Manual of
HISTOLOGIC STAINING METHODS
of the
Armed Forces Institute of Pathology

Third Edition

American Registry of Pathology

Lee G. Luna (Editor)

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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

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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.

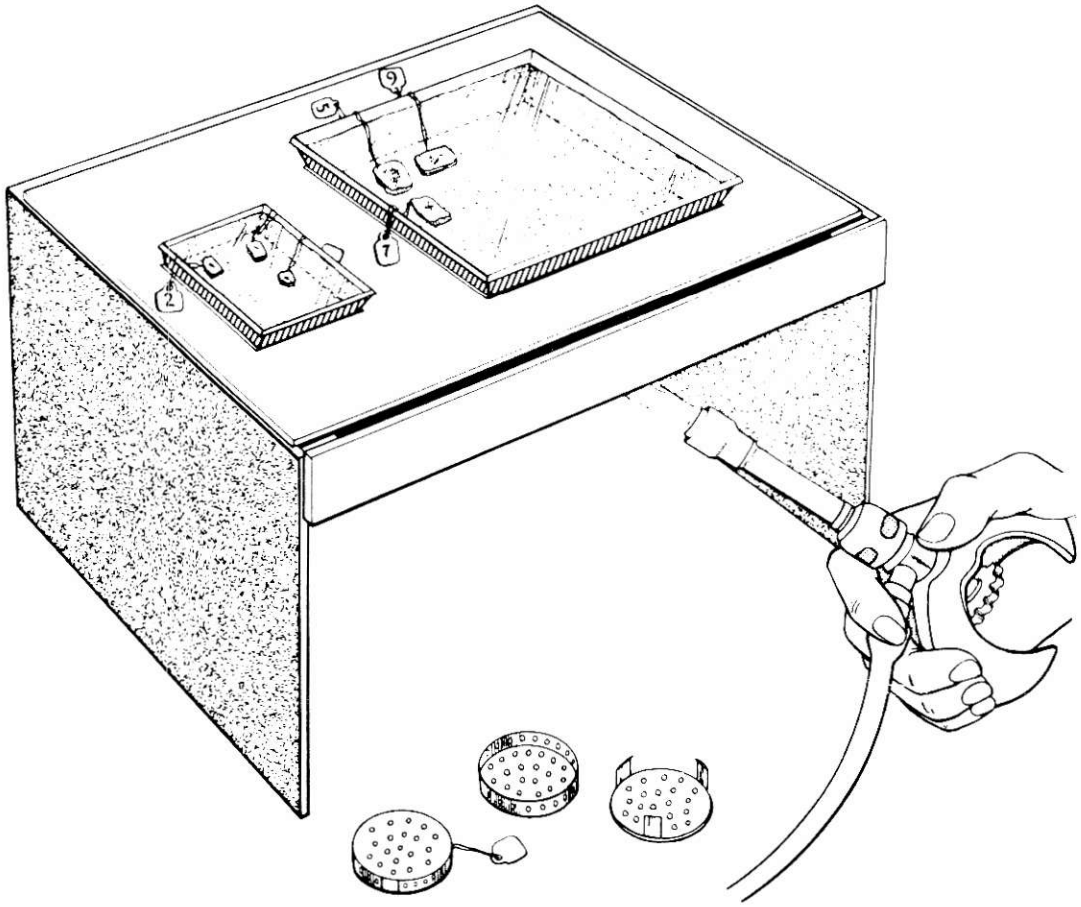


Fig. 1. Multiple block embedding in paraffin.

*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.

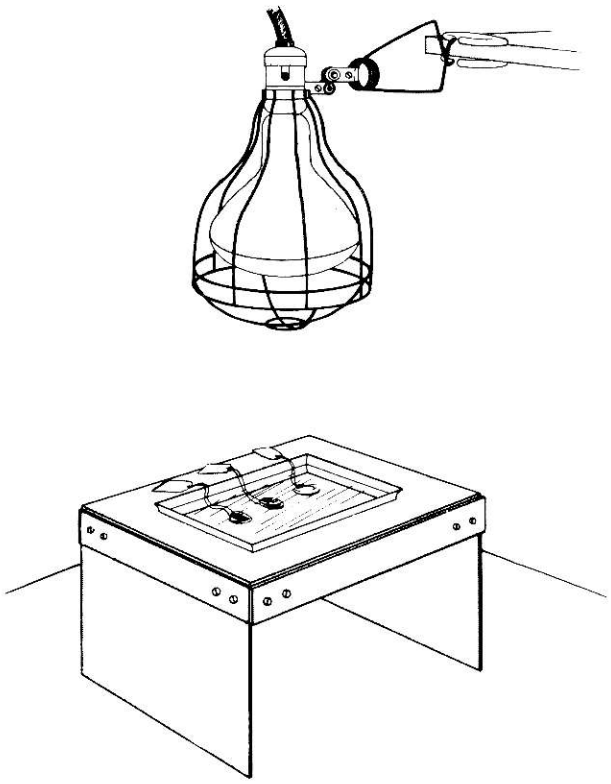


Fig. 2. 250-watt reflector infrared heat lamp with squeeze holder for suspending lamp above embedding set-up.

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.

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

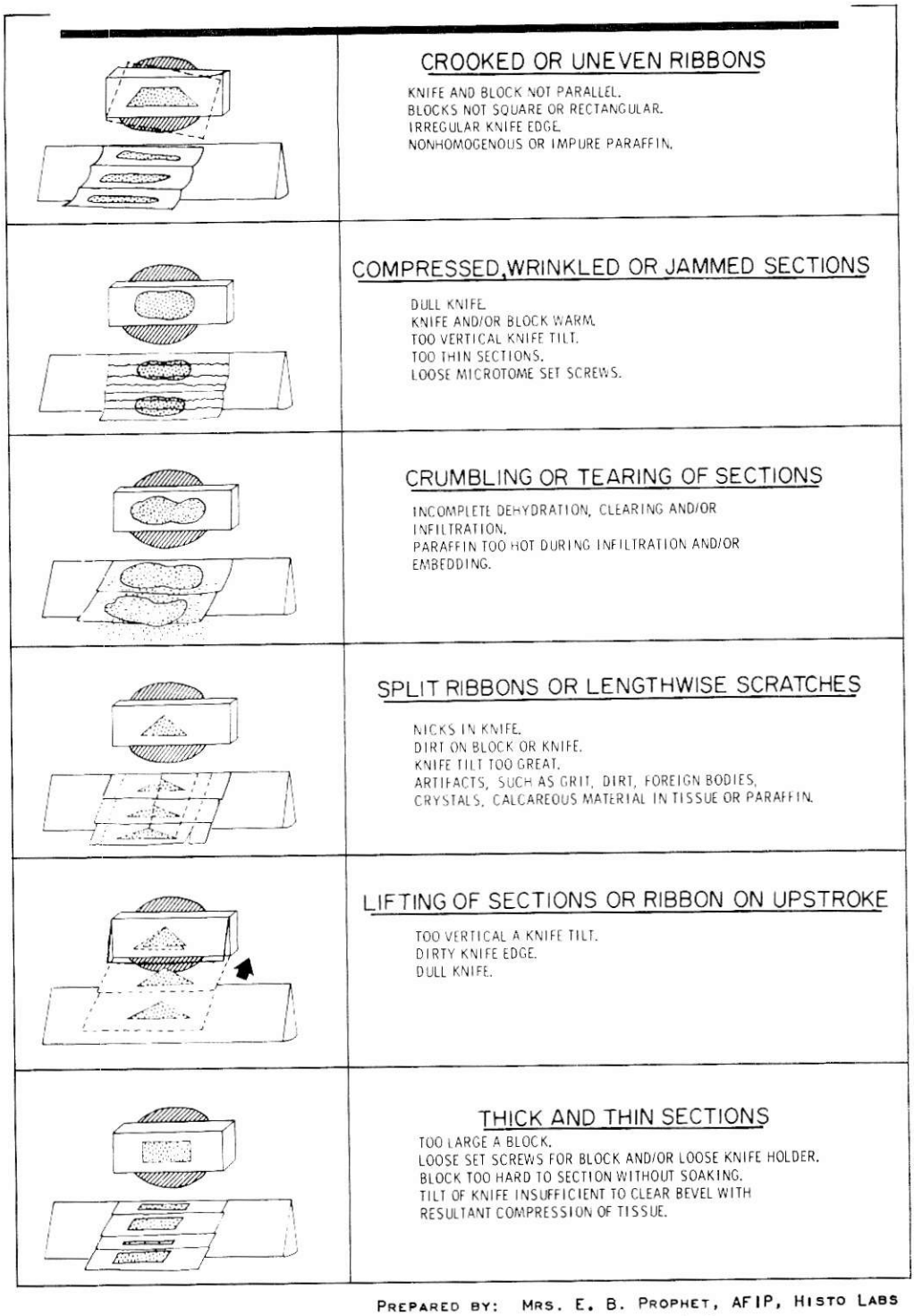


Fig. 4. Commonly encountered problems in cutting and their causes.

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

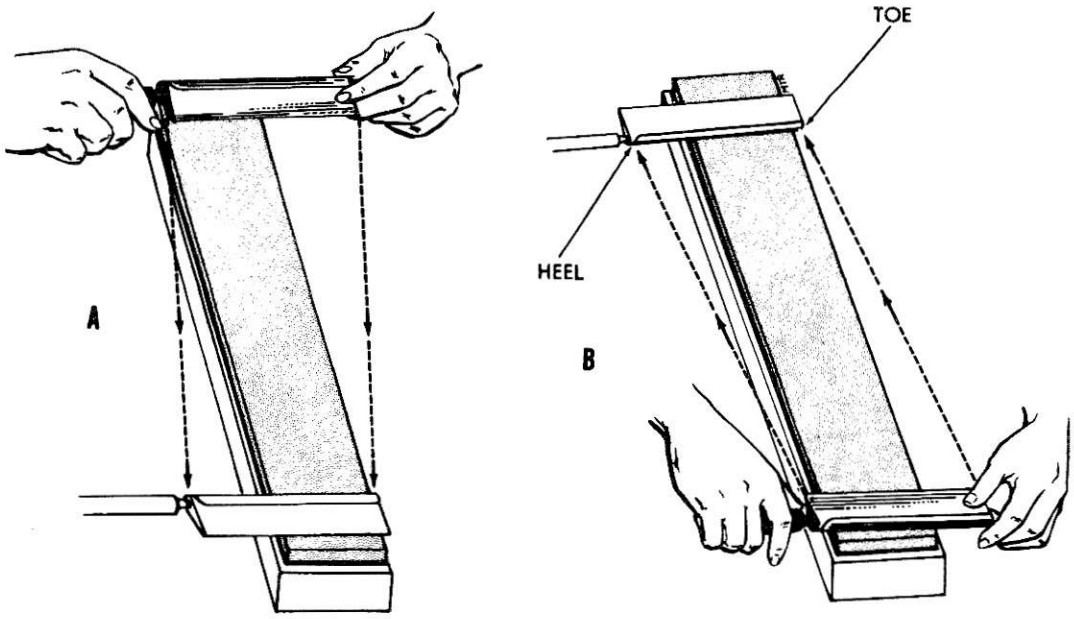


Fig. 5. Diagram of the technique of honing the microtome knife.

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.

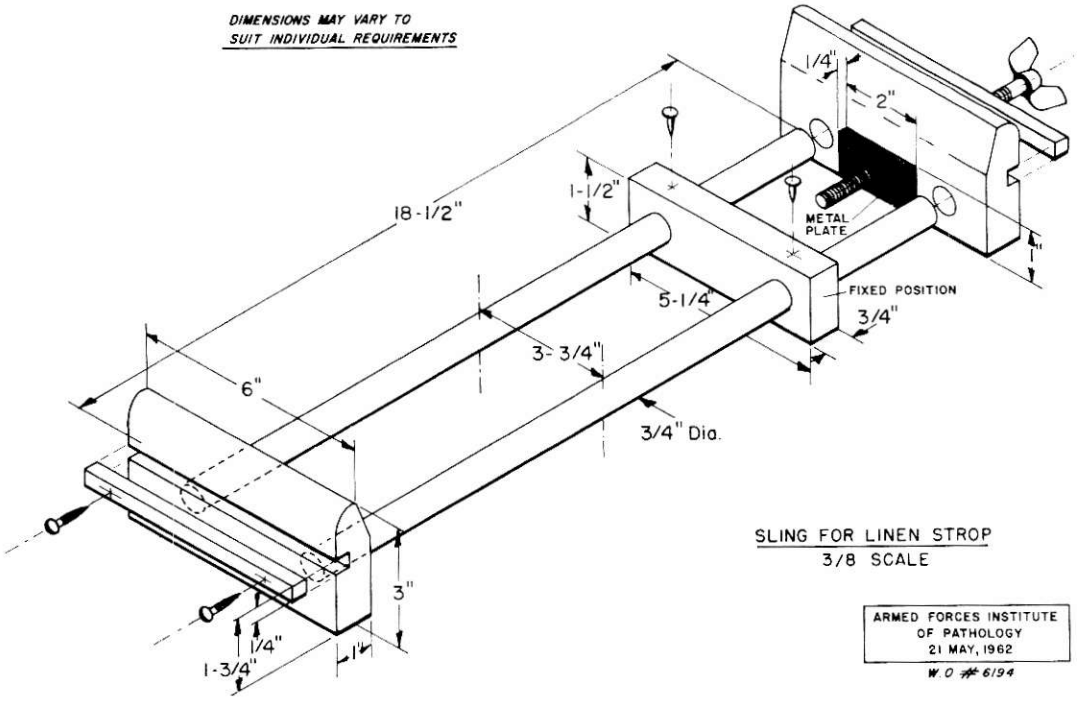


Fig. 6. Sling for a linen strop. Linen for the strop can be purchased at most fabric stores. It should be cut to the length and width dictated by the sling with pinking shears and the linen attached to the sling with the wooden slats and screws shown in the drawing. The large screw at the head of the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

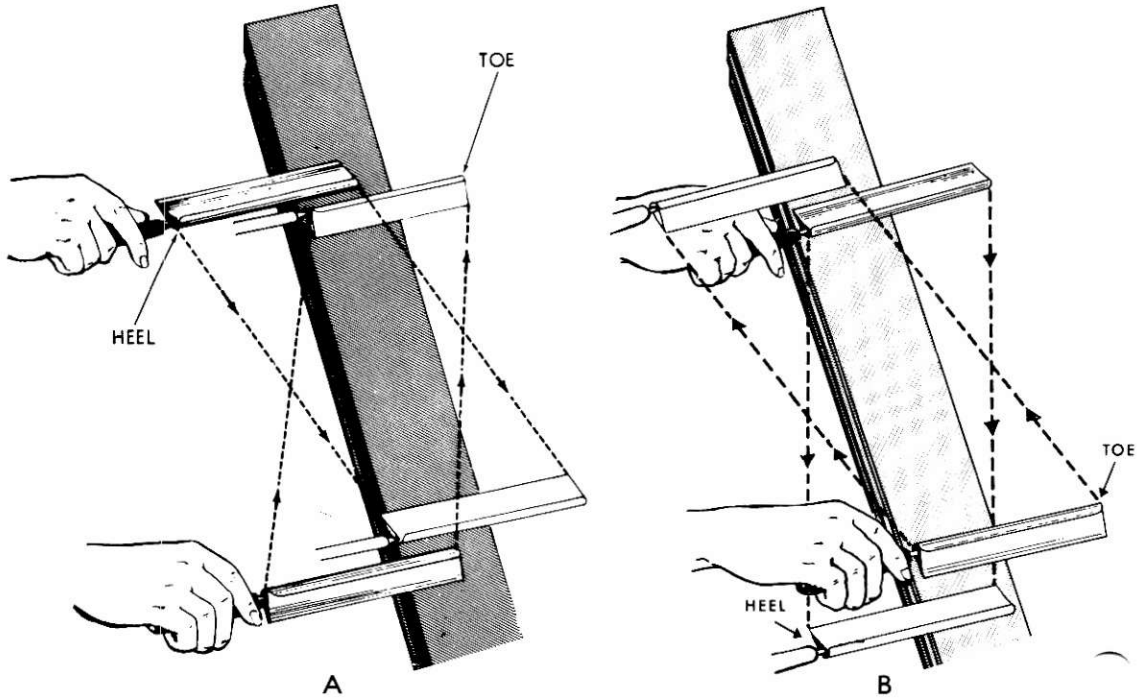


Fig. 7. Diagram of the technique of stropping a microtome knife.

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.

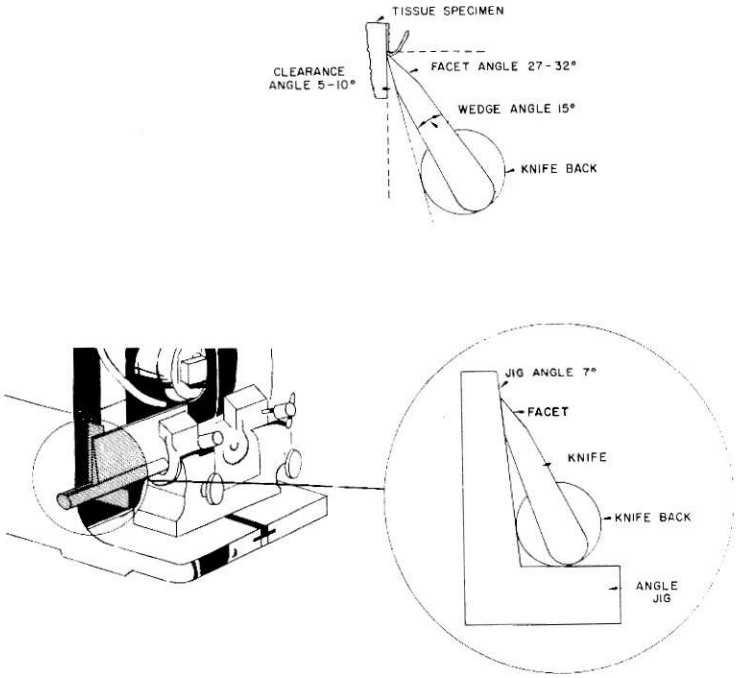


Fig. 8. Diagram showing the use of an angle jig to assure the proper cutting angle.

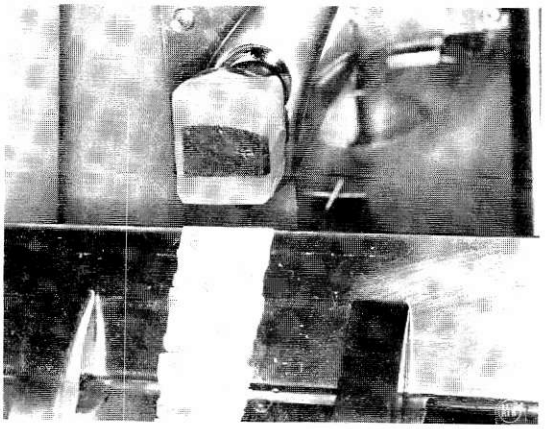
An angle jig is simple to use: Place the knife in the microtome knife clamp making certain it protrudes at least four inches to one side. Place the knife back on the protruding end of the knife. The angle jig is then placed behind the knife making sure

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.



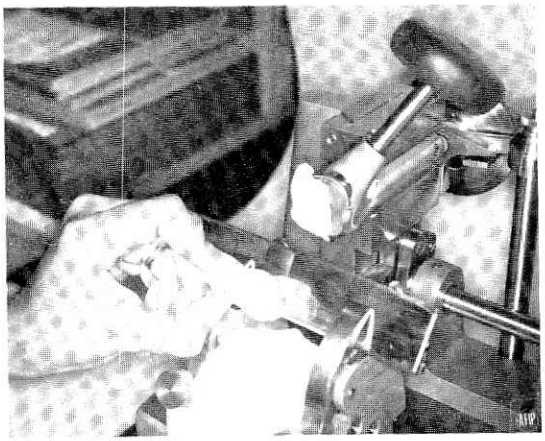


Fig.9. **ORIENTATION OF BLOCK IN MICROTOME AND ROUGH CUTTING**

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.

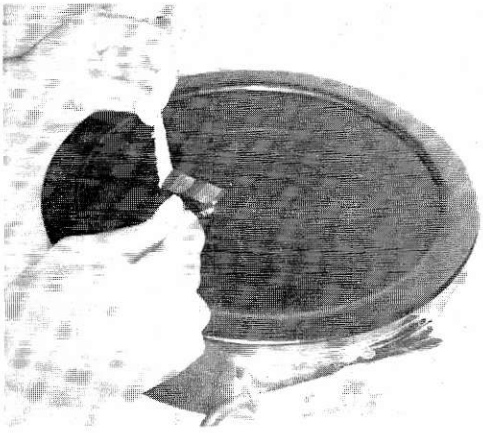


Fig.11. **FLOATING RIBBON ON FLOTATION BATH**

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.

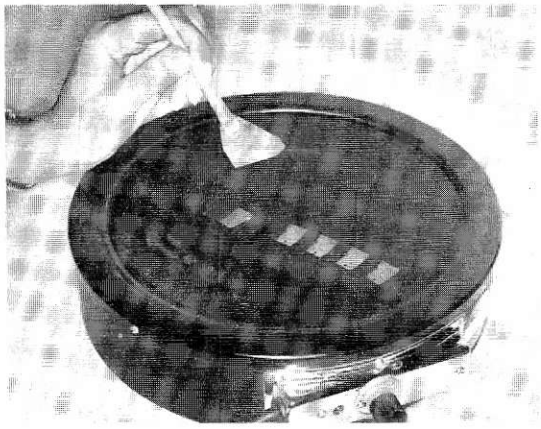


Fig.12. **SEPARATION OF SECTIONS**

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).

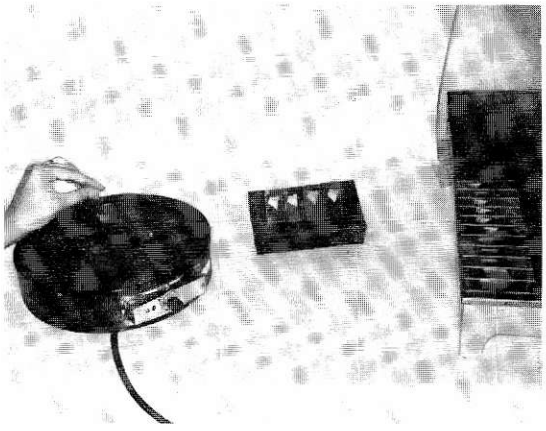


Fig.13. **PICKING UP SECTIONS ON GLASS SLIDES**

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.)

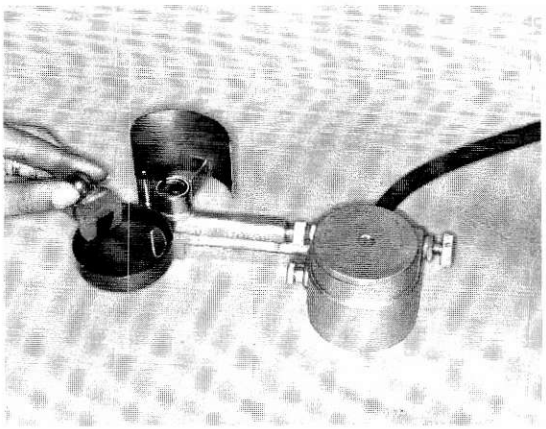


Fig.14. **RESEALING BLOCKS**

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. Alum: Potassium Sulfate	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 20
Stock Phloxine.....10.0 ml 2
Alcohol, 95% .....780.0 ml 156
Glacial acetic acid.....4.0 ml .8

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.

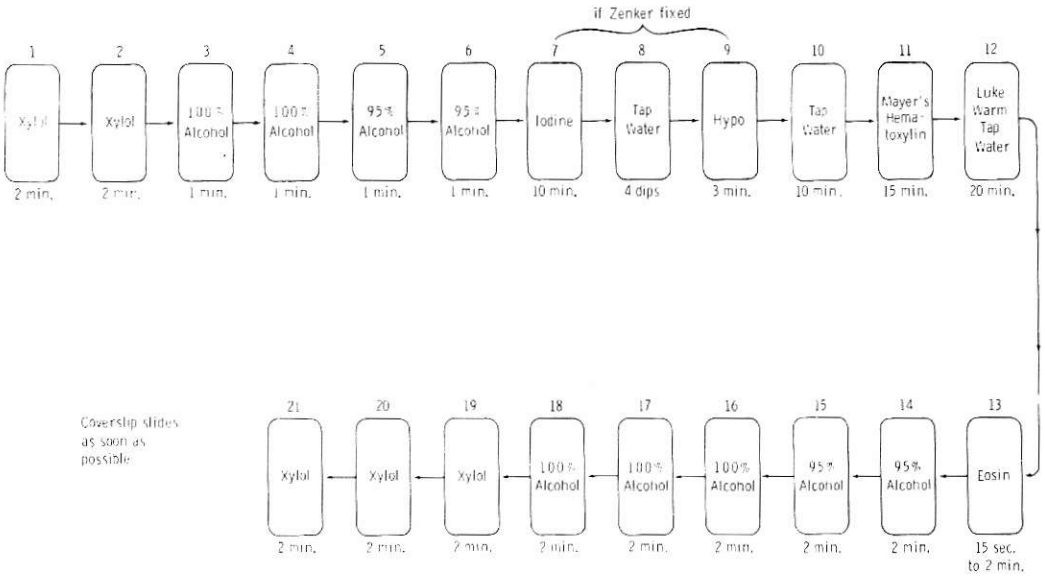


Fig. 15. Steps in staining sections with Mayer's hematoxylin and eosin.

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

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

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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. Alum: Potassium Sulfate	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	20
Stock Phloxine	10.0 ml	2
Alcohol, 95%	780.0 ml	164
Glacial acetic acid	4.0 ml	.8

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.

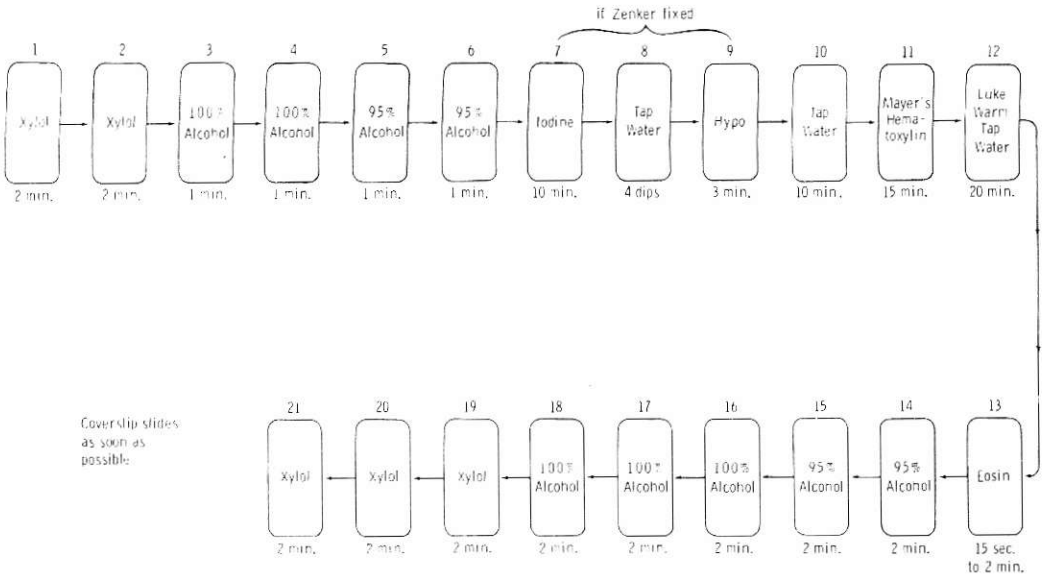


Fig. 15. Steps in staining sections with Mayer's hematoxylin and eosin.

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.

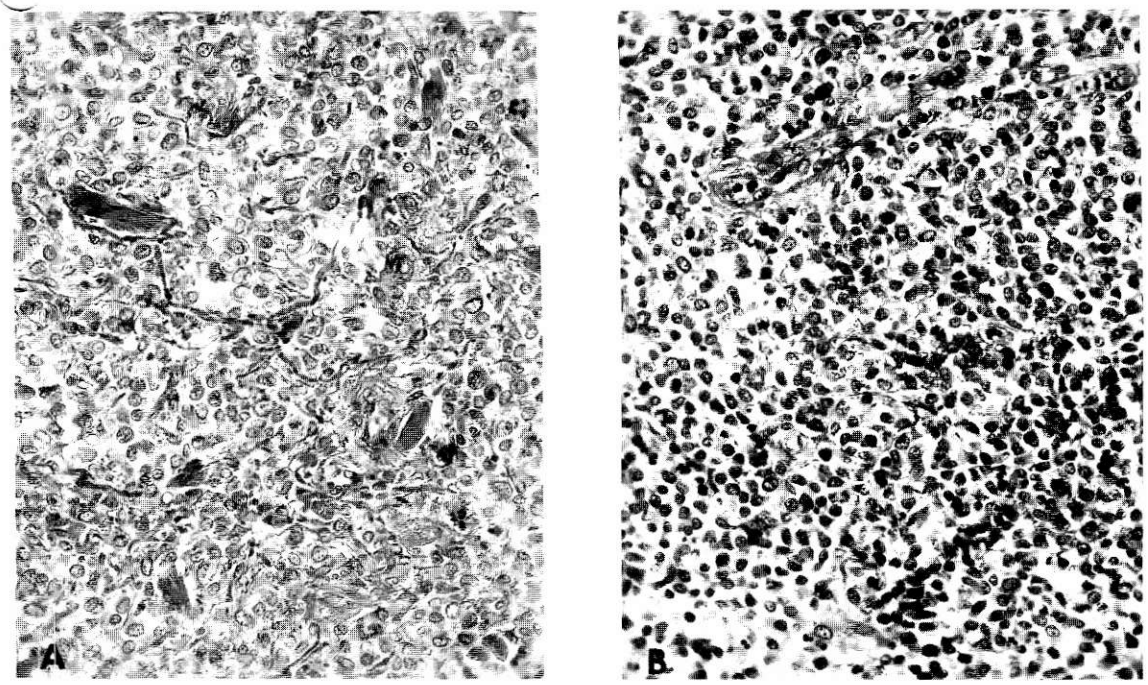


Fig. 16. Comparison of a section stained after overfixation with Zenker's solution (A) and (B) of a section after treatment with basophilic restoration properties Method # I. (A= AFIP 67-3718; B= AFIP 67-3719)

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*

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 Force 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
Chloroform	2 hours	(several changes)
Paraffin (Bioloid, wax of choice for eyes)	2 changes, 1 hour each	5 hours (several changes)
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 ($NaC_2H_3O_2 \cdot 3H_2O$)	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 60 °C oven for 24-48 hours. The final hardening may be done

*Gillings-Hamco sectioning machine, Hamco Machines, Inc., Rochester, N.Y. 14607
†Castolite (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

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

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

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.

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

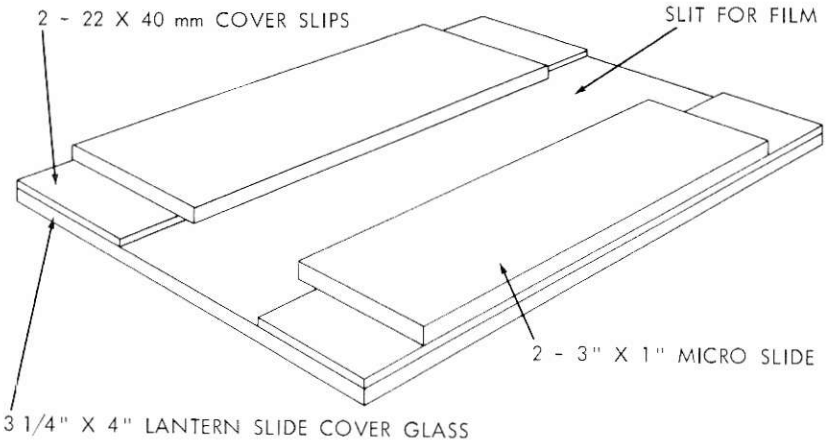


Fig.17. Fabricated film holder for viewing film strips under the microscope.

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.

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*

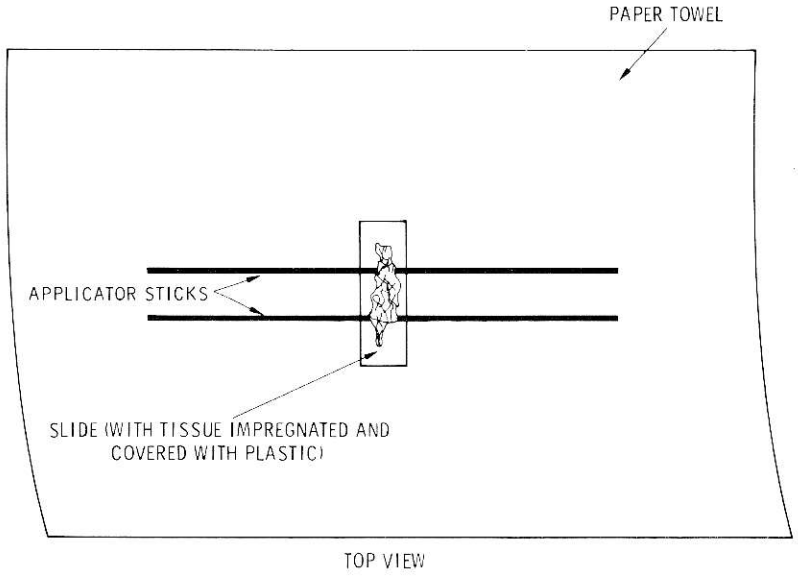


Fig. 18. Plastic drippings

(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).

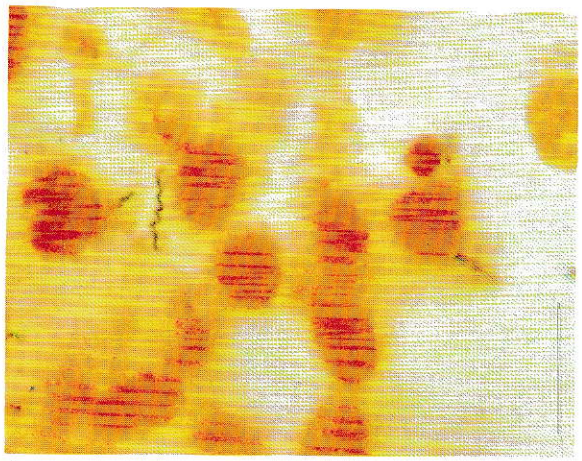
COLOR PLATES

PLATE I

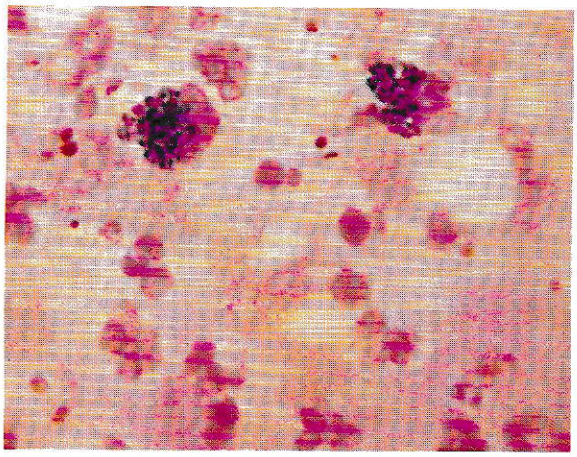
A. Chorioretinal lesion in granulomatous uveitis revealing *Treponema pallidum*. Warthin-Starry Stain: spirochetes—black; background—brownish yellow. (From AFIP Exhibit No. 907. Accession No. 114775.)

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. Grocott 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.)



A



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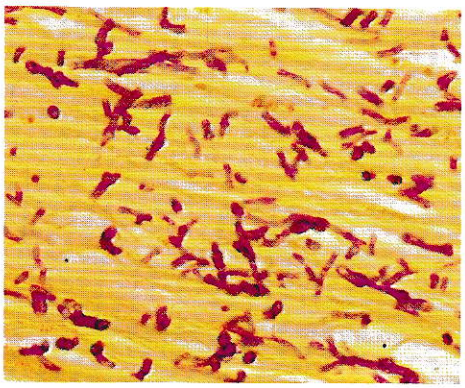
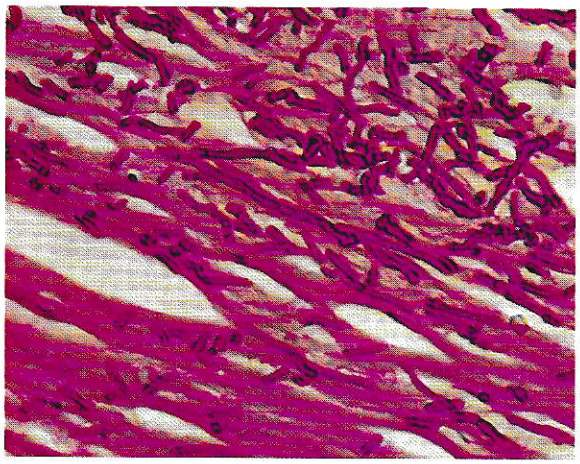
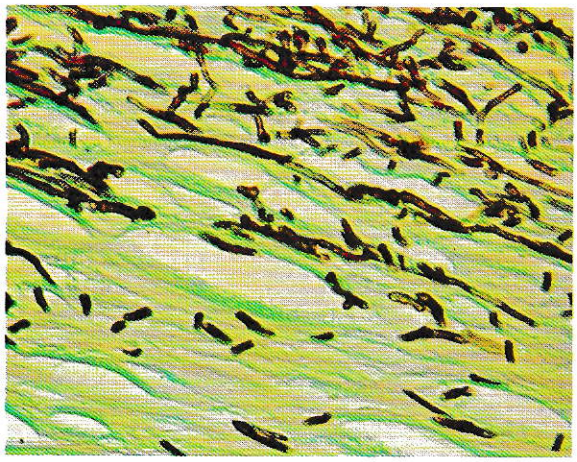


C

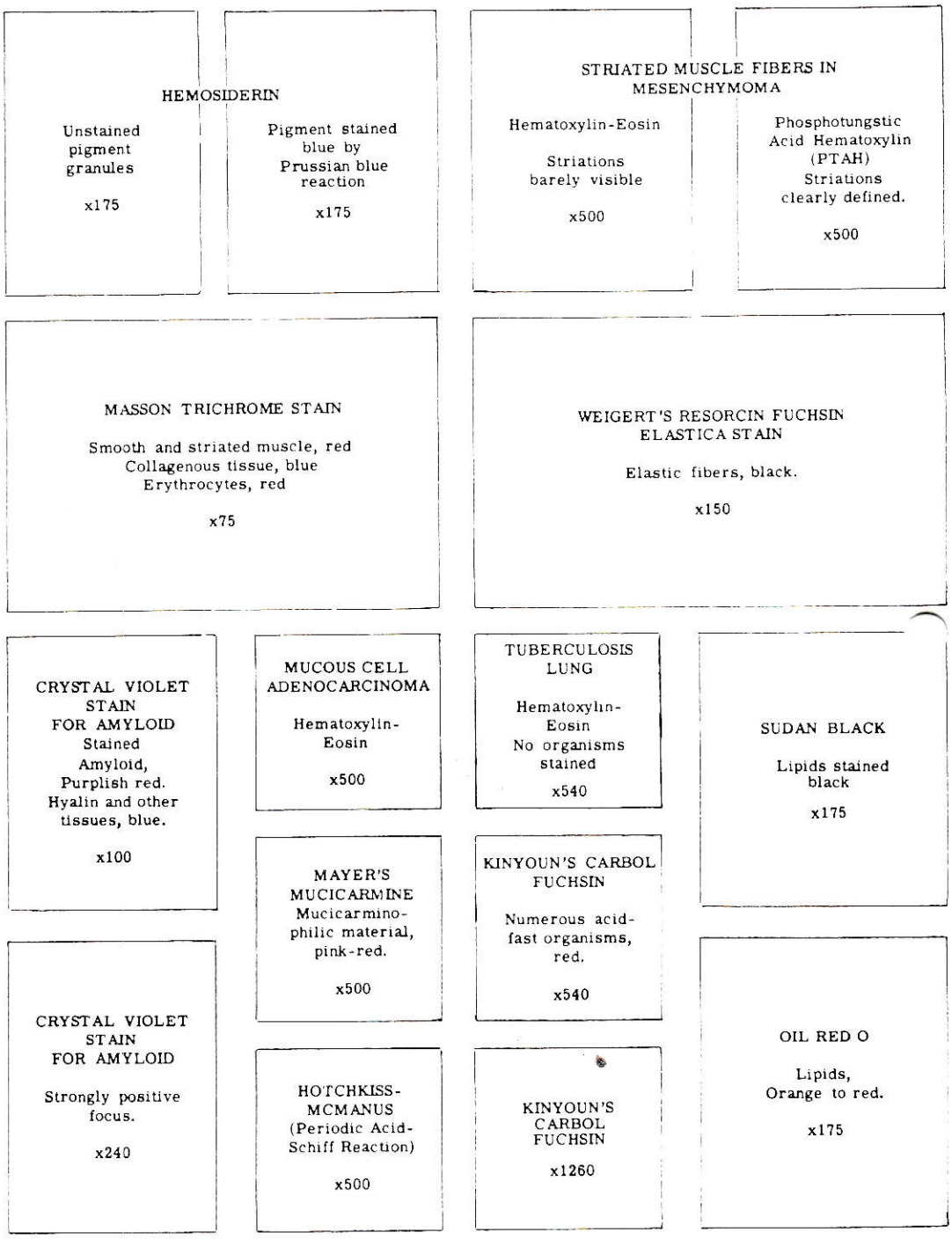


D



E

PLATE II



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

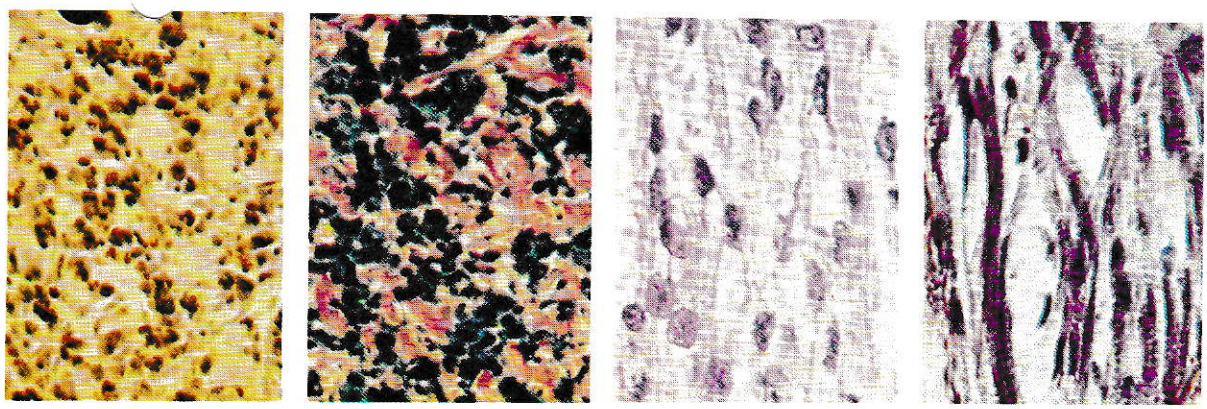
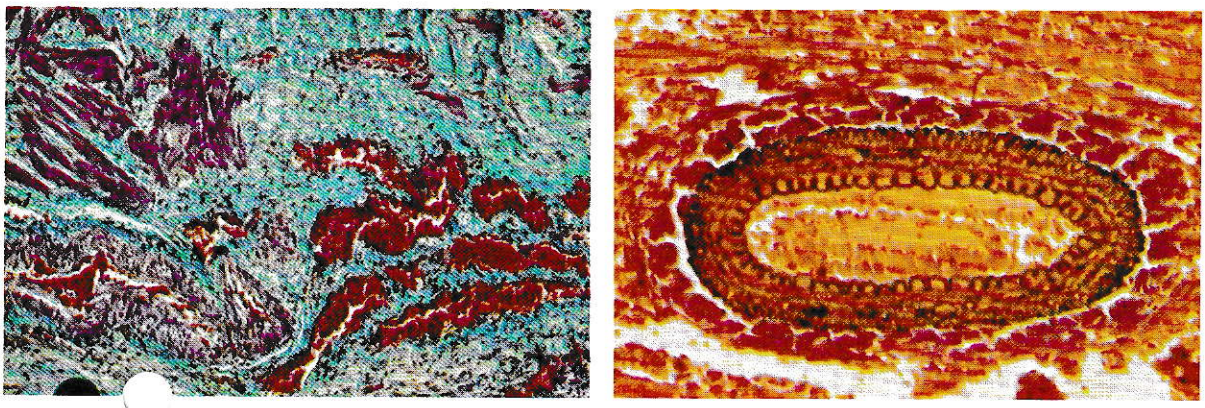
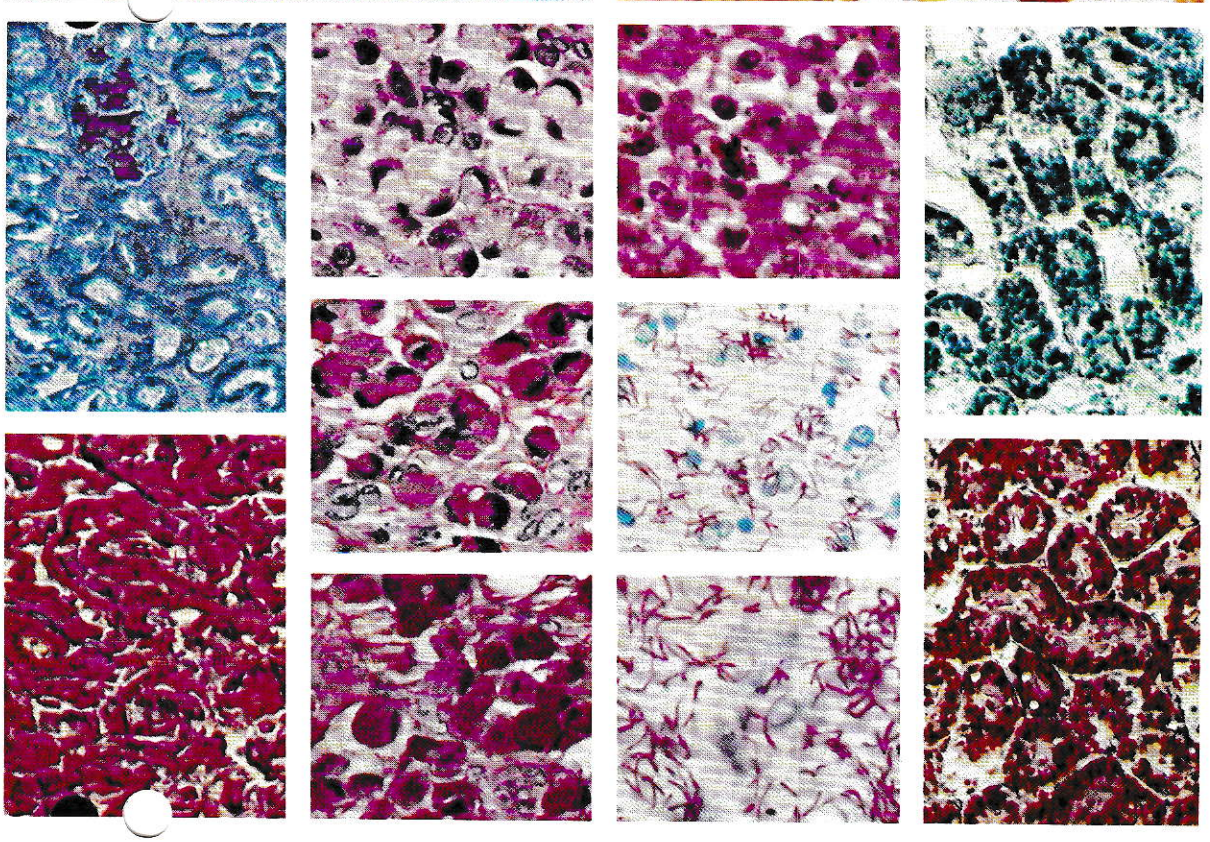
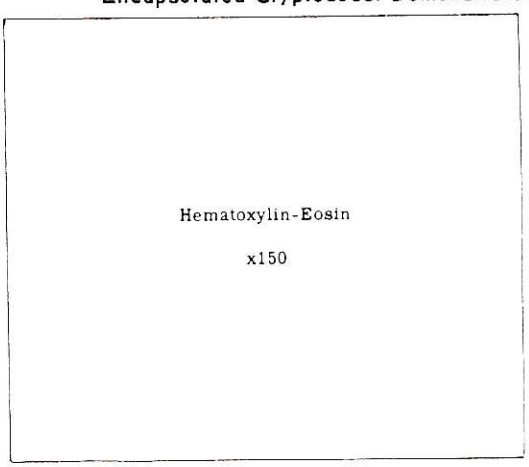




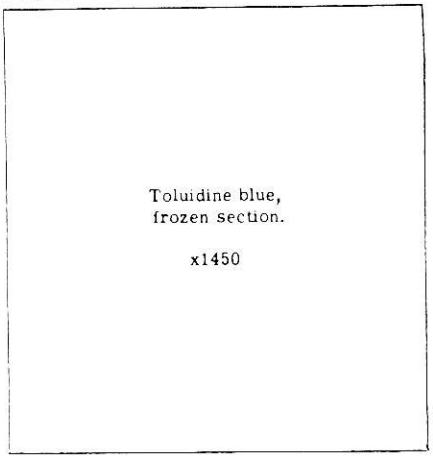
PLATE III

Encapsulated Cryptococci Demonstrated by Special Staining Procedures



Hematoxylin-Eosin

x150



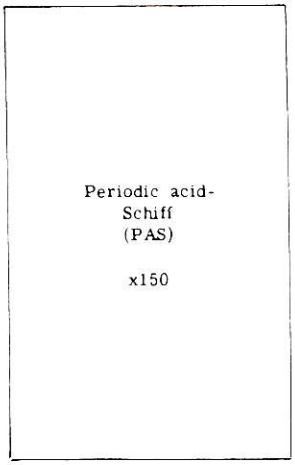
Toluidine blue,
frozen section.

x1450

MAYER'S
MUCICARMINE,

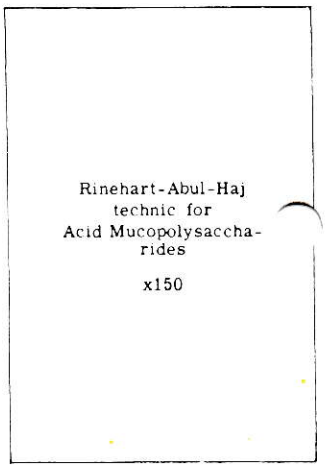
metanil yellow
counterstain

x150



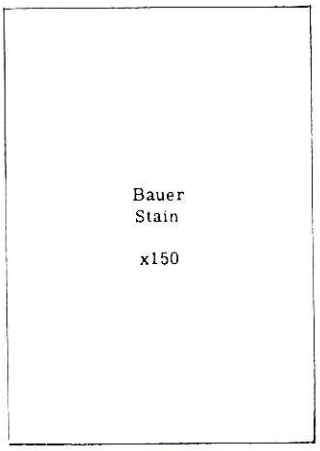
Periodic acid-
Schiff
(PAS)

x150



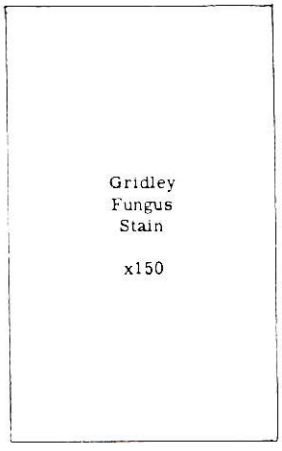
Rinehart-Abul-Haj
technic for
Acid Mucopolysaccha-
rides

x150



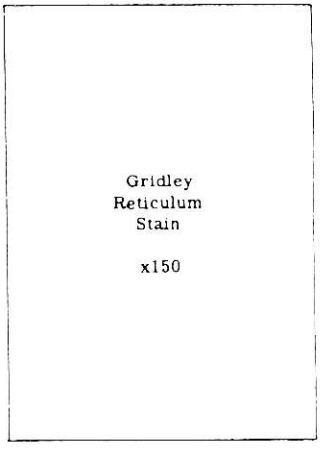
Bauer
Stain

x150



Gridley
Fungus
Stain

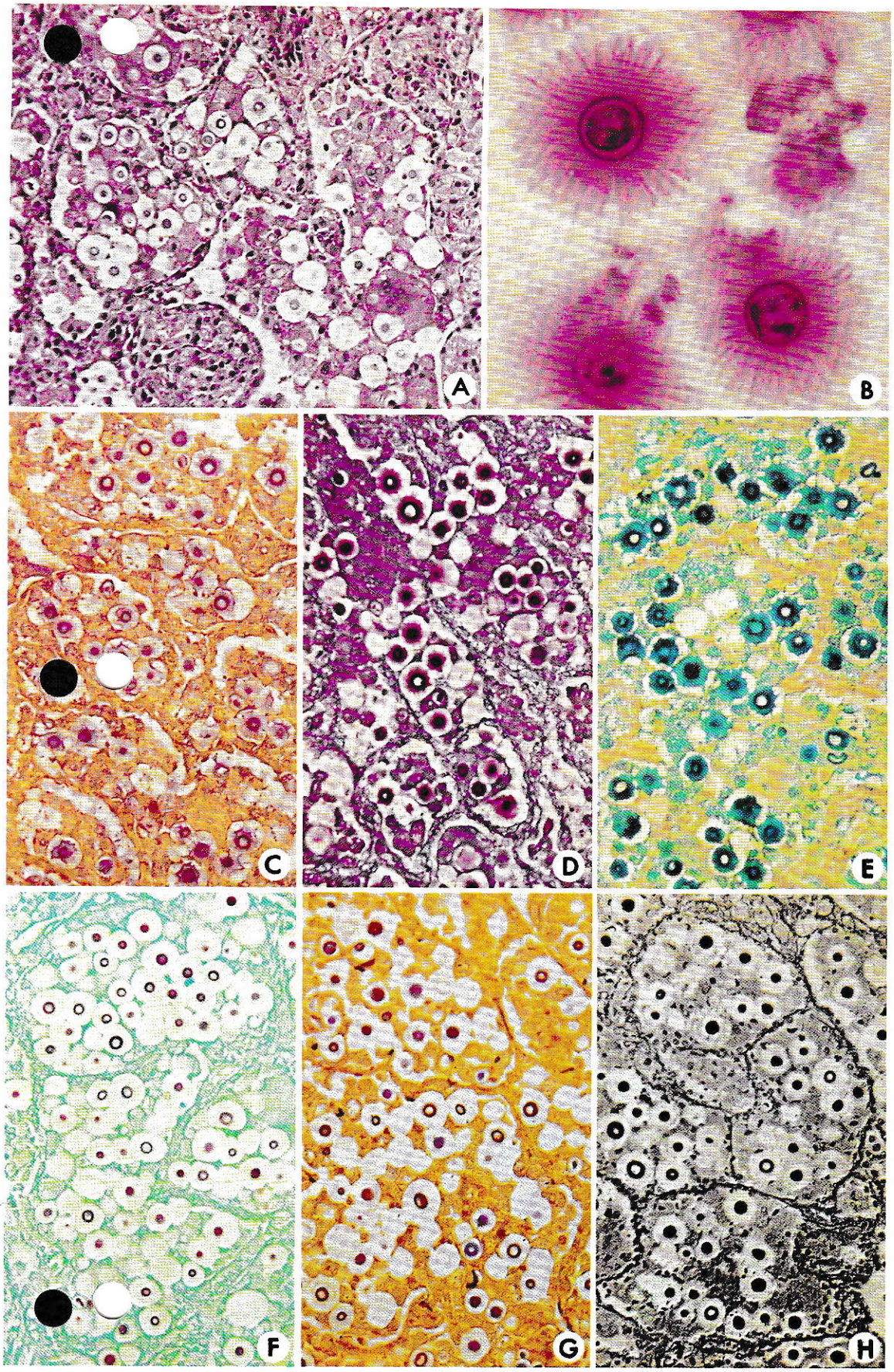
x150



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.

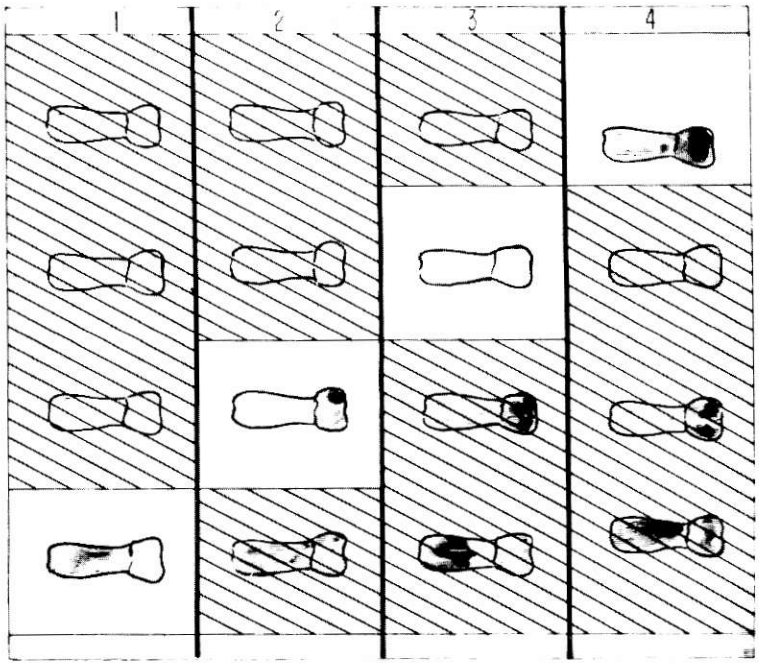


Fig. 19. Plan for multiple staining on one slide.

*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.)

Chapter 6

Methods For Connective Tissue

LILLIE'S ALLOCHROME METHOD FOR CONNECTIVE TISSUE

FIXATION. Any well fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

SCHIFF REAGENT SOLUTION

(See page 159)

0.5% PERIODIC ACID SOLUTION

Periodic acid	0.5 gm
Distilled water	100.0 ml

0.5% SODIUM METABISULFITE SOLUTION

Sodium metabisulfite	0.5 gm
Distilled water	100.0 ml

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

PICRIC ACID-METHYL BLUE SOLUTION

Picric acid, saturated aqueous	100.0 ml
Methyl blue	40.0 mg

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Periodic acid solution for 10 minutes.
3. Wash in tap water for 5 minutes.
4. Schiff's reagent solution for 10 minutes.
5. Sodium metabisulfite solution for two changes, 2 minutes each.
6. Wash in tap water for 10 minutes.
7. Weigert's iron hematoxylin solution for 2 minutes.
8. Wash in tap water for 10 minutes.
9. Picric acid-methyl blue solution for 6 minutes.
10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Nuclei	-black, gray, or brown
Cytoplasm and Muscle cells	-gray, green to greenish yellow
Reticulum and Collagen	-blue
Basement membranes	-red

REMARKS. Valuable for the distinction between basement membrane and reticulum fibers and the demonstration of arteriolosclerotic lesions.

REFERENCE. Lillie, R. D., *Amer. J. Clin. Path.* 21:484-488, 1951. Copyright by Williams and Wilkins Co.

PUCHTLER-SWEAT METHOD FOR BASEMENT MEMBRANES

FIXATION. Carnoy's solution No. 2

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

CARNOY'S SOLUTION NO. 2

Alcohol, 100%	60.0 ml
Chloroform	30.0 ml
Glacial acetic acid	10.0 ml

0.5% PERIODIC ACID SOLUTION

(See page 72)

SODIUM BISULFITE SOLUTION

Sodium bisulfite	20.0 gm
Distilled water	40.0 ml
Alcohol, 100%	10.0 ml

RESORCIN-FUCHSIN SOLUTION

(See page 79)

KERNECHTROT SOLUTION

(See page 89)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Periodic acid solution for 5 minutes.
3. Rinse in distilled water, two changes.
4. Sodium bisulfite solution for 15 hours.
5. Rinse in distilled water, five or six changes.
6. Resorcin-fuchsin solution for 4 hours.
7. Rinse in distilled water, three changes.
8. Counterstain in Kernechtrot solution for 5 minutes.
9. Rinse in distilled water, three changes.

10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Basement membranes	- black in cross sections
	gray in tangential sections
Nuclei	- pink to red

REFERENCE. Puchtler, H., and Sweat, F.: *Stain Techn.* 39: 163-166, 1964.

LUNA-ISHAK METHOD FOR BILE CANALICULI

FIXATION. 10% buffered neutral formalin is preferred.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

CELESTINE BLUE SOLUTION

Celestine blue B	0.5 gm
Ferric ammonium sulfate	5.0 gm
Hydrochloric acid, concentrated	2.0 ml
Glycerin	15.0 ml
Distilled water	85.0 ml

Dissolve the iron alum in the distilled water, then dissolve the celestine blue in this solution and bring to a boil for 3-5 minutes. Cool and add the glycerin and hydrochloric acid.

1% PHOSPHOTUNGSTIC ACID SOLUTION

Phosphotungstic acid	1.0 gm
Distilled water	100.0 ml

1% STOCK ACID FUCHSIN SOLUTION

Acid fuchsin	1.0 gm
Distilled water	100.0 ml

1% STOCK OXALIC ACID SOLUTION

Oxalic acid	1.0 gm
Distilled water	100.0 ml

WORKING ACID FUCHSIN-OXALIC ACID SOLUTION

Acid Fuchsin, stock solution	10.0 ml
Oxalic acid, stock solution	1.0 ml
Distilled water	80.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Celestine blue solution for 30 minutes.

3. Rinse in distilled water.
4. Phosphotungstic acid solution for 15 minutes.
5. Wash in tap water for 3 minutes.
6. Working acid fuchsin solution for 8 minutes.
7. Dehydrate rapidly in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Bile Canaliculi	-pink to red
Nuclei	-blue
Bile	-yellowish-green
Iron	-brown with some red inclusions
Background	-bluish-pink

Note. Water and alcohol will remove red dye on prolonged exposure, thus the reason for rapid dehydration.

REFERENCE. Luna, L. G., and Ishak, K.: *Amer. J. Med. Techn.* 33:459-466, 1967.

MALLORY'S METHOD FOR COLLAGEN

FIXATION. Zenker's

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

0.5% ACID FUCHSIN SOLUTION

Acid Fuchsin	0.5 gm
Distilled water	100.0 ml

ANILINE BLUE-ORANGE G SOLUTION

Aniline blue, water soluble	0.5 gm
Orange G	2.0 gm
Phosphotungstic acid	1.0 gm
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (hypo) (see page 41).
3. Wash in running water for 10 to 20 minutes.
4. Rinse in distilled water.
5. Acid fuchsin solution for 1 to 5 minutes. Staining with acid fuchsin solution may be omitted if it is desired that collagen fibers stand out more sharply.
6. Transfer directly to aniline blue-orange G solution for 30 to 60 minutes or longer.
7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Nuclei	- red
Collagen fibrils	- blue
Ground substance of Cartilage	- varying shades of blue
Mucin	- varying shades of blue
Amyloid	- varying shades of blue
Erythrocytes	- yellow
Basophils (beta cell)	- blue
Acidophils (alpha cell)	- red
Chromophobes	- gray
Myelin	- yellow

REMARKS. Not especially useful for collagen. However, very useful for pituitary cell differentiation.

REFERENCE. Mallory, F. B.: *Stain Techn.* 11:101-102, 1936. Copyright by Williams and Wilkins Co.

VAN GIESON'S METHOD FOR COLLAGEN FIBERS

FIXATION. Any well fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

VAN GIESON'S SOLUTION

Acid fuchsin, 1% aqueous	2.5 ml
Picric acid, saturated aqueous	97.5 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Weigert's hematoxylin solution for 10 minutes. 20 mins
3. Wash in distilled water. Wash in tap water first
4. Van Gieson's solution for 1 to 3 minutes.
5. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
6. Mount with Permount or Histoclad.

RESULTS

Collagen	- red
Muscle and Cornified epithelium	- yellow
Nuclei	- blue to black

REFERENCE. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p. 152.

PINKUS' ACID ORCEIN-GIEMSA METHOD

FIXATION. 10% buffered neutral formalin, formol alcohol, or absolute alcohol.

TECHNIQUE. Cut paraffin sections at 6 microns or 10 microns (See *Note*).

SOLUTIONS

ACID ORCEIN SOLUTION

Orcein, Synthetic Harleco* ..... 0.2 gm
Alcohol, 70% ..... 100.0 ml
Hydrochloric acid, concentrated ..... 0.6 ml

Solution is ready for staining immediately and improves on standing. It is stable for many months.

DILUTE GIEMSA SOLUTION

Giemsa, any good stock solution ..... 1 drop
Distilled water, adjusted to pH 7.0 with a phosphate buffer ..... 20.0 ml

ALCOHOL EOSIN SOLUTION

Eosin, 1% alcoholic ..... 2.0 ml
Alcohol, 95% ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to 70% alcohol.
2. Acid orcein solution for 30 minutes.
3. Wash in running tap water for 15 minutes.
4. Stain overnight in dilute Giemsa solution. Do not hurry this step.
5. Drain off excess fluid. If sections do not have the desired rose-pink color of the collagen, decolorize slightly and counterstain with eosin. Personal preference and experience controls this step.
6. Dehydrate in absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Nuclei	- deep blue
Cytoplasm of epidermis, smooth muscle, and other cells	- light blue
Collagen	- rose pink
Elastic fibers	- dark brown
Mast cell granules and many mucoid substances	- purple
Eosinophilic granules and erythrocytes	- bright red

* Hartman-Ledden Co., Philadelphia, Pennsylvania 19142

Note: Sections cut at ten microns are preferable, since they give a better three-dimensional picture. Step 2 stains elastic fibers specifically. If background becomes overstained; decolorize by dipping in absolute alcohol or 0.1% hydrochloric acid alcohol.

REFERENCE. This stain was modified from the original Unna-Taenzer procedure by Hermann Pinkus *(Arch. Dermat. & Syph.* **49:** 355, 1944) and further modified and simplified by Hermann Pinkus and Rosie Hunter.

GOMORI'S ALDEHYDE FUCHSIN METHOD

FIXATION. 10% buffered neutral formalin, or any well fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

ALDEHYDE FUCHSIN SOLUTION

Basic fuchsin	1.0 gm
Alcohol, 70%	200.0 ml
Hydrochloric acid, concentrated	2.0 ml
Paraldehyde	2.0 ml

Let stand at room temperature for 2 to 3 days or until stain is deep purple in color.

VAN GIESON'S SOLUTION

(See page 76)

0.25% METANIL YELLOW SOLUTION

Metanil yellow	0.25 gm
Distilled water	100.0 ml
Glacial acetic acid	0.25 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Rinse in several changes of 95% alcohol.
3. Aldehyde fuchsin solution for 30 minutes.
4. Rinse off excess stain in 95% alcohol.
5. Rinse in water.
6. Counterstain as desired with Van Gieson's solution, or metanil yellow, or Gomori's trichrome stain.
7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Elastic fibers and mucin - Deep purple

Other tissues are stained according to the counterstain used.

REMARKS. Aldehyde fuchsin has been used quite successfully in the study of mucosaccharides. The details for these methods may be found in Chapter 10.

REFERENCE. Gomori, G.: Techn. Bull. Reg. Med. Techn. 7:115-117, 1946.

HART'S METHOD FOR ELASTIC FIBERS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

0.25% POTASSIUM PERMANGANATE SOLUTION

Potassium permanganate	0.25 gm
Distilled water	100.0 ml

5% OXALIC ACID SOLUTION

Oxalic acid	5.0 gm
Distilled water	100.0 ml

RESORCIN-FUCHSIN SOLUTION (STOCK)

Basic fuchsin	2.0 gm
Resorcinol	4.0 gm
Distilled water	200.0 ml

Mix in a procelain dish, bring to a boil and let boil for 1 minute, then add 25 ml of 29% ferric chloride solution. Cool, filter, and leave precipitate on filter paper until dry. Return to porcelain dish, which should be dry but still contains whatever part of the precipitate remains adherent to it. Add 200 ml of 95% alcohol and heat very carefully dissolving all precipitate from filter paper before discarding it. Add 4 ml of hydrochloric acid. This solution keeps for several months.

RESORCIN-FUCHSIN SOLUTION (WORKING)

Resorcin-fuchsin, stock	10.0 ml
Alcohol, 70%	100.0 ml
Hydrochloric acid, concentrated	2.0 ml

VAN GIESON'S SOLUTION

(See page 76)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Potassium permanganate solution for 5 minutes.
3. Rinse in distilled water.
4. Oxalic acid solution until sections are clear.
5. Wash in tap water for 5 minutes then place in distilled water.
6. Working solution of resorcin-fuchsin overnight.
7. Wash in tap water for 10 minutes.
8. Rinse in distilled water.
9. Van Gieson solution for 1 minute.
10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Elastic fibers	-blue-black to black
Nuclei	-blue to black
Collagen	-pink to red
Other tissue elements	-yellow

REMARKS. This method is especially useful in the demonstration of fine elastic fibers and elacin (degenerated elastic fibers).

REFERENCE. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p. 169. AFIP modification.

WEIGERT'S METHOD FOR ELASTIC FIBERS

FIXATION. Any well fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

WEIGERT'S IRON HEMATOXYLIN SOLUTION
(See page 35)

RESORCIN-FUCHSIN SOLUTION
(See page 79)

VAN GIESON'S SOLUTION
(See page 76)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Weigert's hematoxylin solution for 10 minutes.
3. Wash well in water. Intensifies stain.
4. Resorcin-fuchsin solution for 30 minutes or longer.
5. Rinse in 95% alcohol.
6. Wash in tap water.
7. Van Gieson's solution for 1 minute. Save solution. If overstained, water will remove excess van Gieson's solution.
8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
9. Mount with Permount or Histoclad.

RESULTS

Elastic fibers	-blue-black to black
Nuclei	-blue to black
Collagen	-pink to red
Other tissue elements	-yellow

REFERENCE. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p.168.

FRASER-LENDRUM METHOD FOR FIBRIN

FIXATION. Zenker's.

TECHNIQUE. Cut paraffin section at 6 microns.

SOLUTIONS

CELESTINE BLUE SOLUTION

Ferric ammonium sulfate ..... 2.5 gm
Distilled water ..... 50.0 ml
Dissolve overnight at room temperature. Add:
Celestine blue ..... 0.25 gm
Boil for 3 minutes. Filter when cool, add 7 ml glycerin.

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

ORANGE G-PICRIC ACID SOLUTION

Saturated picric acid in 80% alcohol ..... 200.0 ml
Orange G ..... 0.4 gm

1% ACID FUCHSIN SOLUTION

Acid fuchsin ..... 1.0 gm
Distilled water ..... 99.0 ml
Glacial acetic acid ..... 1.0 ml

DIFFERENTIATING SOLUTION

Orange G-Picric acid solution ..... 30.0 ml
Alcohol, 80% ..... 70.0 ml

MACFARLANE'S SOLUTION (STOCK)

Phosphotungstic acid ..... 25.0 gm
Picric acid ..... 2.5 gm
Alcohol, 95% ..... 100.0 ml

MACFARLANE'S SOLUTION (WORKING)

MacFarlane's solution, stock ..... 40.0 ml
Alcohol, 95% ..... 40.0 ml
Distilled water ..... 20.0 ml

2% LIGHT GREEN SOLUTION

Light green, SF yellowish ..... 2.0 gm
Distilled water ..... 98.0 ml
Glacial acetic acid ..... 1.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant in Zenker's solution overnight if formalin fixed.
3. Wash in running water.
4. Remove mercuric chloride crystal with iodine and clear with sodium thiosulfate (see page 41).
5. Wash in running tap water for 15 minutes.
6. Celestin blue solution for 5 minutes.
7. Wash in tap water.
8. Mayer's hematoxylin solution for 5 minutes.
9. Wash in tap water for 5 minutes.
10. Orange G-picric acid solution for 5 minutes.
11. Wash in tap water for 1 minute.
12. Acid fuchsin solution for 5 minutes.
13. Wash in tap water.
14. Differentiating solution for 10-15 seconds.
15. Wash in tap water.
16. MacFarlane's working solution for 5 minutes.
17. Wash in tap water.
18. Counterstain in light green solution for 1 minute.
19. Wash in tap water.
20. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
21. Mount with Permount or Histoclad.

Note: If fibrin is to be demonstrated, fixation in mercuric chloride fixatives is essential. Ideally, secondary fixation of post mordant mercuric chloride fixatives, Zenker's, etc. is usually beneficial and revitalizes poorly fixed tissues.

RESULTS

Fibrin, keratin, some cytoplasmic granules	- red
Erythrocytes	- orange
Collagen	- green

REFERENCES. Lendrum, A. C., Fraser, D. S., Slidders, W., and Henderson, R.: *J. Clin. Path.* 15:401-413, 1962.

AYOUB-SHKLAR METHOD FOR KERATIN AND PREKERATIN

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

5% ACID FUCHSIN SOLUTION

Acid fuchsin	5.0 gm
Distilled water	100.0 ml

ANILINE BLUE-ORANGE G SOLUTION

(See page 75)

STAINING PROCEDURE. For consistent staining results *use fresh solutions.*

1. Deparaffinize and hydrate to distilled water.
2. Acid fuchsin solution for 3 minutes.
3. Transfer slides directly to aniline blue-orange G solution for 45 minutes.
4. Transfer slides directly to 95% alcohol for three changes.
5. Dehydrate with absolute alcohol and clear in xylene, two changes each.
6. Mount with Permount or Histoclad.

RESULTS

Keratin	- brilliant red
Stratified squamous epithelium	- gray
Stratum spinosum, upper area illustrating pre-keratin-like substances	- orange
Connective tissue	- deep blue
Erythrocytes	- deep red

REMARKS. This is a modification of Mallory's connective tissue stain.

REFERENCE. Ayoub, P., and Shklar, G.: *J. Oral Surg.* 16:580-581, 1963. Copyright by American Dental Association. (Reprinted by permission).

DANE'S METHOD FOR PREKERATIN, KERATIN, AND MUCIN

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

1% PHLOXINE SOLUTION

Phloxine	1.0 gm
Distilled water	100.0 ml

ALCIAN BLUE SOLUTION

Alcian blue, 1%	50.0 ml
Glacial acetic acid, 1%	50.0 ml

ORANGE G SOLUTION

Orange G	0.5 gm
Phosphotungstic acid	2.0 gm
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mayer's hematoxylin solution for 10 minutes.
3. Blue in running tap water for 10 minutes, then rinse in distilled water.
4. Phloxine solution for 3 minutes
5. Wash in running tap water to remove excess phloxine.
6. Rinse in distilled water.
7. Alcian blue solution for 5 minutes.
8. Wash in tap water for 2 minutes, then rinse in distilled water.
9. Orange G solution for 13 minutes.
10. Transfer slides to 95% alcohol, two changes, five dips each.
11. Absolute alcohol, two changes, 15 dips each.
12. Clear in xylene.
13. Mount with Permount or Histoclad.

RESULTS

Acid mucopolysaccharides	turquoise blue
Prekeratin and keratin	orange to red-orange
Nuclei	orange to brown

REFERENCE. Dane, E. T., and Herman, D. L.: *Stain Techn.* 38:97-101, 1963.

MENZIES' METHOD FOR MUSCLE STRIATIONS

FIXATION. 10% buffered neutral formalin

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

BROMPHENOL BLUE SOLUTION

Bromphenol blue*	0.05 gm
Distilled water	100.0 ml
Glacial acetic acid	2.0 ml

0.5% ACETIC ACID SOLUTION

Glacial acetic acid	0.5 ml
Distilled water	100.0 ml

SCOTT'S WATER SOLUTION

Sodium or potassium bicarbonate	2.0 gm
Magnesium sulfate	20.0 gm
Distilled water	1000.0 ml

Dissolve separately and then combine. A few crystals of thymol or 5 to 10 ml of formaldehyde (40%) solution is added to prevent the growth of mold.

*Tetrabromophenolsulphonphthalein

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Bromphenol blue solution 4 to 24 hours.
3. Rinse briefly in water and place in acetic acid solution for 5 to 10 minutes.
4. Wash and blue in Scott's water solution for 30 seconds.
5. Rinse in distilled water.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Cardiac and skeletal muscle - intense blue

Due to the dense background, cardiac and skeletal muscle striations require oil immersion for full resolution.

Note. Scott's water solution used for blueing also differentiates by removing excess dye, but the 30 second treatment should not be exceeded. Immersion for 5 to 10 minutes in acetic acid solution between the primary stain and the Scott's water solution has a definite effect in heightening contrast.

REFERENCE. Menzies, D. W.: *Stain Techn.* 36:285-287, 1961.

MALLORY'S PHOSPHOTUNGSTIC ACID HEMATOXYLIN METHOD (PTAH)

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

PHOSPHOTUNGSTIC ACID HEMATOXYLIN SOLUTION

Hematoxylin	1.0 gm
Phosphotungstic acid	20.0 gm
Distilled water	1000.0 ml

Dissolve the solid ingredients in separate portions of the water, the hematoxylin with the aid of heat. When cool, combine. No preservative is necessary. Ripen by the addition of 0.2 gm potassium permanganate. Stain may be used immediately but the best results are not obtained until the solution is at least two weeks old.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant in Zenker's solution that contains 5% acetic acid for 3-1/2 hours in a 56°-60°C oven.
3. Remove from the oven and let cool for 30 minutes.
4. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).
5. Wash in running water for 10 minutes.
6. Stain in PTAH solution for 90 minutes in a 56°-60°C oven.
7. Remove and allow to cool for 15 minutes.

8. Dehydrate rapidly in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

9. Mount with Permount or Histoclad.

RESULTS

Muscle	- blue, with cross striations well defined
Collagen	- red
Nuclei	- blue
Fibrin	- blue

REMARKS. Excellent technic for all types of tissue (Exception: Central Nervous System, see Chapter 12).

Note. Modifications by Lee G. Randle, HMCS, USN, AFIP. The use of potassium permanganate is an excellent oxidizing media since the PTAH solution can be used immediately. More important is the fact that the staining is more consistent when oxidized in this manner. At step 2 Zenkerization of slides in oven for 3-1/2 hours has stabilized the consistency of the staining results. At step 7, 90 minutes in the oven is necessary for complete staining.

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

HEIDENHAIN'S ANILINE BLUE METHOD

FIXATION. Zenker's, Helly's, Bouin's, Carnoy's.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% AZOCARMINE B SOLUTION

Azocarmine B	1.0 gm
Distilled water	100.0 ml

Bring to a boil, filter at 56 ° C, cool and add 1 ml glacial acetic acid. Must be kept in refrigerator and filtered before use.

1% ANILINE-ALCOHOL SOLUTION

Aniline	1.0 ml
Alcohol, 95%	100.0 ml

5% PHOSPHOTUNGSTIC ACID SOLUTION

Phosphotungstic acid	5.0 gm
Distilled water	100.0 ml

ANILINE BLUE SOLUTION

Aniline blue, water soluble	0.5 gm
Orange G	2.0 gm

Distilled water ..... 300.0 ml
Glacial acetic acid ..... 8.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. If formalin fixed, mordant in Zenker's overnight, then remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).
3. Wash in running tap water.
4. Rinse in distilled water.
5. Place in preheated azocarmine B solution at 56 °C for 15 minutes.
6. Rinse in distilled water.
7. Differentiate in aniline-alcohol solution until cytoplasm and connective tissue are pale pink and nuclei stand out sharply. (Control differentiation by rinsing in 1% acetic alcohol).
8. Mordant in phosphotungstic acid solution for 15 minutes.
9. Rinse in distilled water.
10. Aniline blue solution for 15 minutes.
11. Rinse in distilled water.
12. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes, 2 minutes each.
13. Mount with Permount or Histoclad.

RESULTS

Chromatin, osteocytes, neuroglia	- red
Collagen, reticulum	- blue
Muscle	- red to yellow
Osteoid material in decalcified sections	- red

REFERENCE. Mallory, F. B.: Pathological Technique, New York, Hafner Publishing Co., 1961, p. 154-155.

GOMORI'S METHOD FOR RETICULUM

FIXATION 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

AMMONIACAL SILVER SOLUTION

To 10 ml of 10% silver nitrate solution add 2.5 ml of a 10% aqueous solution of potassium hydroxide, add 28% ammonium hydroxide, drop by drop, while shaking the container continuously, until precipitate is completely dissolved. Add again 4 drops of silver nitrate solution for every 10 ml of silver nitrate used. Make the solution with distilled water to twice its volume. Use acid-clean glassware.

0.5% POTASSIUM PERMANGANATE SOLUTION

Potassium permanagate ..... 0.5 gm
Distilled water ..... 100.0 ml

2% POTASSIUM METABISULFITE SOLUTION

Potassium metabisulfite ..... 2.0 gm
Distilled water ..... 100.0 ml

2% FERRIC AMMONIUM SULFATE SOLUTION

Ferric ammonium sulfate ..... 2.0 gm
Distilled water ..... 100.0 ml

20% FORMALIN SOLUTION

Formaldehyde, 37—40% ..... 20.0 ml
Distilled water ..... 80.0 ml

0.2% GOLD CHLORIDE SOLUTION

(See page 92)

2% SODIUM THIOSULFATE SOLUTION

Sodium thiosulfate ..... 2.0 gm
Distilled water ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Oxidize in potassium permanganate solution for 1 minute.
3. Wash in tap water 2 minutes.
4. Differentiate with potassium metabisulfite solution for 1 minute.
5. Wash in tap water for 2 minutes.
6. Sensitize in ferric ammonium sulfate solution for 1 minute.
7. Wash in tap water for 2 minutes; follow with two changes of distilled water 30 seconds each.
8. Impregnate in the silver solution for 1 minute.
9. Rinse in distilled water for 20 seconds.
10. Reduce in formalin solution for 3 minutes.
11. Wash in tap water for 3 minutes.
12. Tone in gold chloride solution for 10 minutes.
13. Rinse in distilled water.
14. Reduce in potassium metabisulfite solution for 1 minute.
15. Fix in sodium thiosulfate solution for 1 minute.
16. Wash in tap water for 2 minutes.
17. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
18. Mount with Permount or Histoclad.

RESULTS

Reticulum fibers	- black
Background	- gray

REMARKS. This technic is especially useful for demonstrating reticulum in the Central Nervous System.

REFERENCE. Gomori, G.: *Amer. J. Path.* 13:993-1002, 1937.

MANUEL'S METHOD FOR RETICULUM

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% URANIUM NITRATE SOLUTION

Uranium nitrate	1.0 gm
Distilled water	100.0 ml

AMMONIACAL SILVER SOLUTION

In 1000 ml of distilled water dissolve 100 gm of silver nitrate. Pour off 70 ml and save. To the 930 ml of 10% silver nitrate add 60 ml of 28% ammonium hydroxide, shaking the flask vigorously (solution becomes dark then clears). Of the 70 ml saved, add slowly 50 ml to the ammonium hydroxide silver nitrate mixture. Solution should become slightly cloudy, if not, add part or all of the remaining 20 ml of the silver nitrate. Let stand overnight and filter before using. Store in the refrigerator and use as needed.

1% FORMALIN SOLUTION

(See page 90)

1% GOLD CHLORIDE SOLUTION

(See page 90)

5% SODIUM THIOSULFATE (HYPO) SOLUTION

Sodium thiosulfate	5.0 gm
Distilled water	100.0 ml

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

Dissolve 0.1 gm nuclear fast red in 100 ml of 5% solution of aluminum sulfate with aid of heat. Cool, filter, add grain of thymol as a preservative.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Sensitize in uranium nitrate solution for 2 minutes.
3. Dip quickly in running water.
4. Impregnate in ammoniacal silver solution for 1 minute.
5. Wash in running water, two or three quick dips (until no more white precipitate appears in the water).
6. Develop in formalin solution for 1 minute.
7. Wash in running water.
8. Tone in gold chloride solution for 1 minute.
9. Wash in running water.
10. Reduce in hypo for 1 minute.
11. Wash in running water.

12. Counterstain with Kernechtrot solution for 5 minutes.
13. Rinse in distilled water for three changes.
14. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
15. Mount with Permount or Histoclad.

RESULTS

Reticulum	-black
Nuclei and background	-red

REMARKS. Originally, the ammoniacal silver used in this technique was employed by Fontana and Masson to demonstrate argentaffin granules.

REFERENCE. Stain developed and to be published by Mr. Benedicto Manuel, Jr. Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, D.C. 20305.

SNOOK'S METHOD FOR RETICULUM

FIXATION. 10% buffered formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

0.25% POTASSIUM PERMANGANATE SOLUTION

(See page 79)

5% OXALIC ACID SOLUTION

(See page 79)

1% URANIUM NITRATE SOLUTION

(See page 89)

1% FORMALIN SOLUTION

Formaldehyde, 37 – 40%	1.0 ml
Distilled water	100.0 ml

1% GOLD CHLORIDE SOLUTION

Gold chloride	1.0 gm
Distilled water	100.0 ml

5% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page 89)

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

5% SILVER NITRATE SOLUTION

Silver nitrate	5.0 gm
Distilled water	100.0 ml

10% SODIUM HYDROXIDE SOLUTION

Sodium hydroxide	10.0 gm
Distilled water	100.0 ml

AMMONIACAL SILVER SOLUTION

To 20 ml of 5% silver nitrate solution in an acid-clean graduated glass cylinder, add 20 drops of 10% sodium hydroxide solution. Then add ammonium hydroxide, drop by drop, until only a few granules of the resulting precipitate remain on the bottom of the cylinder. Add distilled water to make 60 ml, filter and use at once. The ammonium hydroxide must be fresh.

STAINING PROCEDURE. Use acid-clean coplin jars and paraffin-coated forceps for steps 8 through 18. Replace solutions after 10 or 12 slides have been processed, one slide at a time.

1. Deparaffinize and hydrate to distilled water.
2. Oxidize in potassium permanganate solution for 5 minutes.
3. Wash in tap water.
4. Oxalic acid solution until sections are clear.
5. Wash in tap water, then place in distilled water.
6. Mordant in uranium nitrate solution for 5 seconds.
7. Wash in running water.
8. Ammoniacal silver solution for 1 minute.
9. Dip in running water.
10. 1% formalin solution for 1 minute.
11. Wash in running water.
12. Gold chloride solution until section is grayish black (1 minute).
13. Wahs in running water.
14. Sodium thiosulfate solution (hypo) 30 seconds to 1 minute.
15. Wash in running water.
16. Counterstain (if desired) in nuclear fast red solution for 5 minutes.
17. Rinse in distilled water.
18. Dehydrate through 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
19. Mount with Permount or Histoclad.

RESULTS

Reticulum fibers - gray to black
Background - pink to rose

REMARKS. See Wilder's Reticulum Method

REFERENCE. Snook, T.: *Anat. Rec.* 89 :413 - 427, 1944.

WILDER'S METHOD FOR RETICULUM

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

10% PHOSPHOMOLYBDIC ACID SOLUTION

Phosphomolybdic acid ..... 10.0 gm
Distilled water ..... 100.0 ml

1% URANIUM NITRATE SOLUTION

(See page 89)

AMMONIACAL SILVER SOLUTION

To 5 ml of 10.2% aqueous solution of silver nitrate add 28% ammonium hydroxide, drop by drop, until the precipitate which forms is almost dissolved. Add 5 ml of 3.1% sodium hydroxide and barely dissolve the resulting precipitate with a few drops of ammonium hydroxide. Make the solution up to 50 ml with distilled water. Use at once. Glassware must be acid-clean.

REDUCING SOLUTION

Distilled water ..... 50.0 ml
Neutral formalin, 40%* ..... 0.5 ml
Uranium nitrate, 1% aqueous solution ..... 1.5 ml

Make fresh just before use.*Calcium carbonate in excess.

0.2% GOLD CHLORIDE SOLUTION

Gold chloride solution, 1% ..... 10.0 ml

Break glass vial (15 grains) in graduated cylinder with 100.0 ml distilled water for 1% solution.

Distilled water ..... 40.0 ml

5% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page 89)

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Oxidize in phosphomolybdic acid solution for 1 minute.
3. Rinse well in running water or cells will hold yellow.
4. Sensitize in uranium nitrate solution for 1 minute.
5. Rinse in distilled water for 10 to 20 seconds.
6. Ammoniacal silver solution for 1 minute (change solution frequently).
7. Dip very quickly in 95% alcohol and go immediately into:
8. Reducing solution for 1 minute (change solution frequently).
9. Rinse well in distilled water.

10. Tone in gold chloride solution for 1 minute or until sections lose their yellow color and turn lavender. Too much toning will make sections red. Check individually under microscope.

11. Rinse in distilled water.

12. Sodium thiosulfate solution for 1 minute.

13. Wash well in tap water.

14. Counterstain, if desired, with nuclear fast red solution. Rinse well in distilled water.

15. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

16. Mount with Permount or Histoclad.

RESULTS

Reticulum fibers - black
Collagen - rose
Other tissue elements - red

REMARKS. Well stained reticulum exhibit black, well defined fibers without beading effect and relatively clear background. If beading effect and dark background is noticed replace ammoniacal silver and reducing solution.

REFERENCE. Wilder, H. C.: *Amer. J. Path.* 11:817-821, 1935.

GOMORI'S ONE STEP TRICHROME METHOD

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

BOUIN'S SOLUTION

(See page 5)

TRICHROME STAIN

Chromotrope 2R	0.6 gm
Light green, SF yellowish	0.3 gm
Glacial acetic acid	1.0 ml
Phosphotungstic acid	0.8 gm
Distilled water	100.0 ml

Aniline blue may be substituted for light green if it is more desirable to have collagen stained blue.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Place in Bouin's solution in oven at 56 °C for one hour.

3. Wash well in running water or until yellow color disappears.

4. Stain nuclei with Weigert's iron hematoxylin or Gomori's chromium hematoxylin solution 10 minutes.

5. Wash in water.

6. Trichrome stain for 15 to 20 minutes.

7. Place in 0.5% glacial acetic water for 2 minutes. If sections are too dark, differentiate in 1% glacial acetic water to which 0.7 gm of phosphotungstic acid has been added. Rinse in distilled water.

8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

9. Mount with Permount or Histoclad.

RESULTS

Muscle fibers	- red
Collagen	- green (or blue if aniline blue is used).
Nuclei	- blue to black

REFERENCE. Gomori, G.: *Amer. J. Clin. Path.* 20:661-664, 1950. Copyright by Williams and Wilkins Co.

MASSON'S TRICHROME METHOD

FIXATION. Bouin's or 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

BOUIN'S SOLUTION

(See page 5)

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

BIEBRICH SCARLET-ACID FUCHSIN SOLUTION

Biebrich scarlet, aqueous, 1%	90.0 ml
Acid fuchsin, aqueous, 1%	10.0 ml
Glacial acetic acid	1.0 ml

PHOSPHOMOLYBDIC-PHOSPHOTUNGSTIC ACID SOLUTION

Phosphomolybdic acid	5.0 gm
Phosphotungstic acid	5.0 gm
Distilled water	200.0 ml

ANILINE BLUE SOLUTION

Aniline blue	2.5 gm
Glacial acetic acid	2.0 ml
Distilled water	100.0 ml

2% LIGHT GREEN SOLUTION

(See page 81)

1% GLACIAL ACETIC ACID SOLUTION

Glacial acetic acid	1.0 ml
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant in Bouin's solution for 1 hour at 56 °C, or overnight at room temperature, if formalin fixed.
3. Cool and wash in running water until yellow color disappears.
4. Rinse in distilled water.
5. Weigert's iron hematoxylin solution for 10 minutes. Wash in running water 10 minutes.
6. Rinse in distilled water.
7. Biebrich scarlet-acid fuchsin solution for 2 minutes. Save solution.
8. Rinse in distilled water.
9. Phosphomolybdic-phosphotungstic acid solution for 10 to 15 minutes before aniline blue solution. (Aqueous phosphotungstic acid 5% for 15 minutes before light green counterstain). Discard solution.
10. Aniline blue solution for 5 minutes or light green solution for 1 minute. (for central nervous system 15 to 20 minutes) Save solution.
11. Rinse in distilled water.
12. Glacial acetic solution for 3 to 5 minutes. Discard solution.
13. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
14. Mount with Permount or Histoclad.

RESULTS

Nuclei	- black
Cytoplasm, Keratin, Muscle fibers and Intercellular fibers	- red
Collagen	- blue

Note. To insure proper mordanting Bouin's solution should be preheated to 58 ° – 60 °C.

REFERENCE. Masson, P. J.: *J. Techn. Methods* 12:75-90, 1929. AFIP modification.

MOVAT'S PENTACHROME METHOD

FIXATION. 10% buffered neutral formalin or Bouin's.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

1% ALCIAN BLUE SOLUTION

Alcian blue, 8GS*	1.0 gm
Distilled water	100.0 ml
Glacial acetic acid	1.0 ml

RESORCIN-FUCHSIN SOLUTIONS

(See page 79)

*Roboz Surgical Instruments Co., 810-18th St., N.W., Washington, D. C. 20006

WOODSTAIN SCARLET-ACID FUCHSIN SOLUTION

Stock Solution A

Woodstain Scarlet, N.S. concentrate†	0.1 gm
Distilled water	99.5 ml
Glacial acetic acid	0.5 ml

Stock Solution B

Acid fuchsin	0.1 gm
Distilled water	99.5 ml
Glacial acetic acid	0.5 ml

Working Solution

Mix 8 parts of Solution A with 2 parts of Solution B.

ALCOHOLIC SAFRAN SOLUTION

Safran du Gatinais†	6.0 gm
Alcohol, 100%	100.0 ml

After preparing the solution, place in a tightly corked container to prevent hydration and place in an incubator at 58 °C for 48 hours before use. This is done to extract the safran. Keep the solution in an air-tight dark bottle.

STAINING PROCEDURE

1. Deparaffinize and hydrate to water.
2. Rinse thoroughly in distilled water.
3. Alcian blue solution for 20 minutes.
4. Wash in running water for 3 minutes.
5. Alkaline alcohol (ph over 8) for 2 hours. (Prepared by adding 10 ml ammonium hydroxide to 90 ml 95% alcohol. This converts the alcian blue into the insoluble pigment monastral fast blue).
6. Wash in running water for 10 minutes.
7. Rinse in 70% alcohol.
8. Resorcin-Fuchsin solution for 16 hours.
9. Wash in running water for 10 minutes.
10. Rinse in distilled water.
11. Weigert's hematoxylin solution for 15 minutes.
12. Rinse in running water.
13. Rinse in distilled water.
14. Woodstain scarlet-acid fuchsin solution for 5 minutes. Differentiation of nuclei will take place in this solution.
15. Rinse in 0.5% aqueous glacial acetic acid.
16. Differentiate in 5% aqueous phosphotungstic acid solution for 10-20 minutes, until the collagen is pale pink and the background substance, which is covered by the red, is bluish again.
17. Rinse in 0.5% aqueous glacial acetic acid.

†E. I. duPont de Nemours and Co., Inc., Wilmington, Del. 19898

18. Rinse thoroughly in three changes of absolute alcohol. It is essential not to use low grade alcohols. If low grade alcohols are used, the cytoplasmic stains are dissolved and the tissue will not take on the collagen stain, which is made up in absolute alcohol.

19. Alcoholic safran solution for 5 - 15 minutes.

20. Dehydrate in three changes of absolute alcohol, and clear in several changes of xylene. If the collagen is not sufficiently yellow, repeat the staining with safran.

21. Mount with Permount or Histoclad.

RESULTS

Nuclei	- black
Elastic fibers	- dark purple to black
Collagen and reticulum fibers	- yellow
Ground substance	- blue to bluish green
Fibrinoid	- intense red
Muscle	- red

REFERENCE. Movat, H. Z.: *Arch. Path.*, 60:289 - 295, 1955.

JONES' METHOD FOR KIDNEY

FIXATION. 10% buffered neutral formalin, Bouin's or Zenker's

TECHNIQUE. Cut paraffin sections at 2 microns.

SOLUTIONS

0.5% PERIODIC ACID SOLUTION

(See page 72)

3% METHENAMINE* SOLUTION

Hexamethylenetetramine (methenamine)	3.0 gm
Distilled water	100.0 ml

5% SILVER NITRATE SOLUTION

(See page 91)

BORATE BUFFER SOLUTIONS (STOCK)

Solution A: 0.2 *M* Boric Acid

Boric acid	12.36 gm
Distilled water	1000.0 ml

Solution B: 0.25 *M* Sodium Borate

Sodium borate	19.07 gm
Distilled water	1000.0 ml

*Fisher Scientific Co. or Eastman Kodak Co.

BORATE BUFFER SOLUTION, pH 8.2 (WORKING)

Solution A	6.5 ml
Solution B	3.5 ml

1% GOLD CHLORIDE SOLUTION (STOCK)

(See page 90)

GOLD CHLORIDE SOLUTION (WORKING)

Gold chloride stock solution	10.0 ml
Distilled water	40.0 ml

Solution is stable for approximately 100 slides.

3% SODIUM THIOSULFATE (HYPO) SOLUTION

Sodium thiosulfate	3.0 gm
Distilled water	100.0 ml

METHENAMINE SILVER SOLUTION, pH 8.2 (WORKING)

Methenamine, 3%	42.5 ml
Silver nitrate, 5%	2.5 ml
Borate buffer, pH 8.2	12.0 ml

Prepare fresh just before use and filter. This solution is stable for approximately 60-75 minutes. After this time, there is a breaking down process, which produces a black precipitate and is picked up on the slides.

STAINING PROCEDURE. Chemically clean glassware must be used.

Note. It is absolutely essential that all glassware be acid cleaned with concentrated nitric acid and rinsed in several changes of chloride free distilled water. Distilled water may be checked for free chloride by the addition of several drops of 5% silver nitrate solution. If a white cloud appears upon the addition of the silver nitrate, discard the sample of water and replace.

1. Deparaffinize and hydrate to distilled water.
2. Periodic acid solution for 11 minutes.
3. Rinse in chloride free distilled water.
4. Filter freshly prepared methenamine-silver solution into coplin jar.
5. Place slides in methenamine-silver solution and then place coplin jar in prewarmed 70 °C water bath. Start timing at this point, approximately 60-75 minutes. Check under microscope when slides show macroscopically a medium brown color.

Note. Solution and slides should be allowed to come to 70 °C together. While slides are in the silver solution they may be examined after they begin to show macroscopically a medium brown color reaction. Before checking under the microscope, they are first rinsed in hot 70 °C chloride free distilled water, checked, and then returned to hot water rinse and then returned into hot staining solution. Slides should be checked every 10 minutes when they have reached the dark or medium brown stage. Slides should be checked as rapidly as possible because if the section cools there is an un-

even staining of the section. When the desired staining time has been reached, the slide should be checked as described above, every 1-2 minutes. Strict adherence to the timing is essential in order to obtain a uniform consistency in staining. A properly stained section at this point should have a dark brownish-yellow background; the reticulum fibers will be intense black, as should the basement membranes. An overstained section will be too black. Differentiation will be very difficult as the black will be so intense as to obscure many or all of the tissue elements. The section may be destained with an extremely dilute solution of potassium ferricyanide for one or two dips.

6. Rinse section well in distilled water.
7. Tone in working gold chloride solution for 1 minute.

Note. If sections are overtoned, place in 3% sodium metabisulfite for 1-3 minutes, checking periodically.

8. Rinse well in distilled water.
9. Sodium thiosulfate solution for 1-2 minutes.
10. Wash in running tap water for 10 minutes.
11. Rinse well in distilled water.
12. Counterstain with routine Harris hematoxylin and eosin stain.
13. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, three changes each.
14. Mount with Permount or Histoclad.

RESULTS

Basement membrane	-black
Reticulum fibers	-black
Nuclei	-blue
Cytoplasm, collagen, and connective tissue	-pink to orange

REFERENCE. Jones, D. B.: *Amer. J. Path.* 27:991-1009, 1951. Modified by Avalone, F., G. U. Branch, Armed Forces Institute of Pathology.

Chapter 7

Methods For Cytoplasmic Granules

DIAZO METHOD FOR ARGENTAFFIN GRANULES

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% BRENTAMINE FAST RED B SOLUTION (STOCK)

Brentamine Fast Red B*	1.0 gm
Distilled water	100.0 ml

Keep at 4-5 °C.

LITHIUM CARBONATE SOLUTION (STOCK)

Lithium carbonate	1.36 gm
Distilled water	100.0 ml

Keep at 4-5 °C.

BRENTAMINE FAST RED B SOLUTION (WORKING)

Brentamine Fast Red B (Stock)	5.0 ml
Lithium Carbonate (Stock)	2.0 ml

Mix and let stand for 6-8 minutes at 4-5 °C before use.

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Brentamine working solution at 4-5 °C for 40 to 60 seconds.
3. Rinse in distilled water, two changes.
4. Mayer's hematoxylin solution for 3 minutes.
5. Blue in warm tap water for 5 minutes.
6. Rinse in 95% alcohol, two changes.
7. Dehydrate rapidly in absolute alcohol, then clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Argentaffin granules	- rust red
Background	- yellow
Nuclei	- blue

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

REMARKS. Argentaffin cell granules stain yellow if Brentamine Fast Red B solution has broken down.

REFERENCES. Clayden, E. C.: *Practical Section Cutting and Staining*, 3rd ed., London, W.2, J. and A. Churchill, Ltd., 1955, p. 53.

DEFANO'S METHOD FOR GOLGI APPARATUS

FIXATION. Cobalt nitrate. Use three specimens, not to exceed 1 mm in thickness.
TECHNIQUE. Cut paraffin sections at 2-4 microns.

SOLUTIONS

FIXATIVE SOLUTION

Cobalt nitrate	1.0 gm
Distilled water	100.0 ml
Formalin, 37 – 40%	15.0 ml

1.5% SILVER NITRATE SOLUTION

Silver nitrate	1.5 gm
Distilled water	100.0 ml

RAMON Y CAJAL'S REDUCING SOLUTION

Hydroquinone	1.0 gm
Formalin, neutral	7.5 ml
Distilled water	50.0 ml
Sodium sulfite	0.25 gm

Make fresh.

STAINING PROCEDURE

1. Place the three specimens in fixative solution for 2-4 hours.
2. Rinse in distilled water.
3. Silver nitrate solution, in the dark, for 16-24 hours. Use 300 ml of silver nitrate solution to cover all three specimens.
4. Rinse well in several changes of distilled water.
5. Place the three impregnated specimens in reducing solution and remove the first after 8 hours, the second after 16 hours, and the third after 24 hours.
6. Dehydrate specimen in graded alcohols, clear in xylene or chloroform, ther infiltrate with paraffin.
7. Embed in paraffin.
8. Cut paraffin sections at 2-4 microns.
9. Deparaffinize in xylene, several changes.
10. Mount with Permount or Histoclad.

RESULTS

Golgi elements	-black
Background	-yellow to golden brown

REFERENCE. DeFano, C.: J. Micr. Soc., 157-161, 1920.

SMITH'S METHOD FOR JUXTAGLOMERULAR GRANULES

FIXATION. Smith's fixative.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

SMITH'S FIXATIVE SOLUTION

Formalin, 10%	100.0 ml
Potassium dichromate	5.0 gm
Glacial acetic acid	2.5 ml

Follow fixation by washing in tap water for 8 hours. This fixative is not stable. It should be made fresh just before use.

1% POTASSIUM PERMANGANATE SOLUTION (STOCK)

Potassium permanganate	1.0 gm
Distilled water	100.0 ml

1% SULFURIC ACID SOLUTION (STOCK)

Sulfuric acid, concentrated	1.0 ml
Distilled water	100.0 ml

POTASSIUM PERMANGANATE-SULFURIC ACID SOLUTION (WORKING)

Potassium permanganate (stock)	50.0 ml
Sulfuric acid (stock)	50.0 ml

Mix just before use.

1% SODIUM THIOSULFATE (HYPO) SOLUTION

Sodium thiosulfate	1.0 gm
Distilled water	100.0 ml

ZIEHL'S CARBOL-FUCHSIN SOLUTION

Basic fuchsin	1.0 gm
Phenol crystals	5.0 gm
Alcohol, 95%	10.0 ml
Distilled water	100.0 ml

In a mortar grind basic fuchsin and phenol crystals. Then add 95% alcohol while still grinding. The distilled water is added in 10 ml lots to wash out the mortar. The accumulated washings are filtered before use.

ACID ALCOHOL SOLUTION

Alcohol, 35%	100.0 ml
Hydrochloric acid, concentrated	0.5 ml

BOWIE'S SOLUTION

Biebrich scarlet	1.0 gm
Distilled water	250.0 ml

This is filtered into a 1 liter flask while the following (ethyl violet) solution is being mixed.

ETHYL VIOLET SOLUTION

Ethyl violet	2.0 gm
Distilled water	500.0 ml

The ethyl violet solution is then filtered into the biebrich scarlet while shaking it, until an abrupt color change (red to violet) signals the neutralization end point. The resulting precipitate is separated by vacuum filtering, dried and added to 100 ml of absolute alcohol.

ACETONE-XYLENE SOLUTION

Acetone	50.0 ml
Xylene	50.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Oxidize in freshly prepared potassium permanganate and sulfuric acid solution for 1 minute.
3. Rinse briefly in distilled water.
4. Decolorize in sodium thiosulfate solution for 3 minutes.
5. Wash in tap water for 2 minutes.
6. Ziehl's carbol-fuchsin solution for 1 minute.
7. Wash in tap water for 1 minute.
8. Decolorize in acid alcohol solution until sections are pink. Retention of red color in some nuclei is more desirable than complete extraction.
9. Wash in tap water for 2 minutes.
10. Handle each slide individually in this and subsequent steps. Place the slide flat on a staining rack without draining or shaking off surface water. Drop on sufficient Bowie solution to cover the section without flooding the slide. After 5 to 10 seconds, immerse the slide in a coplin jar of water without draining off the dye. Wait 1 to 2 minutes before dehydrating.
11. Dehydrate rapidly through acetone for two changes and equal parts acetone-xylene for one change.
12. Clear in xylene, two changes.
13. Mount with Permount or Histoclad.

RESULTS

Juxtaglomerular granules	-deep purple
Nuclei	-some pink to red, majority lavender
Cytoplasm	-shades of blue or purple
Elastic tissue	-deep purple
Collagen	-pink
Erythrocytes	-red

Note: The author (C.L.S.) has found the greatest variability in the staining results of this method to be dependent upon the Bowie stock solution. Different dye lots may cause trouble, as well as too low a concentration in the final solution. An easy way to

prepare the solution is to weigh the filter paper prior to filtering the biebrich scarlet-ethyl violet reaction mixture, then filter, dry and weigh the combination. Subtract the original paper weight to determine the amount of dye present. Place the paper and dye in a sufficient quantity of absolute alcohol to yield a 1% solution. There may still be some undissolved dye present, but this does not matter.

The mouse juxtaglomerular granules are the most prominent and easiest to stain. If good results cannot be obtained, try running the unsatisfactorily stained slide back to water and apply a second application of Bowie's solution. This will sometimes result in an improvement, if not try a different dye lot or manufacturer. It is also possible to bleach out all the stain by repeating the procedure from Step 2. This sometimes yields better results and conserves research material.

REFERENCE. Smith, C. L.: *Stain Techn.* 41:291-294, 1966. Copyright by Williams and Wilkins Co.

FONTANA-MASSON SILVER METHOD

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

SILVER NITRATE SOLUTION (FONTANA)

Dissolve 10 gm of silver nitrate in 100 ml of distilled water. To 95 ml of this solution, add ammonium hydroxide until a clear solution with no precipitate is obtained. Add, drop by drop, enough of the remaining 5 ml of silver nitrate solution to cause the clear solution above, to become slightly cloudy. Let stand overnight before using. When ready to use, dilute each 25 ml of silver solution with 75 ml of distilled water and filter.

GOLD CHLORIDE SOLUTION

Gold chloride, 1% aqueous	10.0 ml
Distilled water	40.0 ml

5% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page 89)

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE. Use chemically clean glassware.

1. Deparaffinize and hydrate to distilled water.
2. Silver nitrate solution at 56 °C for 1 hour. Sections should be light brown.
3. Rinse sections in distilled water.
4. Gold chloride solution for 10 minutes.
5. Rinse in distilled water, three changes.
6. Sodium thiosulfate solution for 5 minutes.
7. Rinse in distilled water.

8. Nuclear fast red solution for 5 minutes.
9. Rinse in distilled water, two changes.
10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Silver reducing substances	- black
Nuclei	- pink

REMARKS. In our experience we find that this procedure is nonspecific since many pigments can be demonstrated, including formalin pigment. It is being used widely as an aid for the demonstration of melanin but positive results should not be interpreted as melanin pigment, unless other procedures confirm the results. Argentaffin granules are demonstrated with this method.

REFERENCE. Masson, P.: *Amer. J. Path.* 4:181-212, 1928.

MALDONADO'S METHOD FOR PANCREATIC ISLET CELLS

FIXATION. Bouin's without acetic acid.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% PHLOXINE SOLUTION

(See page 36)

3% PHOSPHOTUNGSTIC ACID SOLUTION

Phosphotungstic acid	3.0 gm
Distilled water	100.0 ml

0.05% AZURE II SOLUTION

Azure II	0.05 gm
Distilled water	100.0 ml

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Phloxine solution for 10 minutes.
3. Rinse in distilled water.
4. Phosphotungstic acid solution for 1 minute.
5. Rinse in distilled water.
6. Azure II solution for 30 seconds.
7. Rinse in distilled water.
8. Weigert's hematoxylin solution for 1 minute.
9. Rinse in distilled water.

10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

11. Mount with Permount or Histoclad.

RESULTS

A cells	-purple
B cells	-violet blue
D cells	-light blue with evident granules
Exocrine cells	-grayish blue with red granules

REFERENCE. Maldonado, R., and San Jose, H.: *Stain Techn.* **42**:11-13, 1967. Copyright by Williams and Wilkins Co.

GOMORI'S METHOD FOR PANCREATIC ISLET CELLS

FIXATION. Bouin's or 10% buffered neutral formalin. If formalin-fixed, the paraffin sections should be mordanted in Bouin's solution for 16 hours before staining.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

BOUIN'S SOLUTION

(See page 5)

POTASSIUM PERMANGANATE SOLUTION

Potassium permanganate	0.3 gm
Distilled water	100.0 ml
Sulfuric acid concentrated	0.3 ml

5% SODIUM BISULFITE SOLUTION

Sodium bisulfite	5.0 gm
Distilled water	100.0 ml

CHROMIUM HEMATOXYLIN SOLUTION

Hematoxylin, 1% aqueous solution	50.0 ml
Chromium potassium sulfate, 3% aqueous solution	50.0 ml

To 100 ml of chromium hematoxylin solution add 0.1 gm of potassium iodate. Boil until a deep blue. The mixture is ripe immediately and can be used as long as a film with a metallic luster forms on its surface in a coplin jar. Filter before use.

1% ACID ALCOHOL SOLUTION

(See page 38)

0.5% PHLOXINE B SOLUTION

Phloxine B	0.5 gm
Distilled water	100.0 ml

5% PHOSPHOTUNGSTIC ACID SOLUTION

(See page 86)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant in Bouin's solution for 16 hours.
3. Wash in tap water to remove picric acid, for 15 minutes.
4. Potassium permanganate solution for 1 minute.
5. Differentiate in sodium bisulfite solution.
6. Wash well in tap water.
7. Chromium hematoxylin solution for 10 minutes or less. Check under microscope and stain until beta cells stand out deep blue.
8. Differentiate in acid alcohol solution for 1 minute.
9. Wash in tap water until the section is a clear blue.
10. Phloxine B solution for 5 minutes.
11. Rinse in distilled water.
12. Phosphotungstic acid solution for 1 minute.
13. Wash in tap water for 5 minutes. The section should regain its red color.
14. Differentiate in 95% alcohol. If the section is too red and the alpha cells do not stand out clearly enough, rinse in 80% alcohol for 15 to 20 seconds.
15. Dehydrate in absolute alcohol, then clear in xylene, two changes each.
16. Mount with Permount or Histoclad.

RESULTS

Alpha cells	-red
Beta cells	-blue
D cells	-from pink to red and indistinguishable from the alpha cells.

REFERENCE. Gomori, G.: *Amer. J. Path.* 17:395-406, 1941.

GOMORI'S METHOD FOR CHROMAFFIN

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

AZOCARMINE G SOLUTION

Azocarmine G	0.05 gm
Glacial acetic acid	1.0 ml
Distilled water	100.0 ml

1% ANILINE-ALCOHOL SOLUTION

Aniline	1.0 ml
Alcohol, 95%	100.0 ml

3% PHOSPHOTUNGSTIC ACID SOLUTION

(See page 105)

ANILINE BLUE-QUINOLINE YELLOW SOLUTION

Aniline blue	0.5 gm
Quinoline yellow or orange G	2.0 gm
Phosphotungstic acid	1.0 gm
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Azocarmine G solution at 58 °C, for 60 to 90 minutes.
3. Wash in tap water, then blot.
4. Rinse in 95% alcohol.
5. Differentiate in aniline-alcohol. Check microscopically to the point where chromaffin cells stand out deep pink against the much paler cortical cells.

Note. Differentiation in aniline alcohol solution may take 5 to 60 minutes, depending on the type of fixation and on the thickness of the section. This is the only critical area in the entire procedure. Judging the correct degree of decolorization requires some experience.

6. Wash briefly in tap water.
7. Phosphotungstic acid solution for 20 minutes.
8. Wash in water for 1 minute.
9. Aniline blue-quinoline yellow solution until connective tissue is deep blue – from 15 to 40 minutes. Better contrast is obtained if quinoline yellow is used in this mixture instead of orange G.)
10. Wash in tap water.
11. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
12. Mount with Permount or Histoclad.

RESULTS

Chromaffin granules - purplish red

Only alpha cells of pancreatic islets, some cells of anterior pituitary and granulation of neutrophils and myelocytes stain similarly.

REFERENCE. Gomori, G.: *Amer. J. Clin. Path.* **16**:115, 1946.

GLENNER-LILLIE METHOD FOR PITUITARY

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

STAINING SOLUTION

Eosin B, 1% aqueous	8.0 ml
Aniline blue, 1% aqueous	2.0 ml

Citric acid, 0.1*M* ..... 1.1 ml
Disodium phosphate, 0.2*M* ..... 0.9 ml
Distilled water ..... 28.0 ml
Store at room temperature.

ACETONE-XYLENE SOLUTION

Acetone ..... 50.0 ml
Xylene ..... 50.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Staining solution in oven at 60 °C for 1 hour.
3. Wash in water for 5 minutes.
4. Dehydrate through 50% acetone, 80% acetone, and 100% acetone, two changes each.
5. Clear in acetone-xylene solution, then xylene, two changes each.
6. Mount with Permount or Histoclad.

RESULTS

Beta cell granules	- blue to black
Acidophil cells	- dark red
Chromophobe granules	- slate gray to pale pink
Colloid	- red to blue violet
Erythrocytes	- orange

REMARKS. This technique is also useful for duodenal enterochromaffin cells and pancreatic islet cells with the following modification:

At step 2: The staining solution is buffered to pH 3.6 (0.1*M* citric acid, 1.4 ml; 0.2*M* disodium phosphate, 0.6 ml) and heated to 60 °C. Sections are then incubated at this temperature for 3 minutes and carried through the rest of the procedure as described above.

RESULTS

Enterochromaffin cells	- blue-black
Goblet cells and mucins	- light blue
Paneth cells	- blue-black
Alpha cells of the Pancreatic islet	- red
Beta cells of the Pancreatic islet	- purple
Background	- varying shades ranging from blue to pale pink

Note. The stain can be extracted by exposure to two 5 minute changes of 1 ml 0.1*N* NaOH in 39 ml 70%

REFERENCE. Glenner, G. G., and Lillie, R. D.: *Stain Techn.* **32**:187 - 190, 1957. Copyright by Williams and Wilkins Co.

HEATH'S METHOD FOR PITUITARY

FIXATION. 10% buffered neutral formalin, Zenker's, or Bouin's.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

PERFORMIC ACID SOLUTION

Hydrogen peroxide, 30%	15.0 ml
Sulfuric acid, concentrated	1.5 ml
Formic acid, 97%	133.5 ml

4*N* SULFURIC ACID SOLUTION

Sulfuric acid, concentrated	112.6 ml
Distilled water	887.4 ml

ALCIAN BLUE SOLUTION

Alcian blue, 4%	100.0 ml
Sulfuric acid, 4*N*	100.0 ml

0.5% PERIODIC ACID SOLUTION

(See page 72)

SCHIFF REAGENT SOLUTION

(See page 159)

0.5% ORANGE G SOLUTION

Orange G	0.5 gm
Distilled water	100.0 ml

1% PHOSPHOTUNGSTIC ACID SOLUTION

(See page 74)

1% GLACIAL ACETIC ACID SOLUTION

Glacial acetic acid	1.0 ml
Distilled water	99.0 ml

Adjust pH to 4.0 -5.0.

STAINING PROCEDURE

1. Deparaffinize and hydrate to 70% alcohol.
2. If Zenker fixed, remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).
3. Performic acid solution for 5 minutes.
4. Wash in tap water for 10 minutes.
5. Alcian blue solution for 1 hour.
6. Wash in tap water for 10 minutes.
7. Periodic acid solution for 5 minutes.
8. Rinse in distilled water.
9. Schiff's solution for 10 minutes.
10. Wash in tepid tap water for 10 minutes.
11. Orange G solution for 30 seconds.

12. Phosphotungstic acid solution for 30 seconds.
13. Glacial acetic acid solution for 30 seconds.
14. Dehydrate in absolute alcohol, then clear in xylene, two changes each.
15. Mount with Permount or Histoclad.

RESULTS

Neurosecretory material in pars neurosa	- clear blue
Basement membranes	- magenta
Erythrocytes	- yellow
Cell cytoplasms	- chromophobic or pale magenta
Colloid	- strong magenta
Basophils	- blue, purple, or red
Acidophils	- orange to yellow

REFERENCE. Heath, E. H.: *Amer. J. Vet. Res.* **26**:368-373, 1965.

LUNA'S METHOD FOR ERYTHROCYTES AND EOSINOPHIL GRANULES

FIXATION. 10% buffered formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

WEIGERT'S IRON HEMATOXYLIN SOLUTIONS

(See page 35)

1% BIEBRICH SCARLET SOLUTION

Biebrich scarlet	1.0 gm
Distilled water	100.0 ml

HEMATOXYLIN-BIEBRICH SCARLET SOLUTION (WORKING)

Weigert's iron hematoxylin solution (working)	45.0 ml
Biebrich scarlet solution	5.0 ml

1% ACID ALCOHOL SOLUTION

(See page 38)

0.5% LITHIUM CARBONATE SOLUTION

Lithium carbonate	0.5 gm
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Stain in working hematoxylin-biebrich scarlet solution for 5 minutes.
3. Differentiate in 1% acid alcohol until desired nuclear detail is achieved (usually eight dips).
4. Rinse in tap water to remove acid alcohol.

5. Dip lithium carbonate solution until section turns blue and erythrocytes are bright red (usually five dips).

6. Wash in running water for 2 minutes.

7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

8. Mount with Permount or Histoclad.

RESULTS

Eosinophil granules	-red
Erythrocytes	-red
Charcott Leyden crystals	-red
Background	-blue

REMARKS. Especially useful or phagocytosis and sometimes useful for demonstrating Negri bodies.

REFERENCE. Luna, L. G.: Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, D.C. 20305.

MONROE-FROMMER METHOD FOR PITUITARY

FIXATION. Zenker's

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

10% TANNIC ACID SOLUTION

Tannic acid	10.0 gm
Distilled water	100.0 ml

1% BASIC FUCHSIN SOLUTION (STOCK)

Basic fuchsin	1.0 gm
Alcohol, 100%	20.0 ml
Distilled water	80.0 ml

BASIC FUCHSIN SOLUTION (WORKING)

Basic fuchsin (stock)	50.0 ml
Distilled water	50.0 ml

Filter before use.

1% ANILINE SOLUTION

Aniline	1.0 ml
Alcohol, 100%	90.0 ml
Distilled water	10.0 ml

1% PHOSPHOMOLYBDIC ACID SOLUTION

Phosphomolybdic acid	1.0 gm
Distilled water	100.0 ml

1% ALCIAN BLUE SOLUTION

Alcian blue, 8GX ..... 1.0 gm
Distilled water ..... 100.0 ml
Filter before use.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).
3. Wash in running water for 10 minutes.
4. Tannic acid solution for 10 minutes.
5. Wash in running water for 5 minutes.
6. Working basic fuchsin solution for 3-5 seconds. Slides should be agitated by dipping in and out of staining solution.
7. Wash in tap water to remove excess stain.
8. Differentiate in aniline solution until acidophils are red and basophils pink.
9. Phosphomolybdic acid solution for 30 seconds.
10. Rinse in distilled water.
11. Alcian blue solution for 30 seconds.
12. Rinse in distilled water.
13. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
14. Mount with Permount or Histoclad.

RESULTS

Acidophils	- red
Beta basophils	- green
Delta basophils	- purple
Collagenous fibers	- bluish green
Chromophobes	- colorless
Colloid	- red
Neurosecretory granules	- brilliant red
Neurohypophysial fibers	- blue

REFERENCE. Monroe, C. W. and Frommer, J.: *Stain Techn.* 41:248, 1966. Copyright by Williams and Wilkins Co.

WILSON-EZRIN METHOD FOR PITUITARY

FIXATION. 10% formol-saline solution.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% PERIODIC ACID SOLUTION

Periodic acid ..... 1.0 gm
Distilled water ..... 100.0 ml

SCHIFF REAGENT SOLUTION

(See page 159)

SULFUROUS ACID SOLUTION

Sodium metabisulfite, 10% aqueous	60.0 ml
Hydrochloric acid, 1*N*	50.0 ml
Distilled water	1000.0 ml

1% ORANGE G SOLUTION

Orange G	1.0 gm
Distilled water	100.0 ml

5% PHOSPHOTUNGSTIC ACID SOLUTION

(See page 86)

1% METHYL BLUE SOLUTION

Methyl blue	1.0 gm
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Periodic acid solution for 5 minutes.
3. Rinse in distilled water.
4. Schiff's solution for 15 minutes.
5. Sulfurous acid rinses, three changes, 3 minutes each.
6. Wash in running water for 10 minutes.
7. Orange G solution for 1 minute.
8. Phosphotungstic acid solution for 30 seconds.
9. Wash in running water for 30 seconds.
10. Methyl blue solution for 1 minute.
11. Wash out excess methyl blue in tap water.
12. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
13. Mount with Permount or Histoclad.

RESULTS

Beta granules	- red
Gamma granules	- purple
Acidophils	- yellow

REFERENCE. Wilson, W. D., and Ezrin, E.: *Amer. J. Path.* 30:891-899, 1954.

LUNA'S METHOD FOR MAST CELLS

FIXATION. 10% buffered neutral formalin

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

ALDEHYDE FUCHSIN SOLUTION

(See page 78)

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

METHYL ORANGE SOLUTION

Methyl orange	0.25 gm
Alcohol, 95%	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to 95% alcohol.
2. Aldehyde fuchsin solution for 30 minutes.
3. Rinse in 95% alcohol.
4. Weigert's hematoxylin solution for 1 minute.
5. Wash in running water for 10 minutes.
6. Rinse in 95% alcohol.
7. Counterstain in methyl orange solution for 5 minutes or until background is light yellow.
8. Dehydrate in absolute alcohol, then clear in xylene, two changes each.
9. Mount with Permount or Histoclad.

RESULTS

Mast cells	-purple
Elastic fibers	-purple
Other cellular elements	-blue
Background	-yellow

REMARKS. Especially useful for demonstration of mast cells in animal tissue.

REFERENCE. Luna, L. G.: Histopathology Laboratories, Armed Forces Institute of Pathology Washington, D. C. 20305.

UNNA'S METHOD FOR MAST CELLS

FIXATION. 10% buffered neutral formalin or formol alcohol.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

POLYCHROME METHYLENE BLUE SOLUTION

Methylene blue	1.0 gm
Distilled water	100.0 ml
Alcohol 95%	20.0 ml
Potassium carbonate	1.0 gm

GLYCERIN-ETHER SOLUTION

Glycerin ..... 50.0 ml
Calcium chloride anhydrous ..... 10.0 gm

Heat the above mixture until the calcium chloride dissolves completely. Cool to room temperature.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Polychrome methylene blue solution for 10 minutes.
3. Rinse in distilled water.
4. Differentiate in glycerin-ether solution, diluted 5 to 10 times with distilled water, for 30 seconds to 1 minute, or until section is a medium blue. Be careful not to differentiate too long.
5. Wash thoroughly in water for 2-5 minutes, then blot with filter paper.
6. Dehydrate rapidly in absolute alcohol.
7. Clear in xylene, two or three changes.
8. Mount with Permount or Histoclad.

RESULTS

Mast cell granules - red
Other cells - greenish blue

REMARKS. This technic, which was originally used for the differentiation of mast cells and plasma cells, has been used in our laboratory for the specific demonstration of mast cells. It is especially useful for highly cellular specimens, such as lymph node and spleen.

REFERENCE. Mallory, F. B.: Pathological Technique, New York, Hafner Publishing Co., 1961, p. 175.

TOREN'S METHOD FOR MAST CELLS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

GIEMSA SOLUTION

Hartman-Leddon Company original azure blend type Giemsa staining solution, 1 drop per ml of distilled water, prepared immediately before use.

0.5% GLACIAL ACETIC ACID SOLUTION

Glacial acetic acid ..... 0.5 ml
Distilled water ..... 100.0 ml

POLLACK'S TRICHROME SOLUTION

Acid fuchsin ..... 0.5 gm
Ponceau 2 R ..... 1.0 gm

Light green, SF yellowish	0.45 gm
Orange G	0.75 gm
Phosphotungstic acid, C. P.	1.5 gm
Phosphomolybdic acid, C. P.	1.5 gm
Glacial acetic acid	3.0 ml
Alcohol, 50% to make	300.0 ml

First, glacial acetic acid is added to the alcohol. Of this acidified alcohol, 50 ml portions are prepared in four beakers: in the first beaker, acid fuchsin and ponceau 2R are dissolved; in the second, light green; in the third, orange G and phosphotungstic acid; and in the fourth, phosphomolybdic acid. The rest of the acidified alcohol is used to rinse out the beakers and to make up the volume. All the ingredients dissolve easily except for the phosphomolybdic acid, which will dissolve after slight warming of the beaker.

The mixture is filtered and then ready for use.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Giemsa solution for 75 minutes.
3. Rinse in distilled water.
4. Glacial acetic acid solution for 5 to 10 seconds.
5. Rinse in distilled water.
6. Pollack's trichrome solution for 10 to 15 seconds.
7. Rinse in glacial acetic acid solution until no more stain washes off, 3 to 5 seconds.
8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
9. Mount with Permount or Histoclad

RESULTS

Mast cell granules	- deep blue
Collagen and cartilage	- blue-green
Muscle and elastic fibers	- red
Fibrin	- purple
Bone	- green
Colloid, keratin and erythrocytes	- orange red to orange

REFERENCE. Toren, D. A.: *Stain Techn.* 38:249-250, 1963.

BERG'S METHOD FOR SPERMATOZOA

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

PUTT'S CARBOL FUCHSIN SOLUTION

New fuchsin	1.0 gm
Phenol	5.0 gm

Alcohol, 100% ..... 10.0 ml
Distilled water ..... 84.0 ml

Filter before use.

SATURATED LITHIUM CARBONATE SOLUTION

(See page 38)

5% GLACIAL ACETIC ACID-ALCOHOL SOLUTION

Glacial acetic acid ..... 5.0 ml
Alcohol, 100% ..... 95.0 ml

0.5% METHYLENE BLUE SOLUTION

Methylene blue ..... 0.5 gm
Alcohol, 100% ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Carbol fuchsin solution for 3 minutes.
3. Place slides directly into saturated lithium carbonate solution for 3 minutes.
4. Decolorize in glacial acetic acid-alcohol solution for 5 minutes.
5. Absolute alcohol, two changes for 1 minute each.
6. Methylene blue for 30-60 seconds.
7. Rinse rapidly in absolute alcohol, two changes.
8. Clear in xylene, two or three changes.
9. Mount with Permount or Histoclad.

Note. Uneven staining can be avoided if slides are gently agitated throughout all stages of the procedure.

RESULTS

Spermatozoa	-brilliant red
Erythrocytes	-pale pink
Other tissue components	-blue to purple

REFERENCE. Berg, J. W.: *Amer. J. Clin. Path.* 23:513-515, 1963.

Chapter 8

Methods For Hematologic And Nuclear Elements

WOLBACH'S GIEMSA METHOD

FIXATION. Zenker's or other well-fixed tissues.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

GIEMSA* SOLUTION (STOCK)

Giemsa powder	1.0 gm
Glycerin	66.0 ml
Alcohol, methyl	66.0 ml

Mix glycerin and Giemsa powder. Place in a 60 °C oven for 2 hours. Finally add the 66 ml methyl alcohol.

GIEMSA SOLUTION (WORKING)

Giemsa solution (stock)	1.25 ml
Alcohol, methyl	1.5 ml
Distilled water	50.0 ml

ROSIN ALCOHOL SOLUTION (STOCK)

Rosin, white	10.0 gm
Alcohol, 100%	100.0 ml

ROSIN ALCOHOL SOLUTION (WORKING)

Rosin solution (stock)	5.0 ml
Alcohol, 95%	40.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).
3. Wash in running water for 15 minutes.
4. Rinse in distilled water.
5. Working Giemsa solution overnight.
6. Differentiate in working rosin alcohol solution until sections assume a purplish pink color. Check under microscope.

*Stain must be National Aniline Certified Giemsa.

7. Dehydrate in absolute alcohol then clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Nuclei	-blue
Collagen and other tissue elements	-pink to rose
Rickettsia	-purple
Bacteria	-blue

REMARKS. Rickettsia can be demonstrated satisfactorily with this method. The demonstration, however, depends on precise differentiation of the section.

Note. Giemsa takes much better if tissue has an acid pH. If tissue is not already acidified by decalcification, etc., place in acid alcohol, wash well, begin stain.

REFERENCE. Wolbach, S. B., Todd, J. L., and Palfrey, F. W.: *The Etiology of Pathology Typhus*, Cambridge, Mass., Harvard University Press, 1922, pp. 13-14.

MAXIMOW'S METHOD FOR BONE MARROW

FIXATION. Zenker-Formol.

TECHNIQUE. Cut parrafin sections at 6 microns.

SOLUTIONS

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

0.1% EOSIN SOLUTION (STOCK)

Eosin Y, yellowish	0.1 gm
Distilled water	100.0 ml

0.1% AZURE II SOLUTION (STOCK)

Azure II	0.1 gm
Distilled water	100.0 ml

EOSIN-AZURE II SOLUTION (WORKING)

Eosin solution (stock)	10.0 ml
Distilled water, triple distilled	100.0 ml
Azure II solution (stock)	10.0 ml
Glacial acetic acid	2 drops

Must be prepared fresh each time with triple distilled water or buffer solution pH 6.8-7.0.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mayer's hematoxylin solution for 15 minutes.
3. Wash in tepid tap water for 15 minutes.
4. Working Eosin-Azure II solution overnight.

5. Differentiate in 95% alcohol until blue ceases to come out into alcohol and erythrocytes and collagen are pink.

6. Dehydrate in absolute alcohol, clear in xylene, two changes each.

7. Mount with Permount or Histoclad.

RESULTS

Nuclei	- blue
Basophile leucocyte, mast cell granules	- purple to violet
Cartilage	- purple
Erythrocytes, eosinophile granules	- pink
Cytoplasm	- blue to pink

REFERENCE. Mallory, F. B.: Pathological Technique, New York, Hafner Publishing Co., 1961, p. 196.

MAY-GRUNWALD GIEMSA METHOD

FIXATION. Zenker's or other well-fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

JENNER SOLUTION (STOCK)

Jenner stain, dry powder*	1.0 gm
Alcohol, methyl	400.0 ml

JENNER SOLUTION (WORKING)

Jenner solution (stock)	25.0 ml
Distilled water	25.0 ml

GIEMSA SOLUTION (STOCK)

(See page 119)

GIEMSA SOLUTION (WORKING)

Giemsa solution (stock)	50 drops
Distilled water	50.0 ml

Make fresh, do not re-use.

1% GLACIAL ACETIC WATER SOLUTION

(See page 94)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).

*National Aniline Certified

3. Wash in running water for 10 minutes.
4. Rinse in distilled water, two changes.
5. Methyl alcohol, two changes for 3 minutes each.
6. Working Jenner solution for 6 minutes.
7. Working Giemsa solution for 45 minutes.
8. Handle each slide individually in this and subsequent steps. Differentiate in glacial acetic water solution then check microscopically for well differentiated nuclei.
9. Rinse in distilled water.
10. Dehydrate quickly in 95% alcohol, absolute alcohol, and clear with xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Nuclei	-blue
Cytoplasm	-pink to rose
Bacteria	-blue

REFERENCE. Strumia, M. M.: *J. Lab. Clin. Med.* **21**:930-934, 1935-1936.

MALLORY'S METHOD FOR HEMOFUCHSIN

FIXATION. Zenker's solution, absolute alcohol or 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

ALUM HEMATOXYLIN SOLUTION

Hematoxylin	1.0 gm
Aluminum ammonium or potassium sulfate	20.0 gm
Distilled water	400.0 ml
Thymol	1.0 gm

0.5% BASIC FUCHSIN SOULTION

Basic fuchsin	0.5 gm
Alcohol, 95%	50.0 ml
Distilled water	50.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Alum hematoxylin solution until the nuclei stand out sharply.
3. Wash thoroughly in water.
4. Basic fuchsin solution for 30 minutes.
5. Wash in water.
6. Differentiate in 95% alcohol until hemofuchsin granules stand out sharply against a gray background.
7. Dehydrate in absolute alcohol, then clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Nuclei	- blue
Hemofuchsin granules	- bright red

REFERENCE. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p. 136.

LISON'S METHOD FOR HEMOGLOBIN

FIXATION. 10% buffered neutral formalin
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

PATENT BLUE B SOLUTION (STOCK)

Patent Blue B	1.0 gm
Glacial acetic acid, 2%	100.0 ml
Zinc dust, granulated	10.0 gm

Boil until pale brown. Filter.

PATENT BLUE B SOLUTION (WORKING)

Patent Blue B solution	100.0 ml
Glacial acetic acid	20.0 ml
Hydrogen peroxide, 3%	10.0 ml

SAFRANIN O SOLUTION

Safranin O	0.1 gm
Glacial acetic acid, 1%	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to 70% alcohol.
2. Working Patent blue B solution for 3 minutes.
3. Rinse in distilled water.
4. Safranin O solution for 1 minute.
5. Rinse in distilled water and blot dry.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Hemoglobin	- dark blue to green
Granules of leucocytes	- dark blue
Nuclei and cytoplasm	- red

Note: This technic may be used for coloring of myeloid white blood cell peroxidase granules.

REFERENCE. McManus, J. F. A., and Mowry, R. W.: *Staining Methods Histologic and Histochemical,* New York, Hoeber Medical Division, Harper and Row, Publishers, 1960, p. 204.

PUCHTLER METHOD FOR HEMOGLOBIN

FIXATION. Zenker's solution.

TECHNIQUE. Cut parrafin sections at 6 microns.

SOLUTIONS

5% TANNIC ACID SOLUTION

Tannic acid ..... 5.0 gm
Distilled water .....100.0 ml

Tannic acid prepared fresh should be allowed to stand for 48 hours before use. (stable).

1% PHOSPHOMOLYBDIC ACID SOLUTION

(See page 112)

BUFFALO BLACK NBR 126% SOLUTION

Buffalo Black NBR, concentrated 126%* .....3.15 gm
Glacial acetic acid ..... 10.0 ml
Alcohol, methyl .....90.0 ml

Solution must be allowed to stand for 48 hours before use. DO NOT FILTER BEFORE USE.

METHANOL-GLACIAL ACETIC ACID SOLUTION

(See page 126)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant in Zenker-formol overnight.
3. Wash in running water for 15 minutes.
4. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).
5. Wash in running water for 5 minutes.
6. Tannic acid solution for 10 minutes.
7. Rinse in distilled water, three changes.
8. Phosphomolybdic acid solution for 10 minutes.
9. Rinse in methanol-glacial acetic acid, two changes.
10. Buffalo black NBR solution for 5 minutes. *DO NOT FILTER.*
11. Rinse in methanol-glacial acetic acid, two changes.
12. Dehydrate in absolute alcohol, then clear in xylene, two or three changes each.
13. Mount with Permount or Histoclad.

*National Aniline Division, 201 W. First St., Charlotte, N. C. 28801

RESULTS

Erythrocytes	-dark blue
Hemoglobin casts	-dark blue
Intracellular hemoglobin droplets	-dark blue
Other tissue structures	-yellow

REMARKS. In our experience we find this technic to be the best available for the demonstration of hemoglobin – only if tissue is Zenker fixed.

Note. Buffalo Black NBR concentrated 126% must be used to insure satisfactory results.

REFERENCE. Puchtler, H., Rosenthal, S., and Sweat, F.: *Arch. Path.* 78:76-78, 1964.

RALPH'S METHOD FOR HEMOGLOBIN

FIXATION. Absolute alcohol, Carnoy's solution or 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

BENZIDINE SOLUTION

Benzidine	1.0 gm
Alcohol, methyl	99.0 ml

PEROXIDE SOLUTION

Hydrogen peroxide, 30%	25.0 ml
Alcohol, 70%	75.0 ml

Handle hydrogen peroxide with care, avoid contact with skin and eyes.

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Flood slide with benzidine solution for 1 minute.
3. Drain off and flood with the peroxide solution for 2 minutes.
4. Rinse in distilled water for 15 seconds.
5. Nuclear fast red for 5 mintues.
6. Rinse in distilled water.
7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Hemoglobin	-dark brown
Nuclei	-pink

REFERENCE. Ralph, P. H.: *Stain Techn.* 16:105, 1941. Copyright by Williams and Wilkins Co.

PUCHTLER-SWEAT METHOD FOR HEMOGLOBIN AND HEMOSIDERIN

FIXATION. Zenker-Formol.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

2% POTASSIUM FERROCYANIDE SOLUTION (STOCK)

Potassium ferrocyanide	2.0 gm
Distilled water	100.0 ml

2% HYDROCHLORIC ACID SOLUTION (STOCK)

Hydrochloric acid, concentrated	2.0 ml
Distilled water	100.0 ml

POTASSIUM FERROCYANIDE-HYDROCHLORIC ACID SOLUTION (WORKING)

Potassium ferrocyanide (stock)	50.0 ml
Hydrochloric acid (stock)	50.0 ml

Mix just before use.

5% TANNIC ACID SOLUTION

(See page 124)

1% PHOSPHOMOLYBDIC ACID SOLUTION

(See page 112)

PHLOXINE B SOLUTION

Phloxine B	5.0 gm
Glacial acetic acid	30.0 ml
Alcohol, methyl	80.0 ml

Adjust pH to 3.7.

METHANOL-GLACIAL ACETIC ACID SOLUTION

Glacial acetic acid	10.0 ml
Alcohol, methyl	90.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant in Zenker-formol, overnight.
3. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).
4. Rinse in distilled water, several changes. Avoid any contact with metal.
5. Potassium ferrocyanide-hydrochloric acid solution for 30 minutes.
6. Rinse in distilled water, three changes.

7. Tannic acid solution for 10 minutes.
8. Rinse in distilled water, three changes.
9. Phosphomolybdic acid solution for 10 minutes.
10. Rinse in distilled water, three changes.
11. Phloxine B solution for 5 minutes. *DO NOT FILTER.*
12. Rinse in methanol-glacial acetic acid solution, two changes.
13. Dehydrate in absolute alcohol and clear in xylene, two changes each.
14. Mount with Permount or Histoclad.

RESULTS

Hemoglobin - red
Hemosiderin - dark blue to blue green

REFERENCE. Puchtler, H. and Sweat, F.: *Arch. Path.* 75: 588-590, 1963.

PRICE'S GIEMSA METHOD

FIXATION. 10% buffered neutral formalin or Zenker's Solution. Avoid fixatives which destroy erythrocytes.

TECHNIQUE. Cut paraffin sections at 4 microns.

SOLUTIONS

GIEMSA SOLUTION (STOCK)

	VOLUME	WEIGHT
Giemsa powder*	1.0 gm	= 1.0 gm
Methanol	65.0 ml	= 51.0 gm
Glycerin	40.0 ml	= 51.0 gm

Measure volume of methanol. Weigh volume of methanol. Weight an equal amount of glycerin. Pour glycerin into an acid clean brown bottle. Use the measured methanol to rinse the glycerin from container and into the bottle. Add sterile glass beads to glycerin and methanol mixture. Combine the methanol and glycerin by shaking slightly. Add giemsa powder, tighten cap, and *shake well.* Label (Record lot no. on label). SHAKE WELL about once an hour every day for THREE DAYS! It is best to make a large volume (100 ml) of this stock and store in this bottle. When ready to use, filter through coarse filter paper into small 50 ml dark dropper bottles for use as working solutions.

GIEMSA STAIN

Label:

Giemsa: 5g. Lot No. ___ Cat. No. _____
Allied Chemical, National Aniline Div.

Glycerin: 200 ml (255 g.)
Cat. No. _____ Lot No. _____
Fisher (A.C.S.)

Methanol: 325 ml (255 g.)
Cat. No. _____ Lot No. _____
Fisher (A.C.S.)

Date _____ Made by: _____

*Must be National Aniline Certified Giemsa Powder

BUFFER SOLUTION

Disodium monohydrogen phosphate anhydrous, Na_2HPO_4 ..... 6.0 gm
Monopotassium di-hydrogen phosphate, KH_2PO_4 ..... 5.0 gm

Mix buffer salts and weigh out 1 gram units and place them in small well stoppered vials or test tubes. One of these units is dissolved in one liter of distilled water to give a buffer solution of approximately pH 7.0.

GIEMSA SOLUTION (WORKING)

Giemsa (Stock) ..... 3.0 ml
Buffer solution ..... 97.0 ml

To avoid contamination of the stock solution *never* introduce a pipette into the stock container. If dropper bottles are not used, pour a small amount of solution from the stock bottle into another container and pipette from this second container the required amount. Discard the unused portion of Giemsa stock solution.

GLACIAL ACETIC ACID WATER SOLUTION

Glacial acetic acid ..... 1.0 ml
Distilled water ..... 499.0 ml

STAINING PROCEDURE

1. Deparaffinize through xylene for 2 changes, 2 minutes each.
2. Absolute alcohol for 2 changes, 2 minutes each.
3. 95% alcohol for 1 minute.
4. 95% alcohol, 2 changes.

If necessary, remove mercuric chloride crystals with alcoholic iodine. (Iodine is removed with subsequent alcohols).

5. 80%, 70%, 50% alcohol for 1 change, 1 minute each.
6. Rinse in distilled water for 15 seconds.
7. Buffer solution for 30 minutes.
8. Working Giemsa solution overnight.
9. Quick rinse in buffer solution.
10. Glacial acetic acid water solution for 1 minute. (See note).
11. Absolute alcohol for 2 changes, 15 seconds each.
12. Xylene for 2 changes, 2 minutes each.
13. Mount with Permount or Histoclad.

RESULTS

Malarial parasites	- blue
Malarial pigment	- black
Nuclei of tissue cells	- blue
Collagen and other elements	- pale pink
Bacteria	- dark blue
Egg shells of schistosomes	- blue
Erythrocytes	- pink to rose

NOTE: *Total time* for steps #10 and #11 should be exactly 90 seconds.

REFERENCE. Price, D. L: Mil. Med. 133: 363-367, 1968.

THOMAS' METHOD FOR MALARIAL PARASITES

FIXATION. Any well-fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

PHLOXINE B SOLUTION

Phloxine B	0.5 gm
Distilled water	100.0 ml
Glacial acetic acid	0.2 ml

A slight precipitate forms but settles on the bottom. Filter before use.

METHYLENE BLUE-AZURE B SOLUTION

Methylene blue	0.25 gm
Azure B	0.25 gm
Borax	0.25 gm
Distilled water	100.0 ml

0.2% GLACIAL ACETIC ACID SOLUTION

Glacial acetic acid	0.2 ml
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Phloxine B solution for 2 minutes.
3. Rinse *well* in distilled water.
4. Methylene blue-azure B solution for 1 minute.
5. Differentiate with glacial acetic acid solution.
6. Complete differentiation in 95% alcohol, three changes.
7. Dehydrate in absolute alcohol, then clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Nuclei	-blue
Plasma cell cytoplasm	-blue
Malarial parasites	-blue
Erythrocytes	-pink
Other tissue elements	-shades of rose to red.

Note. This procedure is being used in our laboratories for the demonstration of malaria parasites in paraffin sections.

REFERENCE. Thomas, J. T.: *Stain Techn.* 28:311-312, 1953.

CAIN'S METHOD FOR MITOCHONDRIA

FIXATION. Regaud's solution.

TECHNIQUE. Cut paraffin sections at 3 microns.

SOLUTIONS

REGAUD'S SOLUTION

Potassium dichromate, 3% ..... 80.0 ml
Formalin, 37 — 40% ..... 20.0 ml

Fix for 4 days, fresh solution each day; follow with 3% potassium dichromate for 8 days, changing solution every 2 days. Wash for 24 hours.

ANILINE-ACID FUCHSIN SOLUTION

Aniline ..... 1.0 ml
Distilled water ..... 20.0 ml
Acid fuchsin ..... 4.0 gm

Shake aniline with distilled water for a couple of minutes. Let stand and then shake again several times at intervals over 24 hours. Filter. Add acid fuchsin and shake at intervals over several hours. This solution keeps only long enough to be used the same day it is made.

0.1% SODIUM CARBONATE SOLUTION

Sodium carbonate ..... 0.1 gm
Distilled water ..... 100.0 ml

1% HYDROCHLORIC ACID SOLUTION

Hydrochloric acid, concentrated ..... 1.0 ml
Distilled water ..... 99.0 ml

0.5% METHYL BLUE SOLUTION

Methyl blue ..... 0.5 gm
Distilled water ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Hot aniline-acid fuchsin solution for 5-10 minutes. Heat aniline-acid fuchsin solution to steaming then remove solution from heat when slides are placed in it.
3. Differentiate in sodium carbonate solution until cytoplasm is pale pink.
4. Dip briefly in hydrochloric acid solution to stop differentiation and also heighten color.
5. Rinse well in distilled water.
6. Counterstain in methyl blue solution for a few seconds.
7. Rinse in distilled water.
8. Dip briefly in hydrochloric acid solution.
9. Rinse in distilled water.
10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Mitochondria - bright red
Nuclei - blue to green

REFERENCE. Cain, A. J.: *Quart. J. Micr. Sci.* 89:229-231, 1948.

SIEGEL'S POLYCHROMATIC METHOD

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

TOLUIDINE BLUE O SOLUTION

Toluidine blue O ..... 0.1 gm
Phosphate buffer, 0.067*M*, pH7.3-7.4 ..... 100.0 ml

NAPHTHOL YELLOW S SOLUTION

Naphthol yellow S ..... 1.0 gm
Glacial acetic acid, 1% ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Toluidine blue O solution for 1 minute.
3. Wash in tap water.
4. Check microscopically for metachromasia.
5. Naphthol yellow S solution, one dip.
6. Wash in tap water.
7. Repeat steps 4 and 5 until brown colors are visible.
8. Dehydrate in tertiary butyl alcohol and clear in xylene, three changes, 2 minutes each.
9. Mount with Permount or Histoclad.

RESULTS

A striking spectrum of colors aid in the differentiation of various cells and tissue constituents.

Note. The optimum time of immersion in each dye solution should be determined empirically. Overstaining with toluidine blue O causes an excessive accumulation of dye in the blue or brown areas, while overstaining with naphthol yellow S can completely remove the basic stain from some structure.

REFERENCE. Siegel, I.: *Stain Techn.* 42:29-30, 1967.

LILLIE'S METHOD FOR NUCLEIC ACIDS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

SCHIFF REAGENT SOLUTION

(see page 159)

NORMAL HYDROCHLORIC ACID SOLUTION

(See page 158)

0.05M SODIUM BISULFITE SOLUTION

Sodium bisulfite ..... 0.52 gm
Distilled water ..... 100.0 ml

0.01% FAST GREEN SOLUTION

Fast green, FCF ..... 0.01 gm
Alcohol, 95% ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Place slides in preheated normal hydrochloric acid solution at 60 °C for 10 to 15 minutes.
3. Schiff reagent solution for 10 minutes.
4. Sodium bisulfite solution, three successive baths for 2 minutes each.
5. Wash in running water for 5 minutes.
6. Fast green solution for a few seconds.
7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Nuclear chomatin	- deep red purple
The chromatin of plasmodia, sarcosporidia, toxoplasmata and histoplasmata	- pale red
Cytoplasm	- light green

Note. The fast green solution does not wash out in alcohol, but if it is too intense it may be removed promptly in water.

REFERENCE. Lillie, R. D.: *Histopathologic Technic and Practical Histochemistry,* 3rd ed., New York, McGraw-Hill Book Co., 1965, pp. 149-150. Used by permission of McGraw-Hill, Inc.

MENZIES' METHOD FOR NUCLEIC ACIDS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

HYDROCHLORIC ACID-TETRAHYDROFURAN SOLUTION

Hydrochloric acid, concentrated ..... 10.0 ml
Tetrahydrofuran ..... 90.0 ml

1% AZURE B SOLUTION (STOCK)

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

0.1% BASIC FUCHSIN SOLUTION (STOCK)

Basic fuchsin ..... 0.1 gm
Distilled water ..... 100.0 ml

AZURE B-BASIC FUCHSIN SOLUTION (WORKING)

Azure B (stock) ..... 30.0 ml
Basic fuchsin (stock) ..... 8.0 ml
Glacial acetic acid ..... 2.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Hydrochloric acid-tetrahydrofuran solution at 37° C for 2-3 minutes.
3. Working Azure B-Basic fuchsin solution for 15-30 minutjs.
4. Rinse in acetone, clear in xylene, two to three changes each.
5. Mount with Permount or Histoclad.

RESULTS

DNA - red
RNA - blue

Note: This method is excellent for rat DNA and RNA. May be used on human tissue but the results are not as good. For human material, the following reference is recommended: Menzies, D. W.: *Stain Techn.* **41**: 165-167, 1966.

REFERENCE. Menzies, D. W.: *Stain Techn.* 38:157-160, 1963. Copyright by Williams and Wilkins Co.

SPICER'S METHOD FOR NUCLEIC ACIDS

FIXATION. Bouin's solution.
TECHNIQUE. Cut paraffin sections at 4 microns.

SOLUTIONS

SCHIFF REAGENT SOLUTION

(See page 159)

0.5% SODIUM METABISULFITE SOLUTION

Sodium metabisulfite ..... 0.5 gm
Distilled water ..... 100.0 ml

0.1M CITRIC ACID SOLUTION

Citric acid, anhydrous ..... 19.21 gm
Distilled water, to make ..... 1000.0 ml

0.2M DISODIUM PHOSPHATE SOLUTION

Disodium phosphate ..... 28.40 gm
Distilled water, to make ..... 1000.0 ml

METHYLENE BLUE SOLUTION

Methylene blue	0.04 gm
Citric acid, 0.2*M*	28.6 ml
Disodium phosphate, 0.2*M*	11.4 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water, wash for 5 minutes.
2. Schiff reagent solution for 10 minutes.
3. Rinse in three changes of sodium metabisulfite solution 2 minutes each.
4. Wash in running water for 10 minutes.
5. Methylene blue solution for 30 minutes. Overstaining by this solution will obscure the red in the DNA.
6. Dehydrate in 70%, 80%, 100% acetone, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

DNA	-red
Chromosomes in mitosis	-red
Chromatin in interphase nuclei	-red
Cytoplasmic RNA	-blue

Note. For viewing sections use slightly yellowish unfiltered light from incandescant light source.

REFERENCE. Spicer, S. S.: *Stain Techn.* 36:337-340, 1961.

TAFT'S METHOD FOR NUCLEIC ACIDS

FIXATION. Carnoy's or absolute alcohol.

TECHNIQUE. Cut paraffin sections at 4 microns.

SOLUTIONS

METHYL GREEN-PYRONIN SOLUTION

Methyl green, purified*	0.52 gm
Pyronin Y†	0.1 gm

Heat 100 ml of distilled water to boiling in 250 ml flask. Remove from heat and add dyes. Mix thoroughly. Stopper flask and store for 5-7 days. Solution is then ready for use and should be stored in an amber glass-stoppered bottle.

DIFFERENTIATING SOLUTION

Tertiary butyl alcohol	30.0 ml
Alcohol, 100%	10.0 ml

*Methyl Green should be extracted several times with chloroform until extracting solution is *blue green* instead of lavender.

†National Aniline Certified

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Methyl green-pyronin solution for 10 minutes. Filter before use.
3. Rinse briefly twice in distilled water.
4. Blot with several thicknesses of smooth filter paper.
5. Differentiating solution for 1-2 minutes.
6. Dehydrate in a fresh change of the differentiating solution. (Slides may be left in this solution for 4-5 minutes without additional differentiation taking place).
7. Clear in xylene, for two changes, 10 minutes each.
8. Mount with Permount or Histoclad.

RESULTS

DNA	- blue green
RNA	- bright red
Remainder of tissue	- pale pink

REMARKS. For more information on Methyl Green-Pyronin staining, see reference books by Gomori and Pearse. Good information may also be found in the following articles:

1. Kurnick, N. B.: *Stain Techn.* **30**:213-230, 1955.
2. Taft, E. B.: *Exp. Cell. Res.* 2:312-326, 1951.

REFERENCE. Taft, E.B.: *Stain Techn.* **26**:205-212, 1951. Copyright by Williams and Wilkins Co.

DEOXYRIBONUCLEASE DIGESTION METHOD

FIXATION. 10% buffered neutral formalin, Carnoy's or Bouins. (Fixatives containing mercuric, chromic, or other heavy metal ions cannot be used).

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

DEOXYRIBONUCLEASE SOLUTION

Deoxyribonuclease, cyrstalline	0.001 gm
0.2*M* Magnesium chloride (4.066% $MgCl_2 \cdot 6H_20$)	22.5 ml
0.2 *M* Calcium chloride (2.22% $CaCl_2$)	2.5 ml
0.1*M* Tris buffer, pH 7.3	20.0 ml
Distilled water	55.0 ml

TRIS BUFFER SOLUTION (STOCK)

Solution A

Trihydroxymethylaminomethane	24.228 gm
Distilled water	1000.0 ml

Solution B

Hydrochloric acid, concentrated (Sp. Gr. 1.1837)	8.35 ml
Distilled water	991.7 ml

TRIS BUFFER SOLUTION pH 7.3 (WORKING)

Solution A	10.0 ml
Solution B	17.0 ml
Distilled water	13.0 ml

DIGESTION PROCEDURE. Run two control sections.

1. Deparaffinize and hydrate two sections to distilled water.
2. (a) Treat one section with deoxyribonuclease solution for 2 hours at 37 °C.
   (b) Treat the second section in Tris buffer solution for 2 hours at 37 °C.
3. Remove both sections and wash together in running water for 10 minutes.
4. Stain as desired.

RESULTS. The material staining in the section incubated in Tris buffer solution and not present in the section treated with deoxyribonuclease solution is presumably *deoxyribonucleic acid.*

REFERENCE. Lillie, R. D.: *Histopathologic Technic and Practical Histochemistry,* 3rd ed., New York, McGraw-Hill Book Co., 1965, p. 147.

RIBONUCLEASE DIGESTION METHOD

FIXATION. 10% buffered neutral formalin, Carnoy's or Bouins. (Fixatives containing mercuric, chromic, or other heavy metal ions cannot be used).

TECHNIQUE. Cut paraffin sections 6 microns.

SOLUTIONS

RIBONUCLEASE SOLUTION

Ribonuclease, crystalline	0.5 gm
Distilled water, pH 6.8	100.0 ml

Adjust pH with 0.1*N* sodium hydroxide or hydrochloric acid.

DIGESTION PROCEDURE. Run two control sections.

1. Deparaffinize and hydrate two sections to distilled water.
2. (a) Treat one section in ribonuclease solution for 3 hours at 37 °C.
   (b) Treat the second section in distilled water for 3 hours at 37 °C.
3. Remove both sections and wash together in running water for 10 minutes.
4. Stain as desired.

RESULTS. The material staining in the sections incubated in distilled water and not present in the section treated with ribonuclease solution is presumably *ribonucleic acid.*

REFERENCE. McManus, J. F. A., and Mowry, R. W.: *Staining Methods Histologic and Histochemical,* New York, Hoeber Medical Division, Harper and Row, Publishers, 1963, p. 80.

GUARD'S METHOD FOR SEX CHROMATIN

FIXATION. Fix immediately in 95% alccohol.

TECHNIQUE. Smears (buccal epithelium).

SOLUTIONS

BIEBRICH SCARLET SOLUTION

Biebrich scarlet, water soluble	1.0 gm
Phosphotungstic acid, C.P	0.3 gm
Glacial acetic acid	5.0 ml
Alcohol, 50%	100.0 ml

FAST GREEN SOLUTION

Fast green FCF (Harleco)	0.5 gm
Phosphomolybdic acid	0.3 gm
Phosphotungstic acid	0.3 gm
Glacial acetic acid	5.0 ml
Alcohol, 50%	100.0 ml

HARRIS' HEMATOXYLIN SOLUTION

Harris' hematoxylin	0.5 ml
Alcohol, 50%	100.0 ml

STAINING PROCEDURE — Technique #1

1. From 95% alcohol fixative transfer to 70% alcohol for 2 minutes.
2. Biebrich scarlet solution for 2 minutes.
3. Rinse in 50% alcohol.
4. Differentiate in Fast green solution for 1 to 4 hours (*See Note).*
5. Place in 50% alcohol for 5 minutes.
6. Dehydrate in 70%, 95%, and absolute alcohol, 2 minutes each.
7. Clear in three changes of xylene, 2 minutes each.
8. Mount with Permount or Histoclad.

RESULTS — Technique #1

Sex Chromatin (Barr body)	-red
Background	-green

Note: During this step, check the differentiation under a microscope at hourly intervals. When all the cells reveal green cytoplasm and the vesicular nuclei are also green, the reaction is complete, which is usually in approximately 4 hours. The pyknotic nuclei, however, will not differentiate and will reveal bright red color.

STAINING PROCEDURE — Technique #2

1. From 95% alcohol fixative transfer to 70% alcohol for 2 minutes.
2. Hematoxylin solution for 15 seconds.
3. Biebrich scarlet solution for 2 minutes.
4. Place in 50% alcohol for 5 minutes.
5. Differentiate in fast green solution for 24 hours.
6. Dehydrate, clear and mount as in Technique #1.

RESULTS — Technique #2

Sex chromatin (Barr body)	- red
Nuclear chromatin	- blue
Cytoplasm	- green

REFERENCE. Guard, H. R: *Amer. J. Clin. Path.* 32:145-151, 1959. Copyright by Williams and Wilkins Co.

KLINGER-LUDWIG METHOD FOR SEX CHROMATIN

FIXATION. Equal parts of 95% alcohol and ethyl ether.

TECHNIQUE. Smears of buccal mucosal cells.

SOLUTIONS

NORMAL HYDROCHLORIC ACID

(See page 158)

THIONIN SOLUTION (STOCK)

Thionin	1.0 gm
Alcohol, 50%	100.0 ml

BUFFER SOLUTION (STOCK)

Sodium acetate	9.714 gm
Sodium barbituate	14.714 gm
Distilled water (CO_2 free) to make	550.0 ml

THIONIN SOLUTION (WORKING)

Hydrochloric acid, 1*N*	32.0 ml
Buffer solution (stock)	28.0 ml
Thionin solution (stock)	40.0 ml

STAINING PROCEDURE

1. Obtain relatively thick smears of buccal mucosal cells on albuminized slides.
2. Fix smears *promptly* in equal parts of 95% alcohol and ethyl ether for 15 minutes.
3. Absolute alcohol for 3 minutes.
4. Immerse in 0.2% parloidin made in equal parts absolute alcohol and ethyl ether for 2 minutes.
5. Air dry for 15 seconds.
6. 70% alcohol for 5 minutes.
7. Distilled water, two changes, for 5 minutes each.
8. Hydrolyse in normal hydrochloric acid solution at 56 °C for 5 minutes.
9. Distilled water, two changes, 5 minutes each.
10. Working thionin solution for 5 minutes.
11. Differentiate in 70% alcohol, 95% alcohol, and absolute alcohol for 1 minute each.
12. Clear in xylene.
13. Mount with Permount or Histoclad.

RESULTS

Sex chromatin (Barr body)	- purplish red
Other cell structures	- light blue

REFERENCE. Klinger, H. P. and Ludwig, K. S.: *Stain Techn.* 32:235, 1957.

Chapter 9
Methods For Fats And Lipids

SCHULTZ'S METHOD FOR CHOLESTEROL

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Frozen sections.

SOLUTIONS

2.5% FERRIC AMMONIUM SULFATE SOLUTION

Ferric ammonium sulfate ..... 2.5 gm
Distilled water ..... 100.0 ml

GLACIAL ACETIC-SULFURIC ACID SOLUTION

Glacial acetic acid ..... 50.0 ml
Sulfuric acid, concentrated ..... 50.0 ml

Add the sulfuric acid slowly, constantly stirring, to the acetic acid in a test tube or flask *cooled with ice. PREPARE FRESH JUST BEFORE USING.*

STAINING PROCEDURE

1. Cut frozen sections and collect in distilled water.
2. Ferric ammonium sulfate solution at room temperature for 3 days.
3. Rinse briefly in distilled water, three changes.
4. Float sections onto the slides and blot dry. Slides may be held at this point until the pathologist is ready for wet reading.
5. Place one drop of *FRESHLY PREPARED* acetic-sulfuric acid solution on the section.
6. Apply coverslip immediately.
7. Examine microscopically within a few minutes.

RESULTS

Cholesterol - green, blue-green, or blue reaction.
Background - colorless

REFERENCE. Schultz, A.: Eine Methode des mikrochemischen Cholesterinnachweises am Gewebsschnitt. *Zbl. Allg. Path.* 35:314, 1924.

OIL RED O IN PROPYLENE GLYCOL METHOD FOR FATS

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Frozen sections.

SOLUTIONS

0.5% OIL RED O SOLUTION

Oil red O ..... 0.5 gm
Propylene glycol, 100% ..... 100.0 ml

Add a small amount of propylene glycol to the oil red O and mix well, crush larger pieces. Gradually add the remainder of the propylene glycol stirring periodically. Heat gently until the solution reaches 95 °C. Do not allow to go over 100 °C. Stir while heating. Filter through coarse filter paper while still warm. Allow to stand overnight at room temperature. Filter through Seitz filter with the aid of vacuum. If solution becomes turbid, refilter. *When using the Seitz filter, put rough surface of filter up.*

85% PROPYLENE GLYCOL SOLUTION

Propylene glycol, 100% ..... 85.0 ml
Distilled water ..... 15.0 ml

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

HARRIS' HEMATOXYLIN SOLUTION

(See page 34)

5% ACID WATER SOLUTION

Hydrochloric acid ..... 5.0 ml
Distilled water ..... 95.0 ml

GLYCERIN JELLY

Gelatin ..... 10.0 gm
Distilled water ..... 60.0 ml

Heat until gelatin is dissolved. Add:

Glycerin ..... 70.0 ml
Phenol ..... 1.0 ml

STAINING PROCEDURE

1. Cut frozen sections and collect in distilled water.
2. Absolute propylene glycol for 2 minutes.
3. Oil red O solution for 1 hour. *Note.* If sections are mounted on glass slides before staining, allow to stand in ORO overnight.
4. Differentiate in 85% propylene glycol solution for 1 minute.
5. Rinse in distilled water, two changes.
6. Stain in Mayer's or Harris' hematoxylin solution for few seconds.
7. Rinse in distilled water, two changes.
8. Differentiate in acid water solution, if overstained.
9. Wash in water.
10. Neutralize in weak ammonia water if differentiated in acid water solution.
11. Wash in water, two changes.
12. Mount with glycerin jelly.

RESULTS

Fat -red
Nuclei -blue

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

FISCHLER'S METHOD FOR FATTY ACIDS

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Frozen sections.

SOLUTIONS

ALCOHOL-ETHYL ETHER EXTRACTION SOLUTION

Alcohol, 100% ..... 50.0 ml
Ethyl ether ..... 50.0 ml

SATURATED CUPRIC ACETATE SOLUTION

Cupric acetate, normal ($Cu(CH_3COO)_2 \cdot H_2O$) ..... 10-12 gm
Distilled water ..... 100.0 ml

WEIGERT'S LITHIUM HEMATOXYLIN SOLUTION

Hematoxylin ..... 1.0 gm
Alcohol 100% ..... 10.0 ml
Dissolve hematoxylin then add:
Distilled water ..... 90.0 ml
Lithium carbonate, saturated aqueous ..... 1.0 ml

WEIGERT'S BORAX-FERRICYANIDE SOLUTION

Borax ..... 2.0 gm
Potassium ferricyanide ..... 2.5 gm
Distilled water ..... 100.0 ml
For use dilute one part of this solution with 10 parts of distilled water.

STAINING PROCEDURE. Run two control sections. Treat one section as follows:

EXTRACTION TEST

1. Dehydrate through 95%, and absolute alcohol, two changes each.
2. Place in alcohol-ethyl ether extraction solution for three changes, 2 minutes each.

Treat both sections as follows:

4. Cupric acetate solution for 2-24 hours at room temperature.
5. Rinse well in distilled water.
6. Weigert's lithium hematoxylin solution for 20 minutes.
7. Differentiate in diluted Weigert's borax-ferricyanide solution until erythrocytes are decolorized.

8. Rinse well in distilled water.
9. Mount with glycerin jelly.

RESULTS

Fatty acids - deep blue-black

Iron, hemoglobin and calcium may also stain and are not removed by the extraction procedure. Free fatty acid will be removed by the extraction procedure.

Note. Following the extraction test, only the calcium soaps will remain.

REFERENCE. Fischler. F.: Über die Unterscheidung von Neutralfetten, Fettsäuren und Seifen im Gewebe, *Zbl. Allg. Path.* 15:913, 1904.

OSMIUM TETROXIDE METHOD FOR FAT (FROZEN SECTIONS)

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Frozen sections.

SOLUTIONS

1% OSMIUM TETROXIDE SOLUTION

Osmium tetroxide..... 1.0 gm
Distilled water..... 100.0 ml

Score a 1 gm ampule of osmium tetroxide with a file and drop into closed cylinder containing the distilled water. Vigorous shaking will break the ampule safely.

Note. Mix under a hood or in a well-ventilated room. Do not inhale fumes.

GLYCERIN JELLY

(See page 141)

STAINING PROCEDURE

1. Cut frozen sections and collect in distilled water.
2. Allow sections to remain in osmium tetroxide solution for 24 hours.
3. Wash in several changes of water for 6 hours.
4. Counterstain as desired with kernechtrot, or hematoxylin and eosin.
5. Place in absolute alcohol for several hours to obtain secondary staining of fat.
6. Rinse well in distilled water.
7. Mount with glycerin jelly.

RESULTS

Fat	- black
Nuclei	- depends on counterstain used
Background	- depends on counterstain used

REFERENCES. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p. 118.

OSMIUM TETROXIDE METHOD FOR FAT (PARAFFIN SECTIONS)

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns. *Note.* (See step 1, staining procedure).

SOLUTIONS

5% POTASSIUM DICHROMATE SOLUTION (STOCK)

Potassium dichromate	5.0 gm
Distilled water	100.0 ml

2% OSMIUM TETROXIDE SOLUTION (STOCK)

Osmium tetroxide	2.0 gm
Distilled water	100.0 ml

Score two 1 gm ampules of osmium tetroxide with a file and drop into cylinder containing distilled water, then close. Vigorous shaking will break ampule safely.

Note. Mix under a hood or in a well-ventilated room. Do not inhale fumes.

POTASSIUM DICHROMATE-OSMIUM TETROXIDE SOLUTION (WORKING)

Potassium dichromate (stock)	50.0 ml
Osmium tetroxide (stock)	50.0 ml

STAINING PROCEDURE

1. Place formalin-fixed specimen, which is no thicker than 4 mm, in working potassium dichromate-osmium tetroxide solution for 8 hours.
2. Wash specimen in running water for 2 hours.
3. Process overnight in conventional automatic tissue processor. Embed and cut sections at 6 microns.
4. Deparaffinize sections and hydrate to distilled water.
5. Counterstain with hematoxylin and eosin or kernechrot.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Fat	- black
Background	- depending on counterstain used.

REMARKS. The following modification to the previous method has proven extremely valuable in our laboratories since it eliminates the many artifacts created in frozen section cutting and/or staining. This method utilizes the fat-preserving qualities of osmium tetroxide and the staining qualities of oil red O. Using the following procedure, we have been able to demonstrate small intracytoplasmic fat droplets not visible with conventional fat stains:

Prepare tissues as above through Step 4. At this point remove osmium tetroxide with a 3% hydrogen peroxide (normally 4 hours is adequate) followed by a 15 minute wash in tap water. Sections are then stained in oil red O for 1 hour. Wash in water and mount in glycerin jelly.

Note. It may be necessary to use a 5% aqueous periodic acid solution for 5 minutes following Step 4 to insure section background is clear enough to accept the counterstain desired.

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

SUDAN BLACK B METHOD FOR FAT

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Frozen sections.

SOLUTIONS

SUDAN BLACK B SOLUTION

Dissolve 0.7 gm Sudan black B in 100 ml of propylene glycol by heating to 100° — 110°C and stirring thoroughly for a few minutes. Do not exceed 110°C. Filter hot through Whatman No. 2 paper to remove excess dye. After cooling to room temperature filter through a Seitz filter with the aid of vacuum.

85% PROPYLENE GLYCOL SOLUTION

(See page 141)

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE

1. Cut frozen sections and collect in distilled water.
2. Absolute propylene glycol for 10 minutes.
3. Sudan black B solution for 30 minutes. *Note.* If sections are mounted on glass slides before staining, allow to stain in Sudan black B overnight.
4. Differentiate in 85% propylene glycol solution for 3 minutes. Agitate sections several times.
5. Rinse in distilled water.
6. Counterstain in nuclear fast red for 5 minutes.
7. Rinse well in distilled water.
8. Mount with glycerin jelly.

RESULTS

Fat - blue-black
Nuclei - red

REFERENCE. Chiffelle, T.L., and Putt, F.A.: *Stain Techn.* **26**:51, 1951. Copyright by Williams and Wilkins Co.

LANDING'S METHOD FOR LIPIDS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns. Cut frozen sections at 10-12 microns.

SOLUTIONS

ACETONE-ETHYL ETHER SOLUTION

Acetone	50.0 ml
Ethyl ether	50.0 ml

PHOSPHOMOLYBDIC ACID SOLUTION

Phosphomolybdic acid	1.0 gm
Alcohol 100%	50.0 ml
Chloroform	50.0 ml

STANNOUS CHLORIDE SOLUTION

Stannous chloride	1.0 gm
Hydrochloric acid, 3*N*	100.0 ml

Make this solution fresh just before use.

3N HYDROCHLORIC ACID SOLUTION

Hydrochloric acid, concentrated	10.0 ml
Distilled water	30.0 ml

AQUEOUS EOSIN SOLUTION

(See page 36)

STAINING PROCEDURE

1. (a) *Frozen*: Place sections on gelatinized slide, drain, blot and expose to formalin fumes for 15 minutes.
   (b) *Paraffin*: Deparaffinize and hydrate to 95% alcohol.
2. Allow sections to dry thoroughly.
3. Dip slides in acetone-ethyl ether solution.
4. Place directly in phosphomolybdic acid solution for 15 minutes.
5. Rinse in equal parts ethanol and chloroform.
6. Rinse in chloroform, two changes.
7. Stannous chloride solution for 3-5 seconds.
8. Wash in water.
9. Counterstain with eosin solution.
10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Gaucher lipid (kerasin)	-deep blue
Niemann-Pick lipid (sphingomyelin)	-lighter blue green
Tay-Sachs' (ganglion cells)	-deep blue
Normal myelin	-deep blue

REFERENCE. Landing, B. H., Uzman, L. L., and Whipple, A.: Lab. Invest. 1:456-462, 1952.

BAKER'S METHOD FOR PHOSPHOLIPIDS

FIXATION. Baker's formol calcium fixative or 10% buffered neutral formalin.

TECHNIQUE. Frozen sections.

SOLUTIONS

POTASSIUM DICHROMATE-CALCIUM SOLUTION

Potassium dichromate	5.0 gm
Calcium chloride	1.0 gm
Distilled water	100.0 ml

BORAX-FERRICYANIDE SOLUTION

Borax	0.25 gm
Potassium ferricyanide	0.25 gm
Distilled water	100.0 ml

Store in refrigerator.

1% SODIUM IODATE SOLUTION

Sodium iodate	1.0 gm
Distilled water	100.0 ml

ACID HEMATEIN SOLUTION

Hematoxylin crystals	0.05 gm
Sodium iodate, 1%	1.0 ml
Distilled water	48.0 ml

Bring to a boil then cool and add 1 ml of glacial acetic acid. Make fresh for use

GLYCERIN JELLY

(See page 141)

STAINING PROCEDURE

1. Cut frozen sections and collect in distilled water.
2. Potassium dichromate-calcium solution at room temperature for 16 hours.
3. Transfer to a second bath of potassium dichromate-calcium solution at 60 °C for 16 hours.
4. Wash in running water for 6 hours, then distilled water for 5 minutes.
5. Acid hematein solution at 37 °C for 5 hours.
6. Rinse in distilled water, then transfer to borax ferricyanide solution at 37 °C for 16 hours.
7. Rinse in distilled water for 10 minutes.
8. Mount with glycerin jelly.

RESULTS

Phospholipids (lecithin, kephalin, sphingomyelin)	- blue black
Galactolipids	- blue-black to pale blue

PYRIDINE EXTRACTION TEST

Pyridine extraction test is designed to distinguish between phospholipids and certain proteins which give a positive reaction with the acid hematein procedure.

SOLUTIONS

WEAK BOUIN'S SOLUTION

Picric acid, saturated aqueous	50.0 ml
Formalin, 37 – 40%	10.0 ml
Glacial acetic acid	5.0 ml
Distilled water	35.0 ml

PROCEDURE

1. Cut frozen sections.
2. Weak Bouin's solution for 16 hours.
3. Place in 70% alcohol for 1 hour.
4. Place in 50% alcohol for 30 minutes.
5. Wash in running water for 30 minutes.
6. Pyridine at room temperature, two changes, 1 hour each.
7. Pyridine at 60 °C overnight.
8. Wash in running water for 2 hours.
9. Proceed at *step* 2 of the acid hematein staining procedure.

RESULTS. With pyridine extraction, phospholipids and galactolipids are unstained. Red cells stain black both with and without pyridine extraction. Nuclei stain after extraction but not before.

REMARKS. This procedure was originally developed for staining thin gross tissue blocks. In our laboratories thin frozen cut sections have been employed satisfactorily. However, the block method as originally presented should be used whenever possible. Only by using the method in this way can one be absolutely certain of the results.

Note. The time suggested at each step in the procedure will suffice for both thin gross tissue or frozen cut section staining.

REFERENCE. Baker, J. R.: *Quart. J. Micr. Sci.* 87:441, 1946.

PEARSE'S METHOD FOR PHOSPHOLIPIDS

FIXATION. Baker's calcium cadmium formol.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

METHASOL FAST BLUE 2G SOLUTION

Methasol fast blue 2G	0.1 gm
Alcohol 100%	100.0 ml

Luxol fast blue MBS may be substituted for Methasol fast Blue 2G.

0.05% LITHIUM CARBONATE SOLUTION

Lithium carbonate	0.05 gm
Distilled water	100.0 ml

1% NEUTRAL RED SOLUTION

Neutral red	1.0 gm
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and place in absolute alcohol.
2. Methasol fast blue solution at 58 °C overnight.
3. Rinse in 70% alcohol.
4. Rinse in distilled water.
5. Differentiate in lithium carbonate solution for 30 minutes to 2 hours.
6. Rinse in distilled water.
7. Counterstain in neutral red solution for 30 minutes.
8. Rinse in distilled water.
9. Blot dry.
10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes, 2 minutes each.
11. Mount with Permount or Histoclad.

RESULTS

Phospholipids	-blue
Early lipofuchsin, eosinophil granules, keratin, keratohyaline, human elastic tissues	-blue to purple
Nuclei and nucleoli	-red

REFERENCE. Pearse, A. G. E.: *J. Path. Bact.* **70**:554-557, 1955.

MUKHERJI'S HISTOCHEMICAL METHOD FOR UNSATURATED LIPIDS

FIXATION. 10% Buffered neutral formalin.

TECHNIQUE. Frozen sections.

SOLUTIONS

3.5% BROMINE SOLUTION

Bromine	3.5 gm
Distilled water	100.0 ml

5% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page 89)

10% SILVER NITRATE SOLUTION

Silver nitrate ..... 10.0 gm
Alcohol, 50% aldehyde free ..... 100.0 ml

REDUCING SOLUTION

Formalin, 37 – 40% ..... 10.0 ml
Distilled water ..... 90.0 ml

STAINING PROCEDURE. Use control slide*.

1. Cut frozen sections and collect in distilled water.
2. Wash in tap water for several hours followed by several changes of distilled water.
3. Blot dry.
4. Expose to Bromine solution fumes in a large closed jar at 37 °C for 1 hour.
5. Sodium thiosulfate solution, two changes, 5 minutes each.
6. Rinse in distilled water, three changes.
7. Silver nitrate solution at 37 °C in the dark until faint brown or pale yellow.
8. Rinse in distilled water.
9. Place in reducing solution until deep brown.
10. Rinse in distilled water.
11. Sodium thiosulfate solution for 2 minutes.
12. Rinse in distilled water.
13. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes, 2 minutes each.
14. Mount with Permount or Histoclad.

RESULTS

Unsaturated lipids - brown to black

REMARKS. If a critical study of unsaturated lipid is desired, sections should be treated with amylase before bromination after step 2 to eliminate any glycogen interfering with the unsaturated lipid.

REFERENCE. Mukherji, M., Deb, C., and Sen, P. B.: *J. Histochem. Cytochem.* 8:189-194, 1960.

SELIGMAN-ASHBEL METHOD FOR ACTIVE CARBONYL GROUPS

FIXATION. 10% buffered neutral formalin (not more than 48 hours).

TECHNIQUE. Cut frozen sections at 15 microns.

SOLUTIONS

0.1% 2-HYDROXY-3-NAPHTHOIC ACID HYDRAZIDE* SOLUTION

2-hydroxy-3-napthoic acid hydrazide ..... 0.1 gm
Glacial acetic acid ..... 5.0 ml

*Control section should be subjected to identical treatment after Baker's pyridine extraction (pyridine at 60 °C for 24 hours), continue at step 2.

Dissolve powder in hot glacial acetic acid then add:

Alcohol, 50% ..... 95.0 ml

5% ACETIC-ALCOHOL SOLUTION

Glacial acetic acid ..... 5.0 ml
Alcohol, 50% ..... 95.0 ml

0.67 *M* PHOSPHATE BUFFER SOLUTION, pH 7.4

1/15 *M* sodium phosphate, dibasic (9.465 gm made up to 1000 ml with distilled water) ..... 80.0 ml
1/15 *M* potassium acid phosphate (9.07 gm made up to 1000 ml with distilled water) ..... 20.0 ml

PHOSPHATE BUFFER-ALCOHOL SOLUTION

Phosphate buffer solution ..... 50.0 ml
Alcohol, 100% ..... 50.0 ml

FAST BLACK B SOLUTION

Fast black B ..... 0.05 gm
Phosphate buffer-alcohol solution ..... 100.0 ml

0.1% SAFRANIN SOLUTION

Safranin ..... 0.1 gm
Glacial acetic acid, 1% aqueous ..... 100.0 ml

STAINING PROCEDURE. Mount sections on glass slides and let dry in 37 °C oven overnight.

1. Wash slide in running water for 2 hours to remove formalin.
2. Place in fresh naphthoic-hydrazide solution for 2 hours at 25 °C.
3. Wash in acetic-alcohol solution, 50% alcohol, and distilled water for four changes, 2 minutes each.
4. Phosphate buffer-alcohol solution for 1 - 2 minutes.
5. Directly into fast black B solution for 2 minutes.
6. Rinse in acetic-alcohol solution.
7. Counterstain with safranin solution for 1 - 2 minutes.
8. Rinse in distilled water.
9. Mount with glycerin jelly.

RESULTS. Active carbonyl lipids -dark blue or greenish blue.

REFERENCE. Seligman, A. M., and Ashbel, R.: *Endocrinology* **50**:338 - 349, 1952.

*K & K Laboratories, 121 Express Street, Engineers Hill, Plainview, New York, 11803.

MENSCHIK'S METHOD FOR PHOSPHOLIPIDS

FIXATION. Baker's formol calcium.

TECHNIQUE. Frozen sections.

SOLUTIONS

NILE BLUE SULPHATE SOLUTION

Nile blue sulphate, saturated aqueous	100.0 ml
Sulphuric acid, 0.05% aqueous	10.0 ml

Boil for 30 minutes using a reflux condenser.

5% GLACIAL ACETIC ACID SOLUTION

Glacial acetic acid	5.0 ml
Distilled water	95.0 ml

0.5% HYDROCHLORIC ACID SOLUTION

Hydrochloric acid, concentrated	0.5 ml
Distilled water	99.5 ml

GLYCERIN JELLY

(See page 141)

STAINING PROCEDURE

1. Cut frozen sections and collect in distilled water.
2. Nile blue sulphate solution at 60 °C for 90 minutes.
3. Rinse in distilled water.
4. Preheat acetone to 50 °C, then remove from source of heat and immediately place sections in this solution for 30 minutes.
5. Differentiate in glacial acetic acid solution for 30 minutes.
6. Rinse in distilled water.
7. Differentiate in hydrochloric acid solution for 3 minutes.
8. Rinse in distilled water.
9. Mount with glycerin jelly.

RESULTS

Phospholipids - blue

REFERENCE. Menschik, Z.: *Stain Techn.* **28**:13-18, 1953.

Chapter 10

Methods For Carbohydrates And Mucoproteins

BENNHOLD'S METHOD FOR AMYLOID (CONGO RED)

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns (see Remarks).

SOLUTIONS

1% CONGO RED SOLUTION

Congo red	1.0 gm
Distilled water	100.0 ml

1% SODIUM HYDROXIDE SOLUTION

Sodium hydroxide	1.0 gm
Distilled water	100.0 ml

ALKALINE ALCOHOL SOLUTION

Sodium hydroxide, 1%	1.0 ml
Alcohol, 50%	100.0 ml

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Congo red solution for 1 hour.
3. Rinse off excess stain in water, two or three changes.
4. Differentiate in alkaline alcohol solution for 3-5 seconds. Agitate constantly until the background appears clear.
5. Wash in running water for 5 minutes.
6. Counterstain in Mayer's hematoxylin solution for 5 minutes.
7. Wash in running water for 15 minutes.
8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
9. Mount with Permount or Histoclad.

RESULTS

Amyloid	-pink to red
Nuclei	-blue

REMARKS. Sections of suspected amyloidosis may be cut at 12 microns if a brighter color is desired. This thick section technic is also being used to advantage in our laboratories to demonstrate minute amyloid deposits. Twelve micron sections can also be used to advantage in the crystal violet and Sirius Red methods for amyloid.

REFERENCE. Mallory, F. B.: Pathological Technique, New York, Hafner Publishing Co., 1961, p. 133-134. (AFIP modification)

LIEB'S METHOD FOR AMYLOID (CRYSTAL VIOLET)

FIXATION. 10% buffered neutral formalin or alcohol.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

CRYSTAL VIOLET SOLUTION (STOCK)

Crystal violet to saturate	approx. 14.0 gm
Alcohol, 95%	100.0 ml

CRYSTAL VIOLET SOLUTION (WORKING)

Crystal violet (stock)	10.0 ml
Distilled water	300.0 ml
Hydrochloric acid, concentrated	1.0 ml

APATHY'S MOUNTING MEDIA

(See page 156)

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Stain in working crystal violet solution for 5 hours.
3. Wash well in running water for 15 minutes.
4. Mount with Apathy's mounting media.

RESULTS

Amyloid	-purplish violet
Other tissue elements	-blue

REMARKS. Five hours in working crystal violet solution should be used as the minimum staining time. In our laboratories, 16 hours staining time demonstrated the optimum of staining qualities. (See remarks, Bennhold's method.)

REFERENCE. Lieb, E.: *Amer. J. Clin. Path.* 17: 413-414, 1947. (AFIP Modification)

SWEAT-PUCHTLER METHOD FOR AMYLOID (SIRIUS RED)

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% SODIUM HYDROXIDE SOLUTION

(See page 153)

ALKALINE ALCOHOL SOLUTION

Alcohol, 80%	100.0 ml
Sodium hydroxide, 1%	1.0 ml

SIRIUS RED SOLUTION

Sirius Red, F3BA*	1.0 gm
Distilled water	100.0 ml

Dissolve and then add:

Sodium chloride	0.5 gm

Let stand overnight before use. *DO NOT FILTER.*

0.1M BORATE BUFFER SOLUTION pH 9.0

Sodium borate	38.143 gm
Distilled water	1000.0 ml

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Wash in running water for 5 minutes.
3. Mordant in buffered neutral formalin overnight.
4. Wash in water for 15 minutes.
5. Alkaline alcohol solution for 1 hour.
6. Rinse in distilled water for 10 seconds.
7. Preheated Sirius Red solution in a 60 °C oven for 90 minutes.
8. Rinse briefly in borate buffer solution for two changes.
9. Wash in running water for 5 minutes.
10. Mayer's hematoxylin solution for 5 minutes.
11. Wash in running water for 15 minutes, then drain slides.
12. Dehydrate rapidly in absolute alcohol, then clear in xylene, three changes each.
13. Mount with Permount or Histoclad.

RESULTS

Amyloid	- pink to red
Nuclei	- blue
Background	- unstained
Elastica	- pink to red

*Roboz Surgical Instrument Co., Washington, D.C.

REMARKS. Sweat and Puchtler used seven direct cotton dyes for the demonstration of amyloid. In studies conducted in our laboratories Sirius Red F3BA has been found to be the most specific. (See remarks, Bennhold's method).

Note. Sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) must be used as other borates will give a different pH.

REFERENCE. Sweat, F. and Puchtler, H.: *Arch. Path.* 80:613-620, 1965.

HIGHMAN'S METHOD FOR AMYLOID (METHYL VIOLET)

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

0.1% METHYL VIOLET SOLUTION

Methyl violet	0.1 gm
Distilled water	97.5 ml
Glacial acetic acid	2.5 ml

APATHY'S MOUNTING MEDIA

Acacia (gum arabic)	50.0 gm
Cane sugar (sucrose)	50.0 gm
Distilled water	150.0 ml
Sodium chloride	10.0 gm
Thymol	0.1 gm

Mix the acacia, cane sugar, and distilled water in a flask. Place the flask in a pan of boiling water until ingredients are dissolved. Dissolve the sodium chloride and thymol in this solution. Filter with a Seitz filter while the solution is still warm, or use coarse filter paper and place in 60°C oven, changing filter paper frequently. Refrigerate to remove air bubbles. Sodium chloride is added to prevent bleeding and thymol is added as a preservative.

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Working Weigert's hematoxylin solution for 5 minutes.
3. Wash in running water for 10 minutes.
4. Methyl violet solution for 15-30 minutes.
5. Wash in running water then to distilled water.
6. Mount in Apathy's mounting media.

RESULTS

Amyloid	- red purple
Nuclei	- blue to black

REMARKS. (See remarks, Bennhold's method).

REFERENCE. Highman, B.: *Arch. Path.* 41:559, 1946.

VASSAR-CULLING METHOD FOR AMYLOID (THIOFLAVINE T)

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% THIOFLAVINE T SOLUTION

Thioflavin T*	1.0 gm
Distilled water	100.0 ml

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

DIFFERENTIATING SOLUTION

Glacial acetic acid	1.0 ml
Distilled water	100.0 ml

APATHY'S MOUNTING MEDIA

(See page 156)

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Mayer's hematoxylin solution for 5 minutes. Do not differentiate.
3. Wash in running water for 1 minute.
4. Thioflavine T solution for 3 minutes.
5. Wash in running water for 10 minutes.
6. Differentiating solution for 10 minutes.
7. Wash in running water for 2 minutes.
8. Mount in Apathy's mounting media.

RESULTS

Amyloid white fluorescence

Note. Since this paper was published the authors (P.S.V.-C.F.A.C.) have found that increased specificity of staining is achieved by using a shorter wave length of light (Zeiss UG5 excitor filter) and a simple (colorless) U.V. filter in the eyepiece. Using this system only Amyloid is yellow, all other tissue constituents are blue.

REFERENCE. Vassar, P. S. and Culling, C. F. A.: *Arch. Path.* 68:487-498, 1959.

BEST'S CARMINE METHOD FOR GLYCOGEN

FIXATION. Carnoy's or Formol-Alcohol.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

*Roboz Surgical Instrument Co., Washington, D. C.

CARMINE SOLUTION (STOCK)

Carmine	2.0 gm
Potassium carbonate	1.0 gm
Potassium chloride	5.0 gm
Distilled water	60.0 ml

Boil in an evaporating dish gently and cautiously for several minutes. When cool add 20 ml of 28% ammonium hydroxide. Store in refrigerator.

CARMINE SOLUTION (WORKING)

Carmine solution (stock)	10.0 ml
Ammonium hydroxide, 28%	15.0 ml
Alcohol, methyl.	15.0 ml

DIFFERENTIATING SOLUTION

Alcohol, 100%	20.0 ml
Alcohol, methyl	10.0 ml
Distilled water	25.0 ml

STAINING PROCEDURE. For digestion procedure, see page 171.

1. Deparaffinize and hydrate to distilled water.
2. Mayer's hematoxylin solution for 15 minutes.
3. Wash in running water for 15 minutes.
4. Working carmine solution for 30 minutes.
5. Differentiating solution for a few seconds.
6. Rinse quickly in 80% alcohol.
7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Glycogen	- pink to red
Nuclei	- blue

REFERENCE. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, pp. 126-129.

McMANUS' METHOD FOR GLYCOGEN (PAS)

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

NORMAL HYDROCHLORIC ACID SOLUTION

Hydrochloric acid, sp. gr. 1.19	83.5 ml
Distilled water	916.5 ml

COLEMAN'S FEULGEN SOLUTION

Dissolve 1.0 gm basic fuchsin in 200.0 ml hot distilled water. Bring to boiling point. Cool and add 2.0 gm potassium metabisulfite, 10.0 ml normal hydrochloric acid. Let bleach for 24 hours, then add 0.5 gm activated carbon (Norit). Shake for 1 minute and filter through coarse filter paper. Repeat filtration until solution is colorless. *Store in refrigerator.*

SCHIFF REAGENT SOLUTION

Dissolve 1.0 gm basic fuchsin in 200.0 ml hot distilled water. Bring to boiling point. Cool to 50°C. Filter and add 20.0 ml normal hydrochloric acid. Cool further and add 1.0 gm anhydrous sodium bisulfite, or sodium metabisulfite. Keep in the dark for 48 hours until solution becomes straw colored. *Store in refrigerator.*

Test For Schiff Reagent Solution

Pour a few drops of Schiff reagent solution into 10 ml of 37 – 40% formaldehyde in a watch glass. If the solution turns reddish purple rapidly, it is good. If the reaction is delayed and the resulting color deep blue-purple, the solution is breaking down.

0.5% PERIODIC ACID SOLUTION

Periodic acid	0.5 gm
Distilled water	100.0 ml

0.2% LIGHT GREEN SOLUTION (STOCK)

Light green, SF yellowish	0.2 gm
Distilled water	100.0 ml
Glacial acetic acid	0.2 ml

LIGHT GREEN SOLUTION (WORKING)

Light green (stock)	10.0 ml
Distilled water	50.0 ml

HARRIS' HEMATOXYLIN SOLUTION

(See page 34)

STAINING PROCEDURE. For digestion procedure see page 171.

1. Deparaffinize and hydrate to distilled water.
2. Oxidize in periodic acid solution for 5 minutes.
3. Rinse in distilled water.
4. Coleman's Feulgen *or* Schiff reagent solution for 15 minutes.
5. Wash in running water for 10 minutes for pink color to develop.
6. Harris' hematoxylin for 6 minutes, *or* light green counterstain for a few seconds.

Light green is recommended for counterstaining sections in which fungi are to be demonstrated. Omit steps 7 through 11 if light green is used.

7. Wash in running water.
8. Differentiate in 1% (HCL) acid alcohol-three to ten quick dips.
9. Wash in running water.
10. Dip in ammonia water to blue sections.

11. Wash in running water for 10 minutes.
12. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
13. Mount with Permount or Histoclad.

RESULTS

Glycogen, mucin, reticulin, fibrin or thrombi, colloid droplets, hyalin of arteriosclerosis, hyalin deposits in glomeruli, granular cells in the renal arterioles where preserved, most basement membranes, colloid of pituitary stalks and thyroid, amyloid infiltration may show a positive reaction - rose to purplish red

Nuclei	- blue
Fungi	- red
Background	- pale green (with light green counterstaining).

REMARKS. For excellent additional information on colloidal iron, Alcian blue 8GX and their combinations with periodic acid Schiff reaction see: "The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins with revised directions for the colloidal iron stain, the use of alcian blue 8GX and their combinations with the periodic acid-Schiff reaction," Mowry, R. W. *Annals of the New York Academy of Sciences,* **106**:402-423, 1963.

Note: A solution of 5% aqueous Clorox bleach will reduce overstaining by leucofuchsin. Running tap water or ammonia water will decolorize the light green.

REFERENCE. McManus, J. F. A.: *Stain Techn.* 23:99-108, 1948. (AFIP modification).

LAQUEUR'S METHOD FOR ALCOHOLIC HYALIN

FIXATION. Zenker's or 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

ACID FUCHSIN-ANILINE SOLUTION

Acid fuchsin	20.0 gm
Aniline	2.0 ml
Distilled water	100.0 ml

Shake the aniline in distilled water thoroughly before adding the acid fuchsin.

ALCOHOLIC PICRIC ACID SOLUTION

Picric acid, saturated in 100% alcohol	10.0 ml
Alcohol, 20%	70.0 ml

1% PHOSPHOMOLYBDIC ACID SOLUTION

(See page 112)

1% LIGHT GREEN SOLUTION

Light green, SF yellowish ..... 1.0 gm
Distilled water ..... 100.0 ml
Glacial acetic acid ..... 1.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mayer's hematoxylin solution for 15 minutes.
3. Wash in running water for 15 minutes.
4. Flood the slides with acid fuchsin-aniline solution and heat gently with bunsen burner until aniline fumes escape, let stand for 5 minutes.
5. Wash in water, three dips.
6. Differentiate in alcoholic picric acid solution until only hyalin and red cells remain red, and collagen is faint gray or unstained.
7. Wash in running water for 3 minutes.
8. Mordant in phosphomolybdic acid solution for 4 hours.
9. Counterstain in light green solution for 1 hour.
10. Wash in running water for 1 minute.
11. Differentiate light green in 80% alcohol. Check under microscope. Collagen fibers are seen as distinct fibrils in well differentiated section.
12. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
13. Mount with Permount or Histoclad.

RESULTS

Mallory bodies	-bright red
Erythrocytes	-red
Cytoplasm	-pale brown
Bile pigment	-green
Proteinaceous material seen occasionally in some liver cells	-red
Hemosiderin and lipofuchsin	-unstained

REFERENCE. Laqueur, G. L.: *Amer. J. Clin. Path.* **20**:689-690, 1950.

MAYER'S MUCICARMINE METHOD FOR MUCIN AND CRYPTOCOCCUS

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

MUCICARMINE SOLUTION

Carmine ..... 1.0 gm
Aluminum chloride, anhydrous ..... 0.5 gm
Distilled water ..... 2.0 ml

Mix stain in small evaporating dish. Heat on electric hot plate for 2 minutes. Liquid becomes black and syrupy. Dilute with 100 ml of 50% alcohol and let stand for 24 hours. Filter.

Dilute 1 part mucicarmine solution with 4 parts *tap water* for use.

0.25% METANIL YELLOW SOLUTION

(See page 78)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Working solution of Weigert's hematoxylin for 7 minutes.
3. Wash in running water for 10 minutes.
4. Diluted mucicarmine solution for 60 minutes.
5. Rinse quickly in distilled water.
6. Metanil yellow solution for 1 minute.
7. Rinse quickly in distilled water.
8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
9. Mount with Permount or Histoclad.

RESULTS

Mucin	- deep rose to red
Capsule of cryptococcus	- deep rose to red
Nuclei	- black
Other tissue elements	- yellow

REMARKS. This technic is used in our laboratories for the demonstration of cryptococcus with good results. Hematoxylin and eosins or elastic counterstains may be used with this method.

Note: Carminophilic properties will be obscured if sections are overstained with Weigert's hematoxylin and/or metanil yellow solution.

REFERENCES. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p. 130.

JOHNSON'S METHOD FOR METACHROMASIA (TOLUIDINE BLUE)

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

0.1% TOLUIDINE BLUE SOLUTION

Toluidine blue	0.1 gm
Distilled water	100.0 ml

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.

2. Toluidine blue solution for 2 minutes.
3. Rinse in distilled water.
4. Cover slip section with distilled water.
5. Blot around edge of cover glass and seal with fingernail polish.
6. Examine the sections as soon as possible after preparation.

RESULTS

Metachromatic tissue - pink

REFERENCE. Johnson, F. B.: Histochemistry Branch, Armed Forces Institute of Pathology, Washington, D.C. 20305.

ALCIAN BLUE METHOD FOR MUCOSUBSTANCES pH 2.5 or pH 1.0

Alcian Blue pH 2.5 Method

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

3% ACETIC ACID SOLUTION

Glacial acetic acid	3.0 ml
Distilled water	97.0 ml

1% ALCIAN BLUE SOLUTION

Alcian blue, 8GX	1.0 gm
Glacial acetic acid, 3%	100.0 ml

Adjust the pH to 2.5. Filter and add a few crystals of thymol.

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant in 3% acetic acid solution for 3 minutes.
3. Alcian Blue solution for 30 minutes.
4. Wash in running water for 10 minutes.
5. Rinse in distilled water.
6. Counterstain in Kernechtrot solution for 5 minutes.
7. Wash in running water for 1 minute.
8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
9. Mount with Permount or Histoclad.

RESULTS. Weakly acidic sulfated mucosubstances, hyaluronic acid and sialomucins-dark blue. Nuclei stain weakly in formalin-fixed tissue if Kernechtrot is not used as a counterstain.

Alcian Blue pH 1.0 Method

SOLUTIONS

ALCIAN BLUE SOLUTION

Alcian Blue, 8GX	1.0 gm
Hydrochloric acid, 0.1*N*	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Alcian blue solution for 30 minutes.
3. Blot sections dry with filter paper without rinsing in water.
4. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
5. Mount with Permount or Histoclad.

RESULTS. This method provides a more selective staining of sulfated mucosubstances which stain dark blue.

REFERENCE. Lev, R. and Spicer, S. S.: *J. Histochem. Cytochem.* 12:309, 1964.

ALCIAN BLUE METHOD FOR MUCOSUBSTANCES pH 0.4

FIXATION. 10% buffered neutral formalin or fixative of choice.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

PHOSPHATE-HYDROCHLORIC ACID SOLUTION

Hydrochloric acid, concentrated	42.0 ml
Sodium phosphate monobasic	13.8 gm
Distilled water to make	1000.0 ml

ALCIAN BLUE SOLUTION pH 0.4

Alcian Blue, 8GX	2.5 gm
Phosphate - Hydrochloric acid solution	250.0 ml

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Phosphate-hydrochloric acid solution for 3 minutes.
3. Alcian blue solution for 30 minutes.
4. Wash in running water for 10 minutes.
5. Rinse in distilled water.
6. Counterstain in Kernechtrot for 5 minutes.
7. Wash in running water for 1 minute.

8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
9. Mount with Permount or Histoclad.

RESULTS. Only strongly acidic sulfated muco substances give a positive reaction.

REFERENCE. Johnson, W. C., Graham, J. H., and Helwig, E. B.: *J. Invest. Derm.* 42:215-224, 1964.

ALDEHYDE FUCHSIN METHODS FOR MUCOSUBSTANCES

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

ALDEHYDE FUCHSIN SOLUTION pH 1.7

(See page 166)

STAINING PROCEDURE. For digestion procedures, see pages 171, 172.

1. Deparaffinize and hydrate to distilled water.
2. Rinse in 70% alcohol.
3. Aldehyde fuchsin solution for 30 minutes.
4. Rinse in 70% alcohol.
5. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
6. Mount with Permount or Histoclad.

RESULTS

Sulfated mucosubstances	-dark purple
Elastica	-dark purple
Mast cells	-dark purple
Sialomucins and hyaluronic acid	-faint reaction
Paget's cells	-purple

ALDEHYDE FUCHSIN-ALCIAN BLUE METHOD

SOLUTIONS

ALCIAN BLUE SOLUTION

Alcian blue, 8GX	0.2 gm
Glacial acetic acid, 3% aqueous	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Rinse in 70% alcohol.
3. Aldehyde fuchsin solution for 30 minutes.
4. Rinse in 70% alcohol, rinse in distilled water, several changes, for 5 minutes.

5. Alcian blue solution for 30 minutes.
6. Rinse in distilled water.
7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Strongly acidic sulfated mucosubstances and elastica	- deep purple
Less strongly acidic sulfated mucosaccharide or mixed sulfated and carboxylic mucosubstances	- blue to purple
Nonsulfated acid mucosubstances	- blue

REFERENCE. Spicer, S. S. and Meyer, D. B.: *Techn. Bull. Regist. Med. Techn.* 30:53-60, 1960. Copyright by Williams and Wilkins Co.

ALDEHYDE FUCHSIN pH 1.0

FIXATION. 10% buffered neutral formalin or fixative of choice.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

ALDEHYDE FUCHSIN SOLUTION

Basic fuchsin	1.0 gm
Alcohol, 70%	200.0 ml
Hydrochloric acid, concentrated	2.0 ml
Paraldehyde	2.0 ml

Let stand at room temperature for 2 to 5 days until stain is deep purple in color. Store in refrigerator. This solution has a pH of 1.7. To adjust the pH of the aldehyde fuchsin to 1.0 use concentrated hydrochloric acid.

METANIL YELLOW SOLUTION

(See page 78)

80% ALCOHOL SOLUTION pH 1.0

To adjust the pH of the 80% alcoholic rinse use concentrated hydrochloric acid.

STAINING PROCEDURE

1. Deparaffinize sections and hydrate to distilled water.
2. Rinse in several changes of 80% alcohol (pH 1.0).
3. Aldehyde fuchsin solution (pH 1.0) for 30 minutes.
4. Rinse off excess stain in 80% alcohol solution (pH 1.0).
5. Metanil yellow solution 1 minute or until background is a light yellow.
6. Rinse in distilled water.
7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS. All mucins except the highly acidic sulfated mucosubstances are aldehyde-fuchsin negative at pH values of 1.0 and below.

REFERENCE. Johnson, W. C., Graham, J. H. and Helwig, E. B.: *J. Invest. Derm.* 42:215-224, 1964.

MODIFICATION OF MOWRY'S 1958 COLLOIDAL IRON STAIN FOR ACID MUCOPOLYSACCHARIDES

FIXATION. 10% buffered neutral formalin or 95% alcohol. Bichromate fixatives should be avoided.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

29% FERRIC CHLORIDE SOLUTION

Ferric chloride	29.0 gm
Distilled water	100.0 ml

MÜLLER'S COLLOIDAL IRON OXIDE SOLUTION (STOCK)

Bring 250 ml of distilled water to a boil. While the water is still boiling, pour 4.4 ml of 29% ferric chloride solution (USP XI) and stir. When the solution has turned dark red, remove from heat and allow to cool. *Label: Stock Müller's Colloidal Iron.* This reagent is stable for many months. In the staining procedures, the stock solution of colloidal iron is diluted just before use as follows:

COLLOIDAL IRON SOLUTION (WORKING)

Müller's colloidal iron (stock)	20.0 ml
Distilled water	15.0 ml
Glacial acetic acid	5.0 ml

Mix just before use.

12% ACETIC ACID SOLUTION

Glacial acetic acid	12.0 ml
Distilled water	88.0 ml

5% POTASSIUM FERROCYANIDE SOLUTION

Potassium ferrocyanide	5.0 gm
Distilled water	100.0 ml

5% HYDROCHLORIC ACID SOLUTION

Hydrochloric acid, concentrated	5.0 ml
Distilled water	95.0 ml

POTASSIUM FERROCYANIDE-HYDROCHLORIC ACID SOLUTION

Potassium ferrocyanide, 5%	50.0 ml
Hydrochloric acid, 5%	50.0 ml

Mix just before use.

VAN GIESON SOLUTION

(See page 76)

STAINING PROCEDURE. Use control slide. For digestion procedures, see pages 171, 172.

1. Deparaffinize and hydrate to distilled water.
2. Rinse in glacial acetic acid solution for 3 minutes.
3. Working colloidal iron solution for 1 hour.
4. Rinse in glacial acetic acid solution for four changes, 3 minutes each.
5. Hydrochloric acid-potassium ferrocyanide solution for 20 minutes.
6. Wash in tap water for 5 minutes.
7. Rinse in distilled water.
8. Van Gieson's solution for 5 to 7 minutes.
9. Without washing, pass quickly to 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
10. Mount with Permount or Histoclad.

RESULTS

Acidic mucopolysaccharides and acidic epithelial mucins are colored deep (Prussian) blue.

Background depending on counterstain used.

REFERENCE. Mowry, R. W.: Department of Pathology, University of Alabama Medical Center, Birmingham, Alabama 35233. (AFIP modification).

PAS-ALCIAN BLUE METHOD FOR MUCOSUBSTANCES pH 2.5 or 1.0

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% PERIODIC ACID SOLUTION

(See page 113)

SCHIFF REAGENT SOLUTION

(See page 159)

ALCIAN BLUE SOLUTION pH 2.5

(See page 163)

ALCIAN BLUE SOLUTION pH 1.0

(See page 164)

0.5% SODIUM METABISULFITE SOLUTION

(See page 72)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Alcian blue solution (pH 2.5 or 1.0) for 30 minutes.
3. Blot section dry with filter paper if Alcian blue solution pH 1.0 is used. Wash in water after Alcian blue pH 2.5 for 5 minutes.
4. Oxidize in periodic acid solution for 10 minutes.
5. Wash in running water for 5 minutes.
6. Schiff reagent solution for 10 minutes.
7. Rinse in sodium metabisulfite solution three changes, 2 minutes each.
8. Wash in running water for 10 minutes.
9. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
10. Mount with Permount or Histoclad.

RESULTS. *PAS-Alcian blue pH 2.5.* All polysaccharides and mucosubstances containing hexoses or deoxyhexoses with vicinal glycol groups stain magenta to red. Those mucosubstances staining red include neutral mucosubstances. Hyaluronic acid, sialomucins and all but the most strongly acidic sulfated mucosubstances stain blue.

PAS-Alcian blue pH 1.0. Pas positive same as above. Alcinophilic substances at this pH include only the sulfated mucosaccharides.

REFERENCE: Lev, R., Spicer, S. S.: *J. Histochem. Cytochem.* 12:309, 1964. Copyright by Williams and Wilkins Co.

SAUNDER'S METHOD FOR ACID MUCOPOLYSACCHARIDES

FIXATION. Newcomer's solution for 24 hours (see *Notes* for processing schedule).

TECHNIQUE. Cut paraffin sections at 6 microns (see *Notes* for ribboning technique).

SOLUTIONS

NEWCOMER'S SOLUTION

Alcohol, isopropyl	60.0 ml
Propionic acid	30.0 ml
Acetone	10.0 ml
Dioxane	10.0 ml

1% CETYLTRIMETHYLAMMONIUM CHLORIDE SOLUTION

Cetyltrimethylammonium chloride*	1.0 gm
Distilled water	100.0 ml

RIBONUCLEASE SOLUTION

Ribonuclease	0.1 gm
Distilled water	100.0 ml

Make fresh.

ACRIDINE ORANGE SOLUTION pH 7.2

Acridine orange	0.1 gm
Distilled water	100.0 ml

*K & K Laboratories, Inc., Plainview, N.Y. 11803

ACRIDINE ORANGE SOLUTION pH 3.2

Acridine orange ..... 0.1 gm
Glacial acetic acid, 0.01 *M*
(0.6 ml /1000 ml distilled water) ..... 100.0 ml

DIFFERENTIATING SOLUTION I

Sodium chloride, 0.3 *M* (17.53 gm/1000 ml
distilled water) ..... 50.0 ml
Glacial acetic acid, 0.01 *M* (0.6 ml/1000 ml
distilled water) ..... 50.0 ml

DIFFERENTIATING SOLUTION II

Sodium chloride, 0.6 *M* (35.05 gm/1000 ml
distilled water) ..... 50.0 ml
Glacial acetic acid, 0.01 *M* (0.6 ml/1000 ml
distilled water) ..... 50.0 ml

FLUORMOUNT* (FLUOR-FREE MOUNTING MEDIA)

STAINING PROCEDURE. Run three comparable sections (see step 5).

1. Deparaffinize and hydrate to distilled water.
2. Cetyltrimethylammonium chloride solution for 3 minutes.
3. Wash in tap water for 10 minutes.
4. Ribonuclease solution at 45 °C for 2 hours.
5. *Slide 1* is again treated for 3 minutes with cetyltrimethylammonium chloride, washed 10 minutes in running water, stained 3 minutes in acridine orange pH 7.2 solution, washed in running water for 10 minutes, air dried and mounted with Fluormount. *Slide 2* is stained 5 minutes in acridine orange pH 3.2, washed in running water for 5 minutes, placed in differentiating solution I for 5 minutes, air dried and mounted with Fluormount. *Slide 3* is treated as slide 2 but placed in differentiating solution II then air dried and mounted with Fluormount. *Note:* Slides may be mounted with distilled water and must be read immediately.

RESULTS

Slide 1:	Hyaluronic acid	red fluorescence
Slide 2:	Chondroitin sulfates and heparin	-red fluorescence
Slide 3:	Heparin	-red fluorescence

Notes

PROCESSING SCHEDULE. Place fixed specimen in equal parts of Newcomer's solution and *n*-butyl alcohol for 30 minutes, then place in *n*-butyl alcohol, three changes for 30 minutes each. Transfer specimen to equal parts of *n*-butyl alcohol and paraffin at 60° C for 30 minutes. Follow with three changes of paraffin 45 minutes each then embed.

RIBBONING TECHNIQUE. Ribbons are floated on cellosolve (ethylene glycol monoethyl ether) heated to 35° to 45° C, then picked up on glass slides *without* adhesives. Place slides in 45° C oven for 2 hours.

*Schuler & Co., Ltd., New York, N. Y.

REFERENCE. Saunders, A. M.: *J. Histochem. Cytochem.* 12:164-170, 1964. Copyright by Williams and Wilkins Co.

DIASTASE DIGESTION METHOD

PHOSPHATE BUFFER SOLUTION pH 6.0

Sodium chloride ..... 8.0 gm
Sodium phosphate, dibasic ..... 0.282 gm
Sodium phosphate, monobasic ..... 1.97 gm
Distilled water ..... 100.0 ml

DIASTASE DIGESTION SOLUTION

Diastase of malt ..... 0.1 gm
Phosphate buffer solution, pH 6.0 ..... 100.0 ml
Mix just before use.

DIGESTION PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Place slides in preheated diastase solution at 37 °C for 1 hour.
3. Wash in running water for 5 minutes.
4. Stain with periodic acid Schiff or other appropriate procedure.

RESULTS. Staining attributable to glycogen is selectively eliminated.

HYALURONIDASE DIGESTION METHOD

0.1*M* POTASSIUM PHOSPHATE, MONOBASIC

Potassium phosphate, monobasic ..... 13.61 gm
Distilled water ..... 1000.0 ml

0.1*M* SODIUM PHOSPHATE, DIBASIC

Sodium phosphate, dibasic ..... 14.20 gm
Distilled water ..... 1000.0 ml

BUFFER SOLUTION

Potassium phosphate, monobasic, 0.1 *M* ..... 94.0 ml
Sodium phosphate, dibasic, 0.1 *M* ..... 6.0 ml

HYALURONIDASE DIGESTION SOLUTION

Testicular hyaluronidase* ..... 0.05 gm
Buffer solution ..... 100.0 ml
Mix just before use.

DIGESTION PROCEDURE. Use control slides.

1. Deparaffinize and hydrate duplicate sections to distilled water.
2. Digest slides in hyaluronidase solution at 37 °C for 1 hour. Incubate undigested slides in buffer solution at 37 °C for 1 hour.
3. Wash both sets of slides in running water for 5 minutes.
4. Stain as desired.

*Cal Bio Chemical Co., Los Angeles, Calif.

RESULTS. Staining attributable to hyaluronic acid, chondrotin 4 sulfate or chondrotin 6 sulfate is selectively eliminated.

SIALIDASE DIGESTION METHOD

Use two slides from the same block. One for digestion and one for Buffer Solution #2 treatment.

SOLUTIONS

BUFFER SOLUTION #1

1.0 *M* Sodium Acetate - 0.4 *M* Calcium Chloride

Sodium acetate	26.2 gm
Calcium chloride	8.9 gm
Distilled water	200.0 ml

BUFFER SOLUTION #2

0.1 *M* Sodium Acetate-0.04 *M* Calcium Chloride

Sodium acetate	2.6 gm
Calcium chloride	0.89 gm
Distilled water	200.0 ml

SIALIDASE DIGESTION SOLUTION

Sialidase (neuraminidase)*	1.0 ml
Buffer solution #1	0.1 ml

DIGESTION PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Circle sections to be digested with diamond marking pencil to contain enzyme solution.
3. Air dry slides for 2 hours.
4. Place slides on glass rods close above layer of water in a petri dish. Place sialidase digestion solution on one slide in sufficient quantity to completely cover section. Cover the second slide, to be used as "control", with buffer solution #2. The petri dishes are then incubated for 3 hours at 40 °C.
5. Remove from oven and rinse in distilled water.
6. Stain with any of the mucosaccharide technics, alcian blue or alcian blue-periodic acid Schiff technics preferred.

RESULTS. Staining attributable to the sialomucins will be selectively eliminated.

STAINS FOR MISCELLANEOUS POLYSACCHARIDES

Stains for the miscellaneous polysaccharides are too numerous to list in a text of this kind. Excellent staining information for the substances listed below can be found

*General Biochemical Co., Chagrin Falls, Ohio

in Lillie, R. D.: *Histopathological Technic and Practical Histochemistry*, 3rd ed., New York, McGraw-Hill Book Company, 1965.

Chitin	Inulin
Cellulose	Pectin
Starch	Dextran
Lignin	Corpora amylacea

Chapter 11

Methods For Pigments And Minerals

HALL'S METHOD FOR BILIRUBIN

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

FOUCHET'S REAGENT

Trichloroacetic acid ..... 25.0 gm
Distilled water ..... 100.0 ml
Mix and add:
10% Ferric chloride ..... 10.0 ml

10% FERRIC CHLORIDE SOLUTION

Ferric chloride ..... 10.0 gm
Distilled water ..... 100.0 ml

VAN GIESON'S SOLUTION

(See page 76)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Fouchet's reagent for 5 minutes.
3. Wash in running water, then in distilled water.
4. Van Gieson's solution for 5 minutes.
5. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
6. Mount with Permount or Histoclad.

RESULTS

Biliverdin	-green
Collagen	-red
Muscle	-yellow

REMARKS. Bilirubin is oxidized to biliverdin, and stains olive drab green to emerald green, depending upon the concentration of bilirubin.

REFERENCE. Hall, M. J.: *Amer. J. Clin. Path.* 34: 313-316, 1960.

CARR'S METHOD FOR CALCIUM

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

CHLORANILIC ACID SOLUTION

Distilled water	100.0 ml
Sodium hydroxide	0.4 gm
Chloranilic acid	1.0 gm

Agitate until all the acid is dissolved. Filter through fine filter paper.

1% LIGHT GREEN SOLUTION

Light green, SF yellowish	1.0 gm
Distilled water	100.0 ml

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Chloranilic acid solution for 30 minutes.
3. Wash in running water for 15 minutes.
4. Light green solution for 5 minutes.
5. Rinse in distilled water.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Calcium	-red brown
Background	-green

Brilliantly birefringent under polarized light.

Note. Some crystallization may occur in the chloranilic acid solution, however, this may be removed by filtering without affecting the usefulness of the solution.

REFERENCE. Carr, L. B., Rambo, O. N., and Feichtmeir, T. V.: *J. Histochem. Cytochem.* 9 415-417, 1961. Copyright by Williams and Wilkins Co.

DAHL'S METHOD FOR CALCIUM

FIXATION. 95% Ethyl alcohol or 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% ALIZARIN RED S SOLUTION

Alizarin red S	1.0 gm
Distilled water	100.0 ml

Stir dye into the distilled water so that only a few small grains of dye remain undissolved. *Add 10 ml of 0.1% ammonium hydroxide* slowly with constant stirring. Resulting pH should be 6.36-6.40. Solution is stable for one month.

0.1% AMMONIUM HYDROXIDE SOLUTION

Ammonium hydroxide, C. P. 28% ..... 0.1 ml
Distilled water .....100.0 ml

1% LIGHT GREEN SOLUTION

(See page 161)

STAINING PROCEDURE

1. Deparaffinize and hydrate to 95% alcohol (drain off excess 95% alcohol).
2. Alizarin Red S solution for 2 minutes.
3. Remove excess stain with distilled water, five or six rinses.
4. Counterstain in light green solution for 1 minute.
5. Rinse in distilled water for 5 - 10 seconds.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Calcium salts - intense reddish-orange
Background - pale green

REFERENCE. Dahl, L. K.: *J. Exp. Med.* 95:474-479, 1952. Permission by The Rockefeller University Press.

KOSSA'S METHOD FOR CALCIUM

FIXATION. Absolute alcohol, formol alcohol or 10% buffered neutral formalin. Alcohol fixatives are preferred.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

5% SILVER NITRATE SOLUTION

(See page 91)

5% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page 89)

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE. Use control slide. Use chemically clean glassware.

1. Deparaffinize and hydrate to distilled water.
2. Silver nitrate solution for 60 minutes exposed to direct sunlight, ultra-violet lamp, or a 100-watt lamp light.
3. Rinse in distilled water.
4. Sodium thiosulfate solution for 2 minutes.
5. Rinse well in distilled water.
6. Counterstain in nuclear fast red solution for 5 minutes.

7. Rinse in distilled water.
8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
9. Mount with Permount or Histoclad.

RESULTS

Calcium salts	-black
Nuclei	-red
Cytoplasm	-light pink

REMARKS. Although the use of ultra-violet lamp and a 100-watt bulb for developing the silver is suggested, we strongly recommend direct sunlight be used whenever possible.

REFERENCE. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p. 144.

PIZZOLATO'S METHOD FOR CALCIUM OXALATE

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

SILVER NITRATE-HYDROGEN PEROXIDE SOLUTION

Silver nitrate, 5% aqueous ..... 25.0 ml
Hydrogen peroxide, 30% ..... 25.0 ml
Mix just before use.

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Pour silver nitrate-hydrogen peroxide solution onto slides in sufficient quantities to cover section. Place 60-watt light bulb 6 inches from sections for 30 minutes.
3. Rinse thoroughly with distilled water.
4. Counterstain in Kernechtrot for 5 minutes.
5. Rinse in distilled water.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Calcium oxalate	-black
All other tissue elements	-red

Note. Numerous gas bubbles will develop during the light bulb staining treatment if a large amount of egg albumin section adhesive is present. This tends to inhibit the reaction. Should this happen, however, it will be necessary to pour off the mixture, at this point, and add fresh solution.

REFERENCE. Pizzolato, P.: *J. Histochem. Cytochem.* 12:333-336, 1964.

UZMAN'S METHOD FOR COPPER

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

RUBEANIC ACID (DITHIOOXAMIDE) SOLUTION

Dithiooxamide*	0.1 gm
Alcohol, 70%	100.0 ml

STAINING PROCEDURE

1. Deparaffinize in xylene, two changes.
2. Equal parts of xylene-absolute alcohol, two changes.
3. Absolute alcohol, two changes.
4. Rubeanic acid solution for 20 minutes.
5. Add solid sodium acetate (0.2 gm/100 ml) to the staining jar and allow to settle to the bottom – leave slides for 24 hours.
6. 70% alcohol, two changes of 1-1/2 hours each.
7. Absolute alcohol, 24 hours.
8. Clear in xylene, two changes.
9. Mount with Permount or Histoclad.

RESULTS

Copper - fine granular black precipitate.

REFERENCE. Uzman, L. L.: *Lab. Invest.* 5:299-305, 1956.

TURNBULL BLUE METHOD FOR HEMOSIDERIN

FIXATION. 10% buffered neutral formalin or absolute alcohol.

TECHNIQUE. Cut paraffin sections at 6 microns.

AMMONIUM SULFIDE, YELLOW

Use as Commercially available. (See *Note*).

1% HYDROCHLORIC ACID SOLUTION (STOCK)

(See page 130)

20% POTASSIUM FERRICYANIDE SOLUTION (STOCK)

Potassium ferricyanide	20.0 gm
Distilled water	100.0 ml

HYDROCHLORIC ACID-POTASSIUM FERRICYANIDE SOLUTION (WORKING)

Hydrochloric acid (stock)	50.0 ml
Potassium ferricyanide (stock)	50.0 ml

Mix just before use.

* Eastman Organic Chemicals Distillation Products Industries Rochester, New York

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Ammonium sulfide solution for 4 hours.
3. Rinse thoroughly in distilled water.
4. Hydrochloric acid-potassium ferricyanide solution for 20 minutes.
5. Rinse thoroughly in distilled water.
6. Counterstain in nuclear fast red solution for 5 minutes.
7. Rinse in distilled water.
8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
9. Mount with Permount or Histoclad.

RESULTS

Hemosiderin - blue
Nuclei - red

Note. Sections are frequently lost in ammonium sulfide. The use of one part of ammonium sulfide with 3 parts of 95% alcohol as suggested by F. B. Mallory is a way to avoid this problem.

REFERENCE. Mallory, F. B.: *Pathological Technique*, New York, Hafner Publishing Co., 1961, p. 138.

GOMORI'S METHOD FOR IRON

FIXATION. 10% buffered neutral formalin or absolute alcohol.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

20% HYDROCHLORIC ACID SOLUTION (STOCK)

Hydrochloric acid, concentrated	20.0 ml
Distilled water	80.0 ml

10% POTASSIUM FERROCYANIDE SOLUTION (STOCK)

Potassium ferrocyanide	10.0 gm
Distilled water	100.0 ml

HYDROCHLORIC ACID-POTASSIUM FERROCYANIDE SOLUTION (WORKING)

Hydrochloric acid (stock)	50.0 ml
Potassium ferrocyanide (stock)	50.0 ml

Mix just before use.

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE. Use control slide. Use chemically clean glassware.

1. Deparaffinize and hydrate to distilled water.
2. Hydrochloric acid-potassium ferrocyanide solution for 30 minutes.
3. Rinse thoroughly in distilled water.

4. Counterstain in nuclear fast red solution for 5 minutes.
5. Rinse in distilled water.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Iron pigments - bright blue
Nuclei - red
Cytoplasm - light pink

REFERENCE. Gomori, G.: *Amer. J. Path.* 12:655-663, 1936.

HUKILL-PUTT METHOD FOR IRON

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

BATHOPHENANTHROLINE SOLUTION (STOCK)

Bathophenanthroline*	0.1 gm
Glacial acetic acid, 3% aqueous	100.0 ml

Shake well and place in 60 °C oven overnight.

BATHOPHENANTHROLINE SOLUTION (WORKING)

Bathophenanthroline (stock)	40.0 ml
Thioglycolic acid	0.2 ml

Mix well and filter before use.

0.5% METHYLENE BLUE SOLUTION (AQUEOUS)

STAINING PROCEDURE. Use control slide. Use chemically clean glassware.

1. Deparaffinize and hydrate to distilled water.
2. Working Bathophenanthroline solution for 2 hours.
3. Rinse in distilled water.
4. Counterstain in methylene blue solution for 3 minutes.
5. Rinse in distilled water, three changes of 1 minute each.
6. Blot and dry thoroughly in 60 °C oven.
7. Mount with Permount or Histoclad.

RESULTS

Iron - red
Nuclei - blue

REMARKS. This method is specific for Fe++ (Ferrous iron) if the staining solution is used without thioglycolic acid. It does not permit separate demonstration of Fe+++ (Ferric) in distinction from Fe++ (Ferrous) iron.

*Bathophenanthroline (4, 7-diphenyl-1, 10-penanthroline) may be purchased from: Matheson, Coleman & Bell, P. O. Box 85, East Rutherford, New Jersey 07073

Note. Stock solutions of bathophenanthroline is stable for 4 weeks if kept in the refrigerator.

REFERENCE. Hukill, P., and Putt, A.: *J. Histochem. Cytochem.* 10:490-494, 1962.

JOHNSON'S METHOD FOR IRON (MICROINCINERATION)

FIXATION. 10% buffered formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

5% POTASSIUM FERROCYANIDE SOLUTION (STOCK)

(See page 167)

0.6*N* HYDROCHLORIC ACID SOLUTION (STOCK)

Hydrochloric acid, concentrated	50.0 ml
Distilled water	950.0 ml

HYDROCHLORIC ACID-POTASSIUM FERROCYANIDE SOLUTION (WORKING)

Hydrochloric acid (stock)	50.0 ml
Potassium ferrocyanide	50.0 ml

Mix just before use.

STAINING PROCEDURE. Three* sections are stained in this technique as follows:

a. One section stained with routine H & E.

b. One section stained with the Prussian blue reaction.

c. One section used for microincineration.

1. Microincineration Technique

1. Place uncovered, unstained paraffin section near the center of a sheet of asbestos board.

2. Heat cautiously, using the outer cone from the flame of Fisher or Meker burner. (Flame which is directed down onto the section must be kept in motion to heat slide as uniformly as possible).

3. Continue heating until specimen, which turns brown then white, has almost completely disappeared.

4. Immediately cover slide with an inverted pan (i.e. pie pan) allowing slide to cool gradually. (If no pan is used, cooling is too rapid and the slide may break).

5. When the slide is cool the presence of iron oxide in the ash is demonstrated by direct observation, with or without coverslip. Completely incinerated specimen is examined under strong oblique lighting with dark field illumination.

RESULTS. Iron oxide imparts to the ash a light yellow-orange to dark red color.

Note. Specimen must be completely incinerated before interpreting colored particles as iron oxide, since in dark field illumination, carbon particles can also give a yellowish to red color.

* By comparing these three sections the location of iron deposits and the presence of "non-stainable" iron can be demonstrated.

II. Incinerated Specimen Staining

1. Dip incinerated specimen in a thin celloidin solution (2 gm Parloidion to 50 ml absolute alcohol and 50 ml ethyl ether in tightly stoppered bottle. Take 15 ml of this stock solution and make up to 2000 ml with equal parts absolute alcohol and ethyl ether).
2. Air dry slides for a few minutes, then rapidly hydrate to distilled water.
3. Stain with the Prussian blue reaction for 15 minutes.
4. Rinse in distilled water, several changes.
5. Air dry or dry in 60° C oven.
6. Mount with Permount or Histoclad.

RESULTS

Iron oxide - blue

III. Direct Observation of Prussian blue Reaction on Ash

1. Build a well of paraffin around the specimen.
2. Pour hydrochloric acid-potassium ferrocyanide solution in this well and observe under the microscope.

RESULTS

Iron oxide blue

REFERENCE. Fenton, R. H., Johnson, F. B. and Zimmerman, L. E.: *J. Histochem. Cytochem.* 12:153-155, 1964. Copyright by Williams and Wilkins Co.

LILLIE'S METHOD FOR FERRIC AND FERROUS IRON

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

POTASSIUM FERROCYANIDE SOLUTION

Potassium ferrocyanide ($K_4Fe(cn)_63H_2O$)	0.4 gm
0.06 *N* Hydrochloric acid (1 ml concentrated HCl + 199 ml distilled water)	40.0 ml

POTASSIUM FERRICYANIDE SOLUTION

0.6 *N* Hydrochloric acid (see above)	40.0 ml

Make solutions fresh before use.

BASIC FUCHSIN SOLUTION

Basic fuchsin	0.5 gm
Distilled water	100.0 ml
Glacial acetic acid	1.0 ml

STAINING PROCEDURE. Use control slide. Use chemically clean glassware.

1. Deparaffinize and hydrate to distilled water.
2. For Ferric iron place section in potassium ferrocyanide solution for 1 hour. For Ferrous iron place section in potassium ferricyanide solution for 1 hour.

3. Wash well in 1% aqueous glacial acetic acid.
4. Basic fuchsin solution for 10 minutes.
5. Rinse in distilled water.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Ferric iron -dark prussian blue
Ferrous iron -dark turnbull's blue
Background -light red

Note: For critical work omit counterstain.

REFERENCE. Lillie, R. D.: *Histopathologic Technic and Practical Histochemistry*, 3rd ed. New York, McGraw-Hill Book Co., 1965, p. 407. Permission of McGraw-Hill Book Co.

MALLORY'S METHOD FOR IRON

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

5% HYDROCHLORIC ACID SOLUTION (STOCK)

(See page 167)

5% POTASSIUM FERROCYANIDE SOLUTION (STOCK)

(See page 167)

HYDROCHLORIC ACID-POTASSIUM FERROCYANIDE SOLUTION (WORKING)

Hydrochloric acid..... 50.0 ml
Potassium ferrocyanide ..... 50.0 ml

Mix just before use.

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE. Use control slide. Use chemically clean glassware.

1. Deparaffinize and hydrate to distilled water.
2. Hydrochloric acid-potassium ferrocyanide solution for 10 minutes.
3. Rinse thoroughly in distilled water.
4. Counterstain with nuclear fast red for 5 minutes.
5. Rinse well in distilled water.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Iron pigments -bright blue

Nuclei -red
Cytoplasm -light pink

REFERENCE. Mallory, F. B., and Wright, J. H.: *Pathological Technique,* 8th ed., Philadelphia, W. B. Saunders Co., 1924, p. 207.

PERLS' METHOD FOR IRON

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

10% POTASSIUM FERROCYANIDE SOLUTION (STOCK)

(See page 179)

10% HYDROCHLORIC ACID SOLUTION (STOCK)

Hydrochloric acid, concentrated	10.0 ml
Distilled water	90.0 ml

POTASSIUM FERROCYANIDE-HYDROCHLORIC ACID SOLUTION (WORKING)

Potassium ferrocyanide solution, (stock)	70.0 ml
Hydrochloric acid solution, (stock)	30.0 ml

Mix just before use.

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE. Use control slide. Use chemically clean glassware.

1. Deparaffinize and hydrate to distilled water.
2. Stock potassium ferrocyanide solution for 5 minutes.
3. Working potassium ferrocyanide-hydrochloric acid solution for 20 minutes.
4. Rinse well in distilled water.
5. Counterstain in nuclear fast red solution for 5 minutes.
6. Wash well in running water.
7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Ferric iron -bright blue
Nuclei -red
Cytoplasm -light pink

REMARKS. Additional metals in *large quantities* may be demonstrated with this method. The metals demonstrated and their color reactions are as follows:

Copper -red
Uranium -brown
Nickel -greenish-white

In some instances nickel will stain brown.

REFERENCE. Perls, M.: *Virchow. Arch. Path. Anat.* **39**:42, 1867.

SCHMORL'S METHOD FOR REDUCING SUBSTANCES

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% FERRIC CHLORIDE SOLUTION (STOCK)

Ferric chloride	1.0 gm
Distilled water	100.0 ml

0.1% POTASSIUM FERRICYANIDE SOLUTION (STOCK)

Potassium ferricyanide	0.1 gm
Distilled water	100.0 ml

FERRIC CHLORIDE-POTASSIUM FERRICYANIDE SOLUTION (WORKING)

Ferric chloride (stock)	150.0 ml
Potassium ferricyanide (stock)	50.0 ml

Adjust the pH to 2.4 with a few drops of dilute hydrochloric acid if necessary.

MUCICARMINE SOLUTION

(See page 161)

0.25% METANIL YELLOW SOLUTION

(See page 78)

STAINING PROCEDURE. Use chemically clean glassware.

1. Deparaffinize and hydrate to distilled water.
2. Working ferric chloride-potassium ferricyanide solution for three changes, 7 minutes each.
3. Rinse in distilled water.
4. Mayer's mucicarmine solution for 1 hour.
5. Rinse rapidly in distilled water.
6. Counterstain in metanil yellow solution for a few seconds.
7. Rinse rapidly in distilled water.
8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
9. Mount with Permount or Histoclad.

RESULTS

Argentaffin granules	- blue
Goblet cells, Mucin	- reddish pink
Background	- yellowish green
Reducing substances	- blue

REFERENCE. Barka, T. and Anderson, P.: *Histochemistry Theory, Practice, and Bibliography*, New York, Harper & Row Publishers, Inc., Hoeber Med. Div., 1963, p. 185-186.

AFIP METHOD FOR LIPOFUSCIN

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

KINYOUN'S CARBOL FUCHSIN SOLUTION

(See page 218)

1% ACID ALCOHOL SOLUTION

(See page 38)

PICRIC ACID SOLUTION

Picric acid, approximately	1.18 gm
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Kinyoun's carbol fuchsin solution for 1 hour.
3. Rinse in distilled water.
4. Differentiate in 1% acid alcohol, usually 5-6 dips, or until section is light pink.
5. Wash in running water for 5 minutes, then rinse in distilled water.
6. Counterstain in picric acid solution (see *Note*), five or eight dips or until slide appears yellow.
7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Lipofuscin	-red
Background	-yellow

REMARKS. The periodic acid Schiff and Fontana-Masson methods also stain lipofuscin. These methods should not be regarded as specific since they stain many pigments. Ceroid pigment can also be stained with these methods.

Note. Picric acid is used as a counterstain since it avoids the masking of lipofuscin which may happen in the usual acid fast procedure when methylene blue is used.

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

OIL RED O METHOD FOR LIPOFUSCIN

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

OIL RED O IN PROPYLENE GLYCOL SOLUTION

(See page 141)

85% PROPYLENE GLYCOL SOLUTION

(See page 141)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. ORO in propylene glycol solution for 72 hours.
3. 85% propylene glycol solution for 1 minute. Agitate slide.
4. Distilled water, two changes for 2 minutes each.
5. Mayer's hematoxylin solution for 5 minutes.
6. Wash in running water for 10 minutes.
7. Mount with glycerin jelly.

RESULTS

Lipofuscin	- red
Nuclei	- blue

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

DE GALANTHA'S METHOD FOR URATE CRYSTALS

FIXATION. Absolute alcohol*.

TECHNIQUE. Cut paraffin sections at 8 to 10 microns. From absolute alcohol transfer blocks to xylene-paraffin, equal parts, at 58°C for 2 hours. Paraffin for 1 hour. Embed.

SOLUTIONS

20% SILVER NITRATE SOLUTION

Silver nitrate	20.0 gm
Distilled water	100.0 ml

DEVELOPING SOLUTION

Gelatin, 3% in hot water	10.0 ml
Silver nitrate solution, 20%	3.0 ml
Hydroquinone solution, 2%	2.0 ml

Prepare just before use.

STAINING PROCEDURE

1. Xylene, absolute alcohol, two changes each.
2. Silver nitrate solution, expose to strong sunlight until urates are a bright rose, approximately 3 hours.

* Two changes of absolute alcohol, 4 hours each for thin specimens, longer for thicker specimens. (See *Note*).

3. Prepare developing solution and pour this over slides until the urates turn black and the connective tissues are yellow.

4. Wash quickly in hot water (58° C).

5. Dehydrate in absolute alcohol, and clear in xylene, two changes each.

6. Mount with Permount or Histoclad.

RESULTS

Urates	- black
Background	- yellow

Note. If specimen contains bone, decalcify in absolute alcohol to which 2% nitric acid has been added. Change solution every 24 hours until bone is softened. Wash in absolute alcohol for 24 hours.

REFERENCE. De Galantha, E.: *Amer. J. Clin. Path.* 5:165-166, 1935.

Chapter 12

Methods For Nerve Cells And Fibers

RAMON Y CAJAL'S METHOD FOR ASTROCYTES

FIXATION. Formalin ammonium bromide for 1 day at 37 °C or 10% buffered neutral formalin (see *Note*).

TECHNIQUE. Cut frozen sections at 15 to 30 microns.

SOLUTIONS

FORMALIN AMMONIUM BROMIDE SOLUTION

Formalin, 37 – 40% (Merck's blue label)	15.0 ml
Ammonium bromide	2.0 gm
Double distilled water	85.0 ml

BROWN GOLD CHLORIDE SOLUTION (STOCK)

Brown gold chloride solution (stock)	1.0 gm
Mercuric chloride solution (stock)	100.0 ml

Solution will keep in a dark bottle for many months.

MERCURIC CHLORIDE SOLUTION (STOCK)

Mercuric chloride	0.5 gm
Double distilled water	10.0 ml

Make just before use. Dissolve bichloride of mercury by *gentle* heat.

GOLD SUBLIMATE SOLUTION (WORKING)

Brown gold chloride solution (stock)	10.0 ml
Mercuric chloride solution (stock)	10.0 ml

Mix while mercuric chloride is still warm and add 40 ml double distilled water. A precipitate will form if mercuric chloride solution was not warm enough when mixed. In this case, begin again with the preparation of gold sublimate solution.

5% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page 89)

STAINING PROCEDURE. Use acid clean glassware. Carry frozen sections on glass rod.

1. Rinse in distilled water, two changes.
2. Place sections flat in freshly prepared gold chloride-sublimate solution for 4 to 6 hours in the dark at room temperature.
3. Rinse in distilled water 5 minutes.
4. Sodium thiosulfate solution for 5 minutes.

5. Rinse well in several changes of distilled water.
6. Float onto slide, and air dry.
7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Astrocytes and their processes	- black
Background	- unstained or light brownish purple
Nerve cells	- pale red
Nerve fibers	- unstained

Note. Formalin fixed tissues may be used provided frozen sections are washed well and placed in formalin ammonium bromide solution for 48 hours prior to staining.

Use mercuric bichloride crystals and *brown* gold chloride only.

REFERENCE. Ramon Y Cajal, S.: *Schweiz Arch. Neurol. Psychiat.* 13:187-193, 1923.

GUILLERY'S METHOD FOR DEGENERATING NERVE FIBERS

FIXATION. 10% formol saline by perfusion (see *Notes* for processing schedule).
TECHNIQUE. Cut paraffin sections at 15 microns.

SOLUTIONS

ALCOHOL-AMMONIUM HYDROXIDE SOLUTION

Ammonium hydroxide, 28%	1.0 ml
Alcohol, 50%	99.0 ml

1.5% SILVER NITRATE SOLUTION

(See page 101)

SILVER NITRATE-WHITE PYRIDINE SOLUTION

Pyridine*	5.0 ml
Silver nitrate solution, 1.5%	95.0 ml

4.5% SILVER NITRATE SOLUTION (STOCK)

Silver nitrate	4.5 gm
Distilled water	100.0 ml

2.5% SODIUM HYDROXIDE (STOCK)

Sodium hydroxide	2.5 gm
Distilled water	100.0 ml

*The pyridine can be replaced by higher methylated derivatives of pyridine, or the concentration of these substances can be increased from 5% up to 20% if necessary to improve differentiation. 10% 2, 4, 6-trimethy pyridine (collidine) has been found particularly useful.

AMMONIACAL SILVER SOLUTION (WORKING)

Silver nitrate solution (stock)	20.0 ml
Alcohol, 100%	10.0 ml
Ammonium hydroxide 28%	1.8 ml
Sodium hydroxide (stock)	1.5 ml

This solution should be placed into a warmed dish on a hot plate and covered.

REDUCING SOLUTION

Equal parts of 1% citric acid and 10% formalin, not neutralized	35.0 ml
Alcohol, 10%	450.0 ml

1% SODIUM THIOSULPHATE (HYPO) SOLUTION

(See page 102)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Alcohol-ammonium hydroxide solution for 6 hours.
3. Rinse in distilled water, three changes.
4. Silver nitrate-white pyridine solution for 24 hours in the dark. Do not wash.
5. Preheat the working ammoniacal silver solution to 40 — 45 °C. Place three slides in this solution for 3 minutes. Solution should be changed frequently.
6. Do not wash. Reducing solution for 1 minute.
7. Rinse in distilled water.
8. Sodium thiosulphate solution for 2 to 5 minutes.
9. Wash in water.
10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Degenerating fibers - dark black
Normal fibers - light black

Notes. After fixation add an excess of lithium carbonate to 40% formaldehyde solution. (This is filtered before use and taken as 100%). Store the blocks for 6 weeks to 6 months. Blocks up to 4 mm across can be used. Wash 8 hours in several changes of distilled water. Dehydrate the blocks, clear in cedarwood oil (2-3 days) and transfer to equal parts of cedarwood oil, benzene and paraffin wax (melting point 45 °C) at 37 °C for 2-3 hours. Embed in 54 °C paraffin.

REFERENCE. Guillery, R.W., Shira, B., and Webster, K. E.: *Stain Techn.* 36:9-13, 1961.

NAUTA-GYGAX METHOD FOR DEGENERATING AXON TERMINALS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut frozen sections at 15-25 microns. Collect in 10% formalin.

SOLUTIONS

15% ALCOHOL SOLUTION

Alcohol, 100%	15.0 ml
Distilled water	85.0 ml

0.05% POTASSIUM PERMANGANATE SOLUTION

Potassium permanganate	0.05 gm
Distilled water	100.0 ml

0.5% PHOSPHOMOLYBDIC ACID SOLUTION

Phosphomolybdic acid	0.5 gm
Distilled water	100.0 ml

HYDROQUINONE-OXALIC ACID SOLUTION

Hydroquinone, 1% aqueous	25.0 ml
Oxalic acid, 1% aqueous	25.0 ml

1.5% SILVER NITRATE SOLUTION

(See page 101)

AMMONIACAL SILVER NITRATE SOLUTION

Dissolve 0.9 gm silver nitrate in 20 ml of distilled water. Add 10 ml absolute alcohol, 1.8 ml ammonium hydroxide, and 1.5 ml of 2.5% sodium hydroxide. Mix well. Prepare fresh, just before use.

REDUCING SOLUTION

Distilled water	400.0 ml
Alcohol, 100%	45.0 ml
Formalin, 10% (non-neutralized)	13.5 ml
Citric acid, 1% aqueous	13.5 ml

1% SODIUM THIOSULFATE SOLUTION

(See page 102)

STAINING PROCEDURE. Carry sections through on a glass rod.

1. 15% alcohol solution for 15 minutes.
2. Rinse in distilled water.
3. Phosphomolybdic acid solution for 1 hour.
4. Without washing place sections in 0.05% potassium permanganate solution for 4-10 minutes (*See Note*).
5. Rinse in distilled water and hold sections at this point.
6. Decolorize in hydroquinone-oxalic acid solution for 2 minutes.
7. Rinse well in distilled water, 3 or 4 changes.
8. 1.5% silver nitrate solution for 30 minutes.

From this point on, transfer sections individually.

9. Rinse in distilled water.
10. Ammoniacal silver nitrate solution for 1 minute.
11. Reducing solution. Sections will float out evenly on the surface of this solution and a brown color will appear within 1 minute.
12. Rinse in distilled water.
13. Sodium thiosulfate solution for 1 minute.
14. Rinse in distilled water, three changes.
15. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, three changes each.
16. Mount with Permount or Histoclad.

RESULTS. Degenerating axons stain with silver as fragmented and swollen fibers.

REMARKS. Guillery et al modified this method for use with paraffin embedded sections, see page 190.

Note. This step is highly critical: too long treatment will inhibit subsequent silver impregnation of all fibers, too short treatment will cause impregnation of too many normal fibers. The optimum duration varies from specimen to specimen and can best be established in a trial run, treating three sections for 5, 7, and 9 minutes respectively. During treatment sections must be turned over from time to time in order to insure an even effect.

REFERENCE. Nauta, W. J. H., and Gygax, P. A.: *Stain Techn.* **29**:91-93, 1954.

BIELSCHOWSKY'S METHOD FOR AXIS CYLINDERS AND DENDRITES

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

Celloidinize sections on slides
Dewax sections & rinse in Abs.
Dip in equal pts Alc/Ether in which 1% Celloidin has been dissolved ~ 5 mins. Drain & wipe back of slide. Harden in 70% - 5 mins. → Stain

SOLUTIONS

2% SILVER NITRATE SOLUTION

Silver nitrate	2.0 gm
Distilled water	100.0 ml

10% SILVER NITRATE SOLUTION

Silver nitrate	10.0 gm
Distilled water	100.0 ml

40% SODIUM HYDROXIDE SOLUTION

Sodium hydroxide	40.0 gm
Distilled water	100.0 ml

AMMONIACAL SILVER SOLUTION

Silver nitrate, 10%	5.0 ml
Sodium hydroxide, 40%	5 drops

Dissolve the resulting precipitate by adding concentrated ammonium hydroxide drop by drop until the precipitate is just dissolved. Filter and dilute to 20 ml with distilled water. Make fresh.

20% FORMALIN SOLUTION

Formalin, 37 - 40%	20.0 ml
Tap water	80.0 ml

GOLD CHLORIDE SOLUTION

Gold chloride, 1% aqueous	3 drops
Distilled water	10.0 ml

5% SODIUM THIOSULFATE SOLUTION

(See page 89)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. 2% silver nitrate solution for 48 hours, in the dark.
3. Rinse quickly in double distilled water.
4. Ammoniacal silver solution 10 - 20 minutes or until sections turn a deep brown.
5. Rinse in distilled water.
6. Reduce in 20% formalin solution for 5 minutes. Sections appear a dark brownish black.
7. Rinse thoroughly in distilled water.
8. Tone in gold chloride solution for 1 hour. Sections will be a reddish violet color.
9. Rinse thoroughly in distilled water.
10. Sodium thiosulfate solution for 1 minute.
11. Wash in tap water.
12. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
13. Mount with Permount or Histoclad.

RESULTS

Intracellular neurofibrils	- black
Axis cylinders	- black
Dendrites	- black
Background	- purplish

REMARKS. This is an excellent stain for axis cylinders and dendrites as well as intracellular neurofibrilla network. The nuclei of cells other than neurons are impregnated to variable degrees, a circumstance that calls for caution in the interpretation of the appearance that may be found in tumors and other pathological conditions. In cases of marked gliosis impregnation of the glial fibers may occur.

REFERENCE. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p. 158 - 160.

BODIAN'S METHOD FOR NERVE FIBERS AND NERVE ENDINGS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% PROTARGOL SOLUTION

Protargol	1.0 gm
Distilled water	100.0 ml

Sprinkle the protargol on the surface of the water and allow it to remain undisturbed until it dissolves.

REDUCING SOLUTION

Hydroquinone	1.0 gm
Formalin, 37 – 40%	5.0 ml
Distilled water	100.0 ml

1% GOLD CHLORIDE SOLUTION

(See page 90)

2% OXALIC ACID SOLUTION

Oxalic acid	2.0 gm
Distilled water	100.0 ml

5% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page 89)

ANILINE BLUE SOLUTION

Aniline blue	0.1 gm
Oxalic acid	2.0 gm
Phosphomolybdic acid	2.0 gm
Distilled water	300.0 ml

LISSAMINE FAST RED SOLUTION

Lissamine fast red	1.0 gm
Glacial acetic acid	1.0 ml
Distilled water	100.0 ml

TARTRAZINE SOLUTION

Tartrazine	1.5 gm
Glacial acetic acid	1.5 ml
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Place slides in protargol solution and add 4 to 6 gm of clean copper shot per 100 ml of solution. Let stand at 37 °C for 48 hours.
3. Rinse in distilled water, three changes.
4. Reducing solution for 10 minutes.
5. Rinse in distilled water, three changes.
6. Tone in gold chloride solution for 10 minutes.
7. Rinse in distilled water, three changes.
8. Develop in oxalic acid solution under the microscope until background is gray and nerve fibers appear clearly, approximately 3-5 minutes.
9. Rinse in distilled water, three changes.
10. Sodium thiosulfate for 5 minutes.
11. Rinse in distilled water.
12. Counterstain, if desired, with aniline blue solution (two or three quick dips, to give a light blue background) or lissamine fast red solution (see Technique for Counterstaining with Lissamine Fast Red Solution below).
13. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
14. Mount with Permount or Histoclad.

RESULTS. (If aniline blue solution is used)

Nerve fibers	-black
Background	-blue
Nuclei	-black

TECHNIQUE FOR COUNTERSTAINING WITH LISSAMINE FAST RED SOLUTION

1. Counterstain in lissamine fast red solution for 5 minutes.
2. Phosphomolybdic acid solution, 1% aqueous, for 5 minutes.
3. Rinse briefly in distilled water.
4. Tartrazine solution for 5 minutes.
5. Rinse briefly in 95% alcohol.
6. Dehydrate rapidly in absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS. (If lissamine fast red solution is used)

Nerve fibers	-black
Myelin	-red
Nuclei	-black
Muscle	-red
Erythrocytes	-red
Background	-yellow

Note. Copper wire may be used in place of copper shot, both of which may be reused if cleaned with three alternating changes of nitric acid and distilled water.

REFERENCE. Luna, L. G.: *Amer. J. Med. Techn.* 30:355-362, 1964. (AFIP Modification)

HOLMES METHOD FOR NERVE CELLS AND FIBERS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

20% SILVER NITRATE SOLUTION

(See page 187)

BORIC ACID BUFFER SOLUTION

Boric acid	12.4 gm
Distilled water	1000.0 ml

BORAX BUFFER SOLUTION

Sodium borate, tetra (Borax)	19.0 gm
Distilled water	1000.0 ml

1% SILVER NITRATE SOLUTION

Silver nitrate	1.0 gm
Distilled water	100.0 ml

10% PYRIDINE SOLUTION

Pyridine	10.0 ml
Distilled water	90.0 ml

IMPREGNATING SOLUTION

Boric acid buffer solution	55.0 ml
Borax buffer solution	45.0 ml
Distilled water	394.0 ml
Silver nitrate, 1%	1.0 ml
Pyridine, 10%	5.0 ml

REDUCING SOLUTION

Hydroquinone	1.0 gm
Sodium sulphite, crystals	10.0 gm
Distilled water	100.0 ml

This solution may be used repeatedly but will not keep for more than a few days.

0.2% GOLD CHLORIDE SOLUTION

Gold chloride, 1% aqueous	2.0 ml
Distilled water	100.0 ml

2% OXALIC ACID SOLUTION

(See page 195)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Silver nitrate solution for 2 hours.
3. Rinse in three changes of distilled water for 10 minutes.
4. Impregnation solution at 37 °C in a covered vessel overnight.

5. Remove slides, drain excess solution and place in reducing solution for at least 2 minutes.

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

7. Tone in gold chloride solution for 3 minutes.

8. Rinse briefly in distilled water.

9. Oxalic acid solution for 3-5 minutes. Examine microscopically until axons are thoroughly blue-black.

10. Rinse in distilled water.

11. Sodium thiosulfate solution for 5 minutes then wash in running water for 5 minutes.

12. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

13. Mount with Permount or Histoclad.

RESULTS

Axis cylinders	- blue to black
Nerves and nerve endings	- black
Background	- gray to rose

REFERENCE. Holmes, W.: *Anat. Rec.* 86:157-187, 1943.

HIRANO-ZIMMERMAN METHOD FOR NERVE CELLS AND FIBERS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut parffin sections at 12-15 microns.

SOLUTIONS

10% SILVER NITRATE SOLUTION

Silver nitrate	10.0 gm
Distilled water	100.0 ml

AMMONIA WATER SOLUTION

Ammonium hydroxide, 28%	2 drops
Distilled water	100.0 ml

FORMALIN-TAP WATER SOLUTION

Formalin, 37 – 40%	50.0 ml
Tap water	50.0 ml

GOLD CHLORIDE SOLUTION

Gold chloride, 1% aqueous	10.0 ml
Distilled water	200.0 ml

5% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page 89)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Silver nitrate solution at room temperature, for 2 hours or longer.

3. Place sections directly into ammonia water solution for 3 minutes.
4. Place sections directly in formalin-tap water solution for 3 minutes. Tissue becomes black-brown.
5. Rinse in distilled water, two changes.
6. Place sections again into silver nitrate solution for 3 to 5 minutes.
7. Repeat steps 3 through 5. Tissue becomes darker in color.
8. Tone in gold chloride solution for 20 to 30 minutes.
9. Place sections directly into sodium thiosulfate solution for 1 minute.
10. Rinse in distilled water.
11. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
12. Mount with Permount or Histoclad.

RESULTS

Nucleolus and nuclear membrane	-black
Neurofibrils, dendrites and axis cylinders	-black
Cytoplasm of astrocytes and the cytoplasmic membranes of macrophages	-gray
Senile plaques	-black

Various lipid granules are unstained, but each granule is clearly outlined.

REFERENCE. Hirano, A., and Zimmerman, H. M.: *Arch. Neurol.* 6:114-122, 1962.

PTAH METHOD FOR CENTRAL NERVOUS SYSTEM (C.N.S) TISSUE

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% POTASSIUM PERMANGANATE SOLUTION

(See page 102)

5% OXALIC ACID SOLUTION

(See page 79)

PHOSPHOTUNGSTIC ACID HEMATOXYLIN (PTAH) SOLUTION

Hematoxylin	1.0 gm
Phosphotungstic acid	20.0 gm
Distilled water	1000.0 ml

Dissolve the solid ingredients in separate portions of the water, the hematoxylin with the aid of gentle heat. When cool, combine. No preservative is necessary. Ripening requires several weeks but the addition of 0.2 gm of potassium permanganate will cause the stain to ripen at once.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant sections in Zenker's fluid containing glacial acetic acid (see page 4) overnight or at 60 °C for 1 hour.

3. Wash in running water for 15 minutes.
4. Lugol's iodine solution (see page 42) for 15 minutes.
5. Decolorize in 95% alcohol for one hour or longer. *DO NOT USE HYPO**.
6. Distilled water for 5 minutes.
7. Oxidize in potassium permanganate solution for 5 minutes.
8. Decolorize in oxalic acid solution for 5 minutes.
9. Rinse well in distilled water followed by running water for 5-10 minutes.
10. PTAH solution for 24 hours.
11. 95% alcohol for one quick dip.
12. Dehydrate in absolute alcohol, two quick changes.
13. Clear in xylene, two changes.
14. Mount with Permount or Histoclad.

RESULTS

Nuclei, fibrin, fibroglia, microglia - blue
Coarse elastic fibrils - purplish

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

HOLZER'S METHOD FOR GLIAL FIBERS

FIXATION. 10% buffered neutral formalin or formol alcohol.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

PHOSPHOMOLYBDIC-ALCOHOL SOLUTION

Phosphomolybdic acid, 0.5%	50.0 ml
Alcohol, 95%	100.0 ml

Make fresh.

ABSOLUTE ALCOHOL-CHLOROFORM SOLUTION

Alcohol, 100%	40.0 ml
Chloroform	160.0 ml

CRYSTAL VIOLET SOLUTION

Crystal violet	5.0 gm
Alcohol, 100%	20.0 ml
Chloroform	80.0 ml

POTASSIUM BROMIDE SOLUTION

Potassium bromide	100.0 gm
Distilled water	1000.0 ml

*Sodium thiosulfate should not be used to decolorize sections after treatment with iodine because it is likely to impair subsequent staining.

DIFFERENTIATING SOLUTION

Aniline	120.0 ml
Chloroform	180.0 ml
Ammonium hydroxide, 28%	20 drops

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water. Carry slides through one at a time.
2. Phosphomolybdic-alcohol solution for 3 minutes. Drain.
3. Absolute alcohol-chloroform solution until section becomes translucent.
4. Place slide on staining rack and flood section with crystal violet solution for 30 seconds. Blot dry.
5. Flood section with potassium bromide solution for one minute. Blot dry.
6. Differentiating solution for 30 seconds. If overdifferentiated, restain.
7. Rinse in xylene, several changes. Check with microscope after first xylene.
8. Mount with Permount or Histoclad.

RESULTS

Glial fibers - deep violet
Background - pale violet

Note. If a crystal violet precipitate forms on slide, it may be removed with straigh aniline.

REFERENCE. Holzer, W.: Über eine neve Methode der Gliofaser-Farbung., *Z. ges Neurol. Psychiat.* 69:354, 1921.

PENFIELD'S METHOD FOR OLIGODENDROGLIA AND MICROGLIA

FIXATION. Formalin-ammonium bromide or 10% buffered neutral formalin.

TECHNIQUE. Cut frozen sections at 20 microns. (Place sections in a 1% formalin solution until ready for staining).

SOLUTIONS

5% HYDROBROMIC ACID SOLUTION

Hydrobromic acid	5.0 ml
Distilled water	100.0 ml

5% SODIUM CARBONATE SOLUTION

Sodium carbonate	5.0 gm
Distilled water	100.0 ml

HORTEGA'S SILVER SOLUTION

Silver nitrate, 10% aqueous	5.0 ml
Sodium carbonate, 5% aqueous	20.0 ml

Add 28% ammonium hydroxide, drop by drop, in sufficient quantities to dissolve the precipitate which forms. Shake vigorously during this step.

Distilled water to make	75.0 ml

1% FORMALIN SOLUTION

(See page 90)

GOLD CHLORIDE SOLUTION

Gold chloride	1.0 gm
Distilled water	500.0 ml

5% SODIUM THIOSULFATE

(See page 89)

HORTEGA'S CARBOL-XYLENE-CREOSOTE MIXTURE

Creosote	10.0 ml
Phenol crystals, melted	10.0 ml
Xylene	80.0 ml

STAINING PROCEDURE

1. Place sections in the following solution overnight: (100 ml distilled water, 15 drops 28% ammonium hydroxide).
2. Hydrobromic acid solution at 37 °C for 1 hour.
3. Wash in distilled water, three changes, for 30 seconds each.
4. 5% sodium carbonate solution for 1 hour.
5. Hortega's silver solution 3-5 minutes (see *Note*).
6. 1% formalin solution until section turns a uniform gray color.
7. Rinse in distilled water.
8. Gold chloride solution until section turns a bluish-gray.
9. 5% sodium thiosulfate for 30 seconds.
10. Wash thoroughly in several changes of distilled water.
11. Float section on slides, make certain sections are flat.
12. Dehydrate with several changes of fresh 95% alcohol from a dropper bottle.
13. Clear in Hortega's carbol-xylene-creosote mixture, drain and blot gently.
14. Mount with Permount or Histoclad.

RESULTS

Oligodendroglial processes	-black
Microglia	-black
Background	-yellowish-brown

REMARKS. Faint staining of astrocytes may occur, especially if sections are left too long in the silver solution.

Note. Leave in stain 3-5 minutes, or until sections turn a uniform gray color when transferred to the reducer. Control staining by taking out a section, from the silver-carbonate solution, at intervals of 1-2 minutes and examining with the microscope.

REFERENCE. Penfield, W.: *Amer. J. Path.* 4:153-157, 1928.

CASPER-WOLMAN FLUORESCENT METHOD FOR MYELIN FIGURES

FIXATION. None, fresh tissue must be used.

TECHNIQUE. Cut frozen sections at 4-6 microns.

SOLUTIONS

BOHMER'S HEMATOXYLIN SOLUTION

(See page 34)

1% THIOFLAVIN TFS SOLUTION

Thioflavin, TFS* ..... 1.0 gm
Distilled water ..... 100.0 ml

1% GLACIAL ACETIC ACID SOLUTION

(See page 94)

APATHY'S MOUNTING MEDIA

(See page 156)

STAINING PROCEDURE. Mount frozen sections on glass slide and allow sections to dry for 2 hours.

1. Bohmer's hematoxylin solution for 2 minutes.
2. Wash thoroughly in running water.
3. Thioflavin TFS solution for 3 minutes.
4. Rinse in distilled water.
5. Differentiate in glacial acetic acid solution for 6 minutes.
6. Wash in tap water, then rinse in distilled water.
7. Mount with Apathy's mounting media.

Examine sections with fluorescent microscope.

RESULTS

Myelin figures - bluish white fluorescence

REFERENCE. Casper, J., and Wolman, M.: *Lab. Invest.* 13:27-31, 1964. Copyright by International Academy of Pathologists.

KLÜVER-BARRERA METHOD FOR MYELIN AND NERVE CELLS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 15 microns.

SOLUTIONS

0.1% LUXOL FAST BLUE SOLUTION

Luxol fast blue, MBS† ..... 0.1 gm
Alcohol, 95% ..... 100.0 ml

Dissolve dye in alcohol. Add 0.5 ml of 10% glacial acetic acid to each 100 ml. Solution is stable.

*Scheuler & Co., Ltd., New York, N. Y.
†E. I. Du Pont de Nemours and Co., Wilmington, Del.

0.1% CRESYL ECHT VIOLET SOLUTION

Cresyl echt violet ..... 0.1 gm
Distilled water ..... 100.0 ml

Just before using, add 15 drops of 10% glacial acetic acid. Filter.

0.05% LITHIUM CARBONATE SOLUTION

Lithium carbonate ..... 0.05 gm
Distilled water ..... 100.0 ml

70% ALCOHOL SOLUTION

Alcohol, 100% ..... 70.0 ml
Distilled water ..... 30.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to 95% alcohol.
2. Luxol fast blue solution at 56°-60° C overnight.
3. Rinse in 95% alcohol to remove excess stain.
4. Rinse in distilled water.
5. Begin differentiation by quick immersion in lithium carbonate solution.
6. Continue differentiation in 70% alcohol solution until gray and white matter can be distinguished.
7. Wash in distilled water.
8. Finish differentiation by rinsing briefly in lithium carbonate solution and then putting through several changes of 70% alcohol solution until the greenish blue of the white matter contrasts sharply with the colorless gray matter.
9. Rinse thoroughly in distilled water.
10. Cresyl echt violet solution for 6 minutes. Filter and preheat cresyl echt violet solution to 57°C just before use.
11. Differentiate in several changes of 95% alcohol.
12. Dehydrate in absolute alcohol, and clear in xylene, two changes each.
13. Mount with Permount or Histoclad.

RESULTS

Myelin - blue
Cell products - pink to violet

REFERENCE. Klüver, H., and Barrera, E.: *J. Neuropath. Exp. Neurol.* 12:400-403, 1953.

(Luxol fast blue has been successfully combined with other stains; for these combinations see: Margolis, G., and Pickett, J. P.: *Lab. Invest.* 5:459-473, 1956).

LAPHAM'S METHOD FOR MYELIN AND GLIAL FIBERS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 15 microns.

SOLUTIONS

GALLOCYANIN SOLUTION

Chromium potassium sulfate (chrom alum) ..... 10.0 gm
Distilled water .....200.0 ml
Gallocyanin ..... 0.3 gm

Heat slowly to boiling, boil for 12-25 minutes with frequent stirring. Cool slowly to room temperature. Filter before using.

0.5% PHLOXINE B AQUEOUS SOLUTION

(See page 106)

5% PHOSPHOTUNGSTIC ACID SOLUTION

(See page 86)

1% GLACIAL ACETIC ACID SOLUTION

(See page 94)

0.05% FAST GREEN FCF SOLUTION

Fast green, FCF ..... 0.05 gm
Distilled water ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Gallocyanin solution overnight.
3. Wash in running water for 5 minutes.
4. Phloxine B solution for 5 minutes.
5. Wash in running water for 3 minutes.
6. Phosphotungstic acid solution for 1 minute.
7. Wash in running water for 3 minutes.
8. Glacial acetic acid solution for 2 minutes.
9. Differentiate in 80% alcohol for 2 minutes.
10. Glacial acetic acid solution for 1 minute.
11. Fast green FCF solution for 1 minute.
12. Glacial acetic acid solution for 1 minute.
13. Dehydrate in 80% alcohol, 95% alcohol, and absolute alcohol, and clear in xylene, two changes each.
14. Mount with Permount or Histoclad.

RESULTS

Myelin	- magenta
Glial fibers, cytoplasm of astrocytes	- bright-green
Nuclei	- blue-black to black
Collagen	- blue-green
Erythrocytes	- orange to red

Note. The phloxine is differentiated at step 9. All tissue structures are decolorized at this time except myelin and erythrocytes. Over-differentiation does not occur within limits.

REFERENCE. Lapham, L. W., Johnstone, M. A., and Brundjar, K. H.: *J. Neuropath. Exp. Neurol.* **23**:156-160, 1964.

WOELCKE'S METHOD FOR MYELIN SHEATH

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 15 microns.

SOLUTIONS

2.5% FERRIC AMMONIUM SULFATE SOLUTION

(See page 140)

10% ALCOHOLIC HEMATOXYLIN SOLUTION (STOCK)

Hematoxylin	10.0 gm
Alcohol, 100%	100.0 ml

Solution should be ripened for 6 months.

SATURATED LITHIUM CARBONATE SOLUTION

(See page 38)

HEMATOXYLIN SOLUTION (WORKING)

Mix the following ingredients in order listed just before use:

Distilled water	45.0 ml
Hematoxylin, stock (filter)	10.0 ml
Lithium carbonate (filter)	7.0 ml
Distilled water	45.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant in ferric ammonium sulfate solution overnight.
3. Rinse in distilled water, two changes.
4. Working hematoxylin solution for 2 hours.
5. Rinse in distilled water, two changes.
6. Differentiate in 80% alcohol until background is clear and myelin stands out sharply.
7. Dehydrate with 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Myelin sheath	- blue
Background	- clear
Glial cells	- black
Nucleoli of neurons	- black

REFERENCE. Personal communication with A. Pentschev, M.D.: Armed Forces Institute of Pathology, Washington, D.C. 20305.

BOSCH'S METHOD FOR NEGRI BODIES

FIXATION. Bouin's (see *Note*) or 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

PHENOL-PHLOXINE B SOLUTION

Phloxine B	1.5 gm
Distilled water	5.0 ml

Mix and add, with vigorous shaking:

Phenol, 5%, to make	100.0 ml

Add, drop by drop, with vigorous shaking, 20 – 30% glacial acetic acid until solution becomes cloudy. Place in a closed vessel and heat in a 50 - 56 °C oven for 12 - 24 hours. Use the supernatant as the staining solution.

0.2% LITHIUM CARBONATE SOLUTION

Lithium carbonate	0.2 gm
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant in Bouin's solution for 1 hour at 60 °C if formalin fixed.
3. Wash in running water for 10 minutes, then rinse in distilled water.
4. Phloxine B solution for 5 minutes.
5. Rinse in distilled water for 1 minute.
6. Mayer's hematoxylin solution for 5 minutes.
7. Wash in running water for several seconds.
8. Differentiate and blue in lithium carbonate solution for several seconds until a pale pink cloud comes from the sections and the bluish tone of hematoxylin appears.
9. Wash in water for 5 minutes.

10. Dehydrate in absolute alcohol and clear in xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Negri bodies	-deep red
Nuclei, nucleoli and nissl granules	-deep blue
Background	-light pink
Erythrocytes	-bright red

Note: The best results have been obtained after fixation in *Bouin's* fluid.

REFERENCE. Bosch, R.: *Stain Techn.* **41**:250-251, 1966. Copyright by Williams and Wilkins Co.

MASSIGNANI-MALFERRARI METHOD FOR NEGRI BODIES

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 4 microns.

SOLUTIONS

HARRIS' HEMATOXYLIN SOLUTION (WITHOUT ACETIC ACID)

(See page 34)

0.5% HYDROCHLORIC ACID SOLUTION

(See page 152)

LITHIUM CARBONATE SOLUTION

Lithium carbonate, saturated aqueous	1.0 ml
Distilled water	200.0 ml

EOSIN-PHOSPHOTUNGSTIC ACID SOLUTION

1 gm of eosin and 0.7 gm of phosphotungstic acid are ground together. Add 10 ml of distilled water and make up to 200 ml with absolute alcohol. Add 2 drops of saturated lithium carbonate and stir for 10 minutes. This suspension is filtered. For the best results, it is necessary that the eosin and phosphotungstic acid are ground together in the solid state.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Harris' hematoxylin, for 2 minutes.
3. Wash for 5 minutes in running water.
4. Dip eight times in hydrochloric acid solution and wash 5 minutes in running water.
5. Place in diluted lithium carbonate solution for 1 minute.
6. Wash in running water 10 minutes, and dehydrate in 50% alcohol, 70% alcohol, 80% alcohol, 90% alcohol, and 95% and absolute alcohol, ten dips each.

7. Stain 8 minutes in eosin solution. Wash thoroughly in water and dehydrate by short dips in 50% alcohol, 70% alcohol, 80% alcohol, and 90% alcohol and longer dips in 95% alcohol. Use two or three changes of absolute alcohol, 4 minutes each.
8. Blot with filter paper.
9. Clear in two changes of xylene, 4 minutes each.
10. Mount with Permount or Histoclad.

RESULTS

Negri bodies	-deep red
Nuclei	-light blue

Note. To obtain a good stain it is important not to overstain in Harris' hematoxylin, to differentiate very well in dilute HCl and after eosin, to follow exactly the instructions regarding the dips.

REFERENCE. Massignani, A. M., and Malferrari, R.: *Stain Techn.* 36:5-8, 1961.

SCHLEIFSTEIN'S METHOD FOR NEGRI BODIES

FIXATION. Zenker's solution.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

SCHLEIFSTEIN'S SOLUTION (STOCK)

Basic fuchsin	1.8 gm
Methylene blue	1.0 gm
Glycerin	100.0 ml
Methyl alcohol	100.0 ml

This solution will keep indefinitely.

POTASSIUM HYDROXIDE SOLUTION

Potassium hydroxide	0.01 gm
Tap water	4000.0 ml

(See *Note*)

SCHLEIFSTEIN'S SOLUTION (WORKING)

Schleifstein's solution (stock)	10 drops
Potassium hydroxide solution	20.0 ml

Mix the working solution immediately before use.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).
3. Place sections on ring stand, then flood with freshly prepared working Schleifstein's solution. Gently heat the bottom of the slide until vapor is produced. Allow slide to cool to room temperature.

4. Wash quickly in tap water.

5. Differentiate each slide individually, by gently agitating in 90% alcohol until sections are faint violet color.

6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

7. Mount with Permount or Histoclad.

RESULTS

Negri bodies - deep magenta
Cytoplasm - bluish violet
Erythrocytes - copper

REMARKS. This technic is excellent for demonstrating Negri bodies in Zenker-fixed specimens only. Attempts to post-Zenkerize sections prior to staining so far have proven useless.

Note. The important consideration at this point is to achieve a slightly alkaline solution. It may be necessary to vary the amount of potassium hydroxide to accomplish this.

REFERENCE. Schleifstein, J.: *Amer. J. Public Health* 27:1283-1285, 1937.

ALDEHYDE-THIONIN-PAS METHOD FOR CENTRAL NERVOUS SYSTEM

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

0.5% SULFURIC ACID SOLUTION

Sulfuric acid, concentrated	0.5 ml
Distilled water	99.5 ml

0.5% POTASSIUM PERMANGANATE SOLUTION

(See page 87)

2% POTASSIUM METABISULFITE SOLUTION

Potassium metabisulfite	2.0 gm
Distilled water	100.0 ml

ALDEHYDE THIONIN SOLUTION

Thionin	0.5 gm
Alcohol, 70%	91.5 ml
Paraldehyde	7.5 ml
Hydrochloric acid, concentrated	1.0 ml

This solution should ripen in a tightly stoppered container for 3-5 days at room temperature, before use.

0.5% PERIODIC ACID SOLUTION

(See page 72)

SCHIFF REAGENT SOLUTION

(See page 159)

1% ORANGE G SOLUTION

(See page 114)

1% GLACIAL ACETIC ACID SOLUTION

(See page 94)

5% PHOSPHOTUNGSTIC ACID SOLUTION

(See page 86)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Equal parts of sulfuric acid solution and potassium permanganate solution for 2 minutes.
3. Bleach with potassium metabisulfite for 1 minute.
4. Rinse well in distilled water.
5. Aldehyde thionin solution in an air stoppered container for approximately 50 minutes.
6. Rinse in distilled water.
7. Periodic acid solution for 5 minutes.
8. Rinse in distilled water.
9. Schiff's reagent solution for 15 minutes.
10. Wash in running water for 15 minutes.
11. Orange G solution for 3 minutes.
12. Phosphotungstic acid solution for 1 minute.
13. Rinse briefly in glacial acetic acid solution.
14. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
15. Mount with Permount or Histoclad.

RESULTS

Nissl substances	-blue-black
PAS positive materials	-red
Background	-various shades depending on amount of differentiation

REMARKS. This technic has been used to demonstrate alpha, beta, gamma, and delta cells of the pituitary and has been modified slightly to demonstrate Nissl substances.

Note. Schiff reagent should be checked and renewed frequently.

REFERENCE. Author unknown.

EINARSON'S METHOD FOR NISSL SUBSTANCE

FIXATION. 10% buffered neutral formalin, Zenker's, or Zenker-formol.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

GALLOCYANIN SOLUTION

Gallocyanin, (Hartman, Leddon)	0.15 gm
Chromium potassium sulfate	5.0 gm
Distilled water	100.0 ml

Dissolve the chrome alum in warm water, add gallocyanin, and boil gently for 10 minutes. Cool and filter. This solution keeps well for about one week.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Place sections in gallocyanin solution for 1 hour at 56°C, or leave overnight at room temperature.
3. Rinse in water.
4. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
5. Mount with Permount or Histoclad.

RESULTS

Nissl substance - blue

REFERENCE. Einarson, L.: *Amer. J. Path.* 8:295-309, 1932.

VOGT'S METHOD FOR NERVE CELL PRODUCTS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

2% CRESYL ECHT VIOLET SOLUTION (STOCK)

Cresyl echt violet (stock)	2.0 gm
Distilled water	100.0 ml

BUFFER SOLUTION

Sodium acetate	2.0 gm
Distilled water	1000.0 ml
Glacial acetic acid	3.0 ml

CRESYL ECHT VIOLET SOLUTION (WORKING)

Cresyl echt violet (stock)	1.0 ml
Buffer solution	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and carry through absolute alcohol, two changes.
2. Let stand in absolute alcohol for 2 hours.
3. Working cresyl echt violet solution for 40-60 minutes.
4. Differentiate rapidly in 95% alcohol, then dehydrate in absolute alcohol, and clear in xylene, two changes each.
5. Mount with Permount or Histoclad.

RESULTS

Nissl substance - intense purple
Nuclei - purple
Background - clear

REFERENCE. Vogt, O.: Institut für Hirnforschung und Allgemeine Biologie, Neustadt/Schwarzwals, Germany. Personal Communication. (AFIP modification).

GWYN-HEARDMAN METHOD FOR MOTOR END PLATES

FIXATION. Formol saline.

TECHNIQUE. Cut frozen sections at 50 microns. Collect in distilled water.

SOLUTIONS

0.1M COPPER SULFATE SOLUTION (STOCK)

Cupric sulfate ($CuSO_4 \cdot 5H_2O$) ..... 24.9 gm
Distilled water ..... 1000.0 ml

0.5M AMINOACETIC ACID SOLUTION (STOCK)

Aminoacetic acid ..... 3.7 gm
Distilled water ..... 100.0 ml

ACETATE BUFFER SOLUTION, pH.5 (STOCK)

0.1 *N* glacial acetic acid, (6 ml to 1000 ml dist. water) ..... 3.0 ml
0.1*N* sodium acetate, (13.6 gm to 1000 ml dist. water) ..... 6.0 ml

2% ACETYLTHIOCHOLINE IODIDE SOLUTION (STOCK)

Acetylthiocholine iodide ..... 2.0 gm
Distilled water ..... 100.0 ml

SUPERNATANT SOLUTION

Copper sulfate solution (stock) ..... 10.0 ml
Acetylthiocholine iodide (stock) ..... 30.0 ml

Centrifuge this mixture until solution is clear, about 5-10 minutes.

SUBSTRATE SOLUTION (WORKING)

Copper sulfate solution (stock)	0.2 ml
Aminoacetic acid solution (stock)	0.2 ml
Acetate buffer solution (stock)	5.0 ml
Supernatant solution	0.8 ml

5% AMMONIUM SULPHIDE SOLUTION

Ammonium sulphide, yellow	5.0 ml
Distilled water	100.0 ml

10% SILVER NITRATE SOLUTION

(See page 198)

AMMONIACAL SILVER SOLUTION

To 10 ml of 10% silver nitrate add 28% ammonium hydroxide, drop by drop, shaking the flask vigorously until the brown precipitate disappears. Add 3 drops in excess.

1% AMMONIA WATER

Ammonium hydroxide, 28%	1.0 ml
Distilled water	99.0 ml

3% SODIUM THIOSULFATE SOLUTION

(See page 98)

1% GLACIAL ACETIC ACID SOLUTION

(See page 94)

FORMALIN-PYRIDINE SOLUTION

Pyridine	2.0 ml
Formalin, 10% neutral	98.0 ml

NEUTRALIZED FORMALIN SOLUTION

Magnesium carbonate	10.0 gm
Formalin, 10%	100.0 ml

STAINING PROCEDURE

1. Working substrate solution at 37 °C for 15 minutes.
2. Wash in distilled water for 5 minutes.
3. Ammonium sulphide solution for 2 minutes.
4. Wash in distilled water, three changes, for 1 hour.
5. Formalin-pyridine solution for 15 days.
6. Wash in tap water, three changes, for 1 hour.
7. 10% silver nitrate at 37 °C in the dark for 1-1/2 hours.

8. Wash in 10% formalin until the white precipitate no longer forms, about six changes.

9. Ammoniacal silver solution until impregnation of the terminal portion of the nerve fibers has occurred. May take several hours.

10. Ammonia water solution for 2 minutes.

11. Glacial acetic acid solution for 2 minutes.

12. Distilled water for 2 minutes.

13. Sodium thiosulfate solution for 5 minutes.

14. Distilled water for 30 minutes.

15. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

16. Mount with Permount or Histoclad.

RESULTS

Motor end plates and associated nerve fibers	- black
Background	- pale grey

REFERENCE. Gwyn, D. G., and Heardman, V.: *Stain Techn.* **40**:15 - 18, 1965. Copyright by Williams and Wilkins Co.

SEVIER - MUNGER METHOD FOR NEURAL TISSUES

FIXATION. 10% buffered neutral formalin. Avoid chromate fixatives.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

20% SILVER NITRATE SOLUTION

(See page 187)

10% SILVER NITRATE SOLUTION

(See page 198)

FORMALIN SOLUTION

Formalin, 37 – 40%	2.0 ml
Tap water	98.0 ml

5% SODIUM THIOSULFATE SOLUTION

(See page 89)

SODIUM CARBONATE SOLUTION

Sodium carbonate (hydrated)	8.0 gm
Distilled water	30.0 ml

AMMONIACAL SILVER SOLUTION (WORKING)

To 50 ml of 10% silver nitrate solution add ammonium hydroxide (28-30%) drop by drop until the dark brown precipitate which forms has almost disappeared. Shake

vigorously between drops. Avoid complete decolorization. The end point is a slightly cloudy solution. Add to this solution 0.5 ml of sodium carbonate solution. Shake well. Add 25 drops of ammonium hydroxide. Shake well. Solution should now be crystal clear. Filter into a 125 ml Erlenmeyer flask. Cover.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Preheat 20% silver nitrate solution to 60 °C for 15 minutes. Add slides to warm silver solution and let them remain in the oven for 15 minutes.
3. Rinse one slide at a time in distilled water, then place in a clean coplin jar.
4. To working ammoniacal silver solution add 10 drops of the formalin solution, shaking gently while adding formalin. Pour quickly over slides, let develop for 5 to 30 minutes until golden brown. Keep in motion to avoid precipitate.
5. Examine microscopically. *Do not wash.* Developing should be neither too fast nor too slow.
6. Rinse well in fresh tap water, three changes.
7. Sodium thiosulfate solution for 2 minutes.
8. Wash in water.
9. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
10. Mount with Permount or Histoclad.

RESULTS

Large and small peripheral neurities	- black
Axons	- black
Myelin sheath	- light brown
Collagen and muscle	- brown
Argentaffin granules	- black

Note. Sections of the central nervous system have also been stained with this technic. The cerebellar basket cell and axons of the Purkinje cell became beautifully impregnated. Within the brain stem and spinal cord, large fibers are stained but small fibers are not visualized. The optic and olfactory nerves do not stain.

REFERENCE. Sevier, A. C., and Munger, B. L.: *J. Neuropath. Exp. Neurol.* **24**: 130 - 135, 1965.

Chapter 13

Methods For Bacteria, Fungi, And Inclusion Bodies

FITE'S METHOD FOR ACID FAST ORGANISMS

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

XYLENE - PEANUT OIL SOLUTION

Peanut oil* ..... 1 part
Xylene ..... 2 parts

ZIEHL - NEELSEN CARBOL FUCHSIN SOLUTION

(See page 220)

1% SULFURIC ACID SOLUTION

(See page 102)

METHYLENE BLUE SOLUTIONS

(See page 218)

STAINING PROCEDURE. Use control slide.

1. Deparaffinize through two changes of xylene-peanut oil solution for 12 minutes each.
2. Drain, wipe off excess oil and blot to opacity. The residual oil helps prevent shrinkage and injury of section.
3. Zeihl-Neelsen carbol fuchsin solution for 30 minutes.
4. Wash in tap water for 3 minutes.
5. Differentiate slides individually with sulfuric acid solution until sections are faint pink, about 1 minute.
6. Wash in running water for 3 minutes.
7. Counterstain lightly with working methylene blue solution.
8. Rinse off excess methylene blue in tap water.
9. Blot and let stand for a few minutes to air dry thoroughly.
10. Dip slides in xylene before mounting.
11. Mount with Permount or Histoclad.

*Matheson, Coleman and Bell, Norwood, Ohio

RESULTS

Acid fast bacilli	- red
Nocardia filaments	- red
Lepra bacilli	- red
Background	- pale blue

REMARKS. This technic is used in our laboratories for the demonstration of certain strains of Nocardia and lepra bacilli. We find this technic to be the most useful of the acid fast procedures for the demonstration of these pathologic entities for the following reasons: (1) The protection, afforded by the combination of peanut oil and xylene, coats the micro-organisms, (2) the oil coating, applied around the filaments and lepra bacilli, is not dissolved due to the elimination of alcohol from both the hydration and dehydration process, and (3) the decolorization of the background with a weak solution of aqueous sulfuric acid instead of the usual 1% hydrochloric acid-alcohol does not differentiate too rapidly.

REFERENCE. Fite, G. L., Cambre, P. J., and Turner, M. H.: *Arch. Path.* 43:624-625, 1947.

KINYOUN'S METHOD FOR ACID FAST BACTERIA

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

KINYOUN'S CARBOL FUCHSIN SOLUTION

Basic fuchsin	4.0 gm
Phenol crystals, melted	8.0 ml
Alcohol, 95%	20.0 ml
Distilled water	100.0 ml

1% ACID ALCOHOL SOLUTION

(See page 38)

METHYLENE BLUE SOLUTION (STOCK)

Methylene blue	1.4 gm
Alcohol, 95%	100.0 ml

METHYLENE BLUE SOLUTION (WORKING)

Methylene blue solution (stock)	10.0 ml
Tap water	90.0 ml

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Kinyoun's carbol fuchsin solution at 56 °C for 1 hour.
3. Wash well in running water.
4. Differentiate in two changes of acid alcohol solution until tissue is pale pink.
5. Wash well in running water.

6. Counterstain in working methylene blue solution for 2 seconds.
7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Acid-fast bacteria - bright red
Background - light blue

REFERENCE. Kinyoun, J. J.: *Amer. J. Public Health* 5:867, 1915. (AFIP modification)

TRUANT'S FLUORESCENT METHOD FOR ACID FAST ORGANISMS

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

AURAMINE-RHODAMINE SOLUTION

Auramine	1.5 gm
Rhodamine B	0.75 gm
Glycerin, U.S.P.	75.0 ml
Phenol crystals, (melted)	10.0 ml
Distilled water	50.0 ml

1% ACID ALCOHOL SOLUTION

(See page 38)

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Weigert's iron hematoxylin solution for 10 minutes.
3. Wash in running water 10 minutes.
4. Auramine-Rhodamine solution at 60 °C for 10 minutes.
5. Wash in tap water.
6. Differentiate in acid alcohol solution to remove excess stain.
7. Wash in running water.
8. Rinse in distilled water.
9. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
10. Mount with Permount or Histoclad.

RESULTS

Bacilli will fluoresce reddish yellow.
Artifacts will not have the reddish color and will remain yellow.

REMARKS. The Weigert's hematoxylin used as a fluorescence inhibitor, also gives a good cell stain. The use of thinner sections will reduce the background staining.

REFERENCE. Truant, J. P.: *Henry Ford Hospital Med. Bull.* 10:287, 1962.

ZIEHL-NEELSEN METHOD FOR ACID-FAST BACTERIA

FIXATION. Any well-fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

CARBOL FUCHSIN SOLUTION

Phenol crystals, (melted)	2.5 ml
Alcohol, 100%	5.0 ml
Basic fuchsin	0.5 gm
Distilled water	50.0 ml

Filter before use.

10 mls Water
10 " D.M.S.O

1% ACID ALCOHOL SOLUTION

(See page 38)

1% SULFURIC ACID SOLUTION

(See page 102)

METHYLENE BLUE SOLUTIONS

(See page 218)

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Carbol fuchsin solution for 30 minutes.
3. Wash well in running water.
4. Decolorize with acid alcohol solution or sulfuric acid solution until sections are pale pink.
5. Wash thoroughly in running water for 8 minutes.
6. Counterstain by dipping one slide at a time in working methylene blue solution. Sections should be pale blue.
7. Wash with tap water, then rinse in distilled water.
8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
9. Mount with Permount or Histoclad.

RESULTS

Acid-fast bacilli	-bright red
Erythrocytes	-yellowish orange
Other tissue elements	-pale blue

REFERENCE. Mallory, F. B.: *Pathological Technique*, New York, Hafner Publishing Co., 1961, p.275. (AFIP modification)

WADE'S METHOD FOR ACID FAST ORGANISMS

FIXATION. Zenker's or 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

DEPARAFFINIZING SOLUTION

Turpentine, rectified ..... 2 parts
Paraffin oil (liquid petrolatum), heavy ..... 1 part

CARBOL-NEW FUCHSIN SOLUTION

New fuchsin, Magenta III ..... 0.5 gm
Phenol crystals, melted ..... 5.0 ml
Alcohol, 100% ..... 10.0 ml
Distilled water to make ..... 100.0 ml

5% SULFURIC ACID SOLUTION

Sulfuric acid, concentrated ..... 5.0 ml
Distilled water ..... 95.0 ml

1% POTASSIUM PERMANGANATE SOLUTION

(See page 102)

2% OXALIC ACID SOLUTION

(See page 195)

MODIFIED VAN GIESON SOLUTION

Acid fuchsin ..... 0.01 gm
Picric acid ..... 1.0 gm
Distilled water ..... 100.0 ml

STAINING PROCEDURE. Use control slide.

1. Deparaffinizing solution, two changes, 5 minutes each.
2. Drain, wipe off excess fluid, blot to opacity, and place in water.
3. Carbol-new fuchsin solution, overnight.
4. Wash in water for 2 minutes.
5. Full strength formalin for 5 minutes (see *Note*). The bacilli become blue. The sections may turn blue or remain predominantly reddish.
6. Wash in running water for 2 minutes.
7. Sulfuric acid solution for 1 minute, then wash well in running water for 5 minutes.
8. Potassium permanganate solution 3 minutes. Rinse in tap water.
9. Oxalic acid solution to bleach for 30-60 seconds, then wash thoroughly in running water. Use 5% oxalic acid for specimens that do not bleach readily.
10. Modified van Gieson solution for 3 minutes.
11. Dehydrate rapidly in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
12. Mount with Permount or Histoclad.

RESULTS

Acid-fast bacilli	-deep ultramarine blue, or blue-black
Connective tissue elements	-red
Background	-yellowish

Note. The quality of the formalin is critical. Fite specified particularly freshly redistilled formalin, but the reagent grade is much more convenient and thoroughly dependable. The blue color of the bacilli is permanent. If it is suspected that there is not total demonstration of bacilli by this method, step 1 may be prolonged for 4-6 hours for "restoration" of poorly staining bacilli.

REFERENCE. Wade, H. W.: *Amer. J. Path.* 28:157, 1952.

BROWN AND BRENN METHOD FOR GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% CRYSTAL VIOLET SOLUTION

Crystal violet	1.0 gm
Distilled water	100.0 ml

5% SODIUM BICARBONATE SOLUTION

Sodium bicarbonate	5.0 gm
Distilled water	100.0 ml

GRAM'S IODINE SOLUTION

(See page 41)

ETHYL ETHER - ACETONE SOLUTION

Ethyl ether	50.0 ml
Acetone	50.0 ml

0.25% BASIC FUCHSIN SOLUTION (STOCK)

Basic fuchsin	0.25 gm
Distilled water	100.0 ml

BASIC FUCHSIN SOLUTION (WORKING)

Basic fuchsin solution (stock)	10.0 ml
Distilled water	100.0 ml

PICRIC ACID-ACETONE SOLUTION

Picric acid	0.1 gm
Acetone	100.0 ml

ACETONE-XYLENE SOLUTION

Acetone	50.0 ml
Xylene	50.0 ml

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Place slides on a staining rack. Pour on approximately 1 ml (or 20 drops) of crystal violet solution and add 5 drops of sodium bicarbonate solution for 1 minute. Agitate gently. (If preferred, the two solutions may be mixed just before use).
3. Rinse in tap water.
4. Flood slides with Gram's iodine solution for 1 minute.
5. Rinse in water and blot with filter paper to complete dryness.
6. Decolorize with ethyl ether-acetone solution dropped on slides until no more color runs off.
7. Working basic fuchsin solution for 1 minute. Wash in water. Blot gently, but do not allow sections to dry completely.
8. Dip in acetone to start differentiation reaction.
9. Differentiate immediately with picric acid-acetone solution until sections are yellowish pink.
10. Rinse quickly in acetone, then in acetone-xylene solution.
11. Clear in xylene, several changes.
12. Mount with Permount or Histoclad.

RESULTS

Gram positive bacteria	-blue
Gram negative bacteria	-red
Filaments of Nocardia and Actinomyces	-blue
Nuclei	-red
Other tissue elements	-yellow

REMARKS. This method is invaluable in the demonstration of the filaments of Nocardia and Actinomyces. It must be realized, however, that these filaments are not completely or strongly Gram positive. It is possible to obtain either Gram positive or Gram negative results depending on the degree of differentiation. It is suggested therefore, that at least two slides be run with varying degrees of differentiation in order to lessen the possibility of over-differentiation at step 8 of the staining procedure. For the study of Gram positive and Gram negative bacteria, R. D. Taylor's modification of Brown and Brenn technic on page 226 is recommended. We find this to be the best technic for demonstrating micro-organisms. This does not imply that this technic (B & B) is not useful for demonstrating Gram positive and Gram negative bacteria.

REFERENCE. Brown, J. H., and Brenn, L.: *Bull. Johns Hopkins Hosp.* **48**:69-73, 1931. (AFIP modification)

BROWN-HOPPS METHOD FOR GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

FIXATION. 10% buffered neutral formalin

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% CRYSTAL VIOLET SOLUTION

(See page 222)

GRAM'S IODINE SOLUTION

(See page 41)

CELLOSOLVE

(Ethylene glycol monoethyl ether).

0.5% BASIC FUCHSIN SOLUTION

Basic fuchsin	0.5 gm
Distilled water	100.0 ml

GALLEGO'S DIFFERENTIATING SOLUTION

Distilled water	50.0 ml
Formalin, 37 – 40%	1.0 ml
Glacial acetic acid	0.5 ml

1.5% TARTRAZINE SOLUTION

Tartrazine	1.5 gm
Distilled water	100.0 ml

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Place slides on staining rack and pour on crystal violet solution for 2 minutes.
3. Rinse in distilled water.
4. Mordant in Gram's iodine solution for 5 minutes.
5. Rinse in distilled water.
6. Differentiate in Cellosolve until blue color no longer streams away from the section (approximately 5-10 seconds) (see *Note*).
7. Quickly rinse in distilled water (see *Note*).
8. Basic fuchsin solution for 5 minutes.
9. Rinse in distilled water.
10. Gallego's differentiating solution for 5 minutes (differentiates and "fixes" the basic fuchsin).
11. Rinse thoroughly in distilled water.
12. Blot lightly, only to remove excess water (not to dryness)
13. Tartrazine solution for 3 seconds. Immediately blot away excess, but not to dryness (see *Note*).
14. Cellosolve, three changes, for six quick dips in each (see *Note*).

15. Xylene, three changes, for ten dips each. Slides may remain in xylene until ready for mounting.

16. Mount with Permount or Histoclad.

RESULTS

Gram positive bacteria	-blue
Gram negative bacteria	-red
Background	-yellow

Notes. Preferably, all stains and solutions are applied to the slide which is in a horizontal position, except for steps 6, 14, and 15, in which case the slide is dipped into the solutions contained in coplin jars. The stain works satisfactorily on tissues fixed in 10% formalin, (Na phosphate or Na acetate buffered), glutaraldehyde (2.5% with phosphate buffer), and formol-Zenker's fluid. It does *not* work well on Bouin's fixed tissues.

Steps 6, 7, 13, and 14 are critical in regard to timing.

REFERENCE. Brown, R. C., and Hopps, H. C.: Geographic Pathology Division, Armed Forces Institute of Pathology, Washington, D.C. 20305. (Method to be published in detail at a later date).

MACCALLUM-GOODPASTURE METHOD FOR GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

FIXATION. Any well-fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

GOODPASTURE'S SOLUTION

Basic fuchsin	0.59 gm
Aniline	1.0 ml
Phenol crystals (melted)	1.0 ml
Alcohol, 30%	100.0 ml

GRAM'S IODINE SOLUTION

(See page 41)

STIRLING'S GENTIAN VIOLET SOLUTION

Gentian violet (crystal violet)	5.0 gm
Alcohol, 100%	10.0 ml
Aniline	2.0 ml
Distilled water	88.0 ml

SATURATED PICRIC ACID SOLUTION

(See page 39)

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Goodpasture's solution for 10 minutes.

3. Rinse in distilled water.
4. Differentiate in full strength formalin for a few minutes (fixes Goodpasture stain).
5. Wash in running water for 3 minutes.
6. Saturated picric acid solution for 3 to 5 minutes.
7. Rinse in distilled water.
8. Differentiate in 95% alcohol for 30 seconds.
9. Rinse in distilled water.
10. Stirling's gentian violet solution for 3 minutes.
11. Rinse in distilled water.
12. Gram's iodine solution for 1 minute.
13. Rinse in distilled water. Blot, but leave moist.
14. Differentiate in a solution of equal parts of aniline and xylene, several changes until section appears light purplish red.
15. Xylene, two changes.
16. Mount with Permount or Histoclad.

RESULTS

Gram-positive organisms - blue
Gram-negative organisms - red
Other elements - various shades of red to purple

REFERENCE. MacCallum, W. G.: *JAMA* 72:193, 1919.

TAYLOR'S METHOD FOR GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

HARRIS' HEMATOXYLIN SOLUTION

(See page 34)

1% ACID ALCOHOL SOLUTION

(See page 38)

HUCKER'S SOLUTION

Gentian violet, 10% alcoholic	2.0 ml
Distilled water	18.0 ml
Ammonium oxalate, 1% aqueous	80.0 ml

Mix and filter before use.

BASIC FUCHSIN SOLUTION (STOCK)

Basic fuchsin	0.1 gm
Methyl alcohol	95.0 ml
Distilled water	5.0 ml

BASIC FUCHSIN SOLUTION (WORKING)

Basic fuchsin (stock)	5.0 ml
Distilled water	60.0 ml

ETHYL ETHER-ACETONE SOLUTION

(See page 222)

0.1% PICRIC ACID-ACETONE SOLUTION

(See page 223)

ACETONE-XYLENE SOLUTION

Acetone	1 part
Xylene	2 parts

ACETONE-XYLENE SOLUTION II

Acetone	1 part
Xylene	3 parts

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Harris' hematoxylin solution for 5 - 10 minutes.
3. Wash in running water for 1 minute.
4. Differentiate in acid alcohol solution.
5. Wash in running water for 3 minutes.
6. Wash in saturated lithium carbonate solution to intensify blue. From this point, carry only one slide at a time.
7. Wash in running water for 5 minutes.
8. Hucker's solution for 2 minutes.
9. Wash quickly in water.
10. Mordant in Gram's iodine solution for 1 minute.
11. Wash in water. Blot, but do not allow to dry, using filter paper moistened with water.
12. Decolorize in ethyl ether-acetone until no more blue color comes off. Blot, but do not allow to dry, using filter paper moistened with ethyl ether-acetone.
13. Working basic fuchsin solution for 3 minutes.
14. Wash in water. Blot, but do not allow to dry, using filter paper moistened with water.
15. Dip in acetone until section begins to decolorize.
16. Pass quickly to picric acid-acetone to decolorize and differentiate until section becomes reddish brown-yellow, approximately 15 seconds.
17. Pass quickly through acetone and xylene solution I, then acetone and xylene solution II.
18. Clear in xylene, two changes.
19. Mount with Permount or Histoclad.

RESULTS

Gram-positive organisms	- blue to blue-black
Gram-negative organisms	- bright red
Nuclei	- brownish red
Erythrocytes	- red to greenish yellow
Necrotic tissue	- yellowish green
Cytoplasm	- yellow
Connective tissue	- red

REFERENCE. Taylor, R. D.: *Amer. J. Clin. Path.* 46:472-474, 1966.

GRIDLEY'S METHOD FOR ENDAMOEBA HISTOLYTICA

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

HARRIS' HEMATOXYLIN SOLUTION

(See page 34)

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

ANILINE-EOSIN SOLUTION

Eosin Y	1.5 gm
Alcohol, 80%	100.0 ml
Aniline	3.0 ml
Glacial acetic acid	1.0 ml

NAPHTHOL GREEN B SOLUTION

Naphthol green B	1.0 gm
Distilled water	100.0 ml
Glacial acetic acid	1.0 ml

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Harris' hematoxylin solution for 10 minutes or Weigert's iron hematoxylin solution for 3 minutes.
3. Wash in running water for 5 minutes.
4. Differentiate in 1% acid alcohol solution, see page 38.
5. Wash in running water for 5 minutes.
6. Blue in ammonia water.
7. Wash in running water for 5 minutes.
8. Aniline-eosin solution for 5 minutes.
9. Rinse well in distilled water. Sections should be a deep rose color.
10. Naphthol green B solution for 5 minutes.

11. Differentiate in 95% alcohol, two changes, until erythrocytes in section are bright rose. Check under microscope.
12. Dehydrate in absolute alcohol, and clear in xylene, two changes each.
13. Mount with Permount or Histoclad.

RESULTS

Amoebae	- blue-green
Nuclei of amoebae	- deeper blue-green
Ingested erythrocytes	- deep rose
Connective tissue	- green

REMARKS. This procedure does not stain amoebae differentially but is useful in that it demonstrates the ingested erythrocytes extremely well.

REFERENCE. Gridley, M. F.: *Amer. J. Clin. Path.* 24:243-244, 1954. Copyright by Williams and Wilkins Co.

GRIDLEY'S METHOD FOR FUNGI

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

4% CHROMIC ACID* SOLUTION

Chromic acid	4.0 gm
Distilled water	100.0 ml

COLEMAN'S FEULGEN REAGENT

(See page 159)

ALDEHYDE FUCHSIN SOLUTION

(See page 78)

0.25% METANIL YELLOW SOLUTION

(See page 78)

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Oxidize in 4% chromic acid for 1 hour.
3. Wash in running water for 5 minutes.
4. Coleman's feulgen reagent for 15 minutes.
5. Wash in running water for 15 minutes.
6. Rinse in 70% alcohol, several changes.
7. Aldehyde fuchsin solution for 30 minutes.
8. Rinse off excess stain with 95% alcohol.
9. Rinse in distilled water.
10. Counterstain lightly with metanil yellow solution for 1 minute.
11. Rinse in distilled water.

*Chromium trioxide

12. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

13. Mount with Permount or Histoclad.

RESULTS

Mycelia	-deep purple
Conidia	-deep rose to purple
Background	-yellow

Elastic tissue and mucin also stain deep purple.

REMARKS. An extremely useful technic for fungi. The morphologic detail of yeast forms and hyphae are usually visible, although very old fungi, which were probably nonviable at the time of fixation, are not as well stained as by the Grocott's methenamine-silver technic. The filaments of Nocardia and Actinomyces are *NOT* stained by this method.

Note: Luna, L. G., published "Evaluation of Staining Technic for Pathogenic Fungi." That report can be found in *Amer. J. Med. Techn.* 30:139-146, 1964.

REFERENCE: Gridley, M. F.: *Amer. J. Clin. Path.* 23:303-307, 1953. Copyright by Williams and Wilkins Co.

GROCOTT'S METHOD FOR FUNGI (GMS)

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTION

4% CHROMIC ACID SOLUTION

(See page 229)

5% SILVER NITRATE SOLUTION

(See page 91)

3% METHENAMINE* SOLUTION

(See page 97)

5% BORAX SOLUTION

Borax	5.0 gm
Distilled water	100.0 ml

METHENAMINE-SILVER NITRATE SOLUTION (STOCK)

Silver nitrate, 5% solution	5.0 ml
Methenamine, 3% solution	100.0 ml

A white precipitate forms but immediately dissolves on shaking. Clear solution remains usable for months. Store in refrigerator.

* Hexamethylenatetramine. Eastman Kodak Co., Rochester, N.Y. 14603

METHENAMINE-SILVER NITRATE SOLUTION (WORKING)

Methenamine-silver nitrate solution (stock)	25.0 ml
Distilled water	25.0 ml
Borax, 5% solution	2.0 ml

Make fresh.

1% SODIUM BISULFITE SOLUTION

Sodium bisulfite	1.0 gm
Distilled water	100.0 ml

0.1% GOLD CHLORIDE SOLUTION

Gold chloride, 1% solution	10.0 ml
Distilled water	90.0 ml

This solution may be used repeatedly.

2% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page 88)

0.2% LIGHT GREEN SOLUTION

(See page 159)

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Oxidize in 4% chromic acid solution for 1 hour.
3. Wash in tap water for a few seconds.
4. Sodium bisulfite solution for 1 minute to remove any residual chromic acid.
5. Wash in running water for 5 to 10 minutes.
6. Rinse with three or four changes of distilled water.
7. Place in freshly mixed working methenamine-silver nitrate solution in oven at 58 to 60 ° C for 60 minutes or until section turns yellowish brown.
8. Rinse in six changes of distilled water.
9. Tone in gold chloride solution for 2 to 5 minutes.
10. Rinse in distilled water.
11. Remove unreduced silver with sodium thiosulfate (hypo) solution for 2 to 5 minutes.
12. Wash thoroughly in tap water.
13. Counterstain with working light green solution for 30 to 45 seconds.
14. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
15. Mount with Permount or Histoclad.

RESULTS

Fungi	-sharply delineated in black
Mucin	-taupe to dark gray
Inner parts of mycelia and hyphae	-old rose
Background	-pale green

REMARKS. This technic gives to all forms of fungi, including the filaments of *Actinomyces bovis* and *Nocardia asteroides*, a black-brown coloration. In staining fungi, especially filaments of Nocardia, it has been found that the time of exposure to methenamine-silver nitrate solution for complete development may vary according to type and/or strains suspected. Therefore, if Nocardia is suspected, two slides should be processed, one for 60 minutes and one for 90 minutes, thereby assuring a positive demonstration of any filaments of Nocardia that may exist. Slides previously stained with most other stains may be used by removing cover glasses in xylol and running through alcohols to water. Subsequent chromic acid treatment will remove any remaining stain.

REFERENCE. Grocott, R. G.: *Amer. J. Clin. Path.* **25**:975-979, 1955. Copyright by Williams and Wilkins Co.

PICKETT'S FLUORESCENCE METHOD FOR FUNGI

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

0.1% ACRIDINE ORANGE SOLUTION

Acridine orange	0.1 gm
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Weigert's iron hematoxylin solution for 5 minutes.
3. Wash in running water for 4 minutes.
4. Acridine orange solution for 2 minutes.
5. Rinse in distilled water.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with XAM.*

RESULTS. Fungi fluoresce brightly against a dark background.

REFERENCE. Pickett, J. P., et al: *Amer. J. Clin. Path.* **34**:197-202, 1960. Copyright by Williams and Wilkins Co.

ATTWOOD'S METHOD FOR AMNIOTIC FLUID EMBOLISM

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

* Schueler & Co. Ltd. New York, N.Y.

SOLUTIONS

1% ALCIAN GREEN SOLUTION

Alcian green	1.0 gm
Distilled water	100.0 ml
Glacial acetic acid	2.0 ml

PHLOXINE-CALCIUM CHLORIDE SOLUTION

Phloxine	0.5 gm
Calcium chloride	0.5 gm
Distilled water	100.0 ml

TARTRAZINE-CELLOSOLVE SOLUTION

Tartrazine	2.5 gm
Cellosolve (ethylene glycol monoethyl ether)	100.0 ml

SCOTT'S WATER SOLUTION

(See page 84)

0.5% POTASSIUM PERMANGANATE SOLUTION

(See page 87)

0.5% OXALIC ACID SOLUTION

(See page 79)

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Alcian green solution for 2 minutes.
3. Rinse in distilled water.
4. Potassium permanganate solution for 2 minutes.
5. Rinse in distilled water.
6. Oxalic acid solution for 2 minutes.
7. Rinse in distilled water.
8. Mayer's hematoxylin solution for 3 minutes (see page 33)
9. Rinse and blue in Scott's water solution for 5 minutes.
10. Phloxine solution for 30 minutes.
11. Rinse in distilled water, then in cellosolve.
12. Differentiate each slide with tartrazine-cellosolve solution, checking until the phloxine is removed from erythrocytes.
13. Dehydrate in cellosolve, clear in xylene, two changes each.
14. Mount with Permount or Histoclad.

RESULTS

Nuclei	-blue-black
Mucin, ground substance of cartilage, granules of certain mast cells	-green
Squamous epithelium, fibrin	-red
Erythrocytes, plasma, collegen	-yellow
Inclusion bodies	-red

REMARKS. This method has been used very successfully in our laboratories for the demonstration of inclusion bodies. For this, however, differentiation at step 12 must be rigidly controlled.

Note: The permanganate-oxalic bleach is not strictly necessary but it assists in the subsequent differentiation of the phloxine and tends to remove any background staining by alcian green. Any excess staining with tartrazine can be removed by rinsing in water until the desired intensity is obtained.

REFERENCE. Attwood, H. D.: *J. Path. Bact.* 76:211-215, 1958.

LENDRUM'S METHOD FOR INCLUSION BODIES

FIXATION. 10% buffered neutral formalin or Zenker's.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

0.5% PHLOXINE SOLUTION

Phloxine	1.0 gm
Alcohol, 70%	200.0 ml
Calcium chloride	1.0 gm

2.5% TARTRAZINE SOLUTION

(See page 233)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mayer's hematoxylin solution for 5-10 minutes.
3. Blue in running water for 15 minutes.
4. Phloxine solution for 30 minutes.
5. Rinse briefly in distilled water and drain slides well.
6. Tartrazine solution until inclusion bodies stand out a bright red on a yellow background.
7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Inclusion bodies - red
Nuclei - blue
Background - yellow

REFERENCE. Lendrum, A. C.: *J. Path. Bact.* 59:399-404, 1947.

PAGE-GREEN METHOD FOR INCLUSION BODIES

FIXATION. Any well-fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% ACID ALCOHOL SOLUTION

(See page 38)

SHORR'S STAINING SOLUTION

Alcohol, 50%	100.0 ml
Biebrich scarlet	1.0 gm
Orange G	0.25 gm
Fast green, FCF	0.075 gm
Phosphotungstic acid	0.5 gm
Phosphomolybdic acid	0.5 gm
Glacial acetic acid	2.0 ml

HARRIS' HEMATOXYLIN SOLUTION

(See page 34)

AMMONIA WATER SOLUTION

(See page 38)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Harris' hematoxylin solution for 3 to 5 minutes.
3. Rinse in water.
4. Differentiate in acid alcohol solution until there is no hematoxylin in the cytoplasm of the cells. *Check with microscope.*
5. Wash in water.
6. Blue sections in ammonia water solution. *Check with microscope.*
7. Wash in running water for 10 minutes.
8. Shorr's staining solution for 1 minute.
9. Rinse in 95% alcohol. *Check with microscope.* Connective tissue will be a clear light green when differentiation is complete.
10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Inclusion bodies	- brilliant red
Connective tissue	- light green
Elastic tissue	- purplish red
Muscle	- red
Keratin	- orange
Erythrocytes	- orange red
Nuclei	- blue

REFERENCE. Page, W. G., and Green, R. G.: *Cornell Vet.* 32:265-268, 1942.

GIEMSA METHOD FOR RICKETTSIA

FIXATION. 10% buffered neutral formalin or Zenker's.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

BUFFERED WATER SOLUTION pH 6.8

Buffer salts (pH 6.8)	1.0 gm
Distilled water	1000.0 ml

GIEMSA SOLUTION (STOCK)

Giemsa powder	1.0 gm
Glycerin, reagent grade	66.0 ml
Methyl alcohol, acetone free, reagent grade	66.0 ml

See page 127 (Price's Giemsa) for additional information on compounding of Giemsa solution.

GIEMSA SOLUTION (WORKING)

Giemsa solution (stock)	1.0 ml
Buffered water, pH 6.8	50.0 ml

0.2% GLACIAL ACETIC ACID SOLUTION

Glacial acetic acid	0.2 ml
Distilled water	99.8 ml

ROSIN ALCOHOL SOLUTIONS

(See page 119)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant in buffer water solution for 60 minutes.
3. Working Giemsa solution overnight.
4. Rinse in buffer water solution.
5. 0.2% glacial acetic acid solution for 1 minute.
6. Rinse in buffer water solution.
7. Differentiate sections individually in working rosin alcohol solution. Check with microscope frequently until rickettsiae appear as violet colored granules (may take up to 3 minutes).
8. Dehydrate in absolute alcohol and clear in xylene, three changes each.
9. Mount with Permount or Histoclad.

RESULTS

Rickettsia	- violet
Nuclei	- blue
Cytoplasm, connective tissue	- pink
Erythrocytes	- salmon

REMARKS. The differentiation of step 7 is the most critical and only if this is properly done can one expect good results. Use the microscope as suggested above for this purpose.

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

PINKERTON'S METHOD FOR RICKETTSIA

FIXATION. Zenker's, Regaud's, or 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% METHYLENE BLUE SOLUTION

Methylene blue	1.0 gm
Distilled water	100.0 ml

0.25% BASIC FUCHSIN SOLUTION

Basic fuchsin	0.25 gm
Distilled water	100.0 ml

0.5% CITRIC ACID SOLUTION

Citric acid	0.5 gm
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).
3. Methylene blue solution overnight.
4. Rinse in 95% alcohol for 5 seconds or blue color will be lost.
5. Rinse quickly in distilled water for 2-3 seconds.
6. Basic fuchsin solution for 30 minutes.
7. Decolorize rapidly in citric acid solution for 1-2 seconds, never more than 3 seconds.
8. Continue differentiation in absolute alcohol until nuclei stand out blue and rickettsia clumps red.
9. Clear with xylene, two changes.
10. Mount with Permount or Histoclad.

RESULTS

Rickettsia	- bright red
Nuclei	- blue

REMARKS. This is by far the most reliable method available for demonstrating rickettsia in paraffin sections. Good differentiation can be controlled since only erythrocytes and rickettsia must be red in well stained sections. All other structures are light blue.

REFERENCE. Simmons, J. S., and Gentzkow, C. J.: *Laboratory Methods of The United States Army*, 5th ed. Philadelphia, Penn., Lea and Febiger, 1944, p. 572.

LEVADITI-MANOVELIAN METHOD FOR SPIROCHETES

FIXATION. 10% buffered neutral formalin. Specimen should be 1 mm thick.

TECHNIQUE. Embed in paraffin after staining is completed (see Staining Procedure Step 10).

SOLUTIONS

3% SILVER NITRATE SOLUTION

Silver nitrate	3.0 gm
Distilled water	100.0 ml

REDUCING SOLUTION

Pyrogallic acid	4.0 gm
Formalin, 37 – 40%	5.0 ml
Distilled water	100.0 ml

STAINING PROCEDURE

1. Rinse specimen in tap water, after fixation.
2. Let stand in 95% alcohol for 24 hours.
3. Transfer to distilled water and leave until the tissue sinks to the bottom of the container.
4. Place in freshly prepared silver nitrate solution and keep in 37 °C in the dark for 3 to 5 days, changing the solution three times.
5. Rinse in distilled water.
6. Reducing solution at room temperature, in the dark for 24 to 72 hours.
7. Rinse in distilled water.
8. Dehydrate in 80% alcohol, 95% alcohol, and absolute alcohol, two changes, 30 minutes each.
9. Clear in oil of cedarwood for two changes, 1 hour each and infiltrate with two changes of paraffin 45 minutes each.
10. Embed in paraffin.
11. Cut sections at 5 microns and mount on slides.
12. When dry, deparaffinize with xylene, three changes.
13. Mount with Permount or Histoclad.

RESULTS

Spirochetes	- black
Background	- yellow to light brown

REFERENCE. Mallory, F. B.: *Pathological Technique*, New York, Hafner Publishing Co., 1961, p. 293.

WARTHIN-STARRY METHOD FOR SPIROCHETES AND DONOVAN BODIES

FIXATION. 10% buffered neutral formalin. *Avoid chromate fixatives.*

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS. Use chemically clean glassware.

ACIDULATED WATER

Triple distilled water ..... 1000.0 ml

Add enough 1% aqueous citric acid to bring water to pH 4.0.

1% SILVER NITRATE SOLUTION (For impregnation)

Silver nitrate, C.P. crystals ..... 1.0 gm
Acidulated water ..... 100.0 ml

2% SILVER NITRATE SOLUTION (For developer)

Silver nitrate, C.P. crystals ..... 2.0 gm
Acidulated water ..... 100.0 ml

5% GELATIN SOLUTION

Sheet gelatin, high grade ..... 10.0 gm
Acidulated water ..... 200.0 ml

0.15% HYDROQUINONE SOLUTION

Hydroquinone, crystals, photographic quality ..... 0.15 gm
Acidulated water ..... 100.0 ml

Keep 2% silver nitrate, 5% gelatin, and 0.15% hydroquinone in 50 ml Erlenmeyer flasks, in a flotation bath at 54 °C until developer is made.

DEVELOPER SOLUTION

Silver nitrate solution, 2% ..... 1.5 ml
Gelatin solution, 5% ..... 3.75 ml
Hydroquinone solution, 0.15% ..... 2.0 ml

Combine in the order given in small beaker, making certain solutions are mixed well. *Prepare immediately before use.*

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to triple distilled water.
2. Impregnate with silver nitrate solution heated in a flotation bath to 43 °C for 30 minutes. Prepare the developer solution at this point. (See *Note*)
3. Flood sections, that have been laid across glass rods, with the developer solution which *must be used as soon as it is mixed.* Allow sections to develop until they are light brown or yellow. Check known control under the microscope. The spirochetes should be black and the background light brown or yellow. (See *Note*)
4. Wash quickly and thoroughly in hot tap water, approximately 56 °C.
5. Rinse in distilled water.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Spirochetes, Donovan bodies	- black
Background	- pale yellow to light brown

REMARKS. It may be necessary to prolong development of sections for the demonstration of Donovan bodies. Certain hematogenous pigments, nuclei and melanin have a greater attraction for silver than do spirochetes, and it is difficult to stain the spirochetes in close proximity to these elements. By lowering the pH of the acidulated solution to 3.6 and prolonging the development, the spirochetes may be demonstrated in the areas of competition; however, the part of the section not containing competitive elements may be overstained and useless. Sections can be restained to increase the amount of development if microscopic observation of the known positive tissue shows pale spirochetes or none at all.

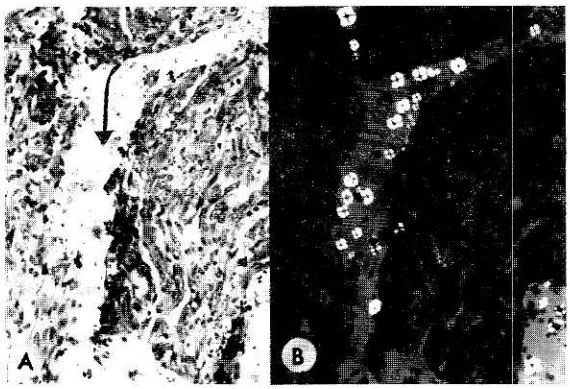
Note. Use paraffin coated forceps, particularly at step 2 and step 3.

REFERENCES. Kerr, D. A.: *Amer. J. Clin. Path. Tech. Suppl.* 8:63-67, 1938. Copyright by Williams and Wilkins Co. (AFIP modification)

C. H. Bridges, and L. G. Luna studied permissible variations of this technic in the AFIP laboratories. Their report can be found in *Lab. Invest.* July-August, 1957.

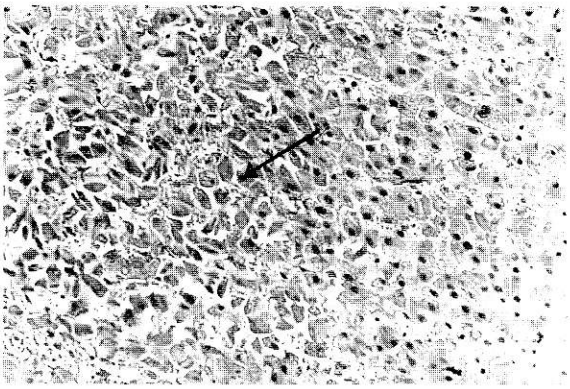
Artifacts

#1. PARTICLES OF STARCH FROM SURGICAL DUSTING POWDER INTRODUCED DURING GROSSING.

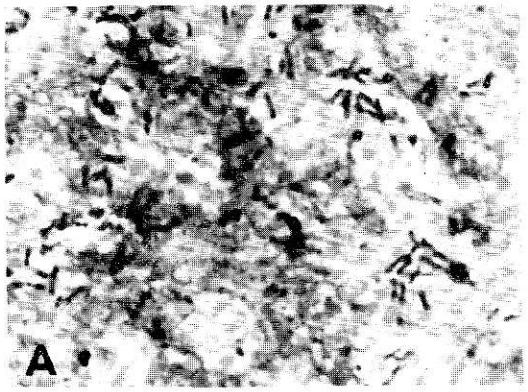


A. H & E showing deposits on final stained section (arrow).

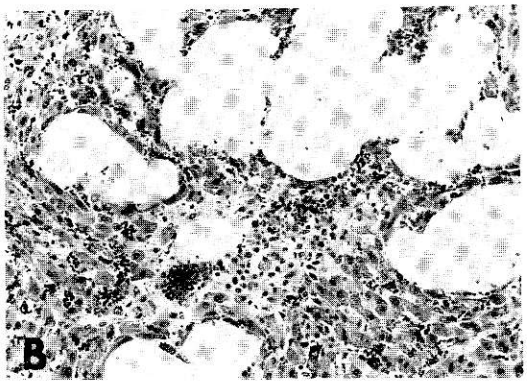
B. Same section polarized.



#2. Extensive autolysis seen on left (arrow), result of tissue being placed in dry container, preceeding addition of fixative.

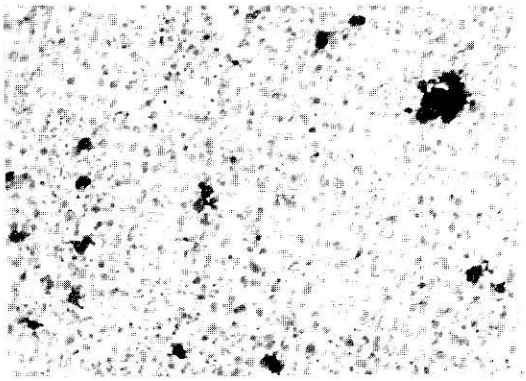


#3. POST-MORTEM AUTOLYSIS.

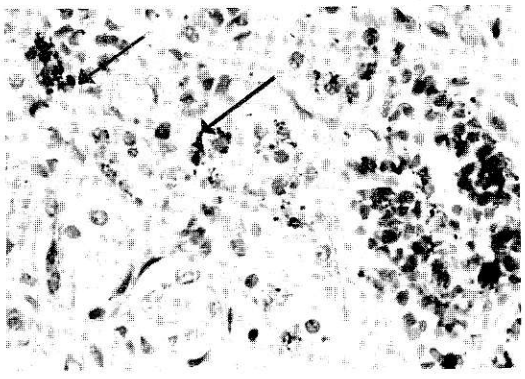


A. Extensive saprophytic growth of gas forming bacteria.

B. Gas bubbles formed by these bacteria.

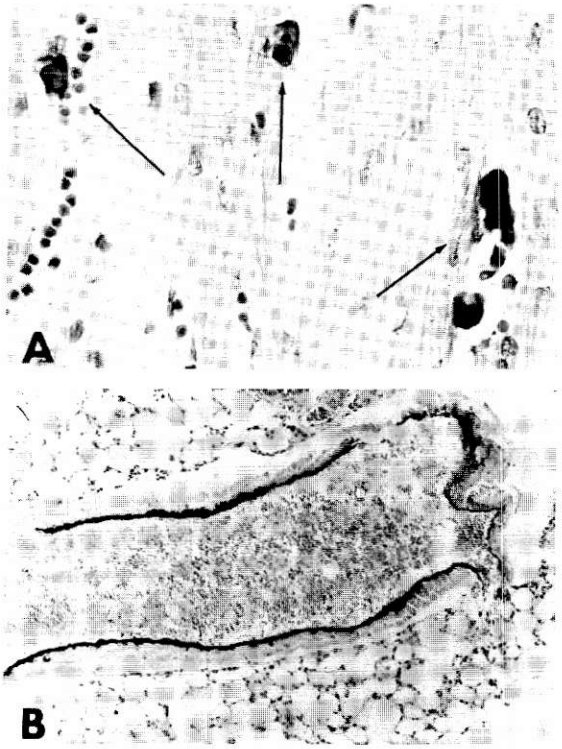


#4. Random deposits of mercuric chloride crystals (Zenker Fixation).



#5. Fine granular pigmentation deposited in and around blood vessels (arrows). (Formalin Fixation — unbuffered).

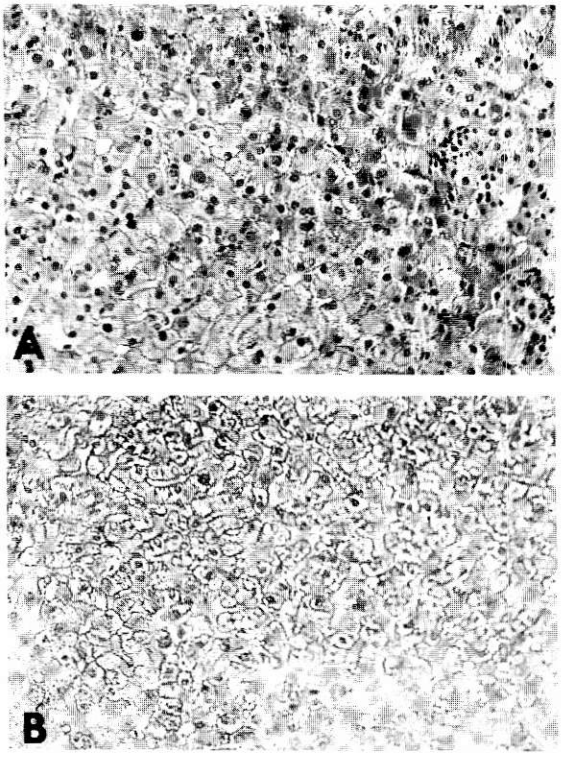
#6. PSEUDO CALCIFICATION — DUE TO CALCIUM ACETATE USED AS BUFFERING AGENT.



A. Heart muscle (arrow). Note absence of true tissue reaction.

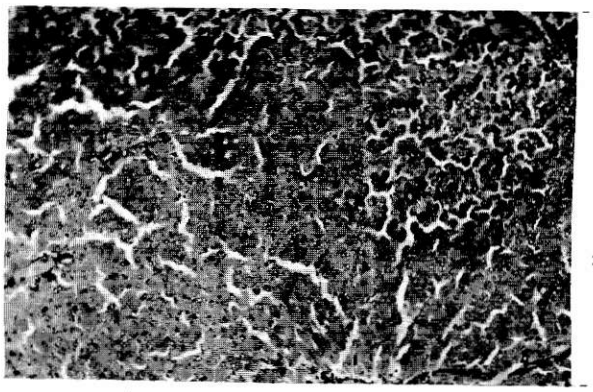
B. Lung. Deposits on intimal layer.

#7. INADEQUATE REMOVAL OF PICRIC ACID AFTER BOUIN'S FIXATION.

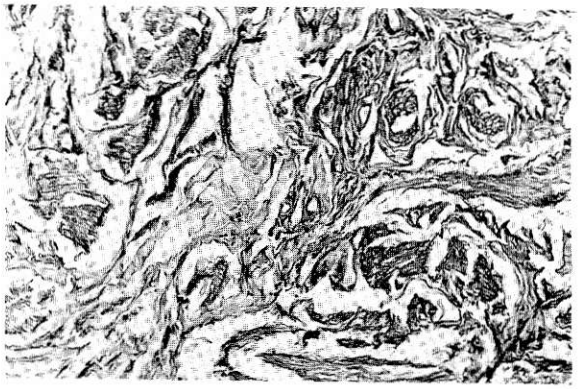


A. Section cut and stained 24 hours after fixation.

B. Section (from same block) cut and stained 4 weeks after fixation. Note destruction of tissue due to action of picric acid.

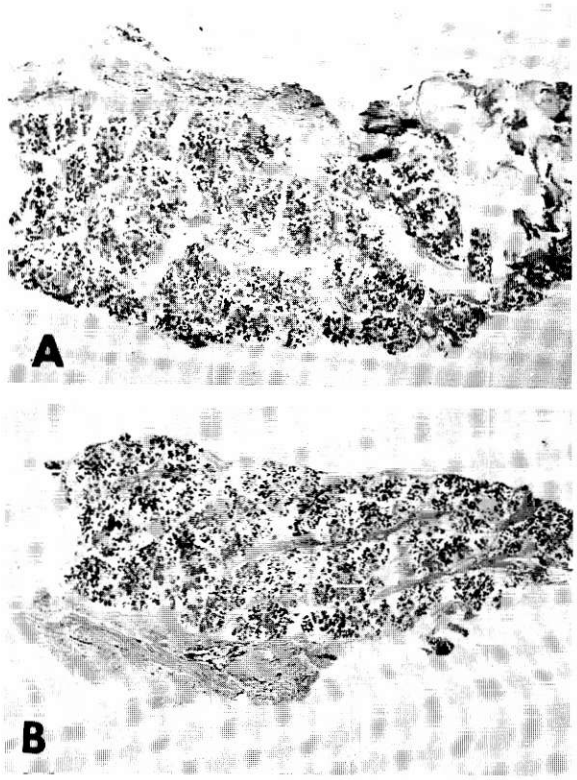


#8. Dry-Earth Effect: Due to inadequate dehydration, clearing and impregnation. This effect takes place in the clearing xylene following H & E Staining.



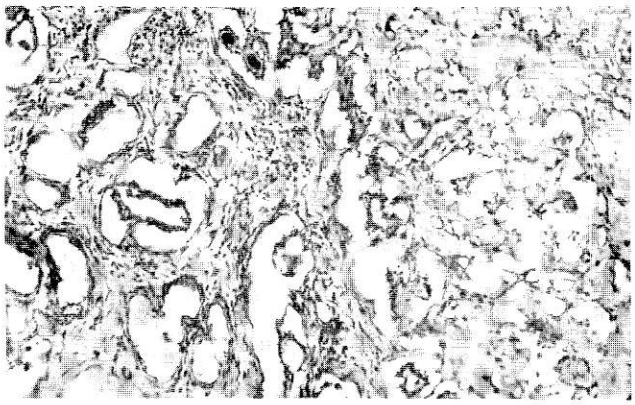
#9. Improper impregnation during processing: Note the uneven staining and spreading effect.

#10. IMPROPER DEHYDRATION, CLEARING AND IMPREGNATION.



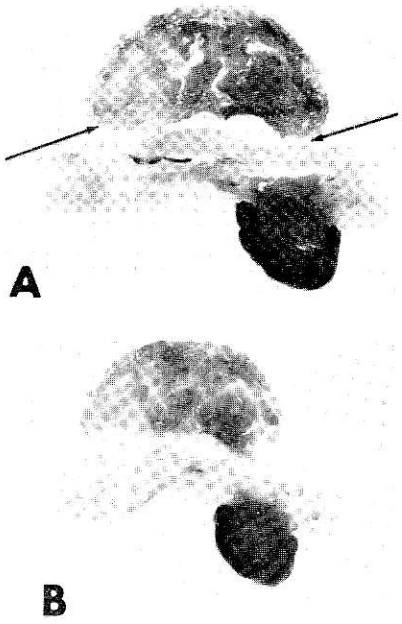
A. Spreading effect created when an improperly processed specimen is placed on tissue floatation bath.

B. Same specimen after reprocessing (*Note*: Lack of spreading).



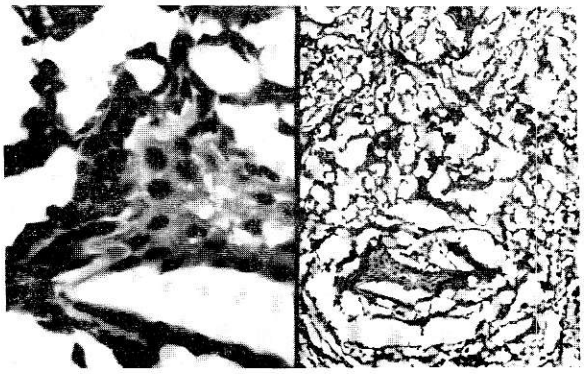
#11. Dry homogeneous effect due to exposure to excessive heat during impregnation. *Note:* This can be readily recognized by grayish-yellow coloration of various parts of section.

#12. IMPROPER PROCESSING.

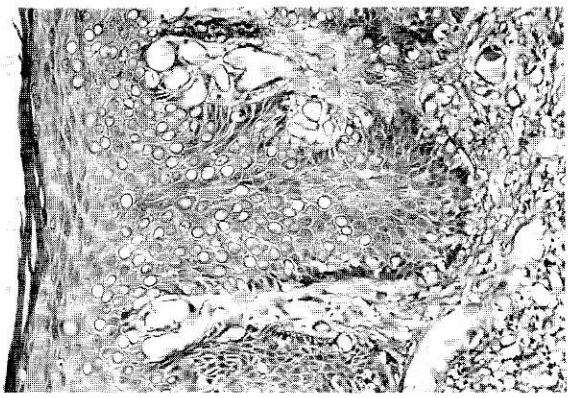


A. Note crevices and hair line separation between tissue and paraffin (arrow). The lack of proper processing of tissue will cause subsequent sectioning problems.

B. Same specimen reprocessed.

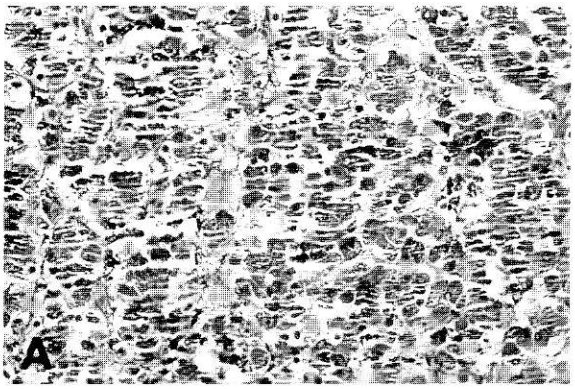


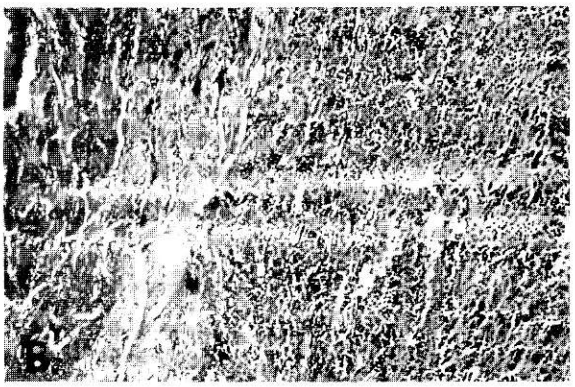
#13. This artifact simulates exposure to excessive heat, however, it is produced by the formation of ice crystals during frozen sectioning and subsequent paraffin processing.

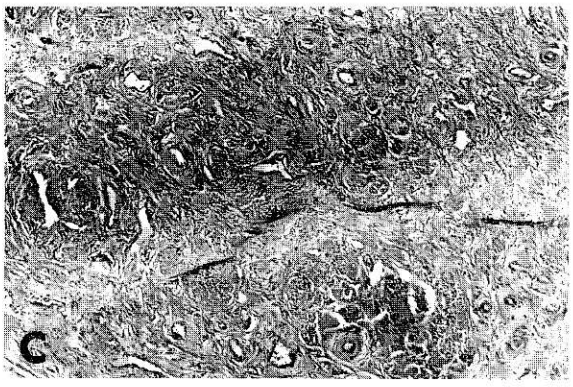


#14. A more dramatic effect is seen on this skin section. Biopsy was taken, put in formalin, then placed in a mail box with the outside temperature below freezing point. Apparently the low temperatures caused ice crystals to form within the cells and the tissues in turn became fixed. Following thawing the vacuoles remained as fixed artifacts.

#15. CUTTING ARTIFACTS.





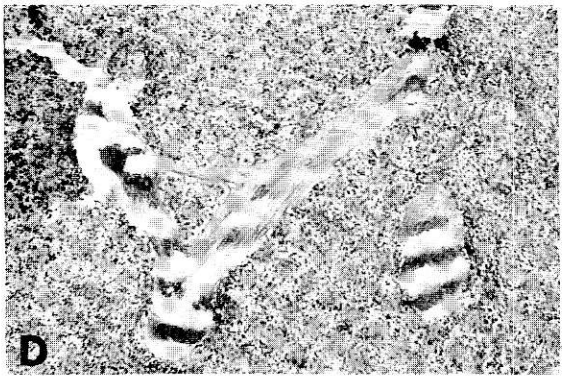


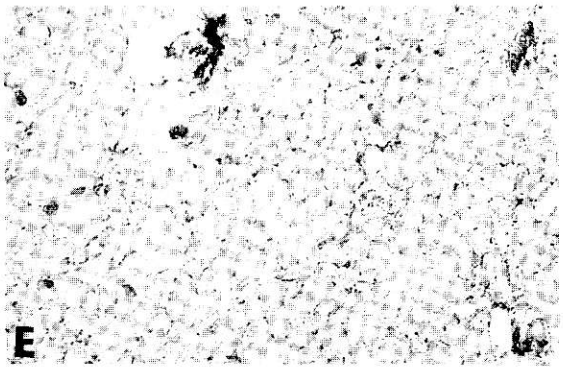
A. Shattering effect due to dull knife. *Note:* It is impossible to recognize this section of a pituitary gland due to the morphologic destruction of the individual cells.

B. Lengthwise scratches may be due to (1) nicks in knife, (2) lint on knife edge, (3) calcified area in the tissue, (4) dirty knife edge, or (5) exogenous pigments.

C. Venetian blind effect (thick and thin areas) produced when an extremely fibrous specimen is cut with a thin microtome knife.

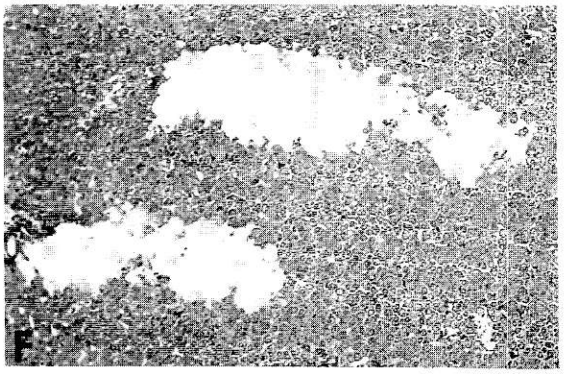
#15. CUTTING ARTIFACTS (continued)



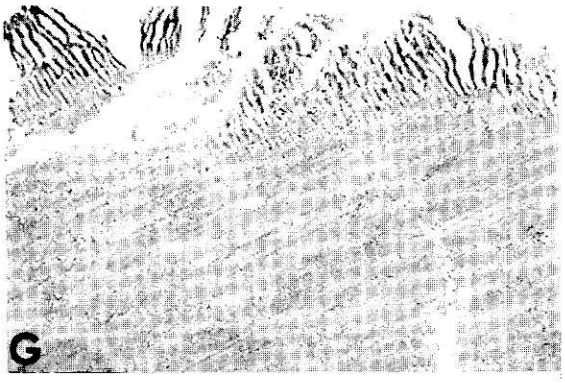


D. Thick and thin areas noted on the collagen of this section are due to microtome knife-holder loose set screw. Note surrounding tissue is not affected indicating knife was sharp.

E. The displacement of blood vessels seen in this section is due to an excessively *acute* knife angle. Note surrounding tissue is not affected indicating knife was sharp.

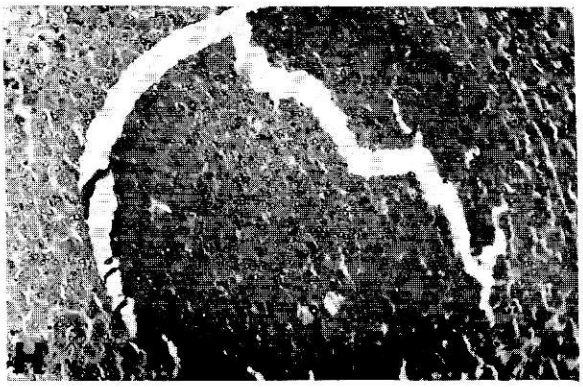


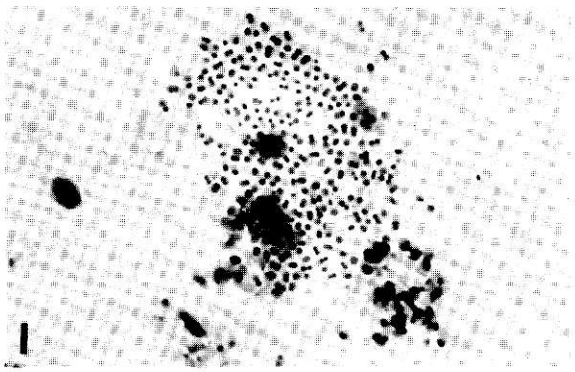
F. Moth-eaten effect is produced when specimens are over-processed causing excessive dryness. The effect is produced while rough-cutting (facing block) since the dry tissue chips out as the knife travels through the specimen. This artifact can be eliminated by soaking the block with cotton, see page 26.

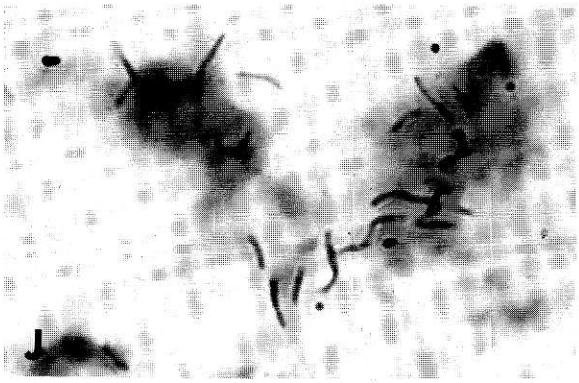


G. Excessive hardening of peripheral margins due to over-processing. Lengthwise scatches are produced when fragments of tissue from the periphery are dragged through the section.

#15. CUTTING ARTIFACTS *(continued)*



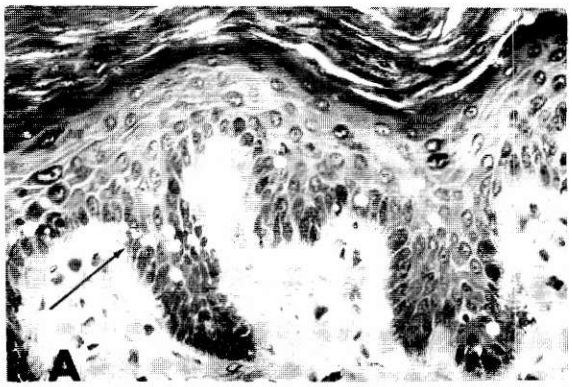


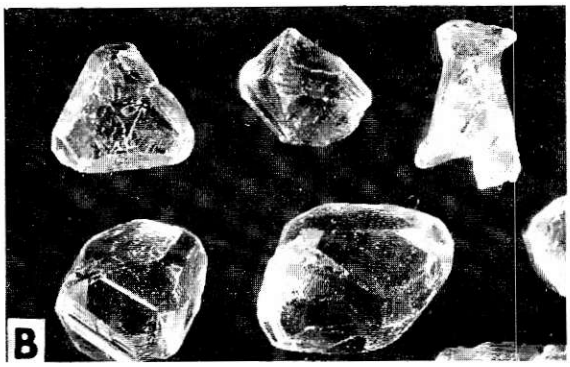


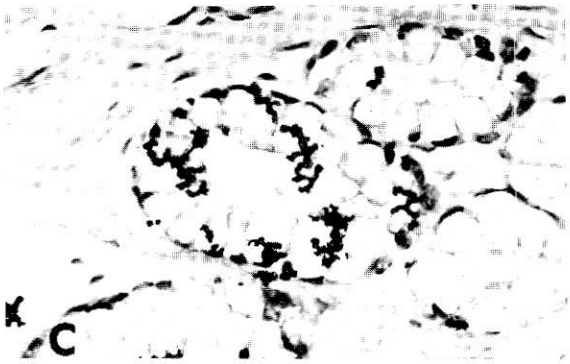
H. Air bubbles produced while laying the ribbon on the floatation bath. These bubbles can be removed by gentle manipulation with a camel-hair brush immediately after sections are picked up on slide.

I.&J. Coccoid and rod-shaped type bacteria produced by the use of gelatin in the floatation bath as a section adhesive. Artifact can be prevented by daily careful washing with good detergent of the water bath and utensils, such as camel-hair brush and forceps used to tease sections onto slides.

#16. STAINING ARTIFACTS





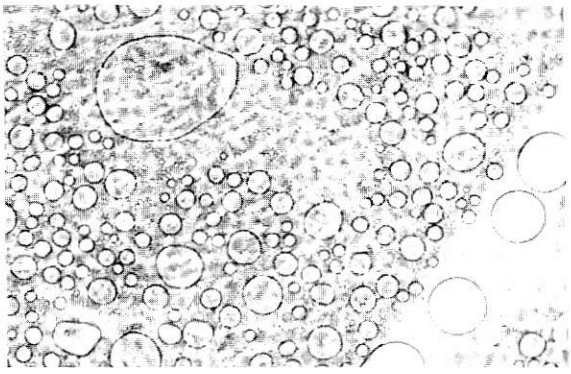


Aluminum or potassium alum crystals are produced in hematoxylin when ingredients are improperly compounded (*Note*: The alum must be mixed thoroughly when making hematoxylin).

A. Partially polarized section of skin showing alum crystals (arrow).

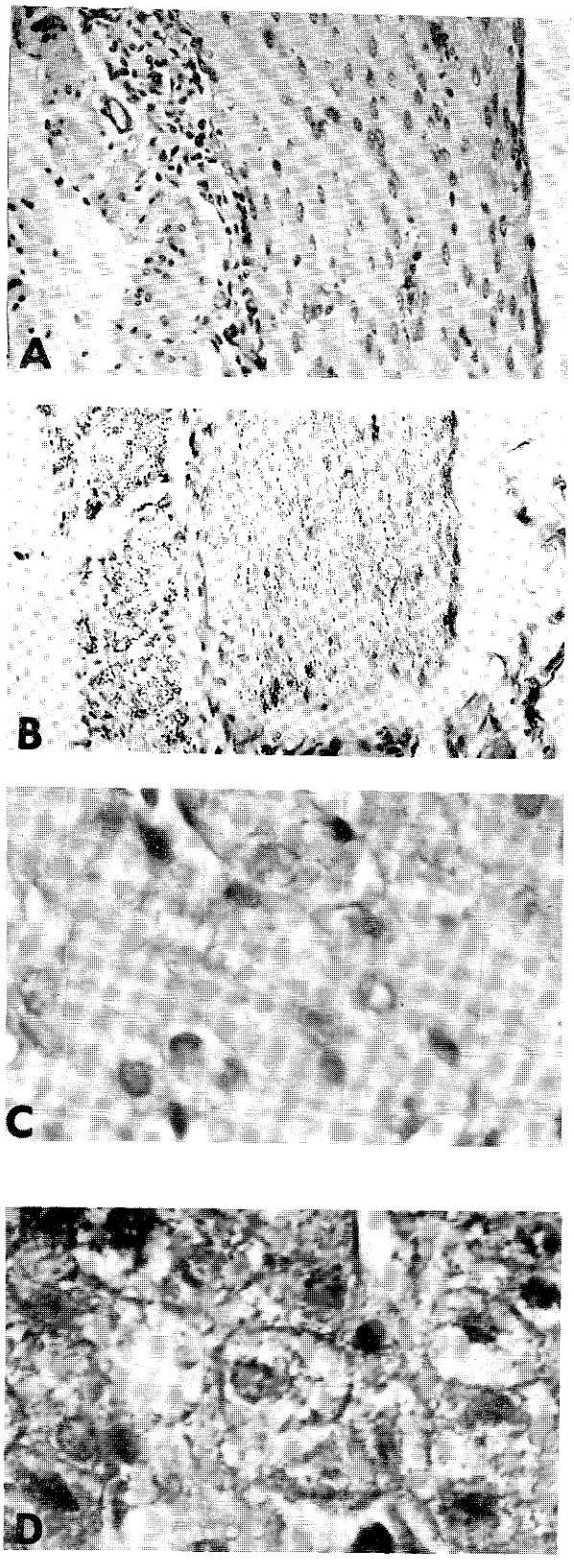
B. Alum crystals removed from bottom of container of improperly compounded hematoxylin solution.

C. Hematein crystals resulting from breakdown of hematoxylin containing alum not completely dissolved.



#17. Moisture deposited on finished slide due to water in the absolute alcohol baths.

#18. COVER SLIPPING ARTIFACTS.



A. Section mounted immediately following removal from xylol.

B. Section allowed to dry before mounting.

C. Section mounted with excessive mounting media giving cloudy result.

D. Section mounted immediately following removal from xylol with correct amount of mounting media.

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