, 38; in gum, 67; in celloidin, 61; free hand, 33.
Sections, affixing, 23; drying of, 55; milky or hazy, 57; plane of, 123, 124; scheme for mounting, 26; to stain by flooding, 58; washing off of, 58.
Semicircular canals, 201.
Seminal vesicle, 210.
Seminiferous tubules, 211.
Serial sections, orienting, 123; see *paraffin* or *celloidin method.*
Sharpey, fibers of, 195.
Shell vials, support for, 30.
Silver, nitrate, 20, 120, 186; formula, 186; for nerve, 73.
Size of microscopic objects, to measure, 153.
Skin and its appendages, 212, 213; blood vessels of, 213.
Slide box, 1.
Slides, 1; passing through reagents, 56; to clean, 57.
Small intestine, 198; epithelium of, 198.
Small objects, to transfer through reagents, 30; to section free hand, 34; to orient in paraffin, 43.
Smear preparations, blood, 98; bacteria, 105.
Smegma bacilli, 110.
Snail, 225; or slug, 84; to obtain eggs, 123.
Sobotta, 122.
Sodium chloride, dissociating fluid, 188.
Solutions, rules for making, 6.
Spawning of fish, 122.
Spectrum, 146; tertiary, 146.
Spencer microscope, 142.
Spermatogenesis, 211.
Spermatozoa, 211.
Spherical aberration, 138.
Spicules of sponges, 217.
Spinal cord, 73, 209.
Spinal ganglia, 209.
Spirillum, 108; of Asiatic cholera, 111.
Spirogyra, 94.
Sponges, 217.
Spores of bacteria, staining, 111.
Sporozoa, 216.
Sputum, to examine for tubercle bacilli, 110.
Staining, 19–21, 49; double or multiple, 20, 21, 51; in bulk, 54; causes of failure in, 56, 60.
Staining jars, Coplin, 4.
Stains, formulae, 170; classification of, 19; cytoplasmic, 20; nuclear, 20; replenishing, 56; precise with hematoxylin, 56; for bacteria, 109.
Standard tube-length, 157, 158.
Staphylococci, 108.
Staphylococcus pyogenes aureus, 111; albus, 111.
Stenders, 4, 49.
Sting of wasp or bee, 77.
Stomach, 200; epithelium of, 198.
Stoppers, to remove, 13.
Storing material, 30.
Streptococci, 108.
Streptococcus pyogenes, 111; capsulatus, 111.
Stropping, 45.
Sublingual gland, 200.
Submaxillary gland, 200.
Sudan III, 186.
Supplies, 1.
Supporting tissue, 194.
Suprarenal gland, 214.
Sweat glands, 213.
Sympathetic ganglia, 209.
Synovial villi, 197.
Syphilis, bacillus of, 110.
Syracuse watch-glass, 4.
Syringe, injection, 82, 85.

Tables of equivalent weights and measures, 227.
Tactile corpuscles, 209.
Tactile menisci, 209.
Taenia, 220, 221.
Tandler, 86.
Tannic acid, 97.
Tapeworm, 94, 220, 221.
Tastebuds, 200.
Teasing, 15, 25, 75, 76.
Teeth and other hard objects, grinding, 80.
Teichmann's crystals, 98.
Teleosts, embryology of, 117; to orient blastoderms of, 117; manipulation of embryonic material, 117, 167; fertilization and early embryonic stages, 122.
Tellyesnicky's fluid, 163.
Temperature of laboratory, 43.
Temporary mounts, 35.
Tendon, 197; cells of, 197; to muscle, 197.
Terminal bars, 202.
Testis, 76, 211.
Tetanus, bacillus of, 111.
Tetracocci, 108.
Thermometers, 228.
Thin sections, 34, 43, 56.

Thionin, 20.
Thymol, 170, 177.
Thymus gland, 191, 212.
Thyroid gland, 212.
Tigroid substance, 182, 208.
Tilt of microtome knife, 40.
Tissues and organs with methods of preparation, 190.
Tissues, killing and fixing, 27; which crumble in paraffin, 43; length of time for staining, 56.
Toluidin blue, 20.
Toluol, 22.
Toisson's solution, 100.
Tongue, 200; papillae and folliculi linguales, 200.
Tonsil, 200.
Tooth, sectioning decalcified, 79, 200; development of, 200; odontoblasts, 201.
Tough objects, sectioning, 47.
Trachea, 212.
Tracts of medullated nerve fibers, 178.
Transparent aquatic organisms, 180.
Transparent larvae, 88.
Trichina, 221, 222; examining alive, 222.
Triplet lens, 138.
Tube-length, 157.
Tubercle bacilli, 110, 111.
Turn-table, 4, 88.
Typhoid, bacillus of, 111.

Umbilicus, 211.
Under-correction, 157.
Unna, 182, 184.
Ureter, 214.
Urethra, 211, 214.
Urinary organs, 213.
Uriniferous tubules, epithelial cells of, 214.
Uterus, 211; and placentation, 121.

Vagina, 211.
Vas deferens, 211.
Valves of heart, 194.
Van Beneden, 120.
Van Giesen's stain, 176.
Variation in thickness of paraffin sections, 47.
Vascular system, double injection of, 84.
Vein, 194.
Vertebrata, 226.
Vials, 4.
Vision, conventional distance of, 144.
Visual purple, 204.
Volvox, 94.
Von Ebner's fluid, 189.
Vorticella, 215.
Vulcanized fiber for celloidin mounts, 62.

Walton, 30, 117, 215.
Wash bottle, 4.
Washing, 17.
Watch-glass, Syracuse, 4.
Water immersion lens, 152.
Water method for affixing sections, 23.
Water mites, 88.
Wax plates for reconstruction, preparation of, 127.
Weigert, hematoxylin, 178; method for bacteria, 107.
Weights and measures, table of equivalent, 227.
Well microtomes, 35.
Wharton, jelly of, 197.
White objects, orientation of, 43.
White spots in paraffin, 38.
Whitman, 118.
Whole objects, mounting, 26, 87.
Wire, copper, 1.
Wollaston's camera lucida, 146.
Worcester, 119, 167.
Working distance, 157.
Work-table, 157.
Wright's stain for blood and for malarial parasite, 102.
Wrinkles in paraffin sections, 42, 46.
Writing on glass, ink for, 58; pencil for, 1, 42.

Xylol, 13, 21, 38, 49; for removing paraffin, 56.

Zenker's fluid, 120, 126; formula, 163.
Zeiss microscopes, 141, 144.
Ziehl-Neelson, carbol-fuchsin, 109.
Zoölogy, material for a course in, 215.

1-94

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