MANUAL OF HISTOLOGIC STAINING METHODS

THIRD EDITION
Mc GRAW – HILL

Institute of Pathology
Armed Forces - 1949

Foreword

The assembling of these various histological methods and special staining techniques for use by the histopathology technician is an outstanding example of the dedicated and superior work continually being accomplished by the many varied members of the staff of the Armed Forces Institute of Pathology.

This new edition represents a drastic revision of the earlier publication but continues to incorporate certain old and proven techniques which time has shown to be invaluable to both beginner and experienced technician. In addition, the revised manual contains many new techniques and special stains which have been developed and published since the 1960 edition as well as many techniques worked out in our own laboratories, some of which have never been published.

The observant reviewer will also find considerably more information in the form of photographs and illustrations, such as the diagrams illustrating problems in cutting and their possible causes, a step by step photographic demonstration of the various facets of cutting, and a chapter which photographically illustrates approximately 32 artifacts of fixation, processing and cutting. These, plus many other features too numerous to mention, make this an invaluable tool for the histopathology technician.

The rapidity with which each of the prior editions has been exhausted attests to both need and popularity of such a collective source of histopathologic techniques. Hence, we are deeply gratified that the McGraw-Hill Book Company has once more accepted the responsibility of making commercially available the *Manual of Histologic Staining Methods.*

BRUCE H. SMITH
Captain, MC, USN
The Director

Preface

The growth of histotechnology is no better demonstrated than by the growth of this manual which was first compiled by the late Mary Francis Gridley in 1953. The immediate success of this manual resulted in a number of mimeographed printings at the Armed Forces Institute of Pathology from 1953 to 1957. The first "published" edition appeared in 1957 and was a revision of Miss Gridley's numerous mimeographed notes by her devoted colleagues at the Armed Forces Institute of Pathology. At the same time her colleagues established a memorial fund in her name and conceived a plan to perpetuate her most outstanding contribution to histotechnology, "The Laboratory Manual of Special Staining Technics."

Although this edition has been modified to the point that most techniques contained bear little resemblance to those published in past editions, it is only proper to give Miss Gridley credit for the original inception of the manual, especially since it has given histotechnology a tremendous boost and has been an excellent aid to many technicians and pathologists. Credit must be shared also by the late Lawrence P. Ambrogi, Chief, Histopathology Laboratories, and Miss Evelyn F. Ballou who were primarily responsible for the 1957 and 1960 editions. It was their dedication and foresight that made the present edition possible.

In response to the great demand for the manual and the many changes in histological and special staining techniques it became necessary to revise the 1960 edition. Our original intentions were to make moderate revisions, but it soon became apparent that a great deal of revision was necessary if the manual was to reflect all the advances which have taken place in histotechnology. One need only to leaf through this manual to find that many techniques presented were developed in our laboratories and have not been published before. Previously published standard techniques have been given new "twists" to simplify and/or better demonstrate the normal structure or pathologic condition.

I am indebted to Dr. Frank Johnson, who was most helpful with his guidance and suggestions and for review of the entire manuscript; to Miss Evelyn F. Ballou, Mrs. Bertha D. Landi, Mrs. Lillian K. Washington, Mr. David Lewis, Miss Eliza Buddo, Mr. Edward Cunningham, and Mr. Peter Emanuele who modified and contributed some of the techniques. Special thanks are due Mr. Benedicto Manuel, Mrs. Marcella Grabner, and Mrs. Edna Prophet who spent many hours abstracting, editing and reviewing. I express my gratitude also to my secretary, Mrs. Sula Hughes, for the endless hours she spent in editing and typing the original manuscript, and to the many technicians who co-operated in various ways in preparing this edition.

Acknowledgement is made to Mrs. Lois O. Runyon, Mrs. Ethel Denis, and Mrs. Ruby Irwin who typed the printer's copy. I wish also to extend my appreciation to the publishing companies who have allowed us to make use of material from their publications.

LEE G. LUNA, HT (ASCP)
Chief, Histopathology Laboratories

General References in Histopathologic Technique

BOOKS

Baker, J. R.: "Principles of Biological Microtechnique," John Wiley and Sons, Inc., New York, N. Y., 1958.

Barka, T., and P. J. Anderson: "Histochemistry: Theory, Practice, and Bibliography," Hoeber Medical Division, Harper and Row, Publishers, Inc., New York, N. Y., 1963.

Conn, H. J.: "Biological Stains," 7th ed., The Williams and Wilkins Company, Baltimore, Md., 1961.

Gurr, E.: "Encyclopedia of Microscopic Stains," The Williams and Wilkins Company, Baltimore, Md., 1960.

Gray, P.: "The Microtomists Formulary and Guide," McGraw-Hill Book Company, New York, N. Y., 1954.

Humason, G. L.: "Animal Tissue Techniques," W. H. Freeman and Company, San Francisco, Calif., 1962.

Lillie, R. D.: "Histopathologic Technic and Practical Histochemistry," 3rd ed., McGraw-Hill Book Company, New York, N. Y., 1965.

Mallory, F. B.: "Pathological Technique," Hafner Publishing Co., New York, N. Y., 1961.

McManus, J. F. A., and R. W. Mowry: "Staining Methods: Histologic and Histochemical, Hoeber Medical Division, Harper and Row, Publishers, Inc., New York, N. Y., 1963.

Pearse, A. G. E.: "Histochemistry: Theoretical and Applied," 2nd ed., Little, Brown and Co., Boston, Mass., 1960.

Preece, A.: "A Manual for Histologic Technicians," 2nd ed., Little, Brown and Co., Boston, Mass., 1965.

Thompson, S. W.: "Selected Histochemical and Histopathological Methods," Charles C Thomas, Publishers, Springfield, Ill., 1966.

JOURNALS

American Journal of Medical Technology, Suite 1600, Herman Professional Bldg., Houston, Tex. 77025

Journal of Histochemistry and Cytochemistry, The Williams and Wilkins Co., 428 East Preston St., Baltimore, Md. 21202

Laboratory Investigation, The Williams and Wilkins Co., 428 East Preston St., Baltimore, Md. 21202

Stain Technology: A Journal for Microtechnic and Histochemistry, The Williams and Wilkins Co., 428 East Preston St., Baltimore, Md. 21202

Contents

Chapter 1

Preparation of Tissue

FIXATION

The foundation of all good histological preparations is adequate and complete fixation. Fixation is required to (1) *prevent* post mortem changes such as putrefaction and autolysis; (2) *preserve* various cell constitutents in as life-like manner as possible; (3) *protect* by hardening the naturally soft tissue, thereby allowing easy manipulation during subsequent processing; (4) *convert* the normal semi-fluid consistency of cells to an irreversible semi-solid consistency; (5) *aid* in the visual differentiation of structure by application of biological dyes and chemicals. To accomplish these objectives the tissue should be placed in the fixative immediately upon removal from the body or as soon after death as possible.

The choice of fixing agent should be determined by the purpose for which the tissue is to be stained or preserved.

Blocks should be cut thin enough so that the fixative will penetrate the tissue within a reasonably short time. To this end the block should not be more than 4mm in thickness and should be immersed in at least ten times its volume of fixative.

Ten percent buffered neutral formalin is the most widely used fixative because it is compatible with most stains. The length of time for fixation depends upon the size of the block and fixative used. It is well to have a clear understanding of the effects of fixation, the time required for complete fixation of specific tissues, and the post fixation handling of tissue specimens.

That many specimens may be ruined, by poor handling subsequent to proper fixation, has been proven. This generally occurs when one fails to realize that different fixatives require varied times to effect complete fixation; and the specimen may require a particular treatment, immediately following fixation, to insure retention of specific staining properties.

Additional useful knowledge, is the action of a simple fixative on different parts of the tissue specimen. A partial list of the characteristics of certain common simple fixatives and their various effects follows, while more detailed information in this regard can be found in: Baker, J. R.: *Principles of Biological Microtechnique,* New York, John Wiley & Sons, Inc., 1958.

CHARACTERISTICS OF CERTAIN AGENTS USED AS FIXATIVES

FORMALDEHYDE, 10% FORMALIN

FORMALDEHYDE, Gas. Formaldehyde is a flammable colorless gas at ordinary temperatures having a pungent suffocating odor. It is very soluble in water (up to 55%) and also is soluble in both alcohol and ether. A very reactive reagent, it combines readily with many substances and polymerizes easily.

FORMALDEHYDE, Solution (Formalin, Formol). A solution of about 37% by weight, of formaldehyde gas in water, usually with 10 – 15% methanol added to prevent polymerization. This solution is the same strength as that known as Formalin 40%.

REFERENCE. *The Merck Index*, 7th edition, page 460, 1960, Merck & Company Inc. Rahway, New Jersey.

The formaldehyde solution, approximately 40% formaldehyde gas in water, called *formalin*, is treated as a 100% solution in making other formalin percent solutions (10 ml formalin and 90 ml H_2O = 10% formalin).

Because it oxidizes to formic acid, it should not have long contact with chromates. In order to counteract the effect of the formic acid, formalin should be *buffered with sodium phosphate, monobasic and dibasic.*

Reactions of the formalin with tissue proteins are numerous and complex, but it is an accepted fact, that it does not precipitate proteins and only lightly precipitates the other constitutents of the cell.

Formalin neither preserves nor destroys fat, and is good for complex lipids, but has no effect on neutral fats.

Formalin is not a fixative for carbohydrates, it preserves the proteins which in turn traps glycogen so that it is not easily dissolved.

GLACIAL ACETIC ACID

Acetic acid causes the constitutents of the cells to swell, therefore, it is *never used alone* but is combined with some other reagent having a shrinking effect.

The penetration is *rapid* and *good*.

The use of acetic acid gives life-like preservation to nuclei and is used when the desire for chromosomes is requested, but it does not aid in staining.

Acetic acid precipitates nucleoproteins, and destroys mitochondria and the Golgi apparatus.

It does not fix lipids or carbohydrates.

It leaves tissue *soft and prevents hardening* by subsequent use of alcohols.

ALCOHOL, ETHYL 70—100 PERCENT

Absolute alcohol is used for the preservation of glycogen.

It acts as a reducing agent, being oxidized to acetaldehyde then to acetic acid; for this reason it cannot be combined with chromic acid.

It is slow of *penetration; hardens and shrinks* the tissue in proportion to the percentage used.

It precipitates soluble nucleic acids. It does not form a compound with protein but abstracts water from it and *denatures* it, (changes the nature of the protein without adding another atom).

It precipitates albumin and globulin making both insoluble in water. It diffuses into the tissue, shrinking the cytoplasm as it precipitates, which action causes a distortion on the cells.

It makes nuclear staining difficult but it does not interfere with cytoplasmic staining.

Tissue so fixed needs no special washing before further processing.

MERCURIC CHLORIDE

This is one of the most useful of the salts employed in fixing tissue. It is rarely used alone because of its shrinking action. The size of tissue is important (not more than 4 mm in thickness) for excessive exposure causes considerable hardening.

It penetrates rapidly without destroying lipids.

It precipitates all proteins without firmly combining with them. These precipitates become soluble in potassium iodide. Removal of these precipitates (crystals) is necessary before staining.

It facilitates staining with most dyes making the colors more brilliant.

It neither fixes nor destroys carbohydrates.

PICRIC ACID

It is not often used in simple solution, because it causes the cells to shrink markedly. It requires damp storage because of its explosive nature.

It penetrates well and fixes rapidly.

It precipitates all proteins, forming picrates easily soluble in water. It traps glycogen in protein.

It leaves tissue soft, and, when used in combination, tends to make for easier staining.

POTASSIUM DICHROMATE

It fixes cytoplasm without precipitation.

It is especially valuable in mixtures for the fixation of lipids, particularly phospholipids.

It preserves phosphatides and is used for mitochondria.

Thorough washing is necessary to avoid forming an oxide in alcohol which cannot be removed later.

For convenience, there are listed here some of the more commonly used fixatives or fixative mixtures. Ideally, all tissues should be washed in running water after the use of most fixatives before processing, but this is particularly true following the various formalin fixatives.

FORMALIN SALINE SOLUTION

37 – 40% formalin	100.0 ml
Sodium chloride	9.0 gm
Tap water	900.0 ml

A tolerant fixative. Long storage does not create excessive hardening or damage. When not buffered may cause formation of formalin pigment. It fixes nuclear chromatin in a diffused homogeneous pattern making it impossible to visualize chromatin distinctly. Therefore, it is not especially useful for routine use. Ideal for the preservation of muco substances.

BUFFERED NEUTRAL FORMALIN SOLUTION

37 – 40% formalin	100.0 ml
Distilled water	900.0 ml
Sodium phosphate monobasic	4.0 gm
Sodium phosphate dibasic (anhydrous)	6.5 gm

The best overall fixative, therefore, strongly recommended for routine use.

FORMALIN - SODIUM ACETATE SOLUTION

37 – 40% formalin	100.0 ml
Sodium acetate	20.0 gm
Tap water	900.0 ml

Formalin with sodium acetate is an excellent fixative and medium in which to store gross blocks of tissue.

FORMALIN-AMMONIUM BROMIDE SOLUTION

37 – 40% formalin, neutralized	15.0 ml
Ammonium bromide	2.0 gm
Distilled water	85.0 ml

An excellent fixative for brain tissue on which the silver and gold techniques are to be performed.

FORMALIN - ALCOHOL - ACETIC ACID SOLUTION

37 – 40% formalin	10.0 ml
Alcohol, 80%	90.0 ml
Glacial acetic acid	5.0 ml

Not an ideal fixative for tissue storage and routine use, however, a good agent for rapid fixation of tissue. Prevents solution of carbohydrates before fixation of the protein component is complete. Addition of acetic acid insures fixation of nucleoprotein providing an improved histological picture. Small pieces (2 mm in thickness) usually are completely fixed in 4 - 6 hours.

FORMOL-CALCIUM SOLUTION

Calcium chloride, anhydrous	1.0 gm
37 – 40% formalin	10.0 ml
Distilled water	90.0 ml

This fixative is especially useful when studies on lipids are to be performed.

ZENKER'S SOLUTION

Distilled water	1000.0 ml
Mercuric chloride	50.0 gm
Potassium dichromate	25.0 gm
Sodium sulfate	10.0 gm

Add 5 ml of glacial acetic acid to 95 ml of Zenker's solution before use. (The solution does not keep well after the addition of the acetic acid).

This mixture has been used since 1894 when Zenker suggested the addition of mercuric chloride to Muller's fluid to improve the fixation of nuclei. Tissues preserved by this method stain well with many technics, but it is suggested that they be post fixed, or placed, in 2.5% aqueous solution of potassium dichromate for 2 hours following Zenker's fixation. (This is especially necessary if thick specimens are to be fixed). Then wash in running water for 12 hours. (Small pieces (4 mm in thickness) usually are fixed completely in 6 - 8 hours).

Note. When staining, a longer time may be required for Zenker-fixed tissues to "take" the hematoxylin stain, while the counterstain may have to be diluted and the staining time decreased.

ZENKER - FORMALIN (HELLY'S) SOLUTION

Zenker-formalin or Helly's solution is a modification of Zenker's solution by the addition of formalin instead of glacial acetic acid (to 95 ml of Zenker's solution add

5 ml of 37 — 40% formalin just before use). Especially good for demonstrating mitochondria. It fixes well for routine processing.

BOUIN'S SOLUTION

Picric acid, saturated aqueous solution	750.0 ml
37 — 40% formalin	250.0 ml
Glacial acetic acid	50.0 ml

Fix blocks from 4 to 12 hours depending on the size. It is important to wash in several changes of 50% alcohol for 4-6 hours, agitating constantly, to insure proper removal of the picric acid. Store in 70% alcohol. *Note*. The removal of picric acid from tissues is most essential in order to insure proper staining of the tissue sections. It has been demonstrated in our laboratories, that tissues undergo deleterious effects, as evidenced in the staining, when the picric acid has not been properly removed and remains in the tissue throughout the entire processing.

This harmful reaction continues in effect, within the embedded specimen for a number of years. An excellent stained section is secured several days after fixation and conversely, a very poorly stained section is demonstrated several months later upon recutting the same paraffin block. Therefore, the necessity of removing picric acid from tissues cannot be over emphasized.

HELLY'S SOLUTION

See Zenker-Formalin, page 4.

CARNOY'S SOLUTION

Absolute alcohol	60.0 ml
Chloroform	30.0 ml
Glacial acetic acid	10.0 ml

One of the best *penetrating and quickly acting* fixatives known. Generally, 3 hours is adequate for normal size tissue. No washing is necessary, and the tissue may be transferred immediately to absolute alcohol. It gives excellent nuclear fixation with preservation of Nissl substances, plasma cells, and glycogen, *but,* it hemolyzes red blood cells.

CLARKE'S SOLUTION

Absolute alcohol	75.0 ml
Glacial acetic acid	25.0 ml

This penetrates rapidly, resulting in good nuclear fixation and reasonably good preservation of cytoplasmic elements. It is an excellent fixative for smears or coverslip preparations of cell cultures. Small pieces (2 mm in thickness) usually are fixed completely in 3 hours. Material so fixed, and not entirely used in processing, may be stored indefinitely in 80% alcohol. Coverslip preparations of cell cultures are fixed in 10-20 minutes. Tissue specimens should be washed in 70% alcohol for 1 hour to remove acetic acid whereas only a five minute wash is necessary for smears and coverslip preparations.

NEWCOMER'S SOLUTION

Isopropanol	60.0 ml
Propionic acid	30.0 ml

Petroleum-Ether	10.0 ml
Acetone	10.0 ml
Dioxane	10.0 ml

Primarily used for chromosome studies, this fixative preserves the chromatin better than Carnoy's and gives an improved Feulgen reaction. Fixation usually is complete in 12-18 hours; small pieces (2mm in thickness) in 2-3 hours. Following the use of Newcomer's solution, place in 60% alcohol for 4 hours.

ORTH'S SOLUTION

Potassium dichromate	2.5 gm
Sodium sulfate	1.0 gm
Distilled water	100.0 ml
Mix and add: formalin, 37-40%	10.0 ml

Place in this fixative for 24 hours. Transfer to a 2.5% aqueous solution of potassium dichromate for 48 hours. Wash in running water overnight. Addition of 5 ml of acetic acid improves this fixative for the demonstration of chromaffin cell granules. This fixative does not keep and must be prepared just before use. *Note*. It penetrates fairly rapidly and evenly, but has a tendency to harden tissues. It is recommended for the demonstration of chromaffin cells and glycogen.

GLUTARALDEHYDE

Limited studies in the use of glutaraldehyde as a fixative for electron microscopy and also as a fixative for routine paraffin sections have been conducted. Studies in this area will continue and more precise information, methods, and technics will be evolved. At the present time only limited information concerning glutaraldehyde and its use as a fixative can be obtained. For this reason no specific instructions for fixation are given in this manual. The following references on this reagent are recommended:

1. Sabatini, D. D., Bensch, K., and Barrnett, R.: Cytochemistry and electron microscopy: and preservation of cellular ultrastructure of enzymes by aldehyde fixation. *J. Cell. Biol.* 17:19-58, 1963

2. Yanoff, M., Zimmerman, L. E., and Fine, B. S.: Glutaraldehyde fixative of whole eyes. *Amer. J. Clin. Path.* **44**:167-171, 1965.

3. Yanoff, M., and Fine, B. S.: Glutaraldehyde fixative of routine surgical eye tissue. *Amer. J. Ophthal.* **63**:137-140, 1967.

PARAFORMALDEHYDE

Paraformaldehyde also has been used recently for the fixation of electron microscopy sections as well as for routine surgical and autopsy material. For further information regarding this fixative see the following reference:

1. Lynn, J. A., Martin, H. H., and Race, G. H.: Recent improvement of histologic technics for the combined light and electron microscopic examination of surgical specimens *Amer. J. Clin. Path.* **45**:704-713, 1966.

DECALCIFICATION

Calcification is the term applied to organic tissues which have been infiltrated with calcium salts. These salts provide hardness and igidity to bone and must be removed

to assure that the specimen is soft enough to allow cutting with the equipment available. The necessity for decalcification makes it impossible to perform many critical studies on bone. In some cases, only when special microtomes are available for sectioning undecalcified specimens could the complete histologic picture be made possible.

Bone and other calcified material should be cut into small pieces (approximately 5mm) with a fine saw before fixation. After adequate fixation, place in a large quantity of decalcifying solution, at least a quart, for blocks of average size. Stirring, agitation, and the use of vacuum hastens decalcification and should be employed when possible. Tissues suspended in the upper third of the fluid during decalcification will allow the calcium salts to sink to the bottom of the container as they are dissolved. Suspension of tissue can be accomplished in a number of ways: (1) Place tissue in a gauze bag suspended with dental floss or string which has been dipped in hot paraffin; or (2) place in a perforated porcelain dish on the bottom of the container. Since decalcification acids continue to act on tissue specimens during any subsequent tissue handling (including paraffin storage) it is important that every trace of decalcifying solution be removed by washing the specimens in running water for several hours before processing can take place. For determining the decalcification end point methods see page 10. Various methods of decalcification follow:

PERENYI'S METHOD

1. Place calcified specimen in large quantities of Perenyi's fluid until decalcification is complete.

PERENYI'S FLUID

10% Nitric acid, aqueous	40.0 ml
Absolute alcohol	30.0 ml
0.5% chromic acid, aqueous	30.0 ml

The various ingredients may be kept in stock, and should be mixed immediately before use. This solution may acquire a blue violet tinge after a short while but this will have no effect on its decalcifying properties.

2. Wash in running water for 2 hours. Store in 95% alcohol if processing cannot continue following this step.

3. Dehydrate, clear and impregnate with paraffin or process as desired.

REMARKS. Perenyi's fluid is slow for decalcifying hard bone, but is an excellent fluid for small deposits of calcium, e.g. calcified arteries, coin lesions, and calcified glands. It may also be an excellent decalcifying fluid for avian eye globes which contain bone normally, at the corneal-scleral junction and for human globes which contain calcium due to pathologic conditions. Little hardening effect of tissue takes place, and excellent morphologic detail is preserved with this technique.

NITRIC ACID METHOD 1

1. Place calcified specimen in large quantities of nitric acid solution until decalcification is complete. (Change solution daily for best results).

5% NITRIC ACID SOLUTION

Nitric acid, concentrated (68—70% Sp. gr. 1.41)	5.0 ml
Distilled water	95.0 ml

2. Wash in running water for 30 minutes.

3. Neutralize for a minimum of 5 hours in 10% formalin to which an excess of calcium or magnesium carbonate has been added.

4. Wash in running water overnight.

5. Dehydrate, clear and impregnate with paraffin or process as desired.

REMARKS. This is a fairly rapid method. Care must be exercised, however, to remove specimen from decalcifying solution shortly after decalcification is complete, since overexposure to nitric acid impairs or destroys nuclear staining. Nitric acid is the acid of choice for decalcifying temporal bones. For this procedure see page 48.

NITRIC ACID METHOD II

1. Place calcified specimen in large quantities of nitric acid-alcohol solution until decalcification is complete. (Change solutions daily for best results).

Alcohol, 80%	95.0 ml
Nitric acid, concentrated (68 – 70% Sp. gr. 1.41)	5.0 ml

2. After specimens are completely decalcified transfer directly to a 4% aqueous sodium sulphate solution for 3 hours.

3. Wash specimens in running water for 2 hours to remove sodium sulphate. When time permits, overnight washing is preferred.

4. Dehydrate, clear and impregnate with paraffin or process as desired.

REMARKS. The decalcification effected with this method is considerably faster than that of method I, since readily soluble fats are dissolved somewhat by the alcohol thereby accelerating the decalcifying process.

FORMIC ACID-SODIUM CITRATE METHOD

1. Place calcified specimen in large quantities of formic acid-sodium citrate solution until decalcification is complete. (Change solutions daily for best results).

SOLUTION A

Sodium citrate	50.0 gm
Distilled water	250.0 ml

SOLUTION B

Formic acid, 90%	125.0 ml
Distilled water	125.0 ml

Mix solutions A and B in equal portions for use.

2. Wash in running water from 4-8 hours.

3. Dehydrate, clear and impregnate with paraffin or process as desired.

REMARKS. This technic gives better staining results than the nitric acid method since formic acid-sodium citrate is less harsh on the cellular properties. Therefore, overexposure of tissue to this solution, after decalcification has been completed is possible with little loss of staining qualities. This method has become the one of choice for all orbital decalcification including the globes (see page 53).

COMMERCIAL DECALCIFYING METHODS

There are several commercial decalcifying solutions available, each of which can be used satisfactorily by following these general directions.

1. Place in "decal" solution until decalcification is complete. (Change solution daily for best results.)
2. Wash in running water for a minimum of 16 hours, 24 hours preferred.
3. Dehydrate, clear and impregnate with paraffin or process as desired.

REMARKS. These methods have proven very useful in our laboratories since by use of any one of them the compounding of solutions is eliminated. Cellular destruction is very rapid, however, when bone specimens are allowed to remain in these solutions for several hours after complete decalcification has been achieved. Therefore, it becomes necessary to determine the decalcification end point precisely.

ELECTROLYTIC METHOD

The electrolytic apparatus is composed of both positive and negative carbon plates for electrodes which are immersed in the solution. The labeled specimen is attached with dental floss to the positive carbon plate.

PROCEDURE

1. Decalcify with electrolytic apparatus in the following solution until decalcification is complete.

DECALCIFYING SOLUTION

Hydrochloric acid, concentrated	80.0 ml
Formic acid, 90%	100.0 ml
Distilled water	1000.0 ml

2. Wash in running water for a minimum of 16 hours, 24 hours preferred.
3. Dehydrate, clear and impregnate with paraffin or process as desired.

CHELATE'S AS DECALCIFYING AGENTS

Organic chelating agents have been recommended for the decalcification of bone specimen. When used, excellent preservation of histologic detail is observed with subsequent demonstration of various tissue components possible. The slow speed at which these agents work dictates that the size of the specimen for decalcification must not exceed 4mm in thickness.

There is presented below one method which is the most useful in routine histology. For other procedures, however, see the following references:

1. Freiman, D. G.: Organic chelating agent in demineralization of bone for histochemical study of alkaline phosphatase. *Amer. J. Clin. Path.* **24**: 227-231, 1954.

2. Trott, J. R.: The presence of glycogen in the rat liver following in vitro processing in decalcifying agents. *J. Histochem. Cytochem.* 9: 699-702, 1961.

3. Balogh, K.: Decalcification with versene for histochemical study of oxidative enzyme systems. *J. Histochem. Cytochem.* 10:232-233, 1962.

VERSENATE METHOD

1. Fix in 10% buffered neutral formalin.
2. Place in saturated solution of Versenate* until decalcification is complete.

*Versenate (Versene) is a proprietary term for ethylenediaminetetraacetate.

VERSENATE SOLUTION

Versenate ..... 10.0 gm
Distilled water (pH 5.5 to 6.5). ..... 100.0 ml

3. Place in 70% alcohol. Washing is unnecessary.
4. Dehydrate, clear and impregnate with paraffin or process as desired.

REMARKS. Most bone specimens (3mm thick) are decalcified in 2-4 days but specimens may be left in Versenate solution for as long as 14 days without noticeable effects on the staining quality of the cell. This is especially true if the solution is between 5.5 and 6.5 pH. Versenate may be used for decalcification of dense calcified materials. This however, may require considerable time; in that event, the Versenate decalcifying solution should be changed at intervals of 3 or 4 days. The advantage of the Versenate method lies in the preservation of the staining affinity of the cells.

REFERENCE. Birge, E. A., and Imhoff, C. E.: Versenate as a decalcifying agent for bone. *Amer. J. Clin. Path* 22: 192-193, 1952.

PROCEDURES FOR DETERMINING END POINT OF DECALCIFICATION

One of the greatest problems encountered in decalcification is the failure of technicians to realize that the selection of a decalcifying fluid is *not* the most important consideration. Most important to be considered, and the one point receiving the least attention, is the removal of calcified specimens from fluids *immediately* after complete decalcification is accomplished. If this is *not* done, the chances of subsequent good staining reactions are reduced 10% for every 2 hours the tissue remains in the decalcifying solution. It must be recognized that 90% of the poor staining qualities demonstrated in decalcified tissues, is due to this one factor. For this reason, one of the following methods should always be employed in all laboratories, to determine the decalcification end point.

CHEMICAL METHOD

Draw approximately 5 ml of decalcifying fluid (from bottom of container) which has been in contact with tissue for 6-12 hours. Add 5 ml each of 5% ammonium hydroxide and 5% ammonium oxalate. Mix, and let stand 15 to 30 minutes. A cloudy solution caused by calcium oxalate indicates that the specimen is not thoroughly decalcified. Such a result indicates the necessity of changing the decalcifying solution and performing the test at a later time or date. When a milky solution is no longer obtained from such a mixture, the specimen is completely decalcified. This test can be performed as frequently as necessary.

X-RAY METHOD

Undoubtedly the best method for determining complete decalcification is by X-raying the specimen.

SPECIMEN FLEXIBILITY METHOD

This method should be used only after considerable experience with decalcification.

Bend specimen gently; if it bends with little resistance it is well decalcified. One may also press the specimen gently with fingernail; areas containing calcium will not give (bend inward) and considerable resistance will be noticed.

KERATIN AND CHITIN SOFTENING PROCEDURES

There are no highly satisfactory procedures for softening keratin and/or chitin which would result in both rapid softening and subsequent good section staining. By use of concentrated sulfuric acid with the aid of heat, keratin can be completely dissolved from the tissue section. However, much tissue destruction also will occur.

For the softening of chitin the following procedure is found to give a satisfactory result.

1. Fix specimens in a fixative of choice.
2. Place specimens in the following solution until completely de-chitinized. Change solution every 2 days for best results.

Mercuric chloride	4.0 gm
Chromic acid	0.5 gm
Nitric acid, concentrated	10.0 ml
Ethyl alcohol, 95%	50.0 ml
Distilled water	200.0 ml

3. Wash in running water for 3 hours.
4. Dehydrate, clear and impregnate with paraffin or process as desired.

Chapter 2

Processing of Tissue

DEHYDRATING, CLEARING, IMPREGNATING, AND EMBEDDING

A specimen brought to the laboratory is usually marked with an identifying number or name. Keep this identification with the specimen throughout processing. All identifying marks should be made with a soft lead pencil. Do not use ink or wax pencils.

The surface from which sections are to be cut may be indicated by notching the opposite surface, or by marking it with India ink. An indelible lead pencil may also be used for this purpose. When the tissue is embedded in paraffin, the marked surface of the block is uppermost.

Fixed tissues must be maintained in position by a firm medium so that thin, uniform sections, can be cut. Media suitable for this purpose are paraffin, celloidin, and carbowax.

Processing by the paraffin technic is accomplished most rapidly and gives the best results when thin sections of soft tissue are desired. Since paraffin is not miscible with water, the tissue must be dehydrated and then cleared in solutions miscible with paraffin before impregnation.

Well processed tissue is achieved by a step by step infiltration of the required reagents each preparing the tissue for the one to follow, so that the end result will be a section closely resembling the living state of the specimen. Every cell should be recognizable as to type, enabling the pathologist to focus all his attention on the cell pattern which determines the diagnosis. Properly fixed tissue is essential, as the following steps of processing build upon it.

It is always advisable to remove fixatives before processing. For various methods note remarks under each fixative in Chapter 1.

Dehydration is the removal of all extractable water by a dehydrant diffusing through the tissue, and in the process diluting itself 2 – 4%. Some dehydrants used are tetrahydrofuran, acetone, dioxane, isopropyl alcohol, and ethanol.

Alcohol is the most commonly used dehydrant usually tarting *with 80%*. Exceptions to this are when processing tissue with cavities, cysts, and embryos, which will have *less shrinkage and distortion when started in 60%*. Compact or fibrous tissue, such as muscle, brain, lymph nodes, and glands infiltrate more rapidly and completely in the 60%, *especially when vacuum is employed*. Allow sufficient time in the starting alcohol for complete infiltration of the tissue. The dehydration process continues by upgrading the alcohols to absolute alcohol. Isopropyl alcohol can be used as a substitute for absolute alcohol if necessary, but absolute alcohol is always preferred. *Note*. Isopropyl alcohol should not be used to dissolve dyes and reagents until it has been tested for each use against absolute alcohol.

Acetone provides a rapid method, used sometimes in hospital laboratories, and when required as a "stat" method. The low cost is an asset, but shrinkage and distortion plus a subsequent dryness and hardness which causes cutting problems, are disadvantages to its use.

Dioxane is a rapid dehydrant, but the fumes are highly toxic and its use requires careful control in a well ventilated area. Clearing is not a necessary step in dioxane processing and when used the tissue will retain a softer texture than when acetone is used. It is recommended when it is necessary to expose tissues to a dehydrant clearing agent up to 48 hours.

Example: Large pieces requiring longer exposure for complete clearing and dehydration.

Tetrahydrofuran possesses essentially the same dehydrating and clearing properties as dioxane.

Clearing reagents must be miscible with the dehydrant and the paraffin. As the dehydrant is removed, the tissue clears, becoming translucent signifying the completion of the process. The one exception is chloroform, since the tissues do not become translucent in it.

Xylene is the most widely used clearing reagent, but it hardens tissue more than chloroform unless the clearing time is controlled. Xylene, toluene, and benzene are difficult to remove during the paraffin impregnation. All clear well, however. Cloroform is the clearing agent of choice in our laboratories.

Impregnation is the complete removal of the clearing reagents, by substitution, as the paraffin penetrates the tissue with use of no less than two, and preferably, three paraffin baths. Most laboratories use paraffins with a melting point of 56 – 58 °C. A frequent check of the temperature of the paraffin baths is a *must*, since more than 5 °C above melting point of the paraffin will cause excessive tissue shrinkage and hardening. Vacuum, when applied during embedding, will remove air, gases, and any remaining clearing agent, and at the same time aid in drawing the paraffin into all areas of the specimen, especially those areas left void by the evacuation of air.

Recommended paraffins for use in embedding are:

Paraplast is perhaps the best embedding medium for use in conventional histological processing. It should not be used, however, when thin-walled circular specimens are to be cut, because it prevents complete expansion of the specimens, such as cross sections of eyes, trachea, cysts, veins, etc.

Bioloid is a good embedding medium in which thin-walled structures can be sectioned satisfactorily. Its elasticity permits its use to give particularly satisfactory results with circular specimens.

Tissuemat possesses somewhat the same properties as *Bioloid*. *Note*. Paraffins have a tendency to crystallize if exposed for long periods at temperatures 10 ° above the melting point. This crystallized paraffin makes sectioning difficult and therefore should be avoided. Paraffins may pick up some moisture and small amounts of the clearing agent during processing. However, these can be evaporated by reheating the paraffin for a short time, not more than 30 minutes at 20 ° above the melting point. Ideally, paraffin should not be re-used but instead, be replaced and changed frequently.

Straight paraffin is not recommended because the cutting consistency of the product is not firm enough. The sections compress and wrinkles are difficult, if not impossible, to remove. It lacks the elasticity that aids in obtaining wrinkle-free ribbons. But it can be used if 10 – 20% beeswax is added to overcome these deficiencies.

EMBEDDING IN PARAFFIN

Embedding is the orientation of tissue in melted paraffin, which when solidified, provides a firm medium for keeping intact all parts of the tissue when sections are

cut. Each laboratory has a preference as to the method used for embedding with the two most often used being lead L's and the Tissue Tech embedding system.

Embedding can be accelerated by the use of a shallow pan which can be purchased or made by any metal worker. This method is used exclusively at Armed Forces Institute of Pathology for embedding of multiple blocks. Pans with slightly sloping sides, ranging from 1 to 2 inches in depth and 8 x 10 inches in length and width, are satisfactory.

Placed on a masonite rack, which holds it about 6 inches above the desk top, (Fig. 1), the pan is warmed gently with a Bunsen burner, or a 250-watt reflector infrared heat lamp* of 115-120 volts, having a squeeze holder attached (Fig. 2). This heat lamp may be purchased at most hardware or drug stores. In the pan filled with filtered, melted paraffin, each piece of tissue is placed in position with the appropriate identifying string tag beside it. When all tissues are oriented and in place, the paraffin is hardened by rubbing an ice cube across the bottom of the pan. In order to achieve a flat surface for cutting, press specimen down gently with forceps, making sure that all parts of the tissue are flat. When the paraffin has cooled sufficiently so that a heavy film forms across the top, the pan is floated on cold water. The paraffin, when hardened throughout will contract from the sides of the pan and the solid block can be lifted out and the tissue blocks cut to the appropriate sectioning size.

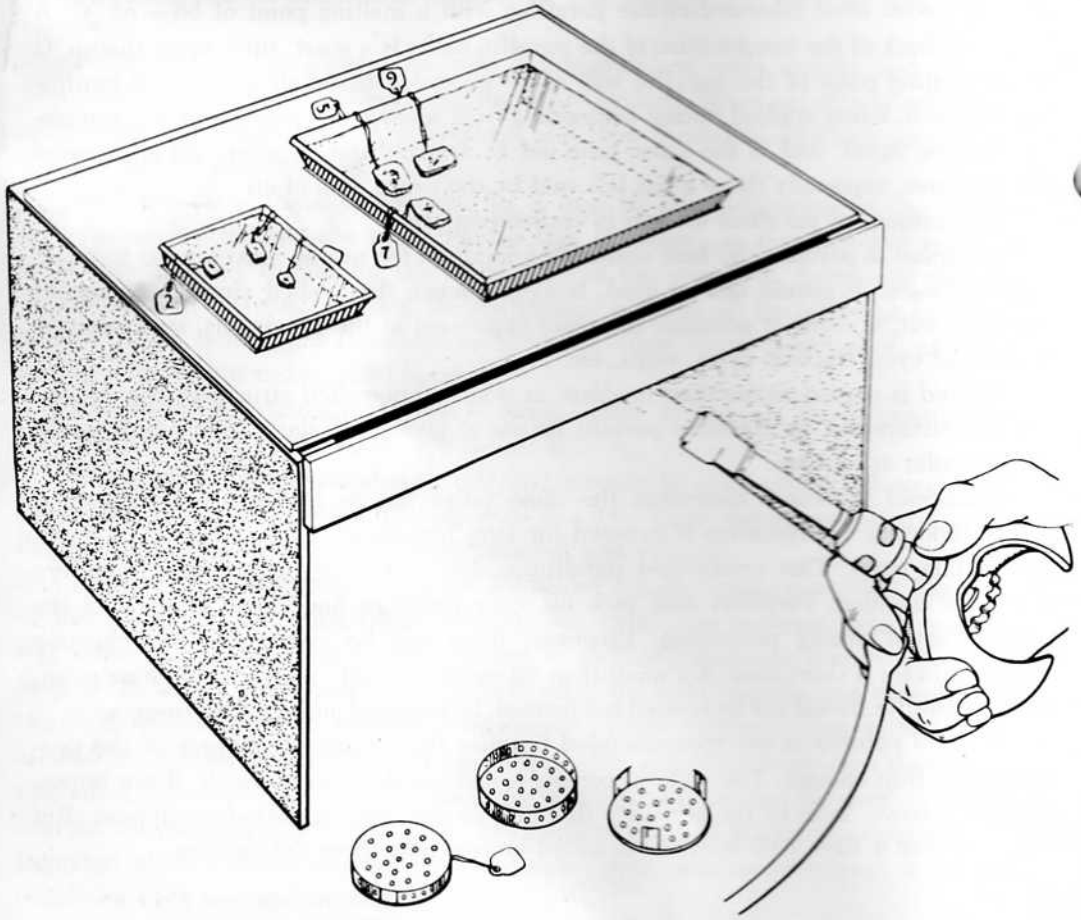


Fig. 1. Multiple block embedding in paraffin.

*Slaughter, E. S., HT(ASCP): The use of the infrared heat lamp for paraffin embedding and blocking, *Amer. J. Clin. Path.* 39: 65-66, 1963.

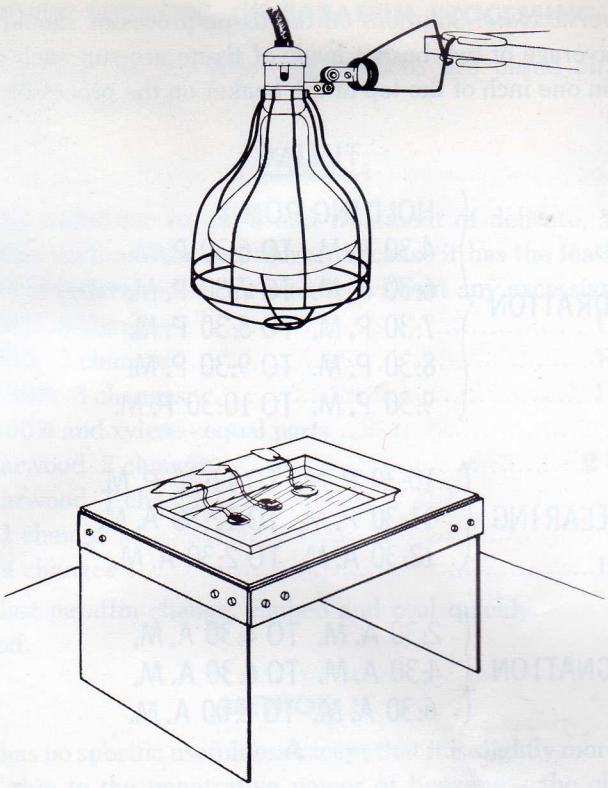


Fig. 2. 250-watt reflector infrared heat lamp with squeeze holder for suspending lamp above embedding set-up.

GENERAL COMMENTS

1. When transferring tissue from impregnating pot to embedding mold, do not allow a thin layer of paraffin to solidfy around the specimen. If by chance it should form, it must be completely melted to avoid a hair line separation between the tissue and the embedding medium in the blocked specimen. 2. Extra care must be used when embedding multiple pieces of tissue in the same block in matching types and densities of tissue. This simple precaution will facilitate tissue cutting. 3. Imcomplete dehydration will result in poor clearing and impregnation; faulty processing in these areas will produce shrinkage and drying of the specimen when placed in the hot paraffin. This effect can be recognized quickly when a depression forms in the surface of the block, and will also be evident when sectioning, since improperly processed tissue turns white on exposure to water. Sections cut from a block such as this can be expected to "explode" or disintegrate when placed on the water bath.

Note. Improperly processed blocks should be taken back through xylene to remove paraffin, through absolute alcohol, 95% alcohol and 80% alcohol. Then they should be dehydrated slowly, cleared and reinfiltrated with paraffin and re-embedded. See artifacts page 245.

Figure 3A is the basic processing schedule utilized at Armed Forces Institute of Pathology and can be altered to suit any specific need. For example, xylene may be substituted for chloroform in the schedule below in instances where chloroform is not

available or vice versa. *Note.* Solutions on the tissue processor should be changed once a week when an average of two basket loads of tissue are run each day. The solutions must be kept within one inch of the top of the beaker on the processor.

STEP		TIMING	SOLUTION
1.	DEHYDRATION	HOLDING POINT	80% ALCOHOL
2.		4:30 P.M. TO 6:30 P.M.	95% ALCOHOL
3.		6:30 P.M. TO 7:30 P.M.	95% ALCOHOL
4.		7:30 P.M. TO 8:30 P.M.	100% ALCOHOL
5.		8:30 P.M. TO 9:30 P.M.	100% ALCOHOL
6.		9:30 P.M. TO 10:30 P.M.	100% ALCOHOL
7.	CLEARING	10:30 P.M. TO 11:30 P.M.	CHLOROFORM
8.		11:30 P.M. TO 12:30 A.M.	CHLOROFORM
9.		12:30 A.M. TO 2:30 A.M.	CHLOROFORM
10.	IMPREGNATION	2:30 A.M. TO 4:30 A.M.	PARAFFIN
11.		4:30 A.M. TO 6:30 A.M.	PARAFFIN
12.		6:30 A.M. TO 8:00 A.M.	PARAFFIN

A

Fig. 3. (A)Schedule of processing in paraffin used at Armed Forces Institute of Pathology.

Figure 3B is the processing schedule for the Central Nervous System used at the Armed Forces Institute of Pathology. The use of one of the three methods is dictated by the thickness of the specimen, for example, handprocessing may be necessary for excessively thick brain specimens.

	OVERNIGHT (Auto Technicon)		WEEK END (2 DAYS) (Auto Technicon)		HAND PROCESSING
WASH	AS TIME ALLOWS				
HOLD 80%	AS TIME ALLOWS				
95%	4:30 PM - 6:30 PM	2 HRS	3:00 PM - 5:00 PM	2 HRS	10:00 AM - 12:00 NOON
95%	6:30 PM - 7:30 PM	1 HR	5:00 PM - 6:00 PM	1 HR	12:00 NOON - 2:00 PM
95%	7:30 PM - 8:30 PM	1 HR	6:00 PM - 7:00 PM	1 HR	2:00 PM - 4:00 PM
ABSOLUTE	8:30 PM - 9:30 PM	1 HR	7:00 PM - 4:00 AM	9 HRS	OVERNIGHT
ABSOLUTE	9:30 PM - 10:30 PM	1 HR	4:00 AM - 12 NOON	8 HRS	8:00 AM - 9:00 AM
ABSOLUTE	10:30 PM - 11:30 PM	1 HR	12 NOON - 3:00 PM	3 HRS	9:00 AM - 10:00 AM
CHLOROFORM	11:30 PM - 12:30 PM	1 HR	3:00 PM - 5:00 PM	2 HRS	10:00 AM - 11:30 AM
CHLOROFORM	12:30 PM - 2:30 AM	2 HRS	5:00 PM - 6:00 PM	1 HR	11:30 AM - 1:00 PM
*PARAFFIN	2:30 AM - 4:30 AM	2 HRS	6:00 PM - 7:00 PM	1 HR	1:00 PM - 3:00 PM
*PARAFFIN	4:30 AM - 6:30 AM	2 HRS	7:00 PM - 4:00 AM	9 HRS	3:00 PM - 4:00 PM
*PARAFFIN	6:30 AM - 8:00 AM	1 1/2 HRS	4:00 AM - 8:00 AM	4 HRS	SOLIDIFY (OVERNIGHT)
*PARAFFIN (Vacuum)	8:00 AM - 10:00 AM	2 HRS	8:00 AM - 10:00 AM	2 HRS	WARM AND THEN VACUUM FOR 2 HOURS

B

Fig. 3. (B)Processing schedules for routine brain tissue. Normal size not to exceed 5 mm in thickness.

*Paraplast preferred 56° – 58°C.

available or vice versa. *Note.* Solutions on the tissue processor should be changed once a week when an average of two basket loads of tissue are run each day. The solutions must be kept within one inch of the top of the beaker on the processor.

STEP		TIMING	SOLUTION
1.	DEHYDRATION	HOLDING POINT	80% ALCOHOL
2.		4:30 P.M. TO 6:30 P.M.	95% ALCOHOL
3.		6:30 P.M. TO 7:30 P.M.	95% ALCOHOL
4.		7:30 P.M. TO 8:30 P.M.	100% ALCOHOL
5.		8:30 P.M. TO 9:30 P.M.	100% ALCOHOL
6.		9:30 P.M. TO 10:30 P.M.	100% ALCOHOL
7.	CLEARING	10:30 P.M. TO 11:30 P.M.	CHLOROFORM
8.		11:30 P.M. TO 12:30 A.M.	CHLOROFORM
9.		12:30 A.M. TO 2:30 A.M.	CHLOROFORM
10.	IMPREGNATION	2:30 A.M. TO 4:30 A.M.	PARAFFIN
11.		4:30 A.M. TO 6:30 A.M.	PARAFFIN
12.		6:30 A.M. TO 8:00 A.M.	PARAFFIN

A

Fig. 3. (**A**)Schedule of processing in paraffin used at Armed Forces Institute of Pathology.

Figure 3B is the processing schedule for the Central Nervous System used at the Armed Forces Institute of Pathology. The use of one of the three methods is dictated by the thickness of the specimen, for example, handprocessing may be necessary for excessively thick brain specimens.

	OVERNIGHT (Auto Technicon)		WEEK END (2 DAYS) (Auto Technicon)		HAND PROCESSING
WASH	AS TIME ALLOWS				
HOLD 80%	AS TIME ALLOWS				
95%	4:30 PM - 6:30 PM	2 HRS	3:00 PM - 5:00 PM	2 HRS	10:00 AM - 12:00 NOON
95%	6:30 PM - 7:30 PM	1 HR	5:00 PM - 6:00 PM	1 HR	12:00 NOON - 2:00 PM
95%	7:30 PM - 8:30 PM	1 HR	6:00 PM - 7:00 PM	1 HR	2:00 PM - 4:00 PM
ABSOLUTE	8:30 PM - 9:30 PM	1 HR	7:00 PM - 4:00 AM	9 HRS	OVERNIGHT
ABSOLUTE	9:30 PM - 10:30 PM	1 HR	4:00 AM - 12 NOON	8 HRS	8:00 AM - 9:00 AM
ABSOLUTE	10:30 PM - 11:30 PM	1 HR	12 NOON - 3:00 PM	3 HRS	9:00 AM - 10:00 AM
CHLOROFORM	11:30 PM - 12:30 PM	1 HR	3:00 PM - 5:00 PM	2 HRS	10:00 AM - 11:30 AM
CHLOROFORM	12:30 PM - 2:30 AM	2 HRS	5:00 PM - 6:00 PM	1 HR	11:30 AM - 1:00 PM
*PARAFFIN	2:30 AM - 4:30 AM	2 HRS	6:00 PM - 7:00 PM	1 HR	1:00 PM - 3:00 PM
*PARAFFIN	4:30 AM - 6:30 AM	2 HRS	7:00 PM - 4:00 AM	9 HRS	3:00 PM - 4:00 PM
*PARAFFIN	6:30 AM - 8:00 AM	1 1/2 HRS	4:00 AM - 8:00 AM	4 HRS	SOLIDIFY (OVERNIGHT)
*PARAFFIN (Vacuum)	8:00 AM - 10:00 AM	2 HRS	8:00 AM - 10:00 AM	2 HRS	WARM AND THEN VACUUM FOR 2 HOURS

B

Fig. 3. (**B**)Processing schedules for routine brain tissue. Normal size not to exceed 5 mm in thickness.

*Paraplast preferred 56° – 58°C.

OTHER METHODS OF PARAFFIN PROCESSING

The processing time for the following methods are based on specimens which do not exceed 3mm in thickness.

METHOD I

This method is useful for research and treatment of delicate, as well as hard or dense fibrous tissues, such as skin and uterus, because it has the least hardening effect. Tissues may be left in cedarwood oil for months without any excessive hardening.

Alcohol, 80% - 2 changes	1 hour each
Alcohol, 95% - 2 changes	1 hour each
Alcohol, 100% - 3 changes	1 hour each
Alcohol, 100% and xylene - equal parts	1 hour
Oil of cedarwood - 2 changes	2 hours each
Oil of cedarwood - 1 change	1 hour
Paraffin - 1 change	2 hours
Paraffin - 4 changes	1 hour each

Vacuum the last paraffin change. Embed and cool quickly.
Cut as desired.

METHOD II

This method has no specific usefulness except that it is slightly more rapid in penetration of the tissue, due to the penetrative power of benzene — the clearing agent. The advantage of benzene is that the tissue becomes more translucent as the alcohol is replaced, making it possible to determine the tissue clearing and end-point, and thereby avoiding overexposure to the drastic hardening effects of this reagent.

Alcohol, 80% - 2 changes	1 hour each
Alcohol, 95% - 2 changes	1 hour each
Alcohol, 100% - 3 changes	1 hour each
Benzene - 2 changes	1 hour each
Paraffin - 4 changes	1 hour each

Vacuum the last paraffin change. Embed and cool quickly.
Cut as desired.

METHOD III

This method has several advantages:

1. Since dioxane is both a dehydrant and clearing agent, it can be used for rapid processing due to the elimination of alcohol.
2. Specimens may be left in dioxane for long periods without excessive hardening.
3. When processing by hand and complete processing cannot be accomplished during the regular working hours, normal sized tissues can be left in this reagent overnight and the routine processing continued in the morning. However, dioxane is toxic and should be used in a well ventilated area.

Dioxane and water - equal parts	1 hour
Dioxane, 100%	1 hour
Dioxane, 100%	2 hours
Paraffin - 4 changes	1 hour each

Vacuum the last paraffin change. Embed and cool quickly.
Cut as desired.

METHOD IV

Tetrahydrofuran is being used in some laboratories with good results. It has the same properties as dioxane in that it serves as both a dehydrant and a clearing agent whereby processing schedules can be accelerated considerably. (We have been able to process thin tissue specimens which do not exceed 3mm in 6 hours). It has also been used very satisfactorily as suggested in advantage 3 of Method III. Tetrahydrofuran is toxic and should be used in a well ventilated area.

Tetrahydrofuran and water - equal parts ..... 1 hour
Tetrahydrofuran, 100% ..... 1 hour
Tetrahydrofuran, 100% ..... 2 hours
Paraffin - 3 changes ..... 1 hour each

Vacuum the last paraffin change. Embed and cool quickly.
Cut as desired.

METHOD V

This method is recommended for biopsies as well as bone marrow and cytological buttons. This method for processing minute pieces of tissue prevents the tissue from becoming hard and brittle due to prolonged periods in the various agents. It is being presented through courtesy of the Walter Reed Army Hospital Pathology Department, Laboratory Service, by Mr. Dan Romeika, Supervisor.

Alcohol, 80% ..... 15 minutes
Alcohol, 95% - 2 changes ..... 15 minutes each
Alcohol, 100% - 2 changes ..... 15 minutes each
Chloroform - 2 changes ..... 15 minutes each
Paraffin - 2 changes ..... 15 minutes each

Embed and cool quickly. Cut as desired.

DOUBLE EMBEDDING METHOD

A double embedding method, in which a combination of celloidin and paraffin is used, may prove advantageous in some instances. The preservation of the morphological relationship during conventional processing is the usual, and often, the only reason, for employing this technic.

The method given below is for specimens not in excess of 3mm in thickness. For thicker specimens, double the time in each solution for each additional 1mm thickness.

Alcohol, 60% ..... 3 hours
Alcohol, 80% - 2 changes ..... 3 hours each
Alcohol, 95% - 2 changes ..... 2 hours each

For convenience, specimen may be left overnight in this solution.

Alcohol, 100% - 3 changes ..... 2 hours each
Ether and alcohol, 100% equal parts - 2 changes ..... 2 hours each

Celloidin, 2% ..... 24 hours
Clearing oil mixture ..... 8 hours

CLEARING OIL MIXTURE

Chloroform ..... 4 parts
Oil of origanum ..... 2 parts
Oil of cedarwood ..... 4 parts
Alcohol, 100% ..... 1 part
Phenol ..... 1 part

Benzene - 2 changes ..... 4 hours each

For convenience, specimen may be left in this solution overnight.

Paraffin - 4 changes 1 hour each.

Embed in paraffin and cool quickly. Cut as desired.

Cutting of sections is done in the usual manner. However, cutting double embedded specimens may present some problems since the normal characteristic of paraffin is to stretch on the flotation bath but it is prevented from doing so by the celloidin. In this event, float section in 95% alcohol for 20 minutes, to soften the celloidin, before placing on flotation bath.

CARBOWAX METHOD

Polyethlene glycols (Carbowax) are water soluble waxes and therefore their use eliminates the need of dehydration and clearing, consequently saving valuable processing time. Most stains can be applied to the tissue section after processing in carbowax. Very little shrinkage and/or tissue distortion, often encountered by conventional methods, is produced with the use of carbowax.

1. Fix small sections of tissue in the desired fixative.
2. Wash well in running water.
3. Impregnate in 56 °C oven for 3 hours in a mixture of carbowax 4000, 9 parts; and carbowax 1500, 1 part. This mixture should be prepared in advance and kept in the incubator. The quantities of each component of the mixture may be varied as required by the individual laboratory and dictated by local conditions, in order to control the hardness of the resulting embedding medium.
4. After 2 - 3 hours in incubated carbowax mixture, place in a fresh mixture of melted carbowax in a small pan or paper boat. Chill in refrigerator for 15 - 30 minutes. Remove block from embedding mold.
5. Cut on rotary microtome at room temperature. Place sections directly on the slide and press down with a gentle rolling motion of the index finger. If this technic is not satisfactory, the sections may be floated and fixed to the slide with the following solution in the flotation bath:

Potassium dichromate ..... 0.2 gm
Gelatin ..... 0.2 gm
Distilled water ..... 1000.0 ml

6. Place slide on warming table in usual manner, temperature not to exceed 40° C.
7. Stain as desired.

REMARKS. All contact with xylene and alcohols must be avoided. Carbowax will become brittle if overheated, and crumbling may occur during cutting. The block must not be allowed to come in contact with ice or water as carbowax is very hygroscopic. If hardening of the block is necessary to eliminate section compression during cutting, it can be hardened with a commercially available freon "Cryokwik"°, or dry ice. A convenient method used in our laboratories is the placement of a 32 oz funnel filled with dry ice, over the specimen, by means of a ring stand. The nipple of the funnel is placed 3-4 inches above the tissue specimen (which is oriented on the microtome ready to be cut). The cold dry-ice fumes being heavier than room temperature atmosphere shoot downward, enveloping the specimen. This method keeps the immediate area of the block and knife in a considerably colder state facilitating carbowax cutting. Store carbowax embedded blocks in a cool, low moisture area. For additional information and uses of carbowax the following references are recommended, especially, if thin sections (1-2 micron) are desired.

REFERENCES. 1. Sidman, R. L., Mottla, P. A., and Feder, N.: Improved polyester wax embedding for histology. *Stain Tech.* **36**:279-284, 1961.

2. Menzies, D. W.: Paraffin-beeswax-stearic acid: An embedding mass for thin sections. *Stain Tech.* **37**: 235-238, 1962.

3. Reid, J. D., and Taylor, D.: An improved method for embedding tissues using polyethlene glycols, with incorporation of low viscosity nitrocellulose. *Amer. J. Clin. Path.* **41**: 513-516, 1964.

°International Equipment Co., 300 Second Avenue, Needham Heights, Mass. 38586

Chapter 3

Preparation of Sections

CARE AND USE OF MICROTOME KNIVES

The cutting of good sections depends greatly upon practical experience and a complete thorough knowledge of the equipment used. Manual dexterity is a *must*; without it one may face a difficult task in handling the fine manipulative detail required in section cutting. Hurried and inadequate introductory and/or initial training will reflect badly for years afterward; conversely a high standard of training will prevail admirably throughout one's career. *Speed* in performing any phase of histologic technique should *never* be a primary objective since it only leads to unsatisfactory processing, cutting, and staining of the tissue sections. A well trained tissue technician will produce first rate sections in a far shorter time than one who always is aiming primarily at speed.

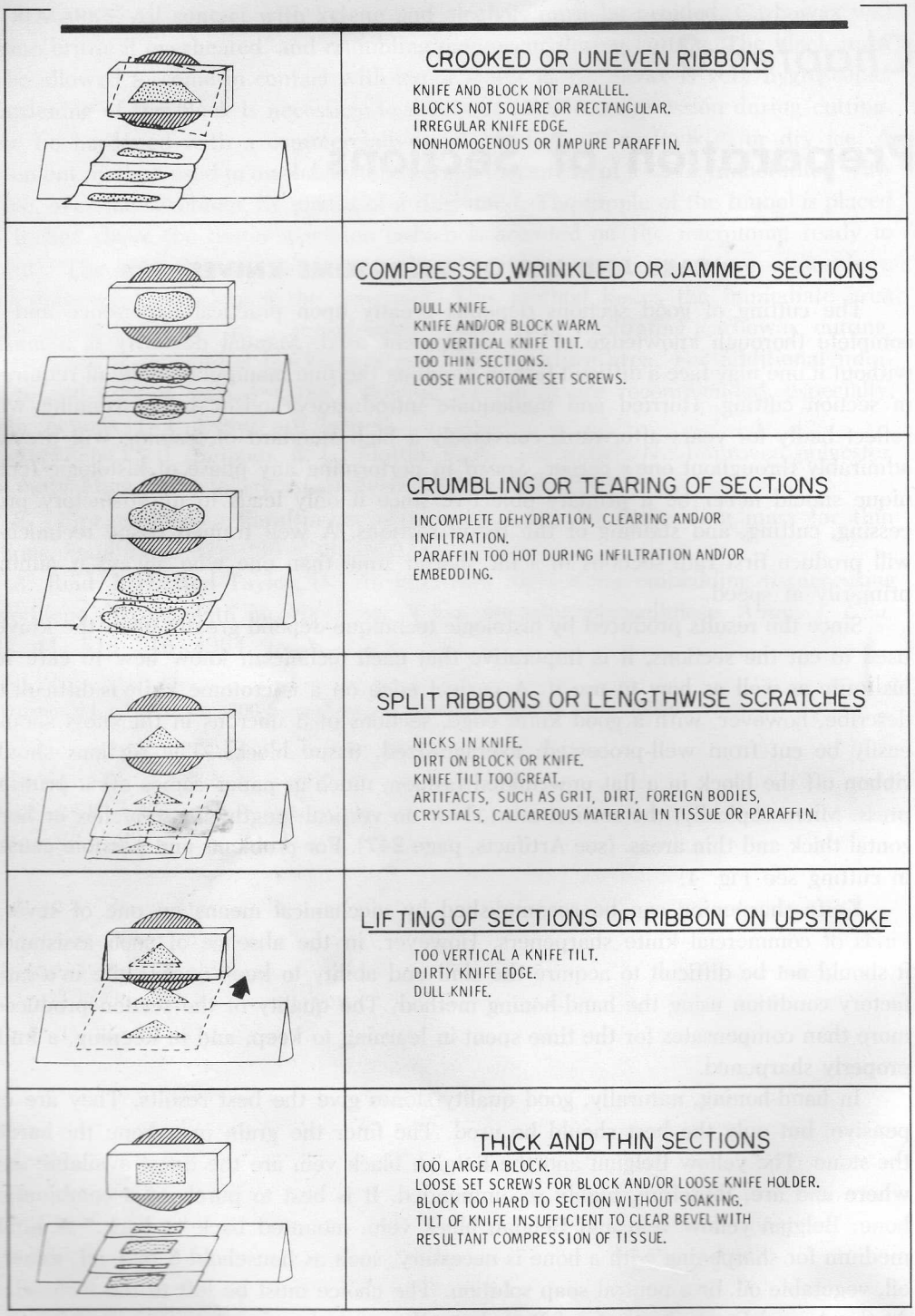
Since the results produced by histologic technique depend greatly upon the knives used to cut the sections, it is imperative that each technician know how to care for his knife as well as how to use it. A perfect edge on a microtome knife is difficult to describe, however, with a good knife edge, sections of 3 microns in thickness should easily be cut from well-processed, average-sized, tissue blocks. The sections should ribbon off the block in a flat unwrinkled fashion, much as paper comes off a printing press. Microscopically, the section must show no vertical lengthwise scratches or horizontal thick and thin areas. (see Artifacts, page 247). For problems and possible causes in cutting see Fig. 4.

Knife sharpening can be accomplished by mechanical means on one of several kinds of commercial knife sharpeners. However, in the absence of such assistance, it should not be difficult to acquire the skill and ability to keep one's knife in a satisfactory condition using the hand-honing method. The quality of the section produced, more than compensates for the time spent in learning to keep, and in keeping, a knife properly sharpened.

In hand-honing, naturally, good quality stones give the best results. They are expensive, but only the best should be used. The finer the grain in a hone the harder the stone. The yellow Belgian and the Belgian black vein are the finest available anywhere and are, therefore, highly recommended. It is best to purchase a combination hone: Belgian yellow vein and Belgian black vein, mounted back to back.* A liquid medium for sharpening with a hone is necessary, such as household 3 in 1 oil, mineral oil, vegetable oil, or a neutral soap solution. The choice must be left to the technician. At the Armed Forces Institute of Pathology, the neutral soap solution is used because it can be made readily by dissolving household (bar) soap in water.

While honing, the knife should be kept flat, held to the hone by its own weight, with its edge facing the direction of the "heel to toe" motion, under continuous but light pressure (Fig. 5).

*Microtome Knife Hone - Yellow Belgian, Fisher Scientific Co., 7722 Fenton St., Silver Spring, Md. 20910



PREPARED BY: MRS. E. B. PROPHET, AFIP, HISTO LABS

Fig. 4. Commonly encountered problems in cutting and their causes.

Whether the sharpening is done by hand, or on any one of the various machines, all knives require and are equipped with a honing back to be used while sharpening in order to maintain the required bevel angle. The edge should be finished on a leather

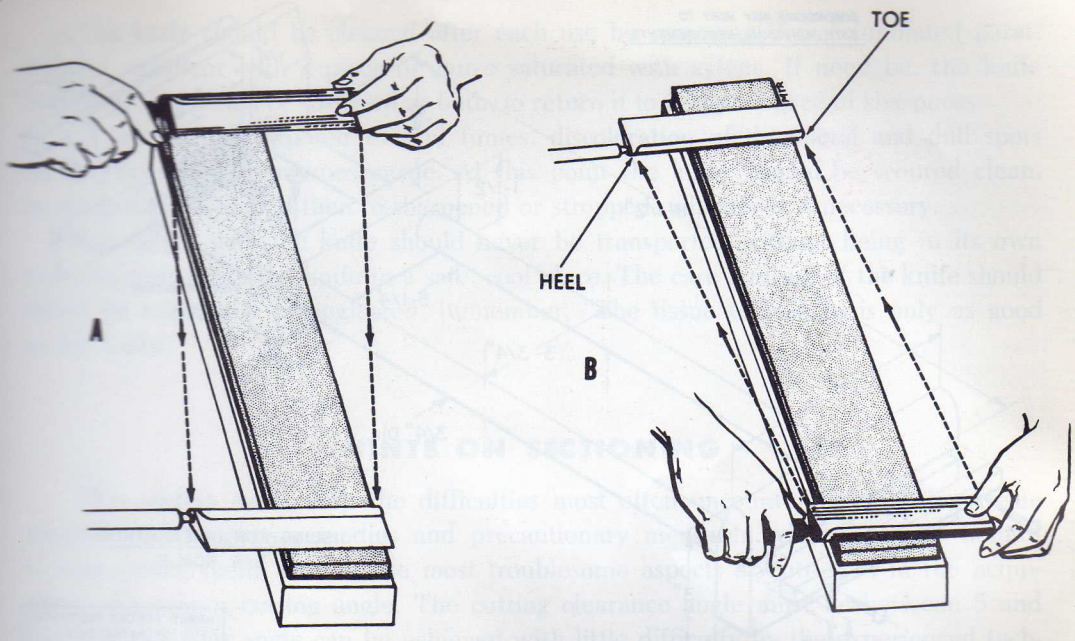


Fig. 5. Diagram of the technique of honing the microtome knife.

or linen strop (Fig. 6) to remove the microscopic knife serrations caused by any one of the various sharpening methods.

This important step is often *minimized*, and is responsible for many cutting problems. Regardless of the stropping device used, stropping should be performed so that all areas of the knife edge are exposed to the strop surface, equally if possible. The movement of the knife should be from toe to heel for 8 to 12 times (Fig. 7A). This movement should then be reversed to from heel to toe 8 to 12 times (Fig. 7B).

However, it must be clearly understood that it is possible to ruin a well-sharpened knife during the stropping phase. The most common defect noted is a turned or rounded edge, which happens when too much pressure is applied to the knife while stropping or as in the case of the linen strop, when the linen is not stretched tautly enough. Theoretically, the cutting edge is not a true edge. It is, in fact, a tiny part of a circumference of a minute circle with the arc measuring approximately 0.25 microns. The length of this arc is increased considerably in a rounded edge, sometimes to as much as 0.75 microns.

This becomes important if one remembers the theoretical mechanism by which sections are cut. The impregnating matrix and tissue in the block are compressed in front of the cutting edge of the knife, which in turn wedges off the section in a tearing crushing manner at submicroscopic levels. It stands to reason, then, that the wider the circumference of the arc the more crushing and tearing effect will prevail and more resistance will be met by the knife edge. This resistance will always result in considerable compression of a section — a sign of a poorly sharpened knife (see page 247).

The microtome knife should always be maintained at its highest degree of sharpness. Only through experience can one know the "feel" of a sharp knife as opposed to that of a dull knife. Many methods for determining sharpness are employed but the ultimate test is in cutting the tissue block. If the block does not section satisfactorily after considerable preparation, assume that the knife is dull and re-sharpen it.

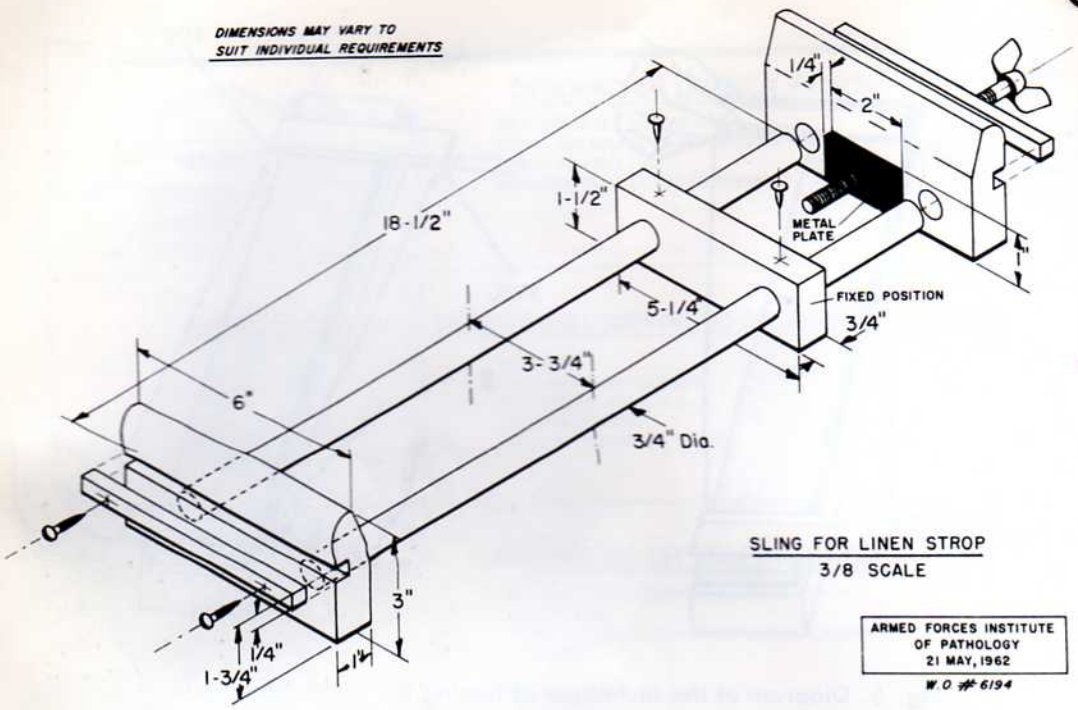


Fig. 6. Sling for a linen strop. Linen for the strop can be purchased at most fabric stores. It should be cut to the length and width dictated by the sling with pinking shears and the linen attached to the sling with the wooden slats and screws shown in the drawing. The large screw at the head of the sling facilitates stretching the linen to the desired tension.

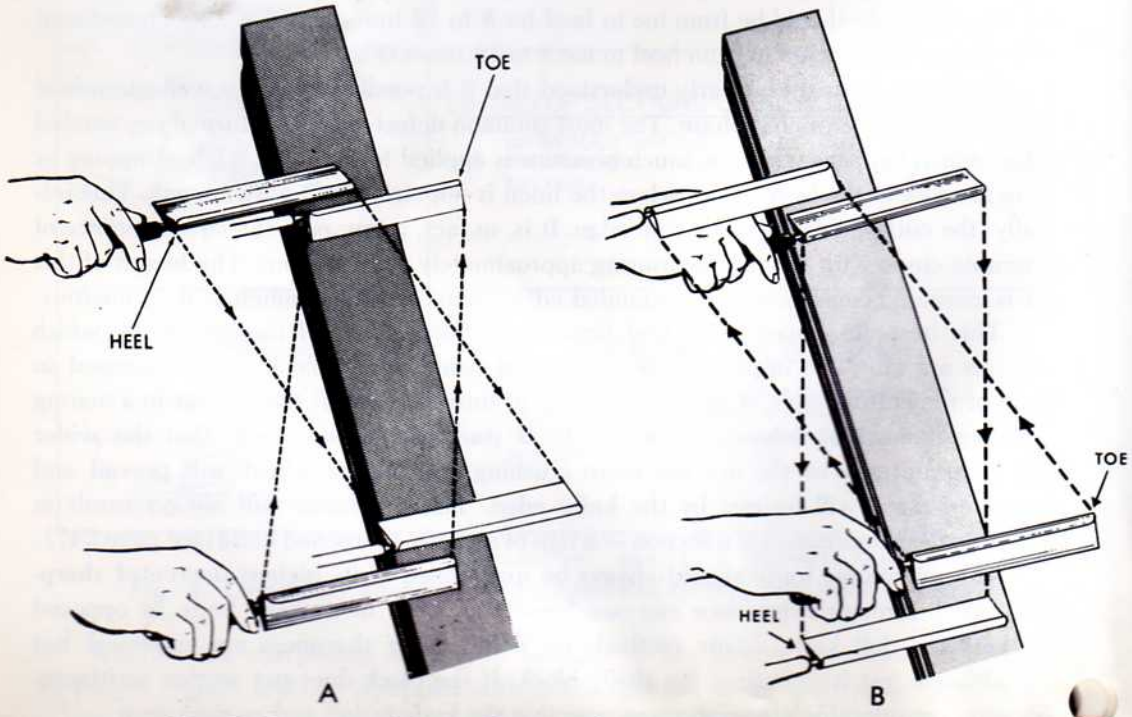


Fig. 7. Diagram of the technique of stropping a microtome knife.

The knife should be cleaned after each use by removing the accumulated paraffin and sediment with a piece of gauze saturated with xylene. If need be, the knife should be sharpened or stropped or both, to return it to a high degree of sharpness.

If the knife is exposed to acid fumes, discoloration of the metal and dull spots on the knife will sometimes ensue. At this point the knife should be scoured clean, to remove the acid, and then re-sharpened or stropped, whichever is necessary.

For safety sake the knife should never be transported without being in its own carrying case. Store the knife in a safe, cool place. The care and use of the knife should never be minimized or neglected. Remember, "The tissue technician is only as good as his knife."

HINTS ON SECTIONING

This section deals with the difficulties most often encountered in cutting tissue sections and suggests remedies and precautionary measures which may be helpful in overcoming them. One of the most troublesome aspects of cutting is in the acquisition of a proper cutting angle. The cutting clearance angle must be between 5 and 10° (Fig. 8). This angle can be achieved with little difficulty by the experienced technician without the use of an angle aid. For those less experienced however, an "angle jig," which can be made simply of aluminum or soft metal, may be useful.

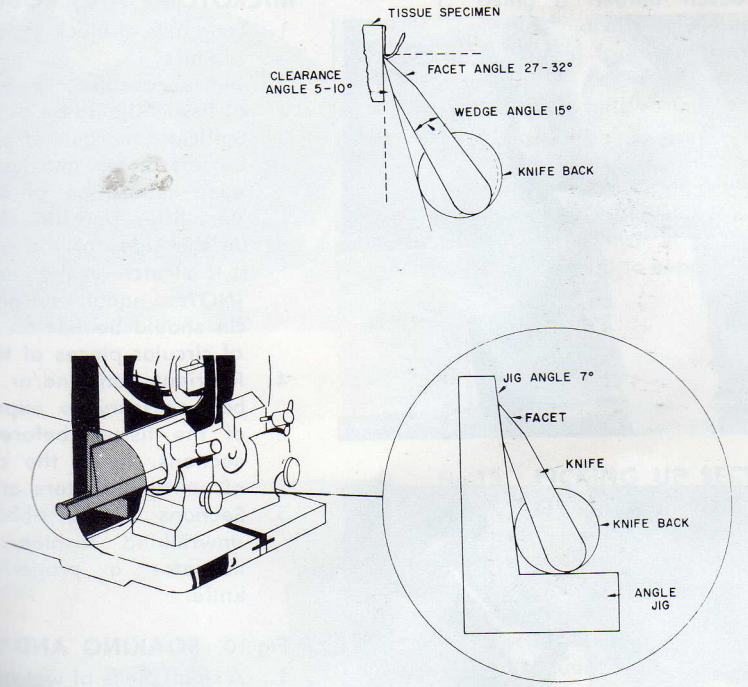


Fig. 8. Diagram showing the use of an angle jig to assure the proper cutting angle.

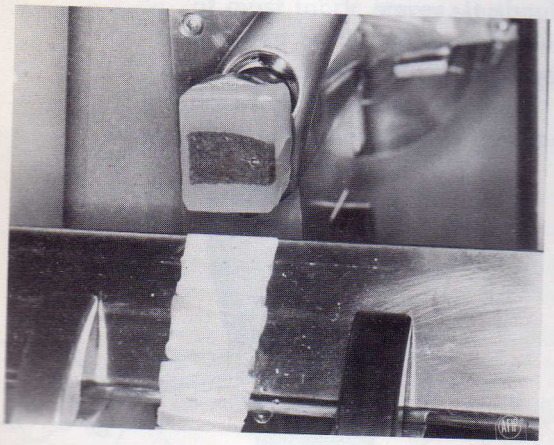
An angle jig is simple to use: Place the knife in the microtome knife clamp making certain it protrudes at least four inches to one side. Place the knife back on the protruding end of the knife. The angle jig is then placed behind the knife making sure

that the knife back rests on the jig (Fig. 8). At this point adjust the knife angle until the facet is flat and rest completely on the angle jig assuring a true 7° setting. It must be remembered that this setting may have to be changed occasionally for different types of tissue. For example hard fibrous tissue such as uterus and bone may require a setting of 15°. These are exceptions however, since most tissue will cut at an angle clearance between 5 – 10°.

A good tissue technician will study each block to determine placement in the microtome so that he may get the most representative sections of the tissue submitted. He will check also the resultant sections to be sure that the best possible sections have been obtained. When dissatisfied with the results he must determine the necessary steps or modifications to make in order to achieve that goal. For example, it is possible to obtain satisfactory (though not the best) sections from tissue which has been poorly processed when ice is applied to both specimen and knife (see fig. 10).

The production of sections of varying degrees of perfection is controlled by the manner in which the microtome is operated. Good sections are the result of a slow even turn of the wheel, whereas uneven (thick and thin) sections can be the result of a fast jerky movement of the microtome wheel.

The various steps in sectioning tissue, with suggestions for orienting the block, separating sections, picking up the sections on glass slides, and resealing the block are shown in Figures 9 – 14.



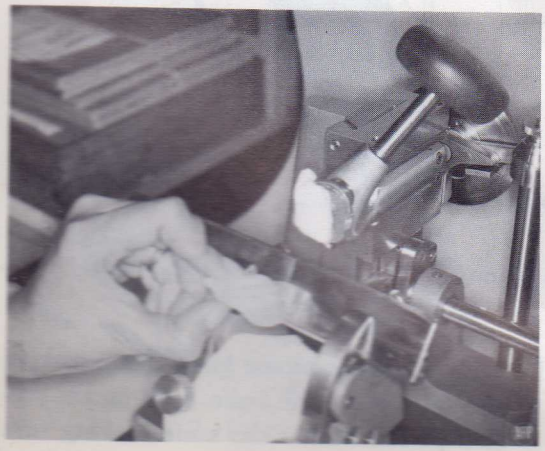


Fig.9. **ORIENTATION OF BLOCK IN MICROTOME AND ROUGH CUTTING**

1. Long axis of block should be parallel to knife.
2. Dense capsuled, or tough surfaces of tissue should be at the top.
3. Sufficient margins of paraffin should be left at top and bottom to allow easy separation of sections later. Very little paraffin should be left on the sides of the block to allow it to stretch on the water bath. (*NOTE.* equal margins of paraffin should be left on all four sides of circular pieces of tissue).
4. Reorientation and/or angling may be necessary to expose all areas of the tissue, before taking final sections. Avoid the common error of omitting centers or corners.
5. Sections should ribbon in a flat unwrinkled fashion. This always indicates a properly sharpened knife.

Fig.10. **SOAKING AND/OR ICING**

1. A small piece of wet cotton is placed over the exposed tissue and block. Warm water is preferred since it penetrates more rapidly.
2. To insure constant temperature of both block and knife, chill each with an ice cube. As a general rule, with tissue from the central nervous system it is necessary to ice the knife only.

Fig.11. **FLOATING RIBBON ON FLOTATION BATH**

1. One end of the ribbon is lowered first, the remainder of the ribbon lowered gradually with a slight pull. If done in this fashion there is less tendency for bubbles to form underneath the section.
2. To remove wrinkles place a finger at one end of the ribbon and with a camel's hair brush pull gently the opposite end thus stretching out the wrinkle.

Fig.12. **SEPARATION OF SECTIONS**

1. Using a heated tissue separator, sections are easily and uniformly separated from each other. In case of serial or step sections, do not separate more than one section at a time.
2. Avoid over-heating or under-heating the tissue separator, since a loss of valuable sections can occur. (*NOTE*. Forceps or glass slides can also be used to separate sections).

Fig.13. **PICKING UP SECTIONS ON GLASS SLIDES**

1. Insert clean, dust free slides into the flotation bath perpendicularly. With the aid of a camel's hair brush orient section so that it is centrally placed allowing free margins for subsequent coverslipping and labeling.
2. If bubbles occur under the sections, they can be brushed out before the slides are dried.
   (*NOTE:* Sections should be drained approximately 1 minute before final drying in a 60°C oven for 30 minutes or on a slide warming table overnight.)

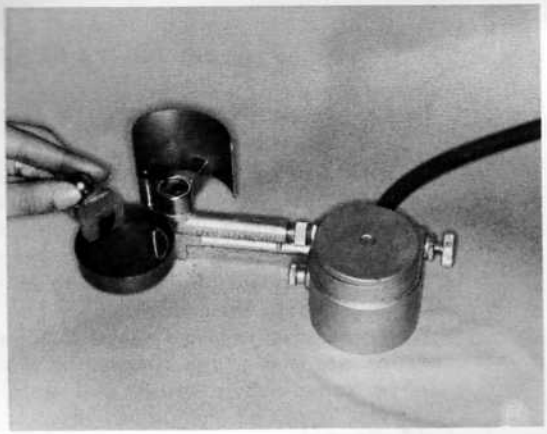


Fig.14. **RESEALING BLOCKS**

1. After the desired number of sections are taken all blocks should be paraffin sealed. This prevents drying and other damage which can alter and sometimes ruin the tissue, making future sectioning difficult or impossible.
2. Using a block sealer as illustrated, eliminates several movements and also the possibility of burning the tissue with an over heated knife or spatula.

ATTACHING SECTIONS TO SLIDES (SECTION ADHESIVES)

Paraffin sections may be attached to slides in several ways. A small drop of Mayer's egg albumin is smeared over the surface of the slide with the finger and the excess rubbed off with the heel of the hand, or it can be applied with a clean foam-rubber sponge. A sponge is usually preferred so that the epithelial cells from the finger will not adhere to the slide and produce artifacts when slides are stained.

MAYER'S EGG ALBUMIN

Egg white ..... 50.0 ml
Glycerin ..... 50.0 ml

Mix well and filter through coarse filter paper or Buchner's funnel with vacuum or through several thicknesses of gauze, add a crystal of thymol to preserve. Commercial egg albumin can be purchased, ready for use.

ADHESIVE FROM DRIED ALBUMIN

Albumin, dried ..... 5.0 gm
Sodium chloride ..... 0.5 gm
Distilled water ..... 100.0 ml

Filter through Buchner's funnel with vacuum, to 50 ml of filtrate, add 50 ml of glycerin. Add a crystal of thymol to preserve.

GELATIN ADHESIVE

The gelatin adhesive added to the flotation bath has proved to be a better medium for affixing sections to the slides than egg albumin. The procedure also has the advantage of saving time since it eliminates smearing each slide. Because the gelatin is a culture medium for bacteria and fungi, it is important that the floatation bath and camel hair brushes be cleaned with a detergent after each day's use to insure against contamination of the section, which might confuse the diagnosis (see Artifacts page 249).

Gelatin, pharmaceutical ..... 5.0 gm
Distilled water ..... 100.0 ml

Dissolve with the aid of heat. Add several crystals of thymol to preserve. For use, thoroughly mix 3 teaspoonfuls of the 5% gelatin solution per 1000 ml, in the flotation bath.

Although the section adhesives presented above have proven to be most satisfactory for routine sections, there frequently arises the need for a more firm section attachment. For example, when sections are treated with potassium permanganate, enzymes, or extremely alkaline solutions, they have a tendency to become separated from the slide. The following methods have proven useful in providing a more firm section attachment in such instances.

GELATIN-FORMALIN

Formalin hardens the protein contained in gelatin and egg albumin. This effect can be accomplished by exposing gelatinized or albuminized slides to formalin and thus achieving a firmer bond between the section and the glass slide.

1. Gelatin or egg albumin is applied as described earlier.
2. Sections are placed on slides from the flotation bath and drained from 30 seconds to 1 minute.
3. Place slides in a coplin jar containing approximately 5 ml of formalin (full strength), making certain the formalin solution in no way comes in contact with the tissue section. The tightly covered coplin jar is then placed in a 60° C oven for one hour.
4. Remove sections from coplin jar and dry in the conventional way i.e., either on slide warmer overnight or in an oven for several hours.
5. Treat and/or stain slides as desired.

THIN CELLOIDIN ADHESIVE FOR PARAFFIN SECTIONS

Paraffin sections which tend to become separated from the slide because of the action of acids and alkalis used in the various staining procedures, and which are extremely dried or which contain bone and keratinized material may be held more firmly to the slide if they are coated with a thin film of 0.5 — 1% celloidin before staining.

1. Deparaffinize the slide in xylene and run through several changes of absolute alcohol.
2. Immerse in 0.5 — 1% celloidin solution for 1 to 2 minutes.
3. Air-dry sections for 30 minutes or until sections turn white.
4. Immerse sections in 80% alcohol for 1 to 2 minutes.
5. Rinse in distilled water and proceed with desired stain.

H. C. Cook conducted a study on section adhesives and their effects with various treatments. This study may be found in *Stain Tech.* 40: 321-328, 1965.

Note. Sections which are to be stained with the following procedures should not be coated with thin celloidin since the celloidin has a strong affinity for the stains used in these procedures: Aldehyde fuchsin, alcian blue, mucopolysaccharide techniques, mucicarmine, Gridley fungus and the periodic acid-Schiff stain.

TECHNIC FOR FROZEN SECTIONS

Frozen sections are necessary for the demonstration of fats and other lipids. They are used also in rapid diagnostic techniques as well as in certain silver impregnation methods.

The Histo-Freeze* and/or Section-Freeze† are excellent units for cutting frozen sections. Each instrument consists of a portable box chamber which generates pressurized liquid freon. These units have become very popular since they obviate the need for carbon dioxide tanks which were necessary when using the clinical microtomes. At the Armed Forces Institute of Pathology the sliding microtome is found to be the most useful. Still another piece of equipment for producing frozen sections is the well known Cryostat. Regardless of the equipment used frozen section procedures must not be taken lightly and every effort made to achieve the best possible results in both cutting and staining.

The following technic is preferred in our laboratory:

1. Fix small blocks of tissue in 10% buffered neutral formalin.
2. Remove fixative by washing specimens in water before cutting.
3. Place a drop of water on the specimen holder and place the block in position parallel to the knife edge.
4. Apply gentle downward pressure on the tissue with a glass slide and release freon until specimen is frozen. (Directions for use of the freon units are provided by the manufacturer, therefore, they will not be repeated here).

Currently there are available embedding compounds which will assist in cutting, and are, therefore, recommended when frozen sectioning. These, embedding matrix M-1† or O. C. T. (Optimum cutting temperatures) ‡ compound provide a surrounding matrix which holds the block and prevents shattering at the free end. It is also possible to impregnate tissue blocks with this material. (Directions for their use are provided by the manufacturer).

5. Start sectioning and continue until a complete section is obtained. If specimen is frozen too hard the section will shatter. In this case thaw the surface of the block by placing a finger on the specimen, but if it becomes too soft sections will be impossible to obtain. The correct temperature can only be judged by experience. It is best to cut slowly.
6. Lift the section from the knife with the middle finger which has been dipped in distilled water and place the section in a dish of distilled water (wipe the knife free of water between sections since water will cause the succeeding section to be uneven or perforated). Sections may be picked up on albuminized slides and dried before staining or they may be stained singly by carrying each section through the various solutions with a bent glass rod. A simple aid in staining frozen sections in quantity is the sieve-like container made from 1 inch sections of plastic or glass tubing with walls 1/8 inch thick and the diameter ranging from 1-3 inches. Over one end of each ring a piece of nylon stocking is stretched tightly and glued to the outside wall with Duco Cement. When the cement is thoroughly dried the excess nylon is trimmed off. These containers are transferred from one solution to another with forceps.

REFERENCE. Albercht, M. H.: *Stain Tech.* 31: 231, 1956.

GELATIN EMBEDDING

If frozen sections of extremely fragile tissue or of small fragments of exudate are to be cut, the material can be embedded in gelatin using the following procedure:

*Scientific Products Inc., 2020 Ridge Ave, Evanston, Ill. 60201

†Lipshaw Manufacturing Co., 7446 Central Avenue, Detroit, Michigan 48210

‡ Arthur H. Thomas Co., Vine Street at Third, Post Office Box 779, Philadelphia, Pennsylvania 19105

GELATIN SOLUTIONS, 5, 10, & 20%

Gelatin solutions of pure pharmaceutical grade gelatin are prepared in strengths of 5, 10, and 20 percent. If made in quantity, add a few crystals of thymol as a preservative.

1. Wash specimens thoroughly in running water.
2. Impregnate with 5% gelatin in 40° C oven for 16 hours.
3. Continue impregnation with 10% gelatin in 40° C oven for 16 hours.

Note. If vacuum is used the time in steps two and three may be cut to 2 hours each.

4. Embed in 20% gelatin.
5. Place in refrigerator overnight.
6. Trim block for sectioning.
7. Harden by immersion in 10% formalin (the length of time in formalin to be determined by the desired firmness of the block).

Chapter 4
Routine Staining Procedures

HEMATOXYLIN AND EOSIN STAINS

Hematoxylin, a natural dye which was first used about 1863, is without doubt the most valuable staining reagent used in histologic work. It has little affinity for tissue when used alone but in combination with aluminum, iron, chromium, copper or tungsten salts it is a powerful nuclear stain. It has polychromatic properties which may be brought out with the proper differentiation. The active coloring agent, hematein, is formed by the oxidation of hematoxylin. This process known as "ripening" takes several days, or weeks, unless it is hastened by the addition of an oxidizing agent such as mercuric oxide, hydrogen peroxide, potassium permanganate, sodium perborate, or sodium iodate. These artificial oxidizers only start the process, so that the solution can be used immediately, but this oxidation process continues slowly over a period of time, during which the hematoxylin retains its staining properties. Once oxidation is complete, the hematoxylin is no longer useful for staining. By increasing the amount of the oxidizing agent the process is much more rapid, thus decreasing the life of the stain so that the amount listed for a given formula should be closely observed. Storage is another factor that has an effect on oxidation. When stored in a dark, tightly sealed container the process is slower, but when in staining dishes exposed to light and air it is markedly increased, therefore, in a staining setup, the solution should be changed at least once a week for consistent staining results.

The most common formulas for staining with hematoxylin are the combinations with aluminum in the form of alum. Those in general use were formulated by Harris, Mayer, Delafield, Ehrlich, Bullard and Bohmer. Sections stained with alum hematoxylins may be counterstained with Eosin, Safranin, Phloxine or other contrasting stains.

The hematoxylins that are combined with iron and tungsten also have their uses. Iron hematoxylin is used in staining myelin and as a nuclear stain in many of the trichrome and other special stain procedures. The one used most extensively in our laboratory was formulated by Weigert. Mallory's phosphotungstic acid hematoxylin (PTAH) is also often used, however not as a nuclear stain.

Although the hematoxylin and eosin stain has been used for nearly a hundred years, a few words of admonition concerning the stain are in order. The word "routine," as applied to the hematoxylin and eosin procedure, should not be used in this connection for Webster defines "routine" as a regular, more or less unvarying procedure. Any one that has worked with the H&E stain knows that many factors contribute to cause some variation in this technic. Examples of this could be: the fixative used, the fixation exposure time, age of staining solution, etc. Therefore, we should not allow the term "routine" to cause the normal amount of laxness usually associated with it.

There are two methods of staining when hematoxylin is employed: Progressive and Regressive.

Progressive staining is accomplished by employing a hematoxylin solution which contains an excess of aluminum salts or acid, thus increasing the selectivity for nuclei. After staining with hematoxylin, the slides are washed well in water and the secondary stain applied.

In *regressive* staining, the sections are overstained in a relatively neutral solution of hematoxylin. The excess stain is removed with an acid alcohol solution, then the sections are neutralized with an alkaline solution such as weak ammonia water or lithium carbonate water. This differentiation is controlled by the use of the microscope, and with experience and a trained eye, the technician can produce excellent results. In a well-differentiated section, the cytoplasm should be colorless and nuclear substances should be clearly visible, showing some metachromatic properties.

Counterstains for hematoxylins are a matter of personal preference, with eosin probably the one most commonly used. Too much time will not be devoted to the discussion of counterstains; but there are a couple of suggestions which should be made. Counterstains are defined as stains applied to render the effects of another stain more discernible. Although this is true, in many cases, it does not apply to the H&E technique since, in this case, it plays a definite diagnostic role in its demonstration of inclusion bodies and cytoplasmic changes. More correctly, it should be considered a secondary stain. With this in mind, one should realize the value of the various steps subsequent to eosin staining.

The most common error often encountered is the improper use of alcohols after the eosin has been applied. The primary purpose of the alcohols is dehydration. But just as important is the removal of excess eosin. By passing rapidly through these alcohol solutions excess eosin will overshadow many diagnostic features, such as pigments and inclusion bodies. It cannot be stressed enough that proper differentiation of the eosin by the alcohol is a *must*, in order to produce H&E sections of high quality. Therefore, it follows that proper differentiation of eosin, or any counterstain, should be considered just as important as proper differentiation of hematoxylin.

REMARKS. Poor hematoxylin staining can often be attributed to improper mixing of the aluminum ammonium sulfate or aluminum potassium sulfate. These chemicals serve as mordants for the hematoxylin and therefore must be in complete solution with the other ingredients. The directions given for mixing hematoxylin should be followed closely. *When mixing hematoxylins be certain that all ingredients are in solution.* For artifacts produced in H&E staining see page 250.

The following is a partial list, with directions for compounding, of the more frequently used hematoxylins.

MAYER'S HEMATOXYLIN

Hematoxylin crystals	1.0 gm
Distilled water	1000.0 ml
Sodium iodate	0.2 gm
Ammonium or potassium alum	50.0 gm
Citric acid	1.0 gm
Chloral hydrate	50.0 gm

Dissolve the alum in water, without heat; add and dissolve the hematoxylin in this solution. Then add the sodium iodate, citric acid, and the chloral hydrate, shake until all components are in complete solution. The final color of the stain is reddish-violet. Stain keeps well for months.

HARRIS' HEMATOXYLIN

Hematoxylin crystals	5.0 gm
Alcohol, 100%	50.0 ml
Ammonium or potassium alum	100.0 gm
Distilled water	1000.0 ml
Mercuric oxide (red)	2.5 gm

Dissolve the hematoxylin in the alcohol, the alum in the water by the aid of heat. Remove from heat and mix the two solutions. Bring to a boil as *rapidly* as possible. (Limit this heat to less than 1 minute and stir often). Remove from heat and add the mercuric oxide slowly. Reheat to a simmer until it becomes dark purple, remove from heat immediately and plunge the vessel into a basin of cold water until cool. The stain is ready for use as soon as it cools. Addition of 2-4 ml of glacial acetic acid per 100 ml of solution increases the precision of the nuclear stain. Filter before use.

DELAFIELD'S HEMATOXYLIN

Hematoxylin crystals	8.0 gm
Alcohol, 95%	50.0 ml
Ammonium or potassium alum, saturated aqueous solution (approx. 15 gm/100 ml)	800.0 ml

Add the hematoxylin dissolved in the alcohol to the alum solution and expose to the light and air in an unstoppered bottle for 3-5 days. Filter and add:

Glycerin	200.0 ml
Alcohol, 95%	200.0 ml

Allow the solution to stand in the light approximately 3 days filter and keep in a tightly stoppered bottle.

BOHMER'S HEMATOXYLIN

Hematoxylin crystals	5.0 gm
Ammonium or potassium alum	100.0 gm
Alcohol, 80%	60.0 ml
Distilled water	1000.0 ml

Dissolve the hematoxylin in the alcohol and the alum in distilled water. Mix and ripen by exposure to light and air for about 2 weeks.

BULLARD'S HEMATOXYLIN

Hematoxylin crystals	8.0 gm
Alcohol, 80%	144.0 ml
Glacial acetic acid	16.0 ml

Dissolve by gentle heat and add mixture of:

Ammonium or potassium alum	20.0 gm
Distilled water	250.0 ml

Heat to boiling and then remove from flame and add slowly:

Mercuric oxide	8.0 gm

Cool quickly, filter and add mixture of:

Alcohol, 95% ..... 275.0 ml
Glycerin ..... 330.0 ml
Glacial acetic acid ..... 18.0 ml
Ammonium or potassium alum ..... 40.0 gm

EHRLICH'S HEMATOXYLIN

Hematoxylin crystals ..... 4.0 gm
Alcohol, 95% ..... 200.0 ml
Ammonium or potassium alum ..... 6.0 gm
Distilled water ..... 200.0 ml
Glycerin ..... 200.0 ml
Glacial acetic acid ..... 20.0 ml

Dissolve the hematoxylin in the alcohol and the alum in distilled water and mix. After these are in complete solution add the glycerin and acetic acid.

Note: Aluminum ammonium sulfate is known as ammonium alum. Aluminum potassium sulfate is known as potassium alum.

WEIGERT'S IRON HEMATOXYLIN

Solution A

Hematoxylin crystals ..... 1.0 gm
Alcohol, 95% ..... 100.0 ml

Solution B

Ferric Chloride, 29% aqueous ..... 4.0 ml
Distilled water ..... 95.0 ml
Hydrochloric acid, concentrated ..... 1.0 ml

Working Solution

Equal parts of Solution A and Solution B.

Not recommended for routine use. A very good and useful nuclear stain for special stains requiring hematoxylin.

Additional information and the formulae for over 60 hematoxylins can be found in: Gray, P.: The Microtomist's Formulary and Guide, New York, McGraw-Hill Book Co., 1954.

COUNTERSTAINS FOR HEMATOXYLIN

Many counterstains could be listed here which would give good and almost identical results. It is for this reason that we will only list those counterstains which give different staining results.

1% STOCK ALCOHOLIC EOSIN

Eosin Y, water soluble ..... 1.0 gm
Distilled water ..... 20.0 ml

Dissolve and add:

Alcohol, 95% ..... 80.0 ml

WORKING EOSIN SOLUTION

Eosin stock solution.....1 part
Alcohol, 80% ..... 3 parts

Just before use and add 0.5 ml of glacial acetic acid to each 100 ml of stain and stir.

EOSIN-PHLOXINE SOLUTION

Stock Eosin

Eosin Y, water soluble .....1.0 gm
Distilled water .....100.0 ml

Stock Phloxine

Phloxine B..... 1.0 gm
Distilled water.....100.0 ml

Working Solution

Stock Eosin .....100.0 ml
Stock Phloxine..... 10.0 ml
Alcohol, 95% .....780.0 ml
Glacial acetic acid..... 4.0 ml

Make up working solution as needed. Working solution should be changed at least once a week.

PHLOXINE AND SAFRAN SOLUTION

These solutions have been used collectively or individually as counterstains for hematoxylin. For these procedures see routine staining methods page 39.

METHOD I

ROUTINE MAYER'S HEMATOXYLIN AND EOSIN STAIN

(Progressive Stain)

Mayer's hematoxylin is used because it eliminates the necessity for differentiation and bluing of the section. It can be considered a progressive stain which produces a stained section with a clearly defined nuclei while the background is completely colorless. The biggest objection to Mayer's hematoxylin as used in the past, has been that stained slides often fade after 1 to 3 years. This problem can be eliminated, however, when the slides are washed, after the hematoxylin, in running water for a minimum of 20 minutes.

This method gives consistent results even when more than one person stains sections from the same block. Also, slides may be left in the hematoxylin for hours without overstaining. Because of the simplicity of the technique, it is possible to teach others to use it within a shorter time as well as a definite reduction in time performance of

the stain itself. See Figure 15 for the steps and timing with use of Mayer's Hematoxylin and Eosin procedure.

FIXATION. Any well fixed tissue.

TECHNIQUE. Paraffin, celloidin, or frozen.

SOLUTIONS

MAYER'S HEMATOXYLIN

(See page 33)

EOSIN SOLUTIONS

(See page 35)

GRAM'S OR LUGOL'S IODINE

(See page 41)

STAINING PROCEDURE

1. Deparaffinize and hydrate to water.
2. If sections are Zenker-fixed, remove the mercuric chloride crystals with iodine and clear with sodium thiosulphate (hypo) (see page 41).
3. Mayer's hematoxylin for 15 minutes.
4. Wash in running tap water for 20 minutes.
5. Counterstain with eosin from 15 seconds to 2 minutes depending on the age of the eosin, and the depth of the counterstain desired. For even staining results dip slides several times before allowing them to set in the eosin for the desired time.
6. Dehydrate in 95% and absolute alcohols, two changes of 2 minutes each or until excess eosin is removed. Check under microscope.
7. Clear in xylene, two changes of 2 minutes each.
8. Mount in Permount or Histoclad.

RESULTS

Nuclei —blue—with some metachromasia.
Cytoplasm —various shades of pink—identifying different tissue components.

REMARKS. The adhesives used to attach sections onto the slides (gelatin, egg albumen) will sometimes stain, in areas around the section, with Mayer's hematoxylin. This will give the slides a slightly dark appearance but in no way affects the nuclear staining. To remedy this, use 10–12% glacial acetic acid in 95% alcohol, to "clean" the slides after Mayer's hematoxylin. Following with a few dips in saturated aqueous lithium carbonate, the nuclei will blue immediately. This is optional, for the 20-minute wash in running water is sufficient to blue the nuclei. This step will in *no way alter* or *minimize* the staining of the nuclei.

REFERENCE. Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, D.C. 20305.

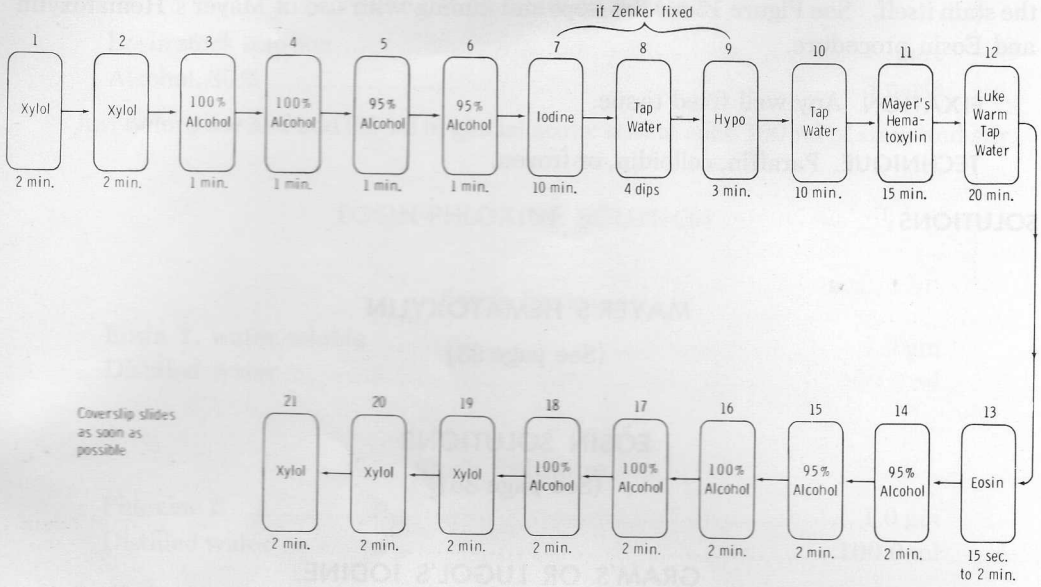


Fig. 15. Steps in staining sections with Mayer's hematoxylin and eosin.

METHOD II

ROUTINE HARRIS HEMATOXYLIN AND EOSIN STAIN

(Regressive Stain)

This is also an excellent staining procedure for the routine hematoxylin and eosin stained slides.

FIXATION. May be used after any fixation.

TECHNIQUE. Paraffin, celloidin, or frozen.

SOLUTIONS

HARRIS' HEMATOXYLIN

(See page 34)

ACID ALCOHOL

Alcohol, 70% ..... 1000.0 ml
Hydrochloric acid, concentrated ..... 10.0 ml

AMMONIA WATER

Tap water ..... 1000.0 ml
Ammonium hydroxide, 28% ..... 2-3 ml

SATURATED LITHIUM CARBONATE

Lithium carbonate ..... 1.0 gm
Distilled water ..... 100.0 ml

EOSIN SOLUTIONS

(See page 35)

STAINING PROCEDURE

1. Deparaffinize and hydrate to water.
2. If sections are Zenker-fixed, remove the mercuric chloride crystals with iodine and clear with sodium thiosulphate (hypo) (see page 41).
3. Harris' hematoxylin for 15 minutes.
4. Rinse in tap water.
5. Differentiate in acid alcohol, three to ten quick dips. Check the differentiation with a microscope. Nuclei should be distinct and the background very light or colorless.
6. Wash in tap water very briefly.
7. Dip in ammonia water or lithium carbonate water until sections are bright blue, (three to five dips).
8. Wash in running tap water for 10 to 20 minutes. If washing is inadequate eosin will not stain evenly.
9. Stain with eosin for 15 seconds to 2 minutes depending on the age of the eosin and the depth of the counterstain desired. For even staining results dip slides several times before allowing them to set in the eosin for the desired time.
10. Dehydrate in 95% and absolute alcohols until excess eosin is removed, two changes of 2 minutes each. Check under microscope.
11. Absolute alcohol, two changes of 3 minutes each.
12. Xylene, two changes of 2 minutes each.
13. Mount in Permount or Histoclad.

RESULTS

Nuclei - blue-with some metachromasia.
Cytoplasm - various shades of pink-identifying different tissue components.

REFERENCE. Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, D. C. 20305.

METHOD III

ROUTINE HEMATOXYLIN-PHLOXINE-SAFRAN STAIN

This method is often requested in our laboratories for color differentiation of various tissue components (see results), and for that reason may be valuable to other laboratories.

FIXATION. May be used after any fixative.

TECHNIQUE. Paraffin, or frozen sections (we have not used this method for celloidin).

MAYER'S HEMATOXYLIN

(See page 33)

SATURATED AQUEOUS PICRIC ACID

Picric Acid	2.0 gm
Distilled water	100.0 ml

1.5% AQUEOUS PHLOXINE B SOLUTION

Phloxine B ..... 1.5 gm
Distilled water ..... 100.0 ml

2.0% ALCOHOLIC SAFRAN*

Safran du Gatinais ..... 2.0 gm
Alcohol, 100% ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to water.
2. Place in saturated aqueous picric acid for 5 minutes.
3. Rinse in tap water until all the picric acid is removed.
4. Stain in Mayer's hematoxylin for 15 minutes.
5. Wash in running tap water for 20 minutes.
6. Stain in 1.5% aqueous solution of phloxine B for 2 minutes.
7. Wash in tap water for 5 minutes.
8. Dehydrate in three changes of absolute alcohol.
9. Stain in 2% alcoholic safran for 5 minutes.
10. Rinse with two changes of absolute alcohol.
11. Xylene, two changes of 2 minutes each.
12. Mount in Permount or Histoclad.

RESULTS

Nuclei	- blue
Red cells	- vermillion pink
Bone	- yellow
Cartilage	- yellowish green
Muscle	- red
Collagen	- yellow

The various effects possible with different fixation are too numerous to be reported here. Only by the use of this technique can these effects be appreciated.

REFERENCE. Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, D.C. 20305.

METHOD IV

ROUTINE METHOD FOR STAINING "RUSH" FROZEN SECTIONS

This method is provided by the Pathology Department, Laboratory Service, Walter Reed Army Hospital. This is the method of choice in that laboratory for staining rush frozen cut sections.

FIXATION. Tissue may be fixed in 10% formalin with the aid of heat or cut unfixed on a cryostat.

TECHNIQUE. Cut frozen section and float on water.

STAINING PROCEDURE

1. Float section onto albuminized slide and blot gently but completely.
2. Alcohol, 100% ..... 1 dip

*Safran du Gatinais, Roboz Surgical Instrument Co., 810 - 18th Street, N.W., Washington, D.C. 20006.

3. Acid alcohol ..... 1 dip
4. Water ..... 1 dip
5. Hematoxylin ..... 1 min.
   (Place hematoxylin on slide with dropper, hold slide near slide warmer.)
6. Warm water ..... 1 dip
7. Acid alcohol ..... 1 dip
8. Water ..... 1 dip
9. Sodium carbonate, saturated aqueous ..... 1 dip
10. Water ..... 1 dip
11. Alcohol, 80% ..... 1 dip
12. Eosin ..... 1 dip
13. Alcohol, 95% ..... 1 dip
14. Alcohol, 100% ..... 1 dip
15. Alcohol, 100% ..... 1 dip
16. Carbol-xylene ..... 1 dip
17. Xylene ..... 1 dip
18. Xylene ..... 1 dip
19. Mount with Permount or Histoclad.

RESULTS

Nuclei - blue
Cytoplasm - pink

REFERENCE. Pathology Department, Laboratory Service, Walter Reed Army Hospital, Washington, D. C. 20305.

REMOVAL OF PIGMENTS AND PRECIPITATES

Our presentation of pigment removal methods will be limited to those specimens containing formalin, mercury, melanin and malaria pigment.

For an excellent review of histochemical methods for the demonstration of pigments see Thompson, S. W.: *Selected Histochemical and Histopathological Method.* Springfield, Charles C Thomas, 1966.

MERCURY PRECIPITATED PIGMENT

When a fixative containing mercuric chloride is employed one can expect a random distribution of brown to black artifactual deposits throughout the tissue section (see page 243). This, the commonly called "Zenker crystal," may be removed by the following method:

1. Deparaffinize and hydrate to water.
2. Place in Gram's *or* Lugol's iodine solution for 15 minutes.

GRAM'S IODINE

Iodine ..... 1.0 gm
Potassium iodide ..... 2.0 gm
Distilled water ..... 300.0 ml

LUGOL'S IODINE

Iodine	1.0 gm
Potassium iodide	2.0 gm
Distilled water	100.0 ml

3. Rinse in tap water.
4. Place in 5% aqueous sodium thiosulfate (hypo) for 3 minutes.
5. Wash in tap water for 10 minutes or longer. Stain as desired.

MELANIN PIGMENT

There are a number of pigments referred to as melanin in both normal and abnormal conditions. These pigments may appear to be closely related, but they are probably different in chemical structure. Regardless of this possibility, we find the use of potassium permanganate a very satisfactory chemical for the removal of melanin pigment.

One must keep in mind however, that this procedure may alter considerably the staining properties since potassium permanganate is very harsh on the tissue section. To prevent loss of sections during staining it may be necessary to coat them with thin celloidin (see page 29).

Alternatively, one can use the gelatin-formalin procedure suggested on page 29.

METHOD I

1. Deparaffinize and hydrate to water.
2. Rince in distilled water.
3. Place in 0.25% aqueous potassium permangenate solution for 30 minutes to 1 hour (time to be determined by the amount of pigment present).
4. Wash well in water.
5. Place in 5% aqueous oxalic acid solution or hydrobromic acid solution (1 part, 3 parts distilled water) until sections become clear (2-5 minutes).
6. Wash in tap water for 10 minutes. Rinse in distilled water and stain as desired.

METHOD II*

This method has proven to be of value in removing melanin from friable or difficult tissues. Especially useful when one experiences difficulty in retaining sections on slides because of the harsh effect of the potassium permanganate.

1. Cut paraffin or frozen sections.
2. Float sections on a conventional flotation bath to allow sections to stretch. The section is transferred to a dish containing an 0.25% aqueous solution of potassium permanganate for 1 hour (stir solution often). Removal of pigment may be accelerated if the solution is kept as 37 °C.
3. Transfer section to distilled water.
4. Float section on a solution of 5% aqueous oxalic or hydrobromic acid solution (see above) until sections become clear.
5. Float sections into dish containing distilled water, three changes for 10 minutes each.

*Emanuele, P. V.: Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, D.C. 20305.

6. Transfer section to albuminized slide and allow to dry on slide warmer overnight or place in a 60 °C oven for 30 minutes.
7. Deparaffinize and stain as desired.

FORMALIN AND MALARIA PIGMENTS

In performing these procedures it must be understood that *no specific time* can be set for the various methods given since much of the results may depend on the length of storage as well as the treatment of the specimen prior to processing. But it must be understood also, that the timing suggested has been based on our experience and is given as the minimum removal time only.

Both formalin and malarial pigments can be removed quite readily (5 - 10 minutes with procedures given) if the tissue specimens are washed for 16 hours in running water prior to exposure to the dehydrating alcohols. If washing is omitted, removal of pigments may require as much as 24 hours.

The following technics serve to remove both formalin and malarial pigments. No satisfactory procedure has been developed to specifically differentiate these pigments in tissue sections by their resistance or solubility in various chemicals. However, several of the methods presented below will remove formalin pigments more rapidly than malarial pigments. Remarks made concerning malaria parasites after the various procedures relate to the staining effect produced with the H&E following exposure of the slides to the different procedures.

METHOD I

1. Deparaffinize and hydrate to water.
2. Rinse well in distilled water.
3. Let stand in saturated alcoholic picric acid solution for 3 hours.
4. Wash well in running tap water.

REMARKS. Formalin and malarial pigments are removed equally well. Overall staining is excellent after this procedure and, due to a yet unknown cause, malaria parasites are demonstrated very well.

METHOD II

1. Deparaffinize and hydrate to water.
2. Wash in distilled water.
3. Immerse slides in 100 ml of 70% alcohol, to which has been added 2 ml of 28% ammonium hydroxide, for 3 hours.
4. Rinse in water.
5. Rinse in a 1% aqueous glacial acetic acid solution.
6. Wash well in distilled water and stain as desired.

REMARKS. The results after using this procedure are identical to those found in Method #1.

METHOD III

1. Deparaffinize and hydrate to water.
2. Rinse well in distilled water.
3. Place in the following bleaching solution for 1 hour or more.

BLEACHING SOLUTION

Acetone	50.0 ml
Hydrogen peroxide, 3%	50.0 ml
Ammonium hydroxide, 28%	1.0 ml

4. Wash well in running tap water and distilled water then stain as desired.

REMARKS. All staining results are similar to method #1 with the malaria parasites *considerably better demonstrated.*

METHOD IV*

1. Deparaffinize and hydrate to water.
2. Rinse well in distilled water.
3. Place in the following solution for 1 hour.

Alcohol, 95%	50.0 ml
Ammonium hydroxide, 28%	15.0 ml

4. Wash well in running tap water and stain as desired.

REMARKS. *This method* is the method of choice in our laboratories because it *removes both malarial* and formalin pigments in 1 hour. The staining qualities are excellent.

METHODS FOR RESTORATION OF BASOPHILIC PROPERTIES

Hematoxylin and eosin stained sections which exhibit poorly stained nuclei even though all stains and chemicals are known to be in proper working condition, could be due to one of the following reasons:

1. Long storage in acid formalin or other fixative.
2. Over exposure to decalcifying solutions.
3. Dried or burned tissue.

Methods which have been used successfully, in our laboratories, for restoring some of this loss of basophilic property are presented herewith. Because no one method solves any specific problem every time, the list presented is in order of our experience. However, if, in any given situation, poor results are encountered in following one method, try another until success is accomplished (Fig. 16 A, B).

METHOD I

1. Deparaffinize and hydrate to water.
2. Place in a 5% aqueous sodium bicarbonate solution overnight. (In most instances 4 hours is sufficient).
3. Wash in tap water for 5 minutes.
4. Stain as desired.

METHOD II

1. Deparaffinize and hydrate to water.
2. Place in a 5% aqueous solution of ammonium sulphide overnight.
3. Wash in tap water for 5 minutes.
4. Stain as desired.

*Washington, Lillian K.: Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, D.C. 20305.

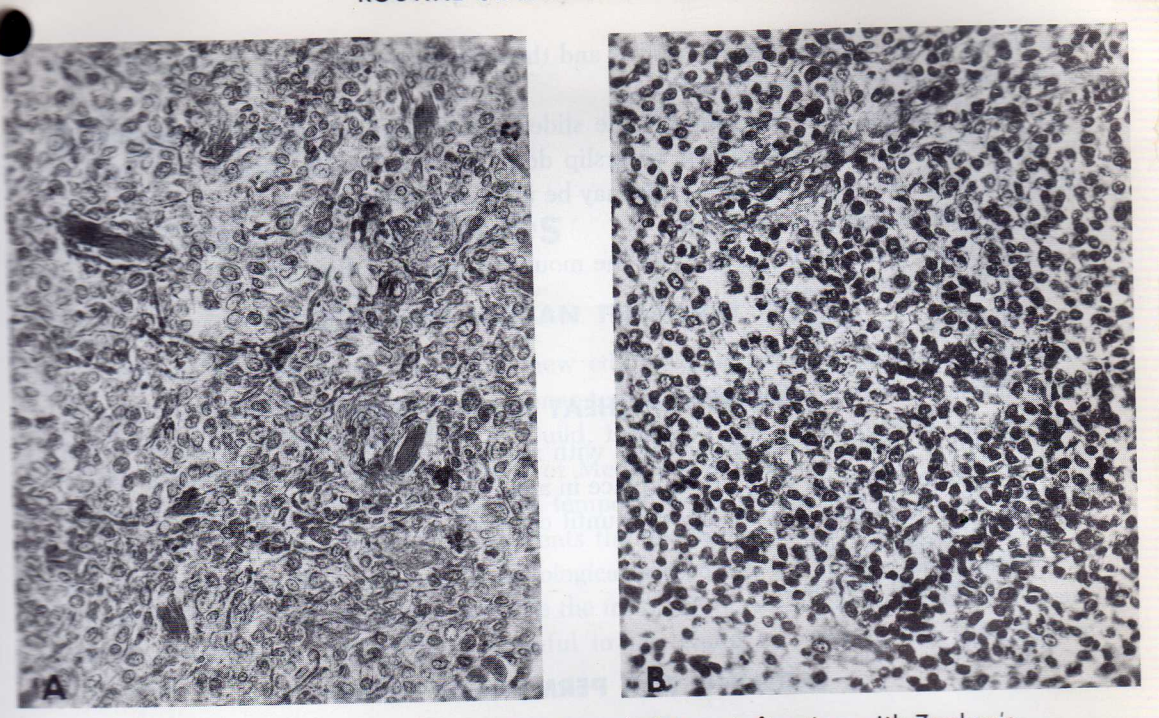


Fig. 16. Comparison of a section stained after overfixation with Zenker's solution (A) and (B) of a section after treatment with basophilic restoration properties Method # I. (A= AFIP 67-3718; B= AFIP 67-3719)

METHOD III

1. Deparaffinize and hydrate to water.
2. Place in 5% aqueous periodic acid overnight.
3. Rinse in distilled water, three changes.
4. Stain as desired.

REMOVAL OF COVERSLIPS

When the need arises for restaining slides it becomes necessary to remove the coverslips and this can be done in one of several ways. When employing any one of these methods, remember that the older the slide, the longer it will take to remove the coverslip.

METHOD I (XYLENE)

1. Place slide in xylene until coverslip detaches of its own accord. (leave slide in xylene until all of the mounting media is dissolved).
2. Hydrate to water.
3. Stain as desired.

METHOD II (HEAT)

1. Heat underside of slide around coverslip edges, by passing over a flame (Bunsen burner, cigarette lighter, etc.) about 2 times a second. *Do not hold slide directly on*

the flame as the section may be burned and the slide will crack if the flame is directed to any one spot for any length of time.

2. Coverslip may be eased off the slide with forceps when the first air bubble is noted beneath the coverslip. If coverslip does not move easily, flame slide until it does. *Do not force the coverslip* as this may be injurious to the section.
3. Cool to room temperature.
4. Place slide in xylene until all the mounting media is dissolved.
5. Hydrate to water.
6. Stain as desired.

METHOD III (HEAT AND XYLENE)

1. Place slide in a coplin jar filled with xylene.
2. Close coplin jar with lid and place in a 60 ° C oven.
3. Check slides every 30 minutes until coverslip is off.
4. Hydrate to water.
5. Stain as desired.

LABELING FOR PERMANENT FILING

If slides are to be filed in an upright position, the use of self-adhesive labels* has several advantages. Not only do the labels supply identification, but when placed on both ends of the slide they separate them thus preventing sticking together with the mounting media. A label measuring 15/16 by 15/16 inches is placed on one end and 1/4 by 15/16 inches on the other.

*Elman Label Inc., 2311 Perkins Place, Silver Spring, Maryland 20910

Chapter 5

Special Techniques

PROCESSING HUMAN TEMPORAL BONES

We are pleased to include in this new edition of histological staining techniques the unaltered procedure for processing human temporal bones. This information was made available to the AFIP by Dr. Stacy R. Guild, Emeritus Associate Professor of Otology of the Johns Hopkins University School of Medicine. A procedure used by Dr. Guild after fifty years experience with human temporal bone histopathology, it is designed to minimize technical artifacts. It represents the distillation of knowledge after experimenting with more than 40 separate histological procedures for human temporal bones. The delicate inner ear membranes require the most careful technical handling to prevent tearing or distortion. The AFIP is grateful to Dr. Guild for making this information available.

INTRODUCTION

The petrous portion of the temporal bone is the housing of the inner and middle ear structures, as well as containing other important tissues such as part of the carotid artery and the facial nerve. Specimens taken for study of the middle ear and inner ear structures should include bone anterior to the internal auditory meatus and posterior into the mastoid region. Between these two landmarks, the desired structures will be found. The specimen block may be removed by making a first cut perpendicular to the tentorial attachment anterior to the internal auditory meatus and a second cut perpendicular to the tentorial attachment 1-1/2 inches postero-lateral to the first. These cuts, if extended 3/4 inch anterolaterally and inferiorly, will contain all of the middle ear and inner ear specimens.

PROCEDURE

1. Place fresh bone specimens in 20% formalin for one hour. Then change to fresh 20% formalin. Tip to allow trapped air to leave the mastoid and other air spaces. Tip slowly, several times, using glass rods or forceps to handle the specimen.

2. Fix in 20% formalin for 24 hours.

Use degassed water to dilute stock formalin. Degassed water is used in order to prevent bubbles from forming on the surfaces of the bone or within the labyrinth. Water may be degassed by subjecting water to a vacuum from a faucet aspirator or a pump for a few hours. Use a thick-walled flask which will withstand the vacuum. For a small number of temporal bones, a 1-liter amount will suffice daily; a 2-gallon jar will be required for large daily usages. After degassing, disconnect slowly from the vacuum; a rapid disconnection will agitate the water with bubble formation. When using the water, transfer by siphon so that it mixes with air as little as possible.

400 ml of fixative is adequate for a pair of bones. In a pint jar add 80 ml of formalin (a 40% solution of formaldehyde in water) to 320 ml of degassed water. Use a tall pint glass fruit jar with glass lid sealed by a rubber ring for each pair of bones. The pair should be kept together.

3. Place bones in fresh 10% formalin for 24 hours. Make this by mixing 40 ml of formalin with 360 ml of degassed water. Tip the bone with each change of solution to remove the trapped air from mastoid and other air spaces.

4. Place in fresh 10% formalin for 24 hours.

5. Place in fresh 10% formalin for 24 hours. Bones may be stored in this.

6. Trim the bone specimen with a circular saw or a band saw. The specimen should be trimmed with vertical cuts made anterior and posterior to the inner and middle ear structures. These cuts are perpendicular to the tentorial ridge. The anterior cut should be as far anterior to the internal auditory meatus as is permitted by the size of block.

7. To remove bone sawdust, carefully grasp the bone with stout toothed forceps and shake rapidly with a back-and-forth motion in 10% formalin in a tall jar. The motion of a vigorous tremor is best. Turn the specimen 90 degrees and repeat. Turn once again and repeat. Each shaking should be vigorous and should last 15 seconds. Use of a tall jar minimizes splashing.

8. Remove specimen and examine it. Gross pathology notes may be dictated at this time.

9. Suspend each bone by a string which passes through the tentorial margin, through soft tissue near the glenoid fossa, or around the bone. Either nylon suture material or white carpet thread (preferably linen) that has been soaked in melted paraffin can be used. Suspend the bones so that the tops are at least one inch below the fluid surface when 400 ml of fluid are in the jar. The bottom of the bone should be at least one inch above the bottom of the jar.

10. Decalcify the bones at room temperature in 1% nitric acid in 10% formalin. Use a separate glass jar for each pair of bones. For decalcification, each jar should contain 356 ml of degassed water, 40 ml of formalin, and 4 ml of nitric acid. Add formalin to the water; then add the acid. Mix carefully to avoid forming bubbles. Transfer each pair of bones to a fresh decalcifying solution daily. When transferring bones to fresh solution, have the fresh solution in a thoroughly washed clean jar standing near the jar containing the bones. Transfer rapidly, to minimize air-trapping. Tip the bones with glass rods to release any trapped air.

Test the decalcifying solution periodically after two or three weeks have passed to determine when decalcification is sufficient. The test is performed just before the bones are transferred to fresh decalcifying solution. Test the fluid that is directly beneath the suspended bone specimen; this has the greatest concentration of dissolved calcium salts when the fluid has not been agitated.

The test is performed as follows: while keeping the upper end of a pipette or glass tube closed with a finger, introduce the pipette into the jar so that the tip is placed on the bottom of the jar beneath the bones. Allow the fluid to flow up the pipette or tube. The amount needed for the test is 5 to 10 ml. Add this to a fresh solution of testing fluid, made by adding about 5 ml of 5% ammonium hydroxide solution to about 5 ml of 5% aqueous ammonium oxalate solution in a test tube. Exact measurements are not essential. If no precipitate forms, allow test tube to stand overnight before reading the test as clear. For each pair of bones, a record should be kept of the tests, using terms such as "distinct," "faint," "very faint," etc., to describe the precipitate. *Decalcification is complete for histological purposes when no precipitate is visible for two days in succession when the tube is closely examined by swirling near a good light source, such as a window.* Thoroughly wash the test tubes between uses. Use a separate test tube for each pair of bones.

This is an empirical test to determine an end-point for histological sectioning; a more delicate chemical test will still indicate the presence of calcium at a stage when the bone is able to be sectioned. Human temporal bones usually require 20 to 30 days for decalcification.

11. When decalcification is complete, specimens may be held in 10% formalin until others are ready for the next step.

12. Place in 5% sodium sulfate for 24 hours. For this, dissolve 20 grams of sodium sulfate in 380 ml of water for each pair of bones. Degas just before using.

13. Change to fresh 5% sodium sulfate for 24 hours.

14. Wash in either (A) running degassed water, 48 hours, or (B) hourly changes of degassed water during working hours of three consecutive days.

15. Place in 35% ethanol for 24 hours. Make this and the following dilutions of alcohol with degassed water.

16. Place in 50% ethanol for 24 hours.

17. Place in 80% ethanol for 24 hours.

18. Place in 90% ethanol for 24 hours.

19. Place in 95% ethanol for 24 hours.

20. Place in 95% ethanol for 24 hours.

21. Place in 100% ethanol for 24 hours.

22. Place in 100% ethanol for 24 hours.

23. Place in a mixture of equal parts of 100% ethanol and ethyl ether for 24 hours.

24. Place in a mixture of equal parts of 100% ethanol and ethyl ether for 24 hours.

25. Transfer specimens to nitrocellulose or parlodion solution for beginning infiltration. Nitrocellulose solutions used in these steps should be mixed as follows from a stock solution of 30% nitrocellulose:

6% nitrocellulose -	50 ml stock 30% nitrocellulose 100 ml absolute alcohol 100 ml ethyl ether
12% nitrocellulose -	100 ml stock 30% nitrocellulose 75 ml absolute alcohol 75 ml ethyl ether
20% nitrocellulose -	200 ml stock 30% nitrocellulose 50 ml absolute alcohol 50 ml ethyl ether
30% nitrocellulose -	Use stock solution, purchased as Tissue Embedding Solution M-4700, Randolph Products Company.
35% nitrocellulose -	Add 5 grams of dry nitrocellulose to each 100 ml of 30% nitrocellulose stock. To dry, expose to air until dry to the touch. *Use ethyl ether safety precautions.*
Thick nitrocellulose -	Old, used 35% nitrocellulose is used for mounting the nitrocellulose block on a fiber or wooden block. Its concentration is not critical.

26. Place bone specimens in 6% nitrocellulose (or 4% parlodion) for three weeks, in a sealed glass jar. This may be re-used if clear. Tip specimens to release trapped air bubbles.

27. Transfer to 12% nitrocellulose (or 8% parlodion) for three weeks. Tip to release trapped air bubbles.

28. Transfer to 20% nitrocellulose (or 12% parlodion) for three weeks. Tip to release trapped air bubbles.

29. Transfer to 30% nitrocellulose (or 16% parlodion) for three weeks. Tip to release trapped air bubbles.

30. Imbed in 35% nitrocellulose (or 16% parlodion), in 100 mm diameter Stender dishes of 50 mm depth, with lid sealed with vaseline and held with weights.

Stender dishes for this purpose should have individually ground covers so that the cover fits exactly the individual dish. After receiving the dishes so purchased, mark the top of the cover and the side of the dish with an identifying number so that the two are identified as belonging to a pair.

To imbed, pour 35% nitrocellulose into the Stender dish and pour a few drops of ether-alcohol onto the surface; put vaseline-rimmed cover on, add weights, and wait for the bubbles to come out (4-5 hours). For weights, use two full glass microslide boxes (50 × 75 mm microslide size). Then remove the weights and the top, cut the string from the bone, and transfer the bone to the 35% nitrocellulose. Tip to release trapped air bubbles. Add a few drops of ether-alcohol to the surface to remove surface bubbles. Reseal the lid in the vaseline; replace weights on the top.

Four bones (two pairs) can be imbedded in one dish. Note the position of the bones in the dish on a sketch; use the number on the side of the dish as a guide to the positions of the specimens.

31. After the bones have been in the sealed dish for three weeks, remove the weights from the top during the daytime and replace them at night. Do this each day for three weeks, providing that the relative humidity is 50% or less. If it is greater than this, do not remove the weights. The nitrocellulose should have an even consistency throughout. If a surface crust forms, reseal and replace weights; solvent will ascend in the sealed dish and will soften the crust.

32. When the surface of the nitrocellulose is no longer "tacky" but yields with gentle finger pressure, flood the surface with chloroform. Replace the top, and add the weights. Allow two to three days for the block to harden.

If more than one specimen is in the dish, before adding the nitrocellulose make a mark on the surface of the nitrocellulose near the mark on the side of the glass dish. This will mark the position of this reference point if the block becomes loosened during hardening. This reference point is important in identifying the specimens.

33. Pour off the chloroform. Pour on 80% alcohol, to fill the dish. Replace the cover and let it stand for one day.

34. Cut block out of dish. Cut blocks by scoring V-shaped grooves on the top of the hardened nitrocellulose between the bones in the dish. Continue deepening these grooves, using a thin bladed scalpel, to separate individual blocks.

35. Nitrocellulose blocks should be kept moist *at all times* with 80% alcohol.

36. Mount nitrocellulose block on fiber or wood block. The top of the fiber or wood block should be grooved. Prepare the block by wrapping a strip of paper around the sides of the fiber or wooden block so that the paper extends above the top of the block by about one centimeter. Secure the paper with a rubber band. This makes a paper-sided container around the top of the block; this container will hold the thick nitrocellulose used to mount the nitrocellulose block on the fiber or wooden block. Place the block with its paper cuff in equal parts of absolute alcohol and ethyl ether; store in this until ready to use.

Soften the bottom of the nitrocellulose block with alcohol-ether in a shallow watch glass while keeping the top of the specimen moist with 80% alcohol by use of a brush.

28. Transfer to 20% nitrocellulose (or 12% parlodion) for three weeks. Tip to release trapped air bubbles.

29. Transfer to 30% nitrocellulose (or 16% parlodion) for three weeks. Tip to release trapped air bubbles.

30. Imbed in 35% nitrocellulose (or 16% parlodion), in 100 mm diameter Stender dishes of 50 mm depth, with lid sealed with vaseline and held with weights.

Stender dishes for this purpose should have individually ground covers so that the cover fits exactly the individual dish. After receiving the dishes so purchased, mark the top of the cover and the side of the dish with an identifying number so that the two are identified as belonging to a pair.

To imbed, pour 35% nitrocellulose into the Stender dish and pour a few drops of ether-alcohol onto the surface; put vaseline-rimmed cover on, add weights, and wait for the bubbles to come out (4-5 hours). For weights, use two full glass microslide boxes (50 × 75 mm microslide size). Then remove the weights and the top, cut the string from the bone, and transfer the bone to the 35% nitrocellulose. Tip to release trapped air bubbles. Add a few drops of ether-alcohol to the surface to remove surface bubbles. Reseal the lid in the vaseline; replace weights on the top.

Four bones (two pairs) can be imbedded in one dish. Note the position of the bones in the dish on a sketch; use the number on the side of the dish as a guide to the positions of the specimens.

31. After the bones have been in the sealed dish for three weeks, remove the weights from the top during the daytime and replace them at night. Do this each day for three weeks, providing that the relative humidity is 50% or less. If it is greater than this, do not remove the weights. The nitrocellulose should have an even consistency throughout. If a surface crust forms, reseal and replace weights; solvent will ascend in the sealed dish and will soften the crust.

32. When the surface of the nitrocellulose is no longer "tacky" but yields with gentle finger pressure, flood the surface with chloroform. Replace the top, and add the weights. Allow two to three days for the block to harden.

If more than one specimen is in the dish, before adding the nitrocellulose make a mark on the surface of the nitrocellulose near the mark on the side of the glass dish. This will mark the position of this reference point if the block becomes loosened during hardening. This reference point is important in identifying the specimens.

33. Pour off the chloroform. Pour on 80% alcohol, to fill the dish. Replace the cover and let it stand for one day.

34. Cut block out of dish. Cut blocks by scoring V-shaped grooves on the top of the hardened nitrocellulose between the bones in the dish. Continue deepening these grooves, using a thin bladed scalpel, to separate individual blocks.

35. Nitrocellulose blocks should be kept moist *at all times* with 80% alcohol.

36. Mount nitrocellulose block on fiber or wood block. The top of the fiber or wood block should be grooved. Prepare the block by wrapping a strip of paper around the sides of the fiber or wooden block so that the paper extends above the top of the block by about one centimeter. Secure the paper with a rubber band. This makes a paper-sided container around the top of the block; this container will hold the thick nitrocellulose used to mount the nitrocellulose block on the fiber or wooden block. Place the block with its paper cuff in equal parts of absolute alcohol and ethyl ether; store in this until ready to use.

Soften the bottom of the nitrocellulose block with alcohol-ether in a shallow watch glass while keeping the top of the specimen moist with 80% alcohol by use of a brush.

Remove the fiber or wooden block from the alcohol-ether container. Pour thick nitrocellulose into the paper container on the top of the block.

Push the nitrocellulose block into the thick nitrocellulose on top of the mounting block quickly, before the mounting medium begins to harden, and push it firmly down onto the grooved surface. Hold it firmly for one minute. Allow it to sit for 5-10 minutes. Then immerse it in 80% alcohol to harden, handling it by the fiber or wood block, not the specimen. On the following day, the paper can be removed. Store the mounted blocks in 80% alcohol.

37. Cut sections on a sliding microtome at 24 micra, keeping the block wet with 80% alcohol. Alcohol should drip onto the block; the sections on the knife should also be kept moist with alcohol. Select every tenth section for staining. Place the other nine, in sets of nine, in order on Bantam manifold paper #9 strips which are numbered with the section number. The paper strips are wrapped in unglazed paper and then in gauze, tied with a string, and stored in 80% alcohol.

38. Stain the sections overnight in Ehrlich's hematoxylin. Arrange the sections in order in the stain in a large glass pie plate.

EHRLICH'S STOCK SOLUTION

Crystals (not powder of hematoxylin)	5.0 gm
Absolute alcohol	250.0 ml
Distilled water	250.0 ml
Glycerin	250.0 ml
Glacial acetic acid	25.0 ml
Potassium alum, to excess	50.0 gm

To prepare, dissolve the hematoxylin crystals in the absolute alcohol, add the glacial acetic acid, then add the glycerin. Dissolve the alum in the water, and add the rest. Let stand in a loosely covered, filter-paper capped flask. The paper cap should be lifted off each day and the flask swirled to mix in the precipitated alum. The flask should be large enough to be only partially full. Allow to stand at room temperature on an open shelf, not in direct sunlight, until ripened (approximately three months). When ripened, the mixture will give off a fruity smell, like Burgundy.

ERHLICH'S HEMATOXYLIN STAIN

Add 10 to 20 drops of Ehrlich's stock to 300 ml of water. The number of drops used will depend on the degree of ripening of the stock. Seldom are more than 20 drops needed. Use this fresh diluted solution for the stain. Because of differences in the stain, it is best to stain a few sections for a trial before staining an entire series. Stain overnight.

39. Blue in: a. dilute ammonium hydroxide (3 drops of ammonium hydroxide in 200 ml of water) or b. cold tap water, or c. dilute lithium carbonate (a few drops of lithium carbonate to a 200 ml dish of water). This will make sections paler as well as bluer.

If too blue, destain with one drop of a dilute hydrochloric acid in 100 ml of water; then re-blue.

40. Wash twice in distilled water if ammonium hydroxide is used. Wash four or more times if lithium carbonate is used to insure removal of all lithium carbonate.

41. Stain in Eosin.

EOSIN STOCK

Dissolve 2 gm Eosin Y in 50 ml of 95% alcohol and 150 ml of water.

EOSIN STAIN

Dilute 6 ml of Eosin Y stock with 150 ml of water. Stain for five minutes.

42. Place in 95% alcohol for 3 minutes.

43. Place in 95% alcohol for 3 minutes.

44. Place in steam-distilled pine oil for at least 15 minutes.

45. Take sections from pine oil and place on slide. Blot the section dry with lint-free Whatman #1 chromatography grade filter paper. Press heavily to remove the oil.

46. Place approximately 2 drops of Damar balsam on a coverglass and place coverglass on the slide. Press gently to remove bubbles. With #1 coverglass, the margins of the coverglasses will bend down and adhere closely to the microslide, decreasing the chance of air coming under the coverglass and forming bubbles. Use this thickness, rather than #2 coverglasses.

47. Label slides with diamond marker.

SUPPLIES. Microtome. American Optical Sliding Microtome #860, with 250 mm Thomas-Schmid knife #950, and Ajustable Knife Clamp AO cat. #862.

Knife Sharpener. Microtome Knife Sharpener, Thomas-Fanz, for 250 mm knife, A. H. Thomas, cat. #7203.

Microslides. Red label corrosion-resistant glass microslides, 50 × 75 mm Arthur H. Thomas, cat. #7033.

Coverglasses. Red label coverglass #1, 35 × 62 mm, A.H. Thomas, cat. #7024. Do *not* use #2 coverglasses.

Stender Dishes. 100 mm diameter × 50 mm deep and 130 mm diameter × 50 mm deep, A. H. Thomas, cat. #4514. Stender dishes should have individually ground covers so that cover matches the individual dish. After receiving dishes so purchased mark the cover and the side of the dish with an identifying number so that the two can be identified as belonging to the same pair.

Whatman #1 Chromatography Paper. A. H. Thomas Company, Vine & Third Street, Philadelphia, Pennsylvania.

Nitrocellulose. (a) Nitrocellulose RS1/2 Second, 2-1/2 lb. package, gallon size. (b) Histological Imbedding Solution, M-4700 (a 30% nitrocellulose solution) 7 lb package, gallon size. Both available from: Randolph Products Company, Carlstadt, New Jersey.

Pine Oil. Fisher Scientific Company, cat. #0134, 7722 Fenton Street, Silver Spring, Md.

Paper, for storing sections in 80% alcohol. (a) Bantam Manifold, Chemical Fine Paper, New White Unglazed, No. 9, 8-1/2″ × 11″. (b) Lucas Copy Paper No. 7, 8-1/2 × 11″ (or a substitute unglazed paper), Lucas Brothers, 219 E. Baltimore Street, Baltimore, Md.

REFERENCES. 1. Guild, S. R.: Emeritus Associate Professor of Otology, The Johns Hopkins University.

2. Gallagher, J. C.: Former Chief, Otolaryngic Pathology Branch, Armed Forces Institute of Pathology Washington, D.C. 20305

A PARAFFIN TECHNIQUE FOR PROCESSING EYES

This is a technique for processing eyes in a wax medium which permits production of completed sections of whole globes ready for reading by the pathologist one week after enucleation. Adaptation is made of various accepted techniques used in fixing, processing, cutting, and staining routine tissue.

The use of this technique also makes possible a greater number of special staining procedures specifically adapted to eye tissue. This in turn aids in the more difficult diagnosis or any further research that may be indicated.

FIXATION. The enucleated eye should be fixed immediately in 10% formalin which has been buffered with sodium phosphate monobasic and sodium phosphate dibasic (anhydrous), (see page 3) in a volume 20 to 25 times that of the eye. Fixation is usually complete in 48 to 72 hours and the specimen is ready for processing or shipment as the case may be. *Cutting windows or injecting the fixative into the globe is neither necessary nor desirable.* The technique after receipt of the globe in the laboratory follows:

1. Wash in running water for 8-24 hours to remove fixative.
2. Place in 60% alcohol until ready to cut, this restores much of the normal color which has been dulled by the fixative; it becomes the first step in dehydration; and it is a good storage solution.
3. Write a good complete description of the specimen including the external appearance, measurements, general aspects of the globe, and note any scars of injury and/or operation.
4. Open the globe with a flat razor blade,[2] starting several millimeters from either side of the optic nerve and passing through the cornea just outside the limbus. (The center block containing the pupil and optic nerve is that portion used for processing while the two sides, calottes, or caps, are held in alcohol for further possible use). Describe internal structures of the center portion indicating known or suspected lesions as well as abnormal appearance. Also incorporate any special instructions to enable the technician to demonstrate any abnormal lesions or conditions in relation to the normal structures.
5. Orientation and embedding instructions should be written on an identification tag which accompanies the specimen through the processing. Place specimen to be processed in 80% alcohol, second step of dehydration, to await processing.
6. If calcified material is observed on "gross" sectioning, the specimen should be placed in decalcifying fluid for several days. The fluid of choice for eye tissue is sodium citrate and formic acid solution (see page 8). Following the use of the decalcifying solution the specimen must be washed in running water for 24 hours before further processing.
7. Processing by an automatic processor or by hand, depending upon either the number of specimens or the size, may be accomplished by the following schedule:

	Overnight (automatic)	By hand (for *large pieces*)
Alcohol, 95%	3 hours	8-24 hours
Alcohol, 95%	2 changes, 1 hour each	(several changes)
Alcohol, 100%	3 changes, 1 hour each	8-24 hours (several changes)

Chloroform	1 hour	5 hours (several changes)
Chloroform	2 hours	5 hours (several changes)
Paraffin (Bioloid, wax of choice for eyes)	2 changes, 1 hour each	
Paraffin	2 hours	
Paraffin (under vacuum)	30-45 minutes	30-45 minutes

8. The embedding is comparable to general embedding prodedures using the multiple embedding technique (see page 13) following specific instructions on the individual tags.

9. Blocking, boxing and holding for completion will be as the accepted plan in the individual laboratory.

SECTIONING. *To obtain satisfactory sections the following instructions must be followed closely:*

a. Cut at 8 microns.

b. Place block with scleral sides parallel to knife, cornea and nerve on either side.

c. Orient block to get as near a complete section as possible without excessive rough cutting so that the required sections will be through the nerve and pupil as well as demonstrating known or suspected abnormal features.

d. Expose the tissue and soak for several minutes, using a piece of cotton saturated with warm tap water. This aids in cutting a cross section of the globe with minimal tearing through lens, gelatinous exudate, and/or blood.

e. Chill both the block and the knife with ice and cut using a smooth slow turn of the microtome wheel.

f. Use two flotation baths at this point: one of distilled water at room temperature, upon which the ribbon of sections is placed; and a second maintained at 55° to 55.5 °C approximately 10 degrees higher than normally used for general tissue flotation, to which has been added gelatin adhesive, 3 teaspoonful of 5% gelatin solution per 1000 ml of water. Upon this flotation bath each individual section is stretched to conform in size and shape with the tissue in the original block.

g. At the close of each day's work, carefully clean with detergent and dry the flotation bath that contained the gelatin, to insure against bacterial growth which could adhere to slides and thus cause an artifact giving a confused diagnosis.

STAINING. After drying, the completed sections are ready for staining as desired, routine H&E and by special staining techniques.

REFERENCES. 1. Ballou, E. F.: *Amer. J. Med. Techn.* 32:287-291, 1966.

2. Ackerman, L.V.: *Surgical Pathology*, 3rd ed., St. Louis, The C.V. Mosby Company, 1964, pp. 1218-1219.

TRYPSIN DIGESTION TO DEMONSTRATE RETINAL VESSELS

FIXATION. 10% buffered neutral formalin. Remaining portions of the globes (see page 53) which have been in alcohol should be placed in formalin for 24 hours then washed in water overnight.

TECHNIQUE. Digestion with enzymes.

SOLUTIONS

0.15 M TRIS BUFFER (pH 7.8)

Tris Buffer, (Sigma* 7-9, M. W. 121. 136) ..... 18.17 gm
Distilled water ..... 1000.0 ml
Hydrochloric acid, concentrated, (to adjust pH if necessary)

TRYPSIN DIGESTION SOLUTION

Trypsin, (Difco † 1:250) ..... 3.0 gm
Tris Buffer ..... 100.0 ml
Merthiolate (as a preservative) ..... 1 pinch
Make fresh for immediate use.

PROCEDURE. Material usually used is taken from the two sides of the globe, or the calottes, after the center portion containing pupil and optic nerve has been processed for routine pathology evaluation.

DISSECTION OF THE RETINA. Work under the dissecting microscope with the calotte immersed in distilled water in a finger bowl. Starting at the posterior pole, with the "heel" of the dissecting needle, separate the retina proper from the choroid-sclera which is held firm with jeweler's forceps. Separate at the pars plana. When the choroid adheres to the retina, it is often easier to partially digest both before attempting to separate them. (An initial digestion of the entire calotte sector for 10-20 minutes may be helpful).

DIGESTION. Suspend the retinas in the trypsin digestion solution at 37 °C for 1/2 to 1-1/2 hours. Incubation time varies with each eye. Terminate digestion when the medium becomes cloudy and the tissue shows signs of disintegration, by transferring to distilled water. This is accomplished by picking up the retina on the heel of the dissecting needle. The vessels are surprisingly strong.

PREPARATION OF THE RETINAL VESSELS. Work with the dissecting microscope, and with the retina submerged in a finger bowl of distilled water. The internal limiting membrane can be peeled off in one sheet, by careful manipulation. Hold the internal limiting membrane with a jeweler's forcep, and disengage the retinal vessels from it with the heel of the dissecting needle. (The point of the needle may adhere to and tear the vessels). Grasp the internal limiting membrane at a point close to the line of separation. The network of vessels can be freed of retinal tissue by gentle shaking. If undigested tissue remains, it may be necessary to incubate longer. The final retinal vessel preparation will appear as an open meshwork. The veins and arteries branch and fan out, and are interconnected by a capillary plexus.

MOUNTING. Replace the water with clean distilled water. Dust should be skimmed from the surface, float the vessels on a clean slide, and with the slide preparation partly immersed, use the heel of the dissecting needle and the surface tension of the water to arrange the vessels in their original configuration. Air dry.

STAINING. PAS-hematoxylin for demonstration of vessel walls and their cellular nuclei. (Arteries stain more intensely than veins).

Hematoxylin-eosin for visulization of red blood cells. (It is necessary to increase the staining time in eosin).

REFERENCE. Kuwabara, T., and Cogan, D. G.: *Arch. Ophthal.* 64:904-911, 1960 (AFIP Modification).

*Sigma Chemical Corp., St. Louis, Mo. 63118
†Difco Co. Inc., Detroit, Mich. 48201

STAINING METHOD FOR RETINAL VESSELS (PAS)

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. See page 53.

SOLUTIONS

M/5 SODIUM ACETATE

Sodium acetate ($NaC_2H_3O_2$-$3H_2O$) ..... 13.6 gm
Distilled water ..... 500.0 ml

PERIODIC ACID SOLUTION

Periodic acid ($HIO_4 \cdot 2H_2O$) ..... 0.8 gm
Distilled water ..... 20.0 ml
Dissolve and add:
M/5 sodium acetate ..... 10.0 ml
Alcohol, 95% ..... 70.0 ml
Prepare fresh each time.

REDUCING RINSE SOLUTION

Potassium iodide ..... 2.0 gm
Sodium thiosulfate ..... 2.0 gm
Distilled water ..... 40.0 ml
Dissolve and add:
Alcohol, 95% ..... 60.0 ml
2N hydrochloric acid ..... 1.0 ml
Prepare fresh each time.

SCHIFF'S LEUCOFUCHSIN SOLUTION

(see page 159)

COLEMAN'S FEULGEN SOLUTION

(see page 159)

MAYER'S HEMATOXYLIN

(see page 33)

STAINING PROCEDURE

1. 70% alcohol for 5 minutes.
2. Oxidize in periodic acid solution for 5 minutes.
3. 70% alcohol, several changes, 2 minutes each
4. Reducing rinse solution for 5 minutes.
5. 70% alcohol, several changes, 2 minutes each.
6. Rinse in distilled water.
7. Coleman's or Schiff's leucofuchsin solution for 35-60 minutes.
8. Running tap water to develop color for 10 minutes.
9. Mayer's hematoxylin for 10 minutes.
10. Wash in running water for 15 minutes.

11. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, 2 changes each.

12. Mount with Permount or Histoclad.

RESULTS

Vessel walls - magenta
Nuclei - blue

REFERENCE. Hotchkiss, R. D.: *Arch. Biochem.* **16**: 131-141, 1948 (AFIP modification). Copyright by Academic Press.

SECTIONING UNDECALCIFIED TISSUES

There may be times when it becomes particularly essential that sections be made of undecalcified tissues so that no portions of the bone architecture are changed or obliterated by any decalcifying procedure. Under such circumstances either of the two following methods may be employed:

SECTIONING OF UNDECALCIFIED TISSUES USING GILLINGS-HAMCO* THIN SECTIONING MACHINE

FIXATION. Any well fixed tissue, however, 10% formalin preferred. After fixation, cut specimen to desired thickness (5mm in thickness is suggested).

DEHYDRATION.

Place specimen in the following solutions for times indicated.

Alcohol, 80%	2 hours
Alcohol, 95%	2 hours
Alcohol, 100%	2 hours
Acetone	1 hour

Remove specimen from acetone and allow the acetone to evaporate, 15 minutes.

IMPREGNATION. Place specimen in a jar of castolite† (plastic without the catalyst) with enough plastic to cover specimen, impregnate under vacuum at least 24 hours. *Note.* This plastic can be re-used, after specimen is removed, if plastic is placed in the refrigerator.

CASTOLITE PLASTIC SOLUTION

Castolite	100.0 ml
Catalyst	5 drops

Mix just before use. Stir carefully until well mixed, try to avoid bubbles.

BLOCKING. Pour about 1/4 inch castolite plastic solution into a mold made with Reynolds wrap or other suitable material. Place tissue in remaining catalyzed plastic ready to be poured into mold. Allow mold to stand at room temperature for 2 hours then place in a 60 °C oven until polymerized but with top still tacky. This provides the base of the block. Pour remaining plastic with specimen into mold. Orient the specimen and allow to stand 3-4 hours at room temperature. When all bubbles have disappeared place in 36 °C oven for 24-48 hours. The final hardening may be done

*Gillings-Hamco sectioning machine, Hamco Machines, Inc., Rochester, N.Y. 14607
†Castulite (liquid casting plastic), Read Plastic, Inc., 317 Cedar Street, Washington, D.C. 20012

at room temperature after the block has been removed from the mold. (Refrigeration will aid in removing block from the mold). Trim the hardened block with a saw, unless the mold was made to exactly fit the block.

Using Duco cement, attach the plastic block in the desired position to a plastic or masonite base. Thirty minutes in a 60 °C oven, followed by room temperature cooling is all that is required for a firm attachment. The base and attached specimen is screwed to the machine specimen mount.

SECTIONING. Regulate water supply making certain it is steady before the cutting is started. While sectioning keep constant check to be sure the water pressure does not vary. Too little water will burn the tissue. Align block and set for section thickness desired. Start machine.

When sectioning is completed, turn off the machine and water and disengage the blade. Extreme care is required in handling the section to prevent fracturing. Blot section between filter paper, and store in dust free container until ready for mounting.

MOUNTING. Dehydrate in 95%, absolute alcohol and clear in xylene, 2 changes each. Mount with Permount or Histoclad.

RESULTS. The structure of teeth and cortical bone is clearly demonstrated.

REFERENCE. Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, D.C. 20305.

PREPARATION OF HAND GROUND SECTIONS OF CORTICAL BONE

FIXATION. Any well fixed tissue, however 10% formalin preferred.

TECHNIQUE.

1. Cut bone into thin slices with hand or band saw.
2. Place bone in a thick soap solution (strong laundry soap) and boil for 4 hours to remove organic material.
3. Wash in running water overnight.
4. Place the bone in absolute alcohol for 7 hours.
5. Suspend bone over ether, benzene, or chloroform for 48 hours to remove fats. Change solution twice during this step.
6. Bleach in 30% hydrogen peroxide for 2 days or until bone turns white.
7. Dry specimen at room temperature for 24 hours.
8. Cut with a jeweler's saw to the desired thickness for grinding. It is wise to make several slices.
9. Grind on coarse sandpaper or metal file. When the section becomes too thin to grind further, affix it to the glass slide with Duco cement; continue grinding. When section is very thin, remove it from the slide with acetone, turn it over and reaffix it to the slide. Continue grinding until it is as thin as possible. Finish with fine sandpaper.
10. Remove section from slide with acetone and wash well in soap solution followed by a tap water wash.
11. Remove from the water and dry thoroughly after which the specimen is cleared in xylene overnight and mounted with Permount or Histoclad.

RESULTS. The structure of cortical bone is clearly demonstrated.

REFERENCE. Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, D.C. 20305.

PARAFFIN METHOD FOR LARGE BONES AND WHOLE ORGANS

The following method is used for sections of bone or organs which measure as much as 5 inches in length and 1/2 inch in thickness.

FIXATION. Bone and large soft tissue specimens should be fixed in 10% buffered formalin in a volume of 20 to 25 times that of the specimen, for 5 days.

DECALCIFICATION. After they are thoroughly fixed, the blocks are tied in gauze and a label with the accession number, paraffin dipped for protection, is attached. The bag is suspended in a large quantity of decalcifying solution. Stirring or agitation of the fluid hastens the decalcifying process.

Decalcification may take from 3-14 days depending on the size of the specimen. Change decalcifying solution frequently (at least every other day) until decalcification is complete. When decalcification is complete wash the bone in running water for 24 hours. Tissues are then transferred to 80% alcohol, before routine hand processing is employed.

PROCESSING. All large specimens must be processed by hand and the following schedule is suggested:

PARAFFIN METHOD

1. Alcohol, 80%, 2 changes	12 hours each
2. Alcohol, 95%, 2 changes	12 hours each
3. Alcohol, 100%, 2 changes	8 hours each
4. Chloroform, 3 changes	4-5 hours each
5. Paraffin (Paraplast) 4 changes	8-10 hours each

6. Embed. For proper embedding, flatten tissue with gentle downward pressure while rubbing an ice cube across the bottom of the embedding pan (see page 13).

7. Attach paraffin block to a fiber or wooden block by melting the back of the block with a hot spatula and pressing the block and holder firmly together. Be certain that the back of the paraffin block is even to prevent any air spaces between the paraffin and the mounting block which will produce vibrations during the cutting stage. Immerse the fiber block containing the paraffin block, in a pan of tap water. Do not chill too rapidly or the tissue block will crack away from the fiber block.

8. The blocks are sectioned on the Sartorius model 39 or any microtome which will accommodate large tissue blocks.

9. The larger the block being cut, the more tendency for the sections to roll-up as they come from the knife edge. In this case, they are removed in the rolled state to a room temperature water bath. Transfer the rolled section on a glass slide, with free end of the roll uppermost, to the conventional 40-45 °C flotation bath. Aided by the heat of the water, unroll the section with a gentle pushing pressure using a camel's hair brush, until the flattened section will float free on the water's surface. Pick up the section on a labeled slide which has been treated with Mayer's albumin section adhesive.

10. These slides are thoroughly dried in a 37-40 °C drying oven and are then ready for staining.

STAINING PROCEDURE. For the staining of sections on slides measuring as much as 5 x 7 inches, animal jars 8 inches tall and 8-1/4 inches in diameter are used. The slides are carried through the staining solutions in an especially constructed stainless

steel rack. For routine staining any one of the methods presented on pages 36-40 can be used satisfactorily. An increase of time in most solutions may be desirable, but only by experience and observation of a given slide during the staining procedure, can dictate this necessity.

Any of the many special stains available can be performed on sections of this size by flooding the slide with the various special staining solutions and allowing them to stand for the desired time. When using this technique it is well to place the slides on rods across a pan or staining dish to prevent spillage of excess solution.

REFERENCE. Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, D. C. 20305.

METHODS FOR PROCESSING ORAL TISSUES

TEETH

Proper fixation of a tooth requires the clipping of the apical third of the root or roots immediately after extraction from the host. This simple step permits the fixation solution to penetrate the tooth completely.

FIXATION

1. 10% buffered neutral formalin for 72 hours minimum; wash in water for 4 hours.
2. Place in one of the following solutions until decalcification is complete, changing the decalcifying solution daily for best results. (10-12 days in formic acid solution. Approximately 5 days in nitric acid solution).

FORMIC ACID SOLUTION

Formic acid, 90%	5.0 ml
Distilled water	95.0 ml

NITRIC ACID SOLUTION

Nitric acid, concentrated	10.0 ml
Distilled water	90.0 ml

Decalcify in refrigerator. (20 °C if this method is used).

3. When decalcification appears to be complete, cut a thin slice from each side of the tooth and return to decalcifying solution for an additional 24 hours to insure complete medial decalcification, the end point of which is determined by any of the methods listed on page 10.
4. Wash in running water overnight (16 hours).
5. Place in 60% alcohol for 24 hours, followed by 80% alcohol for 48 hours.
6. Proceed with routine automatic tissue processing schedule (see page 16).
7. Embed and cut as desired.

SPECIAL CUTTING INSTRUCTIONS

It is obvious that teeth will present problems not found in other tissues due to their extremely hard composition, even after complete decalcification. Sections obtained by rough cutting should not exceed 10 microns and should be cut slowly and carefully to prevent the tooth from "chipping" out of the block. The usual water-soak at

intervals during the rough cutting process prevents loss of or damage to tissue (see page 26).

Sections of teeth have tendency to spread to their original size when placed on the water bath, but are bound by the surrounding paraffin. In this case "slit" the paraffin on all four sides with a suitable instrument to allow for natural expansion to original shape. On a section of a particularly stubborn tooth the distention may be aided manually by pulling gently at opposite ends of the tooth until it is completely flat and smooth. Float sections onto albuminized slides and stain as desired.

JAW BONES

FIXATION

1. 10% buffered neutral formalin preferred. Place specimens in a volume 20-25 times that of the specimen for 10-14 days. Change solutions 3 times during this period. *Note.* In animal research it is best to perfuse the animal after which the jaw bone is placed in formalin solution for 1 week.

2. Wash in running water overnight.

3. Place in decalcifying solution until decalcification is complete. (Human size jaw bones decalcify in 6 weeks to 2 months when formic acid is used; 4-6 weeks for nitric acid). Change solutions daily for the first 3 weeks followed by changes every other day for the remaining period. Test with the chemical decalcification end point procedure given on page 10. If not completely decalcified place in 5% aqueous disodiumethylenediaminetetraacetate for 3 days followed by washing in running water for 2 hours. Re-treat with decalcifying solution for 2 days. When decalcification is complete, cut a thin slice from each side of the specimen to assure complete penetration.

PROCESSING

Alcohol, 60%	2 days
Alcohol, 80%, 3 changes	5 days
Alcohol, 95%, 3 changes	7 days
Alcohol, 100%, 3 changes	3 days
Alcohol, 100%, 3 changes	3 days
Chloroform, 2 changes	1 hour each
Chloroform, 2 changes	16-24 hours each
Paraffin of choice, 3 changes	3 hours each
Paraffin of choice, under vacuum	2 hours
Paraffin of choice	3 hours
Paraffin of choice	3 hours
Paraffin of choice under vacuum	2 hours

EMBED. See page 13.

CUT. See page 59.

STAIN. As desired.

SOFT TISSUES

FIXATION

1. Tissues should be cut in half before being placed in a fixative of choice (10% buffered neutral formalin for 30 minutes). Then gross to desired size, and place in

fresh formalin fixative for complete and proper fixation. (12-24 hours, depending on size). Small pieces, not to exceed 4 mm in thickness, are fixed adequately in 24 hours.

2. Wash specimen in tap water before processing.

PROCESSING

Alcohol, 60% ..... 8 hours
Alcohol, 80% ..... 24 hours

Process on an automatic tissue processor, or by hand, following the schedule suggested on page 16.

EMBED. See page 13.

CUT. See page 25.

STAIN. As desired.

REFERENCE. Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, D.C. 20305.

CELLOIDIN METHODS

Celloidin or nitrocellulose is one medium used for processing hard tissues such as bone and teeth as well as for large sections of whole organs. Use of this medium for processing eye tissue and whole globes had been very successful, however, because of the time required by the method, most laboratories prefer to use the paraffin technique for routine diagnostic work. When the block is hardened to a density which permits cutting, sections can be produced which are free from compression, distortion, and yet are thin enough to be studied under a microscope.

Nitrocellulose is the form of cellulose used to make celloidin, and is available in three grades:

Gun cotton - Cheapest, but contains enough explosive to be potentially dangerous.

Nitrocellulose - Refined, but still containing explosives which requires shipment in water or low grade alcohol.

Parlodion* - Most expensive. Most explosives removed. Shipped in dry strips.

The disadvantages in the use of celloidin are overbalanced by the advantages as can be observed by the following:

Disadvantages

1. The procedure is time consuming.
2. The celloidin attracts moisture very quickly on exposure.
3. The use of special stains is very limited.

Advantages

1. Less shrinkage in tissue.
2. Relationship of component parts is preserved with little distortion.
3. Excellent for the study of embryos.
4. Large, compact bones and whole organs section with no compression or shattering.

*Mallinckrodt's proprietary term for the purified pyroxylincelloidin.

The solvent is equal parts of absolute alcohol and ethyl ether. Solution is brought about by the following simple steps:

1. Add alcohol 100% to the desired amount of Parlodion and let stand over night.
2. Add the ether the following morning. Agitate for 10 minutes.
3. Invert container frequently during the day until the celloidin is completely dissolved.

If 12% celloidin is used as a stock solution, dilutions are made by using the following table. It is suggested, however, that the various percentages be made and kept on hand at all times.

Per cent celloidin desired	*Parts 12% celloidin*	*Equal parts Absolute Alcohol and Ethyl Ether*
10	5	1
8	4	2
6	3	3
4	2	4
2	1	5

FIXATION. 10% buffered neutral formalin. Wash in running water for 16 hours before further processing.

DEHYDRATION. This step is accomplished with the use of ascending percentages of alcohol, starting with 80% and allowing sufficient time for complete diffusion through the block of tissue in each solution. With hard pieces of tissue it may be advantageous to change each percentage of the dehydrating solutions at least twice during the processing since removal of all water is necessary to obtain complete impregnation with the celloidin. The last dehydrant should be the solvent for the celloidin and the tissue should not be allowed to remain in it longer than 24 hours.

Depending upon the type of tissue being processed, each piece should be wrapped in a single layer of gauze and fastened with dental floss attached to a label of sufficient length to allow suspension in the container holding the processing solutions. This permits complete and even penetration throughout all areas of the specimen.

IMPREGNATION. Done by using increasing percentages of the celloidin up to and including 12% which also becomes the embedding medium. The following tables will give the suggested times of dehydration and impregnation for each of the techniques.

DOUBLE EMBEDDING METHOD

(See page 18)

WET CELLOIDIN METHOD

(Specimen not to exceed 5 mm in thickness)

1. Alcohol, 80% ..... 24 hours
2. Alcohol, 95% ..... 24 hours
3. Alcohol, 100%, 2 changes ..... 12 hours each
4. Ethyl ether-alcohol, 100%, equal parts ..... 24 hours

5. Celloidin, 4% ..... 2 to 3 days
6. Celloidin, 8% ..... 2 to 3 days
7. Celloidin, 12% ..... 2 to 3 days
8. Embed. See below.

DRY CELLOIDIN METHOD

1. Same as wet celloidin method through step 7.
2. Place mounted tissue in cedarwood oil and chloroform equal parts ..... 24 hours
3. Cedarwood oil 3 parts, chloroform 1 part ..... 24 hours
4. Cedarwood oil until ready to cut ..... 24 hours or longer

EMBEDDING AND MOUNTING ON FIBER BLOCKS. Place oriented tissue, with the desired cutting surface up as close as possible to the bottom of Stender dishes containing thick (12%) celloidin to avoid excessive handling and thus minimizing the formation of air bubbles. The Stender dishes are placed under a sealed bell jar until all of the air bubbles have disappeared, approximately 4 to 6 days. Remove from bell jar and loosen covers of dishes gradually to allow slow hardening of the celloidin by evaporation of the alcohol-ether solvent. Check daily for the proper consistency for cutting into the individual blocks; hard and firm to the touch with slight resiliency upon increased pressure. Cut into individual blocks; tagging with correct numbers. With same surface down, trim into square blocks with parallel sides and affix with thin celloidin to wood or fiber blocks of a size to support all of the tissue block, keeping the amount of celloidin between the tissue and the block at a minimum in order to reduce vibration at the time of cutting. Place in a covered dish in which has been placed a piece of cotton saturated with chloroform, thus using the chloroform vapors for hardening, about 2 hours.

For wet celloidin cutting, remove blocks from chloroform vapor, placing them upside down in 80% alcohol for at least 24 hours to harden to cutting consistency. Blocks and sections may be stored in 80% alcohol indefinitely.

CUTTING (Wet Celloidin). Place the knife at a 30° angle in relation to the oriented block. *Block and knife must be kept wet with 80% alcohol* by use of a drip bottle, a piece of cotton or a 1″ paint brush. Failure to keep the specimen wet will cause shrinkage, and result in thick and thin sections. Irregular sections may also be caused by a loose set screw on the microtome, or bubbles trapped between the embedded tissue and the fiber block. Cover the specimen with cotton wet with 80% alcohol while working with cut sections. Sections to be stained are removed from the knife with the finger and placed in 95% alcohol to straighten. Sections to be stained are floated on two inch squares of smooth filter paper to be wrapped in a package for future work if necessary. For serial work, arrange sections in order on wet knife, pick up by placing a numbered strip of filter paper on the sections and carefully pull them from the knife. Place face up in a dish of 80% alcohol. This is an easy way to pick up all sections for storage. Cover the top sheet of exposed sections with a slip of filter paper before wrapping. Mark with India ink the identifying number on a similar size piece of onion skin paper and cover the number with Duco cement. Wrap sections and block from the same tissue and tie with dental floss and store in 80% alcohol. The dry celloidin method is used for eyes only, for details of this technique see the original article: Ballou, E. F.: *Amer. J. Med. Techn.* 14:202-206, 1948.

ROUTINE CELLOIDIN STAINING PROCEDURE

Staining of all types and sizes of tissue can be accomplished with the following procedure. It may be necessary however, for the time to be increased in certain steps, especially in the hematoxylin and eosin solutions. This change in the procedure can only be determined by the size of the specimen being stained and by microscopic observation of the staining qualities.

1. Float sections from 80% alcohol on a glass slide into distilled water.
2. Pick up sections with a glass rod and stain in Harris' hematoxylin (without acetic acid) for 20 minutes.
3. Wash in tap water to remove excess hematoxylin.
4. Differentiate sections individually in a 0.5% acid alcohol solution until celloidin is clear. (HCL 0.5 ml, 70% alcohol 100 ml). Stop the differentiation in tap water by transfer of sections to water on a slide.
5. Rinse in 95% alcohol until celloidin is clear.
6. Float sections into a weak ammonia water for "bluing."
7. Wash in water and check for nuclear detail with a microscope.
8. Stain in 1% alcoholic eosin for 30-60 seconds.
9. Rinse in 95% alcohol.
10. Rinse in 95% alcohol until clear.
11. Place in carbolxylene solution or isopropyl alcohol for flattening of the section.
12. Pick section up on numbered slide. Rinse in 2 changes of xylene.
13. Mount with Permount or Histoclad. Small weights are sometimes employed to keep the section flat until Permount or Histoclad has hardened.

RESULTS

Nuclei	- blue
Cytoplasm	- pink

REFERENCE. Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, D.C. 20305.

35 MM FILM METHOD

FIXATION. Any fixative may be used.

TECHNIQUE. Paraffin.

SOLUTIONS. Krylon* or Sherwin Williams Plastic Spray Enamel†

EQUIPMENT. Film: 35 mm Cronar, P 40 B Leader Film‡;

Reel: Nikor auto load, from any photographic supply house.

Film holders to hold film in position while viewing film strips on microscope are not commercially available. They can be fabricated and consist of a 3-1/4 × 4 inch glass plate, two 3 × 1 inch slides and four 22 × 40 coverslips and Duco cement (Fig. 17). A strip of paper approximately 1 mm wider than the film is placed in the center of the long axis of the glass slide (the paper can be kept in place with masking tape). The four coverslips are butted against the edge of the paper with Duco cement, the

*Krylon, Inc., Norristown, Pa. 19404
†Sherwin Williams, 1325 14th St., N.W., Washington, D.C. 20005
‡E. I. du Pont de Nemours & Co., Photo Productions Dept., Wilmington, Del. 19898

two 3 × 1 slides are placed and cemented on top of the coverslips and must extend inwardly 2 mm over the coverslip edge.

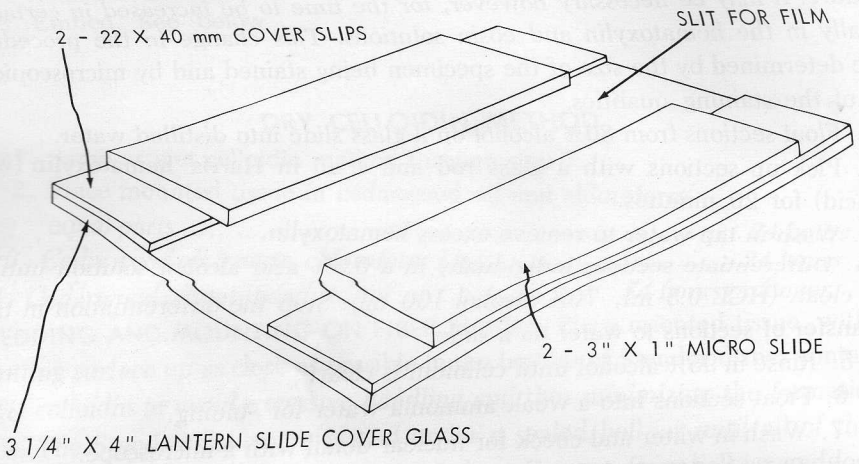


Fig.17. Fabricated film holder for viewing film strips under the microscope.

PROCEDURE

1. Cut paraffin sections in the usual manner and float ribbon onto flotation bath.

2. The film is maneuvered under the ribbon and picked up as on a slide until the entire film is filled. Label film with a diamond pencil or lab ink and place in a 56 °C oven for 30 minutes to dry.

3. Load film into a 35 mm developing reel (three strips of 18 inch length can be accommodated on one reel). Stain as desired.

4. After staining, one strip of film is removed at a time from the clearing xylene and placed face up on a blotter. It must not be allowed to dry. *Note:* The film must lie flat because buckling will result in an uneven coat of plastic. Metal weights may be placed at each end of the plastic to keep it flat.

5. The plastic spray can is held approximately 6 inches above the film and the spray is applied quickly until the surface appears clear and smooth.

6. The plastic is allowed to harden for 10 minutes. The plastic spray is repeated twice and left to dry. The strips may be ready for microscopic examination after 2 hours.

7. If necessary, the plastic coat may be removed by placing in xylene for 10 minutes. This step necessary when spray is uneven.

8. The dry film is labeled with India ink or paper labels.

9. The sections are now ready for study under the microscope. A holder (Fig. 17) is essential for microscopic examination because it keeps the sections flat and facilitates advancement.

10. The sections may be filed in special cabinets, stapled to the record, suspended from racks, or rolled and stored in 35 mm film cans.

11. Before permanent filing, strips should be dried for 2 or more days.

REFERENCES. 1. Pickett, J. P., Sommer, J. R.: *Arch. Path.* 69:236-247, 1960.

2. McCully, R. M., et al: *Amer. J. Clin. Path.* 45:480-482, 1966.

PLASTIC FILM METHODS

The use of plastics, *Cyclon-Lack, farblos Nr. 10830, lufttrocknend**, Diatex† and others, as media for mounting stained microscopic sections in the form of plastic films, can be utilized easily and advantageously for the preparation of serial sections, study sets, large sections, removal of multiple sections from one slide and conversion to several slides, and smears as well as repair of broken slides. Utilization of this method is most practical when considering the problem of storage. Wherein large slide cabinets are used to house one thousand slides, the same number of sections in plastic film, can be maintained in a small card file.

FIXATION. Any fixative.

TECHNIQUE. Paraffin sections and smears.

SOLUTIONS. Plastics as suggested.

PROCEDURE

1. Cut sections and place on the flotation bath in the usual manner.
2. Pick up sections on slides and place on the slide warmer overnight or 60 °C oven for 30-45 minutes.
3. Deparaffinize and stain as desired.
4. Dehydrate in 95% alcohol, absolute alcohol and clear in xylene.
5. Leave in xylene until ready for plastic.
6. (1) *Either* dip slide in plastic, wipe the underside of slide free of plastic and place on applicator sticks, (Fig. 18), *or*

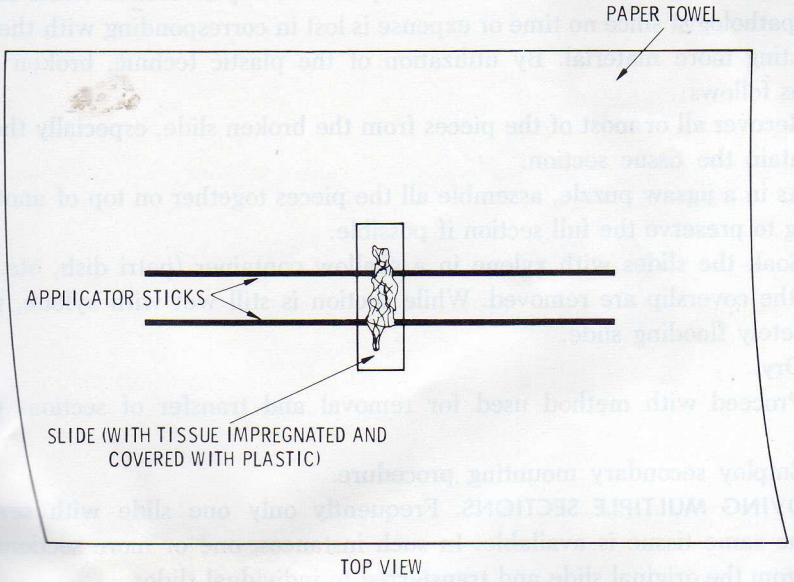


Fig. 18. Plastic drippings

(2) Place slide saturated well with xylene on the applicator sticks, (Fig.18), and immediately, before the section dries, pour the plastic on the center of slide allowing it to spread evenly to all sides.

*Cyclon-Lack, farblos Nr. 10830, lufttrocknend, Lack-u-Farben Fabrick, Bosshard and Co., Zurich, Oberlikon.
†Diatex, Scientific Products Inc., 2020 Ridge Ave., Evanston, Ill. 60201.

7. Dry thoroughly for 2 to 24 hours.
8. Incise film along the edges of the slide with a sharp instrument.
9. Soak slides in *cold* water for 2 hours.
10. Peel film from slide gently and firmly. (If film does not come off easily, return slides to the cold water for additional soaking).
11. Dry film between filter paper.
12. Number film with India ink and store between sheets of tracing paper, cut in the appropriate sizes.

SECONDARY MOUNTING PROCEDURES

1. Cut sections from the plastic film with scissors or with a razor blade.
2. Place on a small drop of Permount on the slide.
3. A second drop is placed upon the film and the coverslip applied immediately. *Avoid air bubbles.*

MICROSCOPY. For microscopy, the film is placed on a glass slide, slightly larger than the film. Good optical quality and minimal thickness are required for good results.

For visual observation, regular dry systems can be used, or oil immersion objective can be used directly on the film. For optimal results, a drop of oil should be placed underneath the section as well as on the top. Do not use xylene or other solvents to remove oil immersion from film since they will dissolve the plastic. Oil can be wiped off with a clean cloth.

REPAIRING BROKEN SLIDES. Slides sent thru the mail become potential broken slides and many are broken. The use of plastic to repair broken slides has greatly aided the pathologist since no time or expense is lost in corresponding with the contributor requesting more material. By utilization of the plastic technic, broken slides are repaired as follows:

1. Recover all or most of the pieces from the broken slide, especially those pieces which contain the tissue section.
2. As in a jigsaw puzzle, assemble all the pieces together on top of another slide, attempting to preserve the full section if possible.
3. Soak the slides with xylene in a shallow container (petri dish, etc.) until all pieces of the coverslip are removed. While section is still wet with xylene, pour plastic, completely flooding slide.
4. Dry.
5. Proceed with method used for removal and transfer of sections (see page 67).
6. Employ secondary mounting procedure.

REMOVING MULTIPLE SECTIONS. Frequently only one slide with several sections of the same tissue is available. In such instances, one or more sections may be removed from the original slide and transferred to individual slides.

PROCEDURE. 1. Original slide with multiple sections is placed in xylene to remove coverslip.
2. When coverslip is removed, let slides dry thoroughly.
3. Proceed with method for removal and transfer of sections (see page 67).

The original technic was developed by numerous collaborators and technicians in the histology laboratories of the Department of Anatomy of the University of Zurich Switzerland (See reference).

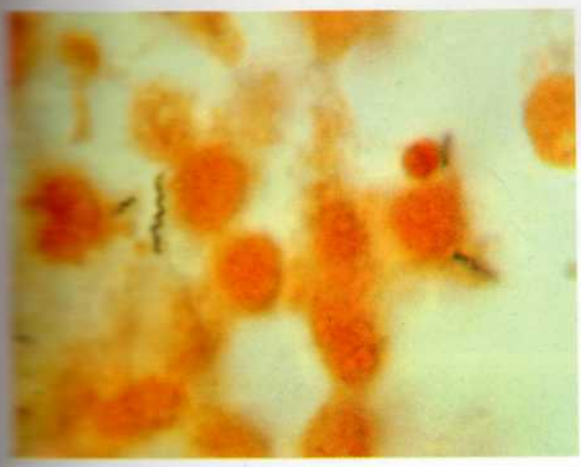
COLOR PLATES

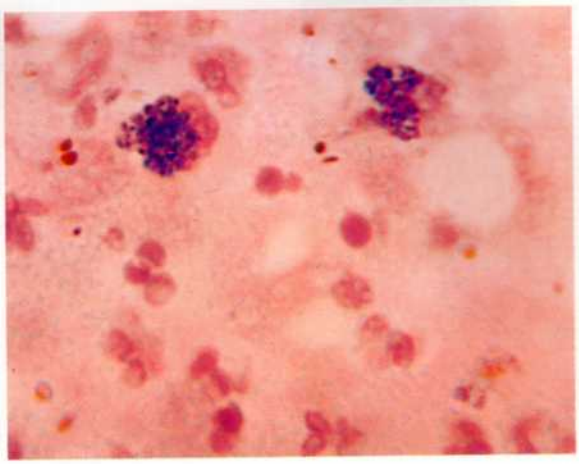
PLATE I

A. Chorioretinal lesion in granulomatous uveitis revealing *Treponema pallidum*. Warthin-Starry Stain: spirochetes—black; background—brownish yellow. (From AFIP Exhibit No. 907. Accession No. 114775.)

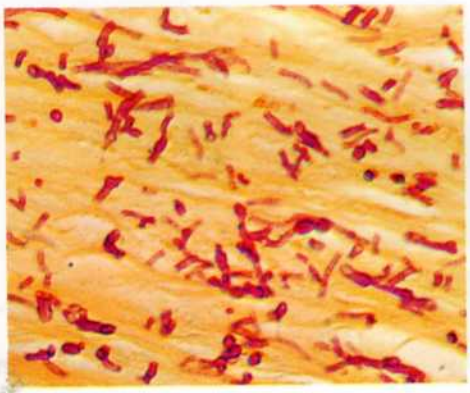
B. Acute endophthalmitis after iridectomy. MacCallum-Goodpasture stain; Gram-positive cocci—blue; other elements—various shades red to purple. (From AFIP Exhibit No. 907. Accession No. 861581.)

Contusion of eye by tree branch, corneal ulcer due to fungus. C. Gridley fungus stain: fungi—red; background—yellow. D. Periodic acid-Schiff stain (PAS) without counterstain: fungi—magenta. E. Gomori's methenamine silver stain (GMS): fungi—black; background—light green. (From AFIP Exhibit No. 907. Accession No. 831164.)

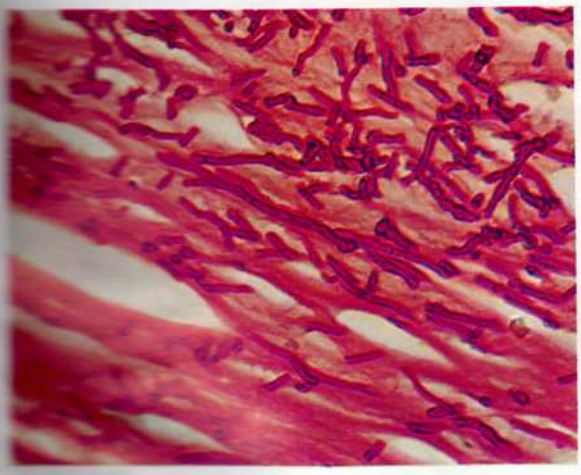




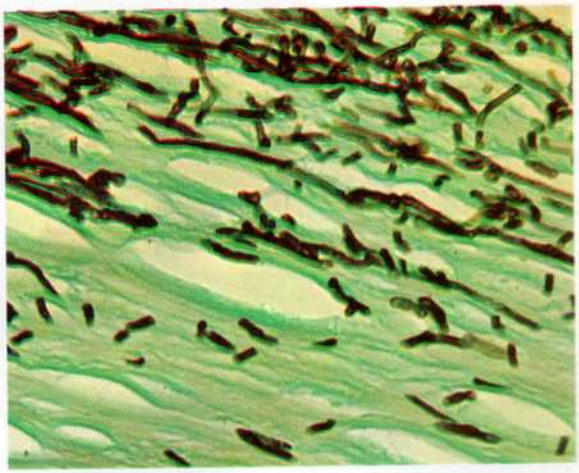
B



C

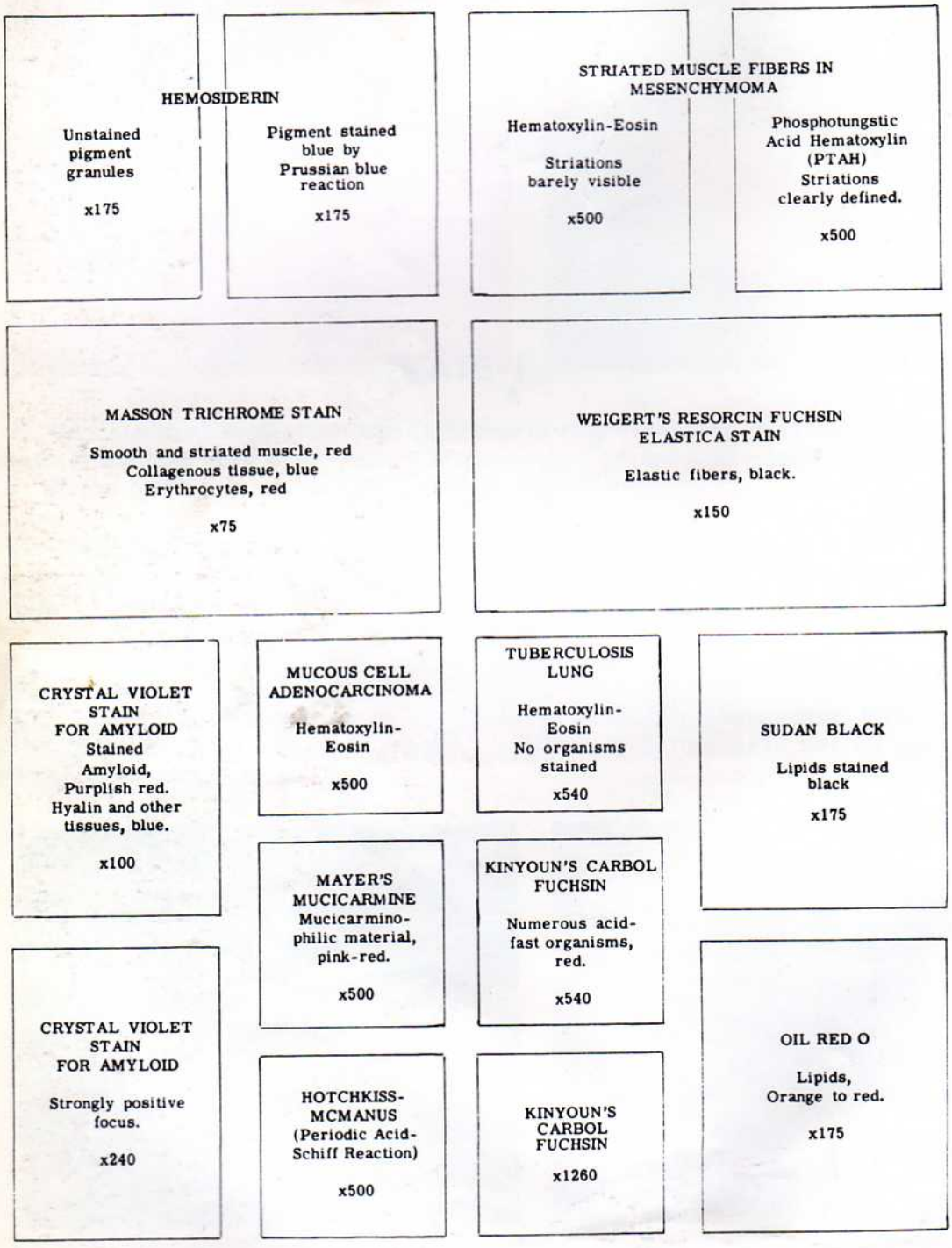


D



E

PLATE II



Reprinted from J. E. Ash and M. Raum: An atlas of Otolaryngic Pathology, American Registry of Pathology. 1956.

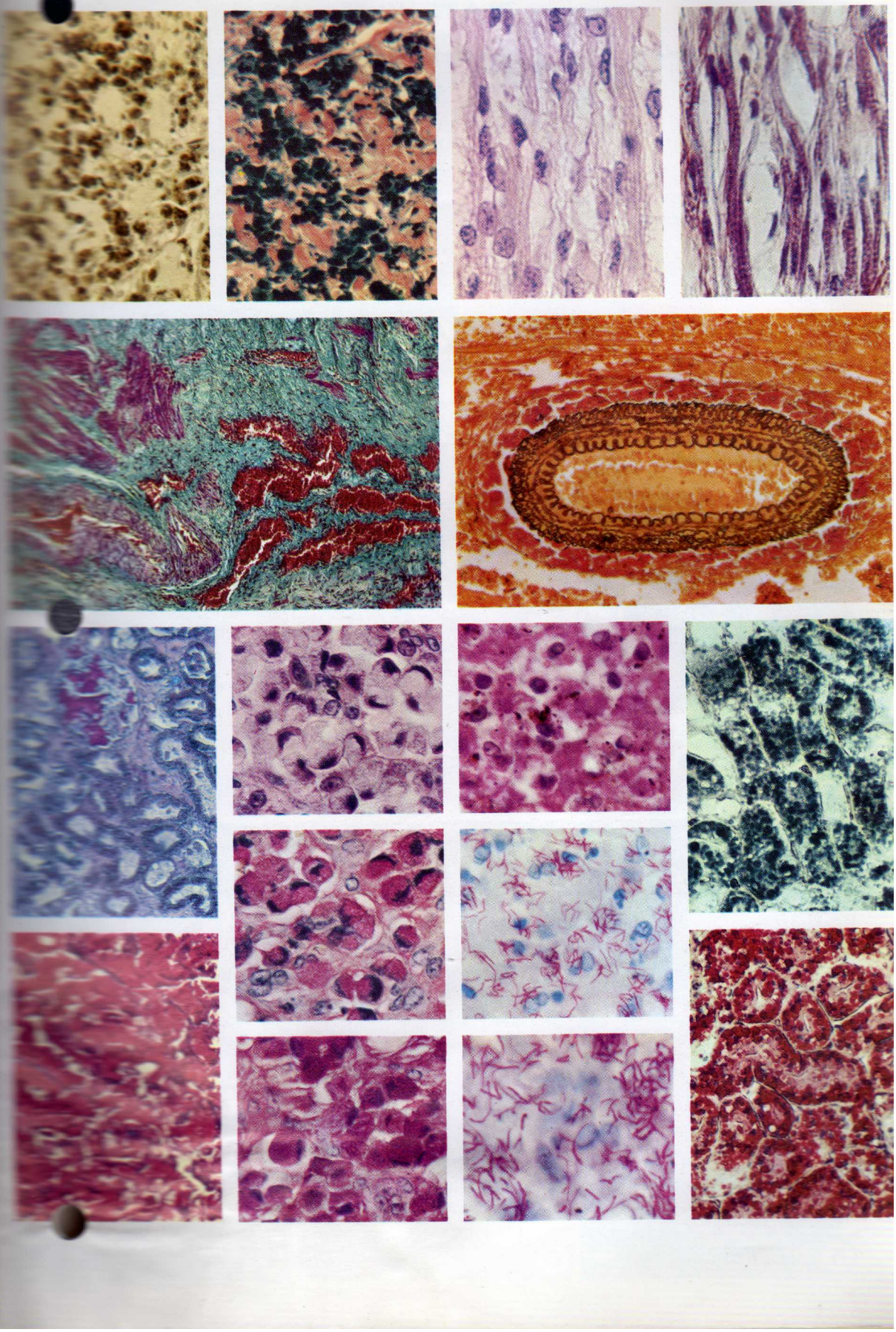
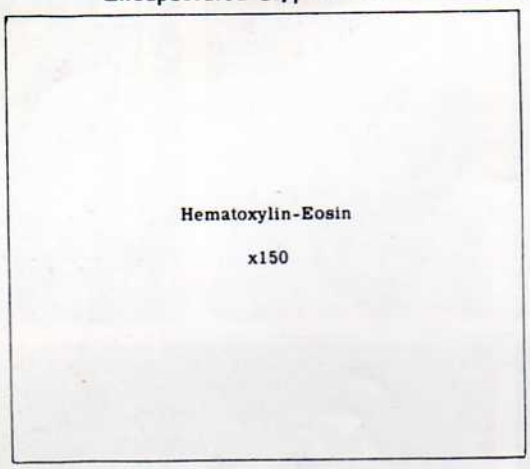


PLATE III

Encapsulated Cryptococci Demonstrated by Special Staining Procedures



Hematoxylin-Eosin

x150

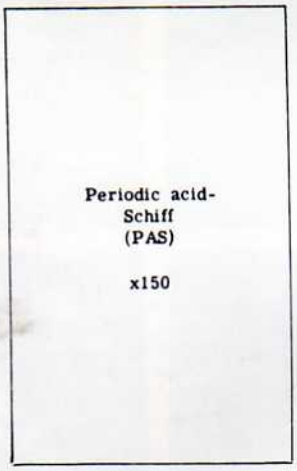
Toluidine blue,
frozen section.

x1450

MAYER'S
MUCICARMINE,

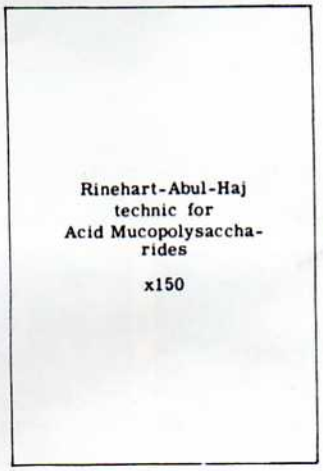
metanil yellow
counterstain

x150



Periodic acid-
Schiff
(PAS)

x150



Rinehart-Abul-Haj
technic for
Acid Mucopolysaccha-
rides

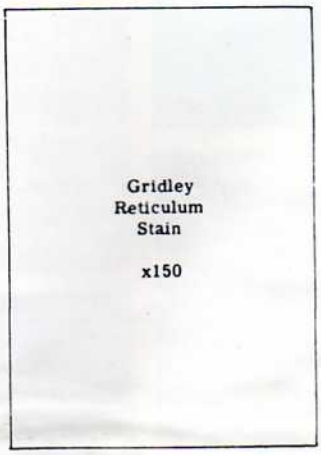
x150

Bauer
Stain

x150

Gridley
Fungus
Stain

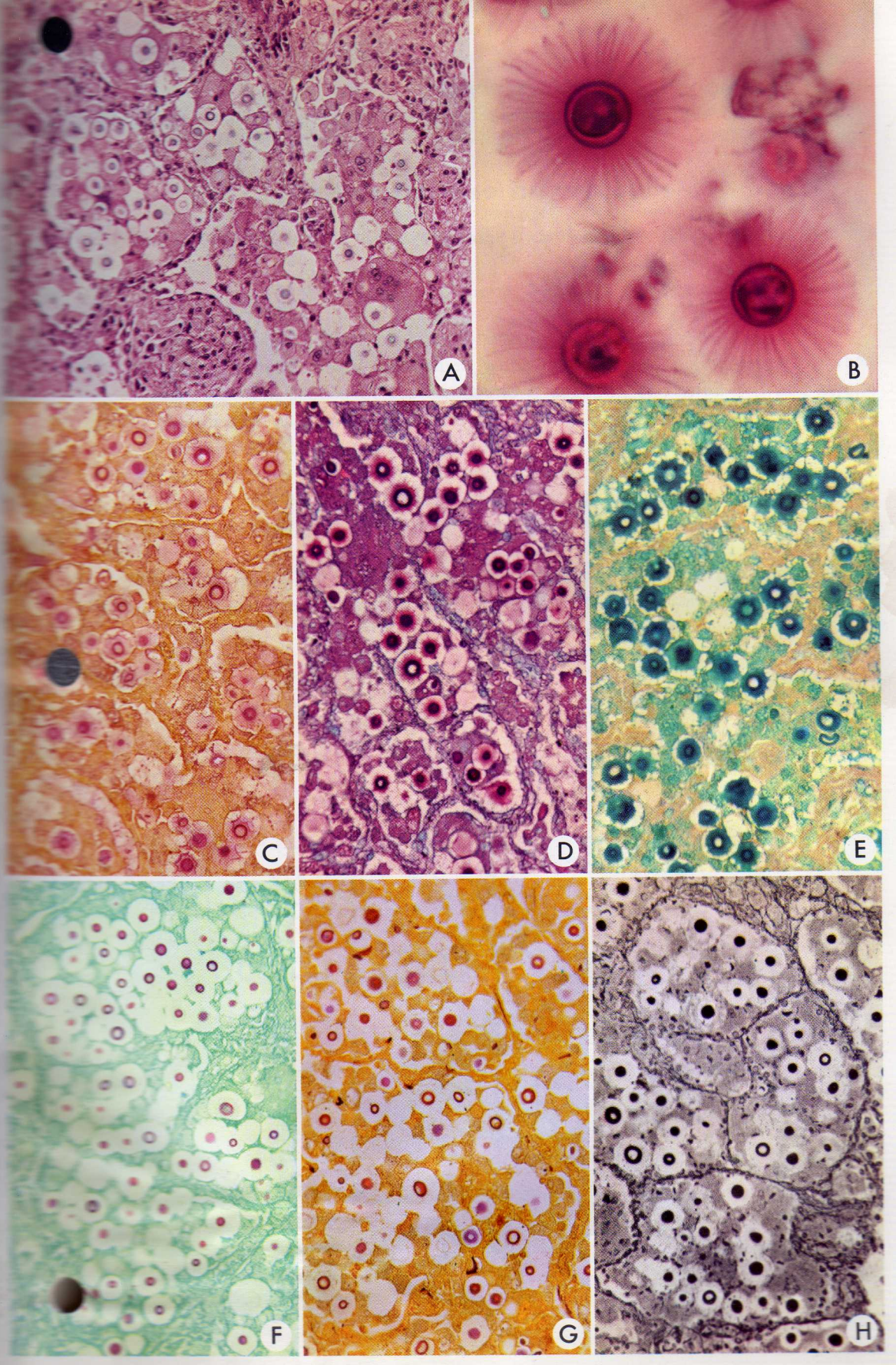
x150



Gridley
Reticulum
Stain

x150

Reprinted from M. L. Littman and L. E. Zimmerman: Cryptococcosis, New York and London, Grune and Stratton, 1956.


A
B
C
D
E
F
G
H

REFERENCE. 1. Weibel, E., Shenk, R. Morger, R., and Toendury, G.: *The preparation of serial microscopic sections in form of plastic films.* 2. Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, D.C. 20305.

MULTIPLE STAINING ON ONE SLIDE

The following technic is useful when one desires multiple special stained sections on one slide.

STAINING PROCEDURE

1. Deparaffinize in three changes of xylene.
2. Cover sections *not* to be stained with: Dow corning 7 Compound* (xylene must evaporate from section before compound is applied), *or* Diatex † (while xylene damp). Permit diatex to harden 30 minutes.
3. Dip in 80% alcohol and distilled water.
4. Perform stain desired. Only the uncovered section will stain.
5. Place in xylene, several changes, until covering media is completely dissolved.
6. Repeat Steps 2 thru 5 for as many stains required or number of sections available.

Figure 19 shows *one* slide as it undergoes application of Dow Corning or Diatex (oblique lines) and subsequent sequential staining of uncoated (unlined) section.

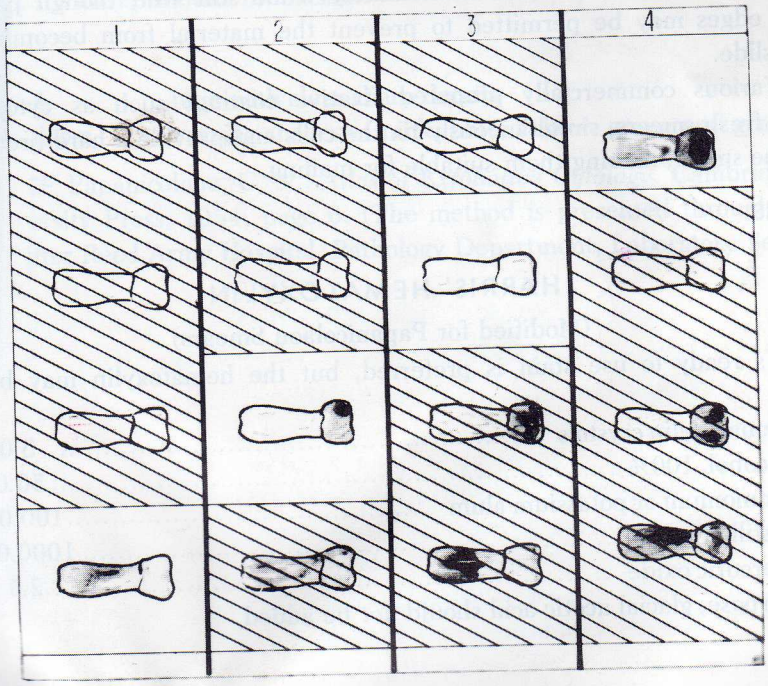


Fig. 19. Plan for multiple staining on one slide.

*Dow Corning 7 Compound, Dow Corning Company, Midland, Michigan. 07605
†Diatex Mounting Media, Scientific Products Inc., 2020 Ridge Ave., Evanston, Ill. 60201

REFERENCE. Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, D.C. 20305.

PAPANICOLAOU METHOD OF STAINING SMEARS

PREPARATION OF SMEARS

1. Exfoliated cells degenerate rapidly; therefore smears should be prepared promptly and fixed immediately. If there is to be any delay, the specimen should be fixed in 95% alcohol and refrigerated until smears can be prepared. Specimens requiring centrifugation (e.g., urine and ascitic fluid) are preserved by adding an equal volume of 50% ethyl alcohol; centrifugation is at 2000 rpm for 30 minutes.

2. Viscid secretions (e.g., vaginal, cervical and prostatic) should be smeared directly onto clean glass slides and fixed immediately.

3. Body fluids and watery exudates (e.g., urine, spinal fluid, pleural fluid, etc.) will not properly adhere to the glass slides unless the slide is first coated with a layer of Mayer's egg albumin (one drop per slide).

4. The sediment of centrifuged specimens is smeared onto glass slides coated with Mayer's egg albumin. Any remaining sediment should be processed as a biopsy specimen for conventional histologic examination.

FIXATION

1. Alcohol, 95%-15 minutes

2. Smears should be fixed immediately while still wet, though partial drying along the edges may be permitted to prevent the material from becoming detached from the slide.

3. Various commercially prepared fixative coatings such as cytospray when applied to fresh smears, simultaneously fix the cells and provide a hard protective coating over the smear, making them suitable for mailing.

SOLUTIONS

HARRIS' HEMATOXYLIN

(Modified for Papanicolaou Smears)

Ortho's ready to use stain is preferred, but the hematoxylin may be prepared as follows:

Hematoxylin crystals	5.0 gm
Alcohol, 100%	50.0 ml
Ammonium or potassium alum	100.0 gm
Distilled water	1000.0 ml
Mercuric oxide	2.5 gm

For this purpose, glacial acetic acid should *not* be added.

ORANGE G6 (OG 6)

Ortho's* ready to use stain is preferred but the stain may be prepared as follows:

Orange G6, 0.5% solution in 95% alcohol	100.0 ml
Phosphotungstic acid	0.015 gm

* Ortho Diagnostics, Raritan, New Jersey. 18869

EOSIN-AZURE 50 (EA 50)

This stain should be obtained from the Ortho Diagnostics, Raritan, New Jersey. It is ready for use after it is filtered and cannot be equalled by mixtures prepared in the laboratory.

STAINING PROCEDURE

1. After fixation, transfer slides without drying directly from 95% alcohol through 80% alcohol, 70% alcohol, 50% alcohol and tap water, to distilled water.
2. Stain in Harris' hematoxylin using either (a) Papanicolaou's modified hematoxylin (Ortho), 2 to 3 minutes, or (b) Harris' alum hematoxylin without glacial acetic acid, 2 to 3 minutes.
3. Rinse gently in tap water.
4. Differentiate in 0.25% hydrochloric acid in distilled water, 1 or 2 dips.
5. Place in gently running tap water for 5 minutes to wash out the acid and thoroughly blue the nuclei.
6. Rinse in distilled water and transfer through 50% alcohol, 70% alcohol, 80% alcohol to 95% alcohol.
7. Stain in OG 6 for 2 minutes.
8. Rinse in three changes of 95% alcohol.
9. Stain in EA 50 for 2 minutes.
10. Rinse in three changes of 95% alcohol, two changes of absolute alcohol and four changes of xylene.
11. Mount with Permount or balsam.

RESULTS

Nuclei	- blue with clear sharp details
Cytoplasm	- varying shades of pink, blue, yellow, green and gray

REFERENCE. Papanicolaou, G. N.: *Atlas of Exfoliative Cytology,* Cambridge, Mass., Harvard University Press, 1954, page 6. (The method is presented through the courtesy of the Walter Reed Army Hospital, Pathology Department, Laboratory Service.)