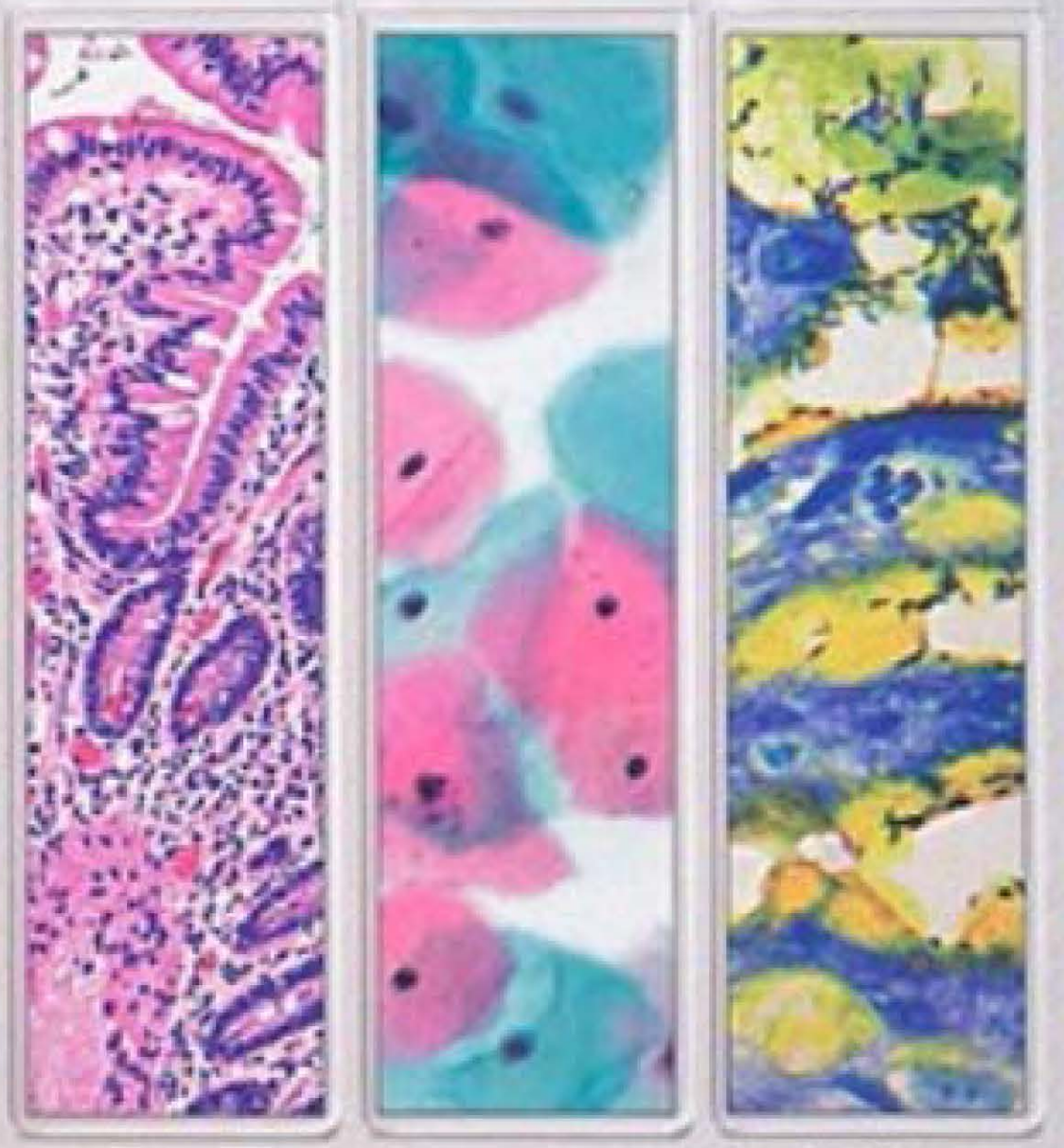
Freida L Carson

Christa Hladik

Histotechnology

A Self-Instructional Text



3rd Edition

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Cover Images

Image (left): Hematoxylin eosin (H&E)—small intestine

Image (middle): Papanicolaou—cervical smear

Image (right): Alcian yellow-toluidine blue—gastric biopsy showing *H pylori*

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Preface

The reception of the first two editions of this text has far exceeded my expectations, and I am very grateful that it has found such a welcome place in the field of histotechnology. The field has changed, especially in the areas of immunohistochemistry and instrumentation, since the publication of the second edition, and there was a need to update the text; therefore I have asked Christa Hladik, AA, HT(ASCP)^{cm}, QIHC, clinical laboratory manager, Neuropathology and Immunohistochemistry, UT Southwestern Clinical Laboratories, University of Texas Southwestern Medical Center at Dallas, TX, to join me as an author of the third edition. My experience in these areas has been limited due to my retirement several years ago. All chapters have been carefully reviewed and most have been updated or expanded. We have attempted to increase the emphasis on troubleshooting in many areas and have added numerous illustrations. We are also pleased to add a chapter on cytopreparatory techniques by Beth Cox, who is certified by ASCP as both a histotechnician and a specialist in cytology.

It is our hope that this updated edition will continue to serve as a basic guide for all students of histotechnology, or for practicing technicians, technologists, residents, and pathologists seeking to gain a better understanding of the technology utilized in the histopathology laboratory.

We are especially grateful to Agatha Villegas and Nicci Duckworth for assisting with the preliminary typing of many chapters; to Charles White III, MD, Director of the Division of Neuropathology and Immunohistochemistry and Histology Laboratories, UT Southwestern Medical Center at Dallas, TX, for assistance with photographs, chapter review, and mentoring for the immunohistochemistry and instrumentation chapters; to Dennis Burns, MD, Division of Neuropathology, UT Southwestern Medical Center at Dallas, TX, for photomicrographs; to all the staff at UT Southwestern Medical Center at Dallas, TX, who work in the Neuropathology, Immunohistochemistry, and Histology Laboratories and in the gross room at St Paul University Hospital for their assistance with tissue preparation and staining. Major contributions were made by the following: Amy Davis, HTL(ASCP), Debra Maddox, HT(ASCP)QIHC, Ping Shang, HT(ASCP)QIHC, Pattie Seward, HT(ASCP), Dawn Bogard, HT(ASCP), Courtney Andrews, HTL(ASCP), Gwen Beasley, HT(ASCP), Eva Osborn, PA(ASCP), and Chan Foong, PA(ASCP), and Steve Lee, BS, HT(ASCP).

Our thanks also go to Maureen Doran, HTL(ASCP), Chair of the Health and Safety Committee of the National Society for Histotechnology, for reviewing the Safety chapter and offering many helpful suggestions, and to Robert Lott, HTL(ASCP), who was able to provide help with images when needed.

Again, to all of you who are students of histotechnology, who continue to search for answers in this field of part art and part science, and who care first and foremost about the quality of your work on the specimens entrusted to you, we dedicate this third edition.

CHAPTER 1

Fixation

OBJECTIVES

On completing this chapter, the student should be able to do the following:

1. Define the purposes of fixation

2. Define:
   a. autolysis
   b. fixation
   c. artifact
   d. pigment
   e. nonaqueous fixative
   f. coagulating fixative
   g. additive fixative → combine protein
   h. hypertonic
   i. isotonic

3. Identify the factors that affect the quality of fixation and describe the effect of each factor on tissue (eg, temperature, size of tissue, time of fixation, or osmolality of fixative)

4. Identify the properties, functions, and actions, and determine whether each action is an advantage or disadvantage of each of the following fixative reagents or solutions:
   a. acetic acid
   b. acetone
   c. alcohols
   d. B-5 fixative
   e. Bouin solution
   f. Carnoy and methacarn solutions
   g. formalin (aqueous, buffered, neutralized, acetate formalin, formalin alcohol, calcium formalin, and formalin ammonium bromide)
   h. Gendre solution
   i. glutaraldehyde
   j. glyoxal
   k. Helly solution
   l. Hollande solution
   m. mercuric chloride
   n. Orth solution
   o. osmium tetroxide
   p. paraformaldehyde
   q. potassium dichromate
   r. Zamboni solution
   s. Zenker solution
   t. zinc formalin

5. Identify the chemicals in:
   a. B-5 fixative
   b. Bouin solution
   c. Carnoy and methacarn solutions
   d. Gendre solution
   e. Helly solution
   f. Hollande solution
   g. Orth solution
   h. Zamboni solution
   i. Zenker solution

6. Identify any special indication for use of each of the fixatives listed in objectives 4 and 5

7. Identify which fixatives require postfixation washing, and identify the preferred washing agent

8. Identify the fixation pigments and the conditions under which the pigment may be formed

9. Identify which of the fixation pigments can be prevented and which of the fixation pigments can be removed

10. For fixation pigments that can be removed, state the method(s) of removal; for fixation pigments that can be prevented, state the method(s) of prevention

11. Explain the difference between buffered and neutralized formalin

12. State how paraformaldehyde differs from formaldehyde

13. Describe the difference between the terms formalin and formaldehyde

14. Identify the percentage and volume of formaldehyde in 1,000 mL of a 10% formalin solution

15. Compare and contrast Zenker and Helly fixatives

16. List 2 methods of fixation other than using chemical reagents

17. Identify the preferred method of fixation (or lack of fixation) for
   a. enzyme histochemistry
   b. immunofluorescence
   c. skeletal muscle cross-striations (nonimmunohistochemical staining)
   d. pheochromocytomas
   e. electron microscopy
   f. urates
   g. immunohistochemical methods
   h. tissue for trichrome stains

18. Identify which fixative reagents are protein coagulants and which are noncoagulants

19. Identify which fixative reagents are additive fixatives and which are nonadditive

20. If the reagent is an additive compound, identify the site or group with which the reagent reacts (if known)

21. Describe the effect of acetic acid on erythrocytes and collagen

22. Identify any reagents that have associated safety hazards and state the hazard and any special precautions required

23. Describe the action of zinc in fixation

24. Give the 2 major problems associated with fixation, and identify at least 3 corrective actions for each

Definition

A fixative alters tissue by stabilizing the protein so that it is resistant to further changes. Baker [1958] uses the following example to explain fixation: When a door is opened, its position can be changed easily, but if the door is fixed open, it is altered in such a way that it is stabilized and is resistant to change. A fixative must change the soluble contents of the cell into insoluble substances so that those substances are not lost during the subsequent processing steps. This change occurs by either chemical (fixative solutions) or physical (heat, desiccation) means in a process called *denaturation*. Denaturation causes the protein molecule to unfold and the internal bonds to become disrupted. In the process known as additive fixation, this disruption enables the protein to combine chemically with a fixative molecule, and the protein then becomes insoluble [Feldman 1980]. With nonadditive fixatives (eg, alcohol, acetone), denaturation causes the protein to become less capable of maintaining an intimate relationship with water and to become more reactive, but the fixative molecule does not combine with the protein.

The older definition of fixative action states that a fixative kills, penetrates, and hardens tissue. Killing will be discussed in the following section. Penetration is extremely important, because adequate penetration of the fixative ensures fixation of the interior of the tissue as well as the few exterior cell layers. Hardening was a very important fixative action in the early days of microtechniques, because much of the sectioning was done freehand. Because of the array of embedding media available today, other than to make the tissue firm for grossing, the hardening action is less important.

Functions of Fixatives

One function of a fixative is to kill the tissue so that the postmortem activities of decay, or putrefaction (bacterial attack), and autolysis (enzyme attack) are prevented. Bacterial attack can be prevented in most fresh tissues by observing very strict antiseptic techniques, but autolysis cannot be prevented. Autolysis occurs because some of the enzymes present in tissue continue their metabolic processes, even after interruption of the blood supply, until something happens to stop the enzyme action. Some of these metabolic processes include breaking down cells and their components. Autolysis is a very common problem, especially if fixation is delayed in tissues that are rich in enzymes. Severely autolyzed tissue will fail to stain.

Another function of fixatives is to help maintain the proper relationship between cells and extracellular substances, such as the connective tissue fibers (collagen, reticulin, and elastin) and amorphous ground substance. This stabilization is very important during the subsequent processing steps which might otherwise distort the tissue elements. A fixative also functions to bring out differences in refractive indexes and to increase the visibility of, or the contrast between, different tissue elements. Refractive index may be defined as the ratio of the velocity of light in air to the velocity of light in a liquid or solid medium. If air and tissue had the same index of refraction, the tissue would be invisible; therefore, enhancing differences in the refractive indexes of various tissue structures will increase the contrast between those structures.

Most staining is enhanced by fixation, and frequently tissue that has not been fixed will stain poorly. Exceptions do exist, as in the masking of antigenic sites by fixation, thereby decreasing or completely obscuring antigen sites, resulting in faint or negative immunohistochemical staining. This effect can be reversed in most cases by using antigen retrieval techniques. Fixatives also aid in rendering cell constituents insoluble, with tissue proteins serving as the primary target for stabilization. Some fixatives will help stabilize or retain lipids and carbohydrates initially, but much of the time these substances will be lost in the subsequent processing. Fixation will make the tissue firmer, so that gross dissection and taking of the thin sections required for processing become much easier.

Actions of Fixatives

Although very similar to fixative functions, fixative actions can be considered a separate topic. Enzymes, which are proteins, are rendered inactive as a result of the protein-stabilizing action of fixatives. This is a very important fixative characteristic, because enzymatic action causes tissue autolysis. Tissues that are rich in enzymes, such as liver, pancreas, and brain, are more subject to rapid autolysis than those tissues with a predominance of connective tissue fibers. Fixatives also kill bacteria and molds, which cause putrefaction. Fixatives make tissue more receptive to dyes and, in many instances, act as mordants, which serve to link the dye to the tissue (mordants are discussed in chapter 6, "Nuclear and Cytoplasmic Staining," p109). Fixatives modify tissue constituents for the maximum retention of form through subsequent processing steps. This is a very important action because the steps following fixation can induce a dramatic change in the tissue. The fixative should stabilize the tissue elements so that the effect of any subsequent procedures will be minimal.

Proteins can be stabilized using various physical and chemical methods. One of the physical methods uses heat. Heat will stabilize and denature protein, as demonstrated by the cooking of an egg. Heat fixation generally has not been used in the histopathology laboratory, but with the advent of the microwave oven, it is finding much more use. Microwaves are a form of nonionizing radiation. When dipolar (charged) molecules, such as water or the polar side chains of proteins, are exposed to microwaves, the molecules oscillate, or swing back and forth, at the rate of 2.5 billion times per second. The result is molecular friction or instantaneous heat. The heat produced is controlled by adjusting the energy levels of the microwaves and the duration of exposure. Early in the process, either 1- or 2-stage microwave fixation was used. With 2-stage fixation, the first step involved fixation by immersion in saline of large specimens such as the stomach, solid organs, and intestinal segments to make the tissue sufficiently firm for gross dissection, and the second step involved the fixation of 2-mm-thick blocks immersed in saline and heated to a temperature of 50°C to 68°C [Leong] or 45°C to 55°C [Hopwood 1993]. These temperature ranges are

critical; if the temperature is allowed to exceed these ranges, or a maximum of 68°C, the tissue will show pyknotic, overstained nuclei. Hopwood [1982] stated that the denaturation of proteins that occurs with overheating can also cause a loss of enzyme activity and antigenicity, false localization of nucleic acids, and frequently, lysis of red cells. If the temperature used is too low, it will result in poor fixation. Although saline was widely used for microwave fixation initially, today the aldehydes, especially formaldehyde, are more commonly used. Microwave ovens are discussed in chapter 2, "Processing," p39, and chapter 3, "Instrumentation," pp66-68.

Desiccation is also a physical method of fixing protein, but is rarely, if ever, used in routine histopathology. Air-drying of touch preparations for Wright staining is probably the most frequent use of this method of fixation.

The primary method of stabilizing protein in the histopathology laboratory involves the use of 1 or more chemical reagents. These reagents can be classified as additive or nonadditive and coagulant or noncoagulant. Additive fixatives chemically link, or add themselves on, to the tissue and change it with this action. When a fixative molecule adds on to a tissue macromolecule, the electrical charge at the site of attachment may be changed. If the electrical charge is changed, and that charge was a force helping to maintain the conformation, or shape, of the protein, then the tertiary structure may be significantly altered. The common additive reagents are mercuric chloride, chromium trioxide, picric acid, formaldehyde, glutaraldehyde, osmium tetroxide, and zinc sulfate or chloride. Nonadditive fixatives, predominantly organic compounds such as acetone and the alcohols, act on tissue without chemically combining with it. For example, methyl and ethyl alcohols precipitate or coagulate protein but do not add to the tissue. The primary mechanism by which these fixatives act is to dissociate bound water molecules from tissue protein groups. As a result, this can cause shrinkage and hardening if overexposure occurs.

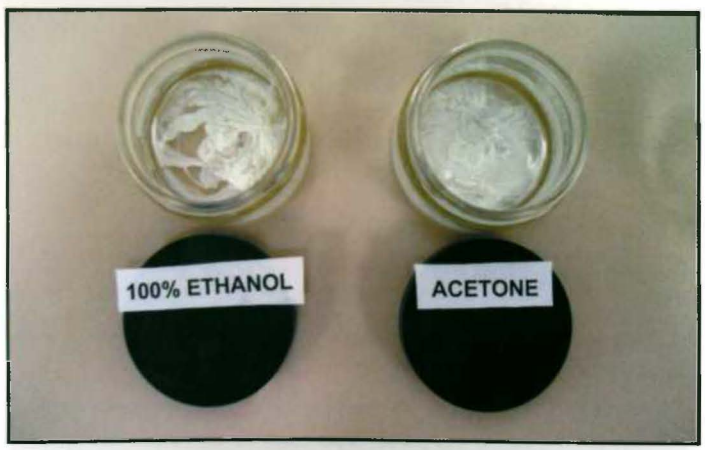
The amino ($-NH_2$) and carboxyl (–COOH) groups on the proteins are very important in staining. If the fixative adds itself to either one of these groups, the staining of the tissue will be markedly affected. At a pH of 7.0, formaldehyde adds on to tissue proteins primarily at the amino group, with the eventual formation of a methylene bridge. This results in an excess of negative charges on the proteins. The heavy metals (chromium, mercury, and osmium) are cations (positively charged) that combine with anionic (negatively charged) groups of proteins [Sheehan 1980]. This results in an excess of positive charges. Some of the groups that combine with cations are sulfhydryl (–SH), carboxyl (–COOH), and phosphoric acid ($-PO_4$). This will be discussed further under each fixative reagent.

To better understand the coagulant and noncoagulant action of fixatives, imagine 2 dishes, with 1 dish containing a piece of gelatin (eg, Jello) and the other dish containing a mesh ball. Which of the substances in the dishes do you think aqueous or alcoholic solutions would penetrate or enter most freely? An aqueous solution would easily enter all of the crevices in the mesh, but would have a difficult time entering, or penetrating, the gelatin. Coagulation establishes a network in tissue that allows solutions to readily penetrate or gain entry into the interior of the tissue. The noncoagulant fixatives act by creating a gel that makes penetration by the subsequent solutions difficult. Because the noncoagulant fixatives do not allow good penetration by the reagents applied after fixation (during processing), Baker [1958] considered these fixatives inferior for paraffin infiltration and embedding. Although the importance of this phenomenon is really seen at the microscopic level, it can be demonstrated at the macroscopic level. Wenk demonstrates this phenomenon with students as follows: take small jars with lids (50-mL beakers will also work) and put 20 mL of a different fixative in each jar; label carefully. Use whatever fixatives are readily available in the laboratory, but be sure to include 10% formalin, aqueous zinc formalin, acetone, alcohol, and acetic acid. Separate a raw egg at room temperature, saving only the white, which is protein; pipette 2 mL of the egg white into each fixative solution. See what happens by watching the change in consistency of the egg white, and the time frame for any changes to occur **[i1.1]**, **[i1.2]**, **[i1.3]**.

The coagulant fixatives are zinc salts, mercuric chloride, cupric sulfate, ethyl alcohol, methyl alcohol, acetone, and picric acid. Baker [1958] classified acetic acid as a coagulant of nucleic acids, but a noncoagulant of cell cytoplasm; however, Wenk [2006] found that acetic acid acted as a coagulant of egg white. The noncoagulant fixative reagents are formaldehyde, glutaraldehyde, glyoxal, osmium tetroxide, and potassium dichromate. For use after a noncoagulant fixative, infiltration or embedding media other than paraffin (eg, plastics) work best.

A summary of fixatives categorized by composition and properties is shown in **[f1.1, p 4]**.

Knowledge of fixatives and fixation has evolved over time, beginning with the biologic effects of mercury and its salts dating back to Hippocrates [Bancroft 1982]. Wine, or alcohol, also has long been recognized as a preservative. Many fixatives that differed only slightly were developed in the 19th century. Gray [1954] listed more than 500 fixatives, with only a few of these being widely accepted. Baker [1958] introduced the convention of naming the fixative solution after its first user and disregarding any minor modifications.



[i1.1] The egg white hardens and turns white almost immediately in the 100% alcohol and in the acetone, similar to raw egg on a hot skillet. The photograph was taken 10 minutes after the egg white was placed in these two solutions, but the change was seen within 1 minute. Within 2 hours, the egg white was so hard it was brittle and would break apart when touched with a wooden stick. (Reprinted with permission from Wenk [2006])

[il.2] In the Bouin solution and the zinc formalin, the egg white hardens a little slower than it does in pure ethanol or acetone, but the egg white has a consistency of a soft-boiled egg after 10 minutes of fixation. There is less hardening in these fixatives than in the pure ethanol or acetone. Hollande solution, mercuric fixatives, and 100% acetic acid behave similarly. (Reprinted with permission from Wenk [2006])

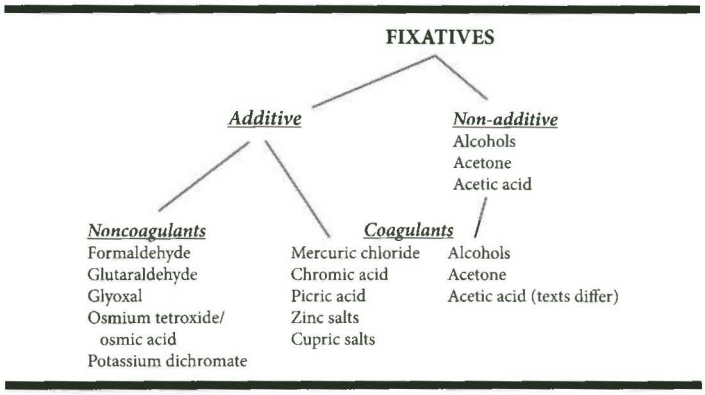
[il.3] The egg white is at the bottom end of the wooden sticks but cannot be seen. The egg whites have not changed color or hardened in the first 10 minutes. The egg white in the 10% formalin looks and acts as if it has been put into room-temperature water; it continues to have the same consistency as raw egg white. Even by the next morning it is not visible, has not hardened, and is not dissolved; it remained clear and could be swirled. Egg white in glutaraldehyde behaves similarly. In the alcoholic formalin, the egg white remains the same for the first few hours, but eventually the 70% alcohol causes the egg to slightly turn white in some areas and harden slightly; however, the alcoholic formalin never hardens the egg completely, no matter how long it is in this fixative. (Reprinted with permission from Wenk [2006])

Factors Affecting Fixation

Fixation factors are those elements that affect the quality of fixation; most of them are easily controlled.

TEMPERATURE

The temperature at which fixation is carried out may affect tissue morphology. In general, an increase in temperature increases the rate of fixation but also increases the rate of autolysis and diffusion of cellular elements. Traditionally, 0°C to 4°C has been considered the ideal temperature for the fixation of specimens



[f1.1] A summary of the additive vs nonadditive fixatives, and coagulant vs noncoagulant fixatives.

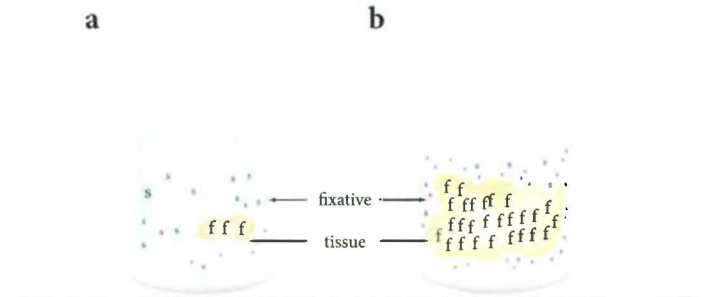
for electron microscopy; however, some laboratories have moved away from the use of cold fixation. Some parts of the cell are less affected when formaldehyde fixation is performed at room temperature instead of refrigerator temperature, and we prefer the ultrastructural preservation yielded by room temperature fixation [Carson 1972]. Today higher temperatures are being used for fixation in both tissue processors and microwave ovens; in general, increasing the temperature of the fixative up to about 45°C is reported to have very little effect on tissue morphology.

SIZE

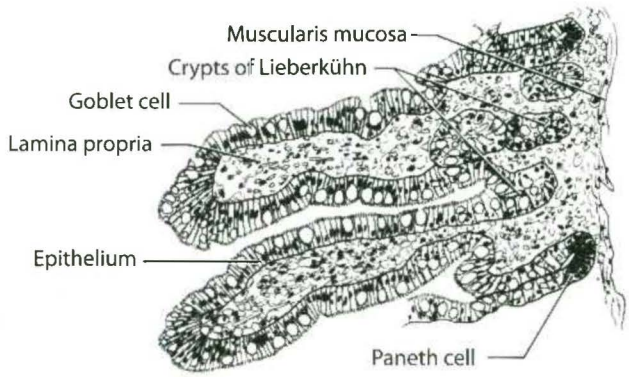
The thickness of the tissue is especially important because of its effect on reagent penetration. Size should be considered when the gross tissue specimens are placed in fixative. If large specimens such as segments of colon or small intestine are held for any extended period without being surgically opened to expose all layers, the fixative will have difficulty penetrating through the entire wall to the inner epithelial surface. The result frequently is autolysis of the epithelium; therefore, specimens of this type should be opened before they are placed in fixative solution. A more common consideration is the size of the sections cut for processing. For routine processing schedules, sections should be no more than 3 mm thick. When processing on a short protocol, the sections must be even thinner or the reagents will not completely penetrate the section. At no time should a section be so thick that it touches both the top and bottom of the tissue-processing cassette.

VOLUME RATIO

The ratio of the tissue volume to the fixative volume is one of the fixation criteria over which there may be limited control. The fixative volume should be at least 15 to 20 times greater than the tissue volume. Effects of many fixatives are additive; fixative molecules are bound chemically to the tissue, and the solution is gradually depleted of these molecules. Tissue also contains soluble salts that are dissolved by the fixative solution. The "2-way exchange" does not greatly alter the characteristics of the fixative if a large volume ratio is used **[f1.2a]**; however, if the volume of the tissue is greater than that of the solution **[f1.2b]**, the fixative composition can be altered; therefore, the volume ratio is a very important consideration. Frequently, staining problems are really the result of poor fixation because of the use of an inadequate volume of fixative.



[f1.2] Soluble salts "s" are dissolved out of the tissue into solution in the fixative, whose molecules "f" attach to the tissue, decreasing fixative concentration. This is of little consequence if the fixative-to-tissue ratio is large **a**, but the fixative's composition can be markedly altered if the ratio is small **b.**



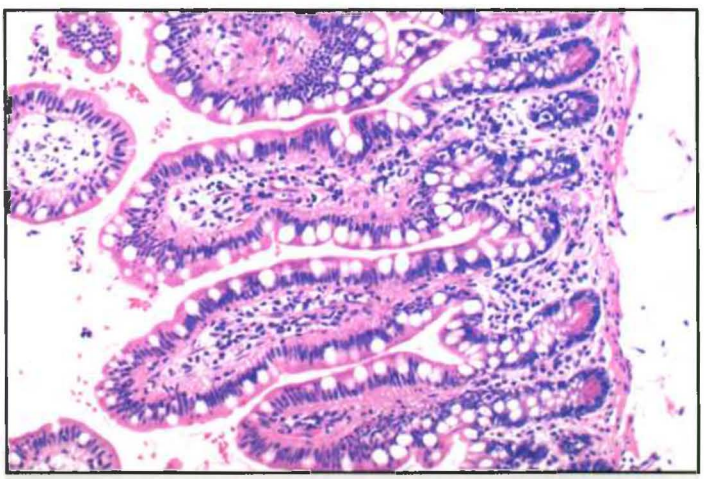
[f1.3] A section of small intestine.

TIME

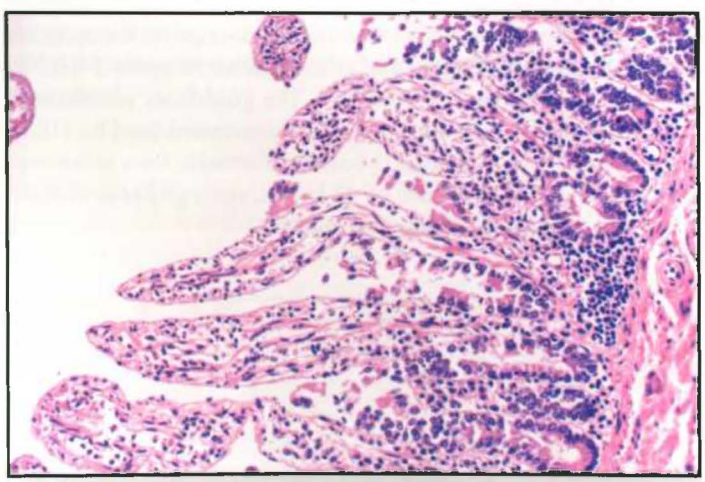
Time is important in 2 respects. The first consideration is the interval between interruption of the blood supply and placement of the tissue in fixative. Ideally, the tissue should be placed in fixative immediately after surgical removal, and autopsies should be performed immediately after death. The more time that elapses between interruption of the blood supply and fixation, the more postmortem changes that can be demonstrated microscopically. **[f1.3]** and **[i1.4]** illustrate well-preserved tissue, and **[i1.5]** illustrates postmortem changes in the same type of tissue.

The cellular detail that can be seen in a well-preserved section of small intestine is illustrated in **[f1.3]**. The outer, relatively monotonous layer of cells is the epithelium, which is defined as a membrane that covers or lines. Notice that the section shows 2 fingerlike projections of the small intestine. These fingerlike projections are called villi, and they are a very distinctive feature of the small intestine. When you see them, you can confidently identify the tissue as small intestine. Intestinal epithelium is composed of a row of simple columnar cells and an occasional goblet cell, 1 of which is identified. Between the fingerlike projections are crypts with cells containing an abundance of secretory granules that usually stain a deep red with eosin; these are Paneth cells. The tissue underlying the epithelium is called the lamina propria. It contains connective tissue cells and fibers, very small blood vessels, and nerve twigs. Although none is illustrated, an aggregate of lymphocytes called a lymph nodule will be present occasionally. Underlying the lamina propria is a layer of smooth muscle, the muscularis mucosa. The epithelium, the lamina propria, and the muscularis mucosa form the mucosa, the first of 4 layers common to the gastrointestinal tract. Because autolytic changes and bacterial decomposition are most pronounced on the mucosa, only this layer is described.

A section of small intestine in which all of the structures identified thus far are well preserved is shown in **[i1.4]**. This section was taken from a surgical specimen that was opened and placed in fixative solution immediately after removal. Therefore, the fixative had early contact with the epithelium and was able to penetrate from both the epithelial and the serosal (outermost) surfaces. This rapidly halted the postmortem changes of autolysis and putrefaction. Notice the well-preserved epithelium and compare this illustration with **[i1.5]** in which the epithelium is entirely gone except in a few deep glands. The lamina propria is completely denuded, a



[i1.4] The mucosa is excellently preserved in this section of small intestine. Note that autolysis is absent and the epithelium is intact.



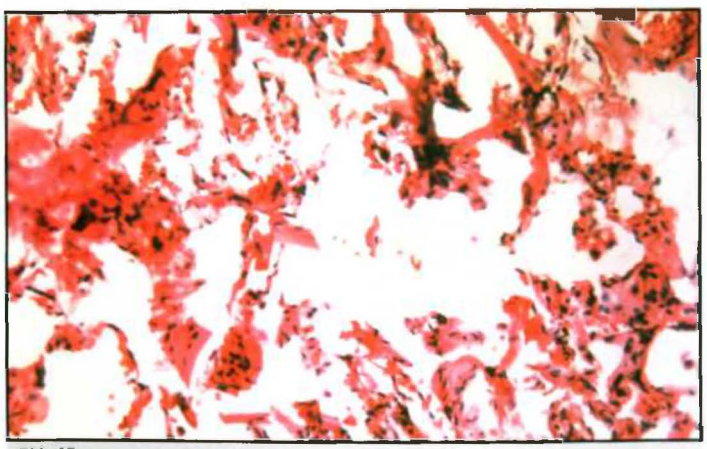
[i1.5] Fixation of this section of small intestine taken at autopsy was delayed. Marked autolysis has occurred, and except for a few glands, or in the crypts, the epithelium is gone. Most of the goblet cells and the argentaffin cells have disappeared. Only the denuded lamina propria of the villi can be seen.

common finding in autopsy sections of the gastrointestinal tract. This section is autolyzed; autolysis will cause desquamation of the epithelium and separation from the basement membrane [Leong 1994]. Because of the bacterial content of the gastrointestinal tract, some of the changes seen in this section are probably the result of putrefaction. When selecting control tissue, one must be very

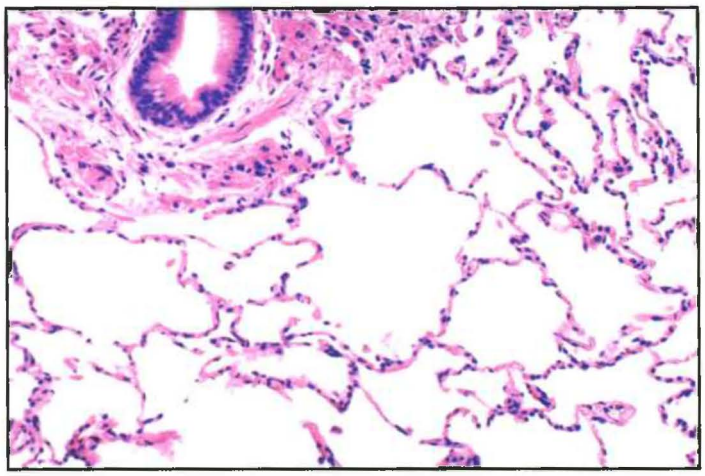
careful about using autopsy tissue. For example, the tissue shown in **[i1.5]** would not be a good control for mucin stains, because the mucin-containing goblet cells have not been preserved.

The duration of fixation is also important. The current trend of decreasing the time allowed for fixation is resulting in many problems. Adequate fixation is needed so that the tissue will not be distorted by the subsequent processing steps. **[i1.6]** shows a tissue specimen that is difficult to identify. It is a section of lung that was not well fixed, and proper relationships of tissue structures have not been maintained during the subsequent processing steps. **[i1.7]** shows a well-fixed section of lung. Tissue that is not well fixed does not process well, and subsequently will not stain well, so adequate fixation time is of primary importance in quality assurance. **[i1.8]** also contrasts poorly fixed, undifferentiated tumor tissue with a well-fixed tissue section from the same tumor **[i1.9]**. The latter section shows nuclear bubbling that is commonly attributed to fixation with formalin alone [Banks 1985]; however, Dapson [1993] attributes it to the specimen not being completely fixed before dehydration is begun. Formalin should have at least 6 to 8 hours to act before the remainder of the processing schedule is begun. Dapson [2004] reported that in a carefully controlled study in his laboratory, artifact-free sections could be produced only after a minimum of 30 to 40 hours of fixation with neutral-buffered formalin, and marked artifacts were present after only 7 hours formalin exposure. Much of the processing occurring today takes place in the dehydrating alcohols, because not enough time is allowed for fixation to occur in the fixative solution. Dapson also stated that with proper fixation, the tissue is almost immune to artifacts; whereas, with incomplete fixation, the specimen is vulnerable to the effects of any subsequent denaturing agent, be it chemical or physical.

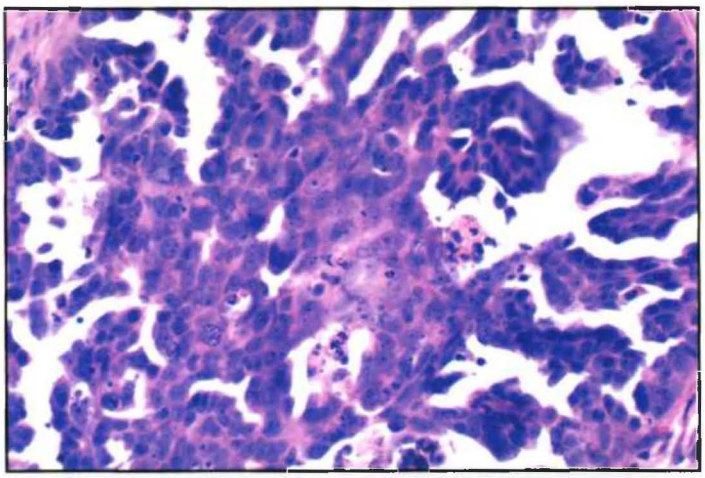
The importance of time in fixation was stressed, when in 2007, the American Society of Clinical Oncology and the College of American Pathologists released guidelines to improve the accuracy of testing for human epidermal growth factor receptor 2 (HER2) in invasive breast cancer [Wolff 2007]. The guidelines recommend that the incisional and excisional biopsy specimens used for HER2 testing be fixed in 10% neutral-buffered formalin for a minimum of 6 hours and a maximum of 48 hours, stating that prolonged fixation may show false-negative results.



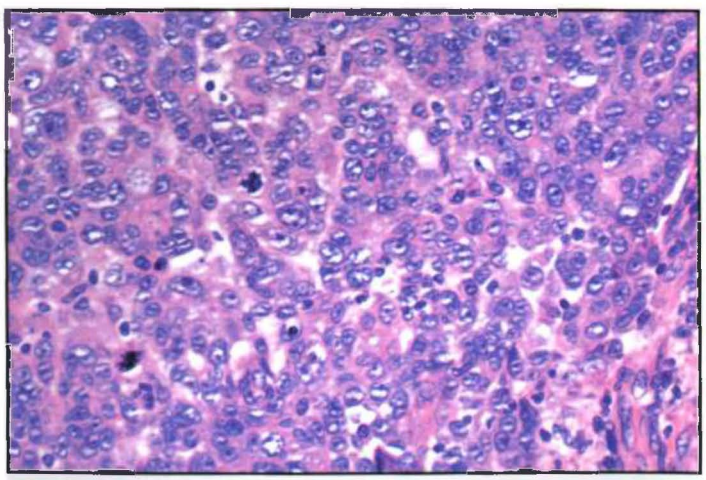
[i1.6] A section of lung that was not completely fixed before processing shows poor stabilization of the tissue structures, and proper relationships are not maintained.



[i1.7] A section of lung that was well-fixed before processing shows the proper relationships of tissue structures. The interalveolar septa are well preserved and the alveolar sacs are clearly seen. A bronchiole with well-preserved epithelium is seen in the upper left corner.



[i1.8] A section of an undifferentiated tumor that has not been well fixed shows that the proper relationship of cellular elements has not been maintained. The staining is poor, with a lack of contrast between the cell nucleus and cytoplasm.



[i1.9] A section from the same tumor as seen in **[i1.8]** that has been well fixed in 10% neutral-buffered formalin. The nuclei show the "bubbling artifact" frequently associated with formalin fixation. Note that the contrast between the cell nucleus and cytoplasm is much better, and crisp nuclear membranes are demonstrated.

While tissue must be left in most fixatives for an adequate length of time to achieve good fixation, tissue cannot remain indefinitely in many fixatives. Tissue must be removed from fixatives such as glutaraldehyde, Helly solution, Zenker solution, and Bouin solution; washed if indicated; and then stored in an appropriate storage solution. If allowed to remain in these fixatives too long, the tissue becomes overhardened and staining may be impaired.

CHOICE OF FIXATIVE

The broad range of fixative choices requires the technician to stop and think, on receipt of the specimen in the laboratory, about which fixative is appropriate. If tissue is improperly fixed for a given technique, frequently no corrective action is possible. Therefore, immediately upon presentation of the specimen, the method of fixation must be chosen. Sometimes no fixation is desired; if an immunofluorescence study or an enzyme profile is needed, the specimen must be frozen without fixation. Although some enzymes can be demonstrated on frozen sections that have been fixed, other enzymes are rapidly inactivated by even brief contact with a fixative. Some antibodies used in immunohistochemical procedures require that tissue be frozen, sectioned, and then left unfixed or briefly fixed in acetone. A more comprehensive discussion of tissue fixation for immunohistochemical studies is found in chapter 12, "Immunohistochemistry," p279.

Often, a particular fixative must be chosen to ensure optimal demonstration of a particular tissue element, such as the choice of Zenker solution when muscle cross-striations are to be stained with phosphotungstic acid-hematoxylin (PTAH) or Bouin solution when the tissues are to be stained with a trichrome technique. To increase the staining reaction, a microscopic section of tissue that has been fixed with 1 reagent frequently can be treated with another fixative reagent. This process is called postfixation or mordanting, and is used in the Masson trichrome technique, in which a microscopic section of formalin-fixed tissue is mordanted with Bouin solution before staining. Although postfixation gives very good results with the Masson technique, superior staining can be achieved with some techniques only when the tissue is fixed appropriately at the outset. Some tissue elements cannot be demonstrated if the original fixation is incorrect. For example, the demonstration of chromaffin granules, found in cells of the adrenal gland, is helpful in the identification of pheochromocytomas, but these granules cannot be demonstrated after formalin fixation. For the subsequent demonstration of chromaffin granules, tissue must be fixed in a primary dichromate fixative such as Orth solution. Urate crystals are water-soluble and require a nonaqueous fixative such as absolute alcohol. The proper fixative also must be used if electron microscopy or ultrastructural studies are required.

PENETRATION

Fixative solutions penetrate at vastly different rates. According to Baker [1958], the factors that determine the minimum length of time that a fixative should act are the rate of penetration and the mode of action. Most coagulant fixatives achieve their full effect on tissue at any particular depth as soon as they have penetrated to that depth at a concentration sufficient to cause coagulation. Formaldehyde, a noncoagulant fixative, penetrates fast, but continues to cross-link proteins for a long time after the penetration is complete. In fact, according to Baker [1958], formaldehyde penetrates faster than any of the common fixative ingredients. Fixatives in order of decreasing speed of penetration are as follows: formaldehyde, acetic acid, mercuric chloride, methyl alcohol, osmium tetroxide, and picric acid. Although the information is not available, ethyl alcohol probably penetrates at a rate similar to methyl alcohol. The rate of penetration is affected by heat, but not by the concentration of the fixative. Because fixation begins at the periphery of the tissue and proceeds inward, most of the interior fixation of larger specimens may be due primarily to only 1 chemical in a compound fixative.

TISSUE STORAGE

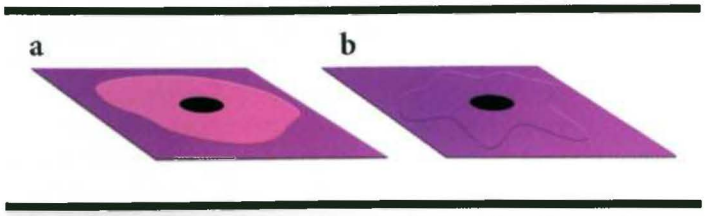
The method of wet tissue storage is very important because the wet tissue often will be needed for additional studies. If the tissue has not been fixed and stored properly, additional studies may be impossible. Storage is not usually a problem with tissue fixed in neutral-buffered formalin because the tissue may remain in this solution indefinitely; this is not true of many other fixatives. However, if immunohistochemical stains are anticipated at a future time, tissue should be transferred from formalin to 70% alcohol to stop cross-linking. Appropriate storage is described in the individual sections on each of the more common fixative solutions.

pH

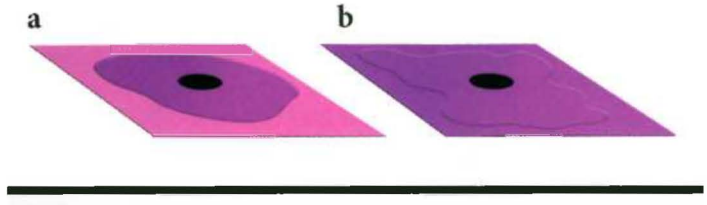
The pH of the fixative is not very important in light microscopy and many fixatives are quite acidic. Varying the pH from 4 to 9 apparently makes little difference in the fine structure produced by formalin fixation; however, a pigment is produced at a lower pH. The pH of the fixative solution is very important in electron microscopy. When ultrastructural preservation is the main purpose of fixation, the solution should be buffered to a pH of 7.2 to 7.4. This is a physiological pH, that is, approximately the pH of tissue fluid.

OSMOLALITY

Osmolality refers to the number of particles in solution and is not as important in light microscopic studies as in ultrastructural studies. Body fluids have an osmolality of about 340 mOsm or 0.3 Osm. A 1-Osm solution may be defined as 1 formula weight of a nondissociating compound (eg, sucrose) per 1,000 g of solution. 1 formula weight of a dissociating compound (eg, sodium chloride) per 1,000 g of solution is equal to a 2-Osm or 2,000-mOsm solution. The terms isotonic, hypotonic, and hypertonic are used frequently; normal (isotonic, physiological) saline solution is sometimes used in histopathology as a holding solution for tissue. What does this mean and why is it important? **[f1.4a]** shows a cell in a solution that is more concentrated or contains more particles than the cell cytosol; this solution is hypertonic to the cell. The cell membrane (plasma membrane) is a semipermeable membrane that allows water molecules to pass through it very readily. Water passes through the cell membrane toward the most concentrated solution in an effort to equalize the concentrations on both sides of the membrane. When surrounded by a hypertonic solution, the water leaves the cell and the cell



[f1.4] The effect of hypertonic solution on cells. **a** A cell in a hypertonic solution; **b** The cell showing shrinkage because water was drawn from the cell into the surrounding solution.



[f1.5] The effect of hypotonic solution on cells. **a** A cell in a hypotonic solution; **b** The cell showing swelling because water was drawn from the surrounding solution into the cell.

shrinks **[f1.4b]**. If the cell is placed in a hypotonic solution or one that contains fewer dissolved particles than the cell cytosol **[f1.5a]**, the cell swells, possible rupturing its membrane **[f1.5b]**.

Often it is the osmolality of the fixative vehicle, or the solution exclusive of the fixative ingredient, that is critical. Water is the most rapidly penetrating component of an aqueous fixative, so the central parts of a specimen are probably in contact with a hypotonic solution before fixation occurs. Unreactive salts with small rapidly diffusing ions (eg, sodium sulfate or sodium chloride) frequently are added to fixative mixtures to prevent the damage caused by these hypotonic solutions [Kiernan 1999]. Formaldehyde is not osmotically active, so although 10% neutral-buffered formaldehyde solutions appear to be very hypertonic (approximately 1,800 mOsm), most of the tonicity is related to the osmotically inactive formaldehyde molecules.

As mentioned before, physiological saline solution can be used as a holding solution, and other isotonic solutions with a salt composition more closely approximating that found in body fluid also may be used. However, even though they are isotonic, these solutions are not without effect on the tissue and should not be used for prolonged holding of tissue. For biopsy specimens that cannot be placed in fixative immediately, it is probably a better practice to dampen a piece of gauze with saline solution, squeeze out the excess, and place the tissue on the dampened gauze. Tissue treated in this way can be sealed in a plastic container and placed on ice for short-term holding. Kidney biopsy specimens for immunofluorescence frequently are held, or even mailed, in Michel transport solution. The formula and directions for use are given on p23,.

The factors that influence fixation are very important in quality assurance because improper fixation cannot be corrected in subsequent processing steps; instead, these subsequent steps further differentiate the products of fixation.

Reactions of the Cell with Fixatives

THE NUCLEUS

Deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and attached protein are found in the nucleus. Much more is known about the effect of fixatives on proteins than on nucleic acids. Although several fixatives are used for nucleic acids, most fixatives do not appear to react chemically with them. Acetic alcohol and Carnoy solution are the preferred fixatives for nucleic acids; formaldehyde does not react with DNA and RNA in their native states until the temperature reaches about 45°C for RNA and 65°C for DNA [Hopwood 1993]. Much of nuclear fixation is probably entrapment of RNA and DNA molecules by the fixed or stabilized nuclear proteins. Banks states that the coagulating or precipitating fixatives render tissue more resilient to the disruptive effects of sectioning, deparaffinization, and staining. This results in shaper, more intact-appearing nuclei. Following formalin fixation, the nuclei often show coalescence of the chromatin into strands with intervening clear spaces. This has been called nuclear bubbling **[i1.9, p6]**; Banks states that nuclear bubbling is introduced in the deparaffinization step on formalin-fixed tissue, because the nuclei are only delicately fixed.

PROTEINS

Most nonnuclear staining occurs because of the proteins present and the particular chemical group or groups with which a fixative reacts. Proteins have a primary, secondary, and tertiary structure. The primary structure is determined by the arrangement of covalent bonds in the amino acid sequence. The secondary structure is determined by hydrogen bonding between various components of the peptide chain, and the tertiary structure is defined as the total 3-dimensional structure. Hydrogen bonds, ionic (electrostatic) bonds, hydrophobic bonds, and disulfide bonds are responsible for the tertiary structure of a protein [Pearse 1980]; these folded conformations are generally very fragile. Additive fixatives can alter the 3-dimensional shape of proteins by changing electrical charges at the site of attachment.

The nonadditive, coagulant fixatives cause proteins to become insoluble by altering their tertiary structure. Pearse [1980] states that methanol and ethanol preserve the secondary structure of proteins while markedly affecting their tertiary structure. The isoelectric point of the proteins may be shifted by the reaction. If they are known, the sites of fixative attachment will be pointed out as each fixative is described. The effects of the attachment on hematoxylin and eosin (H&E) staining are also be discussed in chapter 6, "Nuclear and Cytoplasmic Staining," p114.

LIPIDS

While several of the fixatives will preserve lipids, only 2 chemicals will fix lipids so that they are not lost in the subsequent processing steps. These are osmium tetroxide and chromic acid. The chemical reactivity of lipids is altered by both of these reagents.

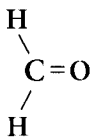
CARBOHYDRATES

Some carbohydrates are lost during fixation, and with many fixatives, the retention of glycogen, the storage form of glucose (blood sugar), is thought to result from entrapment by the fixed proteins.

Simple Aqueous Fixatives or Fixative Ingredients

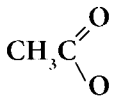
To understand the compound fixatives that constitute the majority of fixatives, we must understand the properties, functions, and actions of each of the individual ingredients. The following are the water-based, or aqueous, fixatives ingredients that are discussed in this text.

1. Acetic acid
2. Formaldehyde (formalin alcohol is included)
3. Glutaraldehyde
4. Glyoxal
5. Mercuric chloride
6. Osmium tetroxide
7. Picric acid
8. Potassium dichromate
9. Zinc salts
10. Others

ACETIC ACID | 

Acetic acid, in a dilute form, is a common household chemical. Vinegar contains about 5% acetic acid and has been used in pickling for many years; however, its use in microtechnique dates back only about 100 years. Concentrated acetic acid is called glacial acetic acid because of its freezing point of 16.6°C. Acetic acid does not fix or destroy carbohydrates, and it does not fix lipids. It penetrates very rapidly and leaves tissue very soft. The major use of acetic acid in fixatives is the precipitation and preservation of nucleoproteins; it is added to many fixative mixtures because of its ability to fix nuclei. Acetic acid also precipitates DNA. Baker classified acetic acid as a noncoagulant, stating that its precipitating action on nucleoproteins is different from that on the nonnuclear proteins. It increases protein swelling more than any other fixative. This is a decided disadvantage, but acetic acid is sometimes added to other fixatives to counteract the shrinking effect of another reagent. Swelling is characteristic of acid fixation as long as the solution pH is below 4.0. Collagen swells dramatically near a pH of 2.5 because links in the proteins are broken and any hydrophilic (water-loving) groups present are exposed. As a result, water is absorbed and the collagen swells. Other short-chain acids such as formic, propionic, and butyric acid behave in much the same way as acetic acid; however, these other acids have found little use in fixatives. Red blood cells are lysed by acetic acid, and thus their preservation is poor in any fixative containing acetic acid.

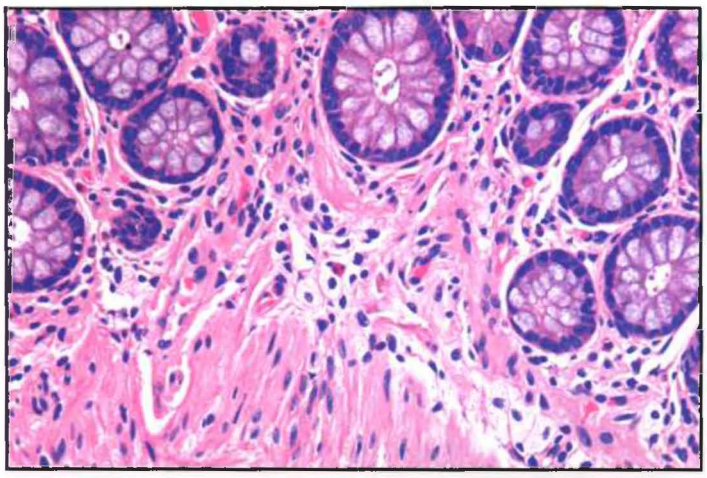
Acetic acid should be stored at room temperature and away from strong oxidizers, nitric acid, and strong caustics. Acetic acid can cause severe burns and has a permissible exposure limit of 10 ppm; therefore it should be transported in an acid carrier and used under a hood. As with all acid dilutions, acetic acid should be added to water, and never the other way around.

FORMALDEHYDE | 

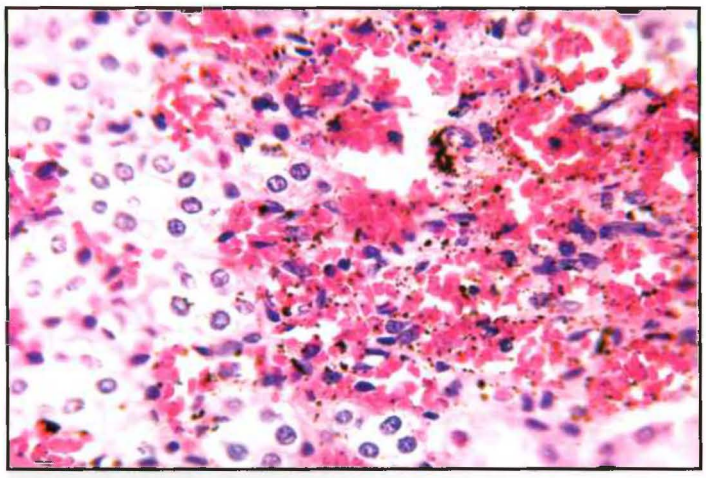
Formaldehyde was introduced in 1893, later than any of the other important fixatives used in microtechnique [Baker 1958]. While using formaldehyde as an antiseptic, Blum accidentally discovered its fixative properties. Formaldehyde is a colorless gas commonly obtained in the histopathology laboratory as a 37% to 40% solution in water. The terms formaldehyde and formalin have been used rather loosely in some of the literature, resulting in a gray area in terminology. Baker [1958] refers to the 37% to 40% solution as "formalin," but bottles obtained from manufacturers are labeled "formaldehyde." It would appear that in the United States today, when the term formaldehyde is used, the actual formaldehyde content is considered. Commercial solutions, or stock solutions, are 37% to 40% formaldehyde but are considered to be 100% formalin. To prepare a 10% formalin solution, we dilute 1 part of the stock solution with 9 parts of water. The resulting solution is 10% formalin or 3.7% to 4.0% formaldehyde. "Formol" and "formal" have also been used in naming some solutions found in older reports, but these terms are not used today except when referring to some of these older solutions, such as calcium-formol.

Ten percent formalin is the most commonly used fixative and is probably one of the most valuable, even though it is not considered the best fixative for subsequent paraffin infiltration [Baker 1958], and there are some safety concerns with its use. In aqueous solution, formaldehyde combines chemically with water to form methylene hydrate ($HO\text{-}CH_2\text{-}OH$). This compound has the same reactivity as formaldehyde; it has a strong tendency to polymerize to dimers and trimers. Only a small percentage of the formaldehyde or methylene hydrate, is present as a monomer in 37% to 40% solutions, but the monomer predominates in 10% solutions. Paraformaldehyde, a highly polymeric form of formaldehyde, may be deposited as a white powder in concentrated solutions. Commercial 37% to 40% formalin contains 10% to 14% methanol added by the manufacturer to help prevent this polymerization.

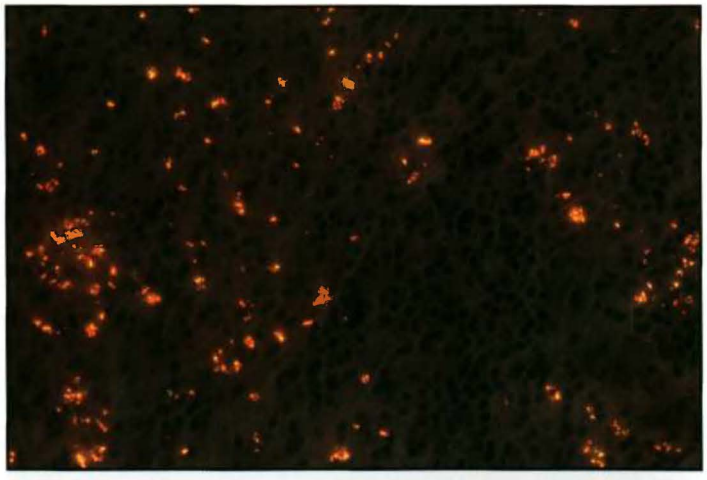
Paraformaldehyde is used in many electron microcopy laboratories for the preparation of fixative solutions because it yields a pure formaldehyde solution; the methanol added to commercial formaldehyde solutions is a coagulant and has been considered undesirable for ultrastructural studies. For depolymerization to



[il.10] This section of gastrointestinal tract was fixed in formalin. It was stained at the same time as the section shown in **[il.19, p 21]**, which was fixed in Zenker solution. Note the difference in cytoplasmic staining due to the attachment of fixative groups to different cytoplasmic groups; formalin decreases the affinity for eosin by attaching predominantly to the amino group, while Zenker solution increases the affinity for eosin by binding at more acidic sites.



[il.11] Formalin pigment is seen in a blood-rich area of the section of kidney. Formalin pigment is a brown microcrystalline pigment that tends to form in tissues when the pH of the formalin solution drops below 6. It is caused by a reaction between the heme part of hemoglobin and the formic acid present in acidic formalin solutions; the pigment is also called black acid hematin. In some sections, formalin pigment may be confused with anthracotic pigment, malarial pigment, and rarely melanin.



[il.12] A section of spleen containing formalin pigment examined by polarization, which provides a good screening method for the presence of formalin pigment.

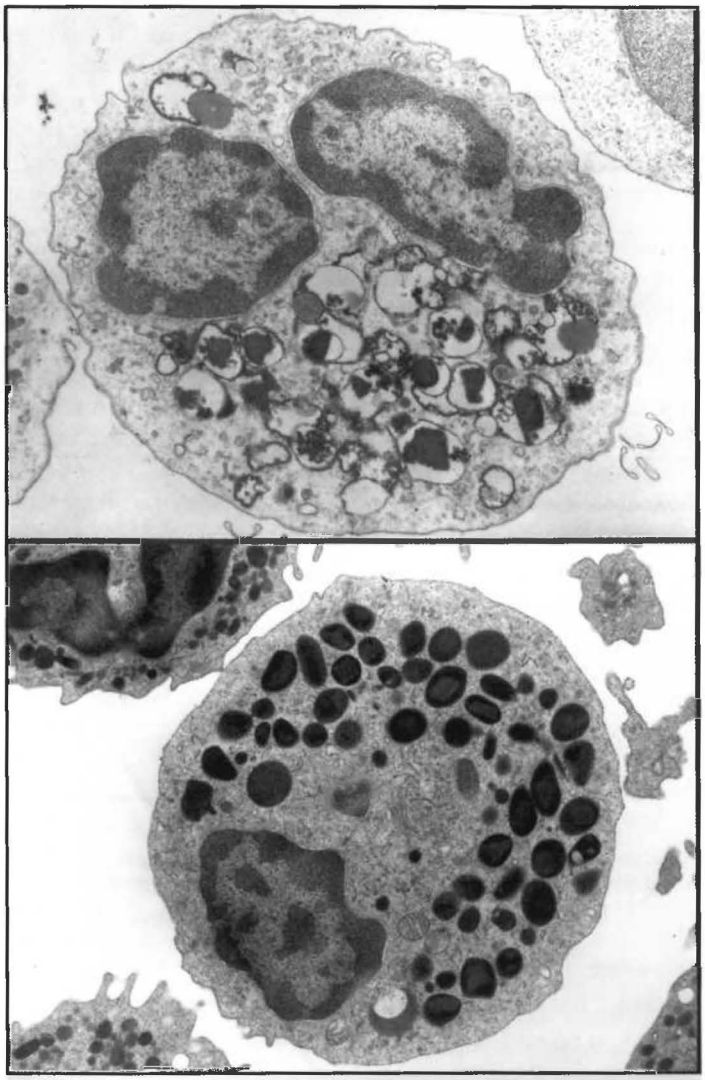
pure formaldehyde, paraformaldehyde must be heated and made slightly alkaline; therefore, it is very difficult to prepare large quantities of solution.

Formaldehyde is both a noncoagulant and an additive fixative. It reacts with tissue groups, primarily groups found in amino acids that contain a reactive hydrogen. A major site of reaction is the amino group on the side chains of amino acids, with the formation of methylene bridges that link protein chains together. The amino (NH_2) group is a positively charged group that is very important in attracting the eosin dye during routine H&E staining. By binding the amino group, formaldehyde alters the ability of certain positively charged tissue elements (cytoplasmic proteins) to bind eosin **[il.10]**. Because binding of hematoxylin by negatively charged groups is unaffected, the tissue reaction is more basophilic. The greatest binding for formaldehyde occurs between pH values of 7.5 and 8.0. Formalin also reacts with the sulfhydryl groups of the amino acid cysteine to form cross-links.

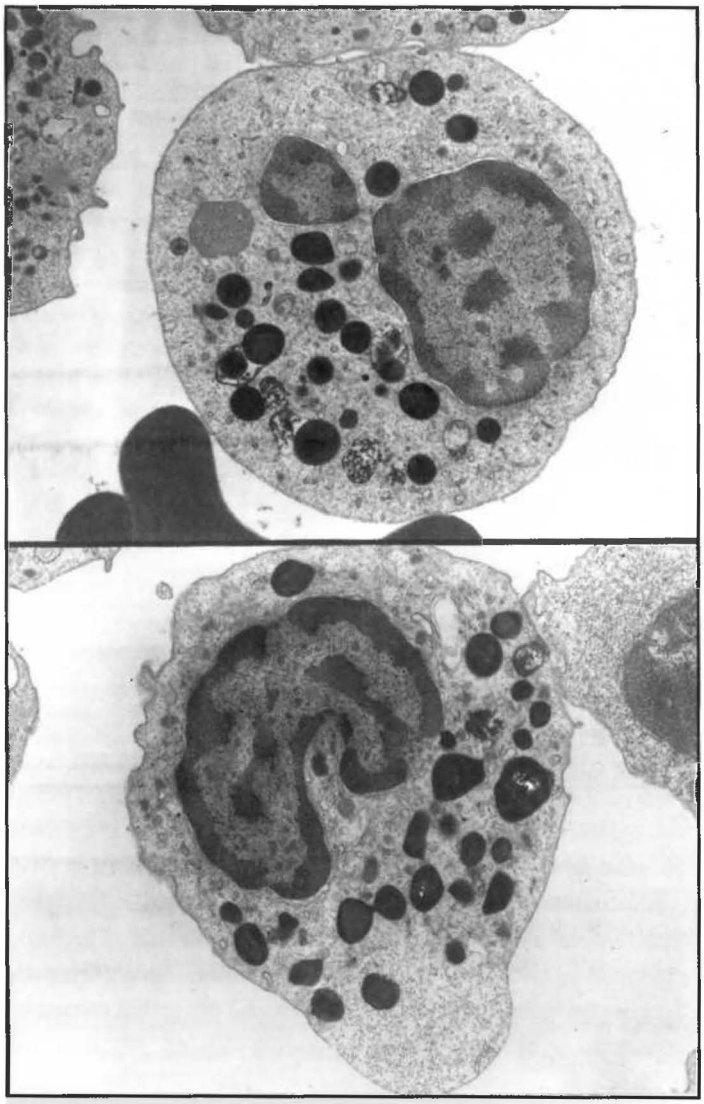
Lipids are preserved by formaldehyde but they are not made insoluble, and prolonged storage in formalin leads to a gradual loss of lipids. When tissue is subsequently processed for embedding in paraffin, the lipids are dissolved by alcohol and xylene. A frozen section can be made of formalin-fixed tissue, and special stains used to demonstrate the lipid. Formaldehyde does not fix carbohydrates, but it stabilizes and fixes the proteins in such a way that much of the glycogen is trapped in the tissue.

Formaldehyde penetrates and adds very quickly, but it fixes very slowly because it takes a long time to cross-link the tissue proteins. Pearse states that although fixation is not complete for at least 7 days, about one half of the formaldehyde is taken up, or added on to the proteins, in about 8 hours. Any loosely bound formaldehyde can be removed easily by washing in water. In more recent studies, Helander [1994], using ^{14}C-formaldehyde, found that at a pH of 7.0 and temperature of 25°C, formaldehyde bonding to tissue increased up to approximately 25 hours. Half-maximal binding was reached in approximately 80 minutes. Using a gelatin/albumin gel, Baker [1958] found that formaldehyde penetrated 3.6 mm in 1 hour, and 7.2 mm in 4 hours; however, the rate-limiting step is apparently not the uptake but the binding of the formaldehyde to the tissue. Werner [2000] considers cross-linking complete in 24 to 48 hours, with the possibility of excessive cross-linking (overfixation) occurring after that. When the tissue is well-fixed in formaldehyde, the many cross-links prevent alteration during processing and staining; however, tissue that is poorly fixed in formaldehyde can be redenatured by alcohol and heat, leading to artifact formation.

Formaldehyde causes less shrinkage than any of the other fixatives. Because much of the shrinkage resulting from fixation occurs in the subsequent processing steps, it really should be stated that formaldehyde allows less shrinkage in the subsequent processing steps than many of the other fixatives. Formaldehyde hardens tissue more than any other fixative except ethanol and acetone.



[i1.13] Top, eosinophil fixed with neutralized 10% formalin (formalin neutralized with calcium or magnesium carbonate). Bottom, eosinophil fixed with 10% modified Millonig formalin. Note the effects of the different buffer ions on the cell cytoplasm and granules.



[i1.14] Top, eosinophil fixed with neutral-buffered 10% formalin. Bottom, eosinophil fixed with acetate formalin. Note the effects of the different buffer ions on the cell cytoplasm and granules.

It is a relatively cheap and stable fixative and allows more special staining techniques than any of the other fixative reagents. The morphologic criteria used for diagnosis have been established primarily on formalin-fixed tissues.

Formaldehyde can be used in a simple aqueous solution, or with the addition of sodium chloride to achieve the correct osmolality, but these solutions become acidic by reacting with atmospheric oxygen and forming formic acid. Black acid hematin, or formalin pigment, is frequently the result. This, the first of 3 possible fixation pigments, is a microcrystalline dark brown pigment that is formed when the acid aqueous solution of formaldehyde acts on tissues rich in blood **[i1.11]**. Black acid hematin, or formalin pigment, tends to form when the pH of the solution drops below 6.0, and the pigment can occur with fixation in any acidic solution containing formaldehyde. Rarely, it may be seen when tissues containing large amounts of blood have been fixed in the more neutral formalin solutions. Formalin pigment can be prevented and it can be removed. The pigment is prevented by maintaining a solution pH near neutrality and using the appropriate volume ratio of fixative solution. It can be removed by treating tissue sections with alcoholic picric acid or alkaline alcohol. The pigment is undesirable because it may react during the staining procedure to mask or simulate microorganisms and pathologically relevant pigments. Formalin pigment will reduce silver solutions used in procedures for staining melanin, fungi, reticulin, and spirochetes. The pigment is birefringent and can be monitored by polarization **[i1.12]** before silver staining or it can be bleached or removed if necessary.

As previously mentioned, formaldehyde solutions are very hypertonic, but the formaldehyde molecule is not osmotically active [Maunsbach 1966]. Therefore, the buffer vehicle is very important, and the tonicity and composition of the buffer ions exert a marked influence on the morphology **[i1.13]**, **[i1.14]**.

Although some laboratories use formaldehyde solutions neutralized with calcium or magnesium carbonate to prevent pigment formation, it is preferable to buffer the solution so that the pH is relatively stable. Some of the more common formalin solutions used in the laboratory are listed below.

10% Aqueous Formalin

Formaldehyde, 37% to 40%	100 mL
Distilled water	900 mL

Because formaldehyde is not osmotically active, this solution is very hypotonic and may also produce formalin pigment.

10% Formalin Saline

Formaldehyde, 37% to 40%	100 mL
Sodium chloride	9 g
Distilled water	900 mL

This solution is isotonic exclusive of the formaldehyde, but may produce formalin pigment.

Calcium Formalin

Formaldehyde, 37% to 40%	100 mL
Calcium chloride	10 g
Distilled water	900 mL

This solution is recommended especially for the fixation and preservation of phospholipids in tissues. According to Baker, phospholipids tend to take up water and extend their surface by growing outward in wormlike myelin forms. Calcium ions have a dramatic effect in preventing the gradual solution and distortion caused by these outgrowths.

Formalin Ammonium Bromide

Formaldehyde, 37% to 40%	150 mL
Ammonium bromide	20 g
Distilled water	850 mL

This solution is recommended for tissue specimens of the central nervous system, especially when the Cajal astrocyte procedure is to be performed. This solution is very acidic, lyses red blood cells, and causes nuclei to give a direct positive Schiff reaction due to Feulgen hydrolysis during fixation.

Acetate Formalin

Formaldehyde, 37% to 40%	100 mL
Sodium acetate	20 g
Distilled water	900 mL

The literature does not specify the sodium acetate to be used; the anhydrous compound yields a solution pH of approximately 7.3, and the trihydrate yields a solution pH of approximately 7.0. This is probably one of the better formaldehyde solutions if one does not wish to prepare the buffered reagent. Lillie and Fullmer [1976] recommend a 2% solution of calcium acetate instead of sodium acetate and prefer it to the traditional calcium formalin for phospholipids fixation; however, a pseudocalcification in the tissue can be caused with the use of calcium acetate. This may be difficult to distinguish from true calcification in some tissues [Luna 1983].

10% Neutralized Formalin

Formaldehyde, 37% to 40%	100 mL
Distilled water	900 mL
Calcium or magnesium carbonate	To excess

Although this has been used widely as a fixative, it is not recommended because the solution becomes acidic after withdrawal from the storage bottle.

10% Neutral-Buffered Formalin

Formaldehyde, 37% to 40%	100 mL
Distilled water	900 mL
Sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$)	4 g
Sodium phosphate, dibasic (Na_2HPO_4)	6.5 g

This solution is the most widely used solution for routine formalin fixation. It has a pH of approximately 6.8 and it is hypotonic in the buffer ions present (approximately 165 mOsm).

Modified Millonig Formalin

Formaldehyde, 37% to 40%	100 mL
Distilled water	900 mL
Sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$)	18.6 g
Sodium hydroxide	4.2 g

Be sure the solution is well mixed. This solution is isotonic in buffer ions (310 mOsm) and has a pH of approximately 7.2 to 7.4 [Carson 1973]. This solution can be used as a dual-purpose fixative, allowing electron microscopy on stored tissue. Because less extraction of cellular elements occurs with this fixative, sectioning of the paraffin-embedded tissues may be slightly more difficult. When placed in solution, the sodium phosphate monobasic and sodium hydroxide immediately form an equilibrium between sodium phosphate monobasic and sodium phosphate dibasic. According to Pease [1964], if one begins with an isotonic solution of sodium phosphate monobasic, the amount of alkali can be varied so that the pH can be adjusted between 5.4 and 8.0 without changing the tonicity of the medium. A method for easily preparing large volumes of this fixative is given in chapter 14, "Electron Microscopy," p336.

Alcoholic Formalin

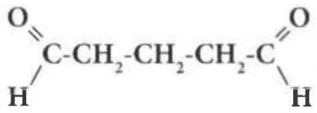
Formaldehyde, 37% to 40%	100 mL
Ethyl alcohol, absolute	650 mL
Distilled water	250 mL

This solution is a compound fixative, but is categorized with the other formalin solutions. It is useful as a fixative on the tissue processors, because in addition to fixation, the dehydration process is also begun. Alcoholic formalin solution should be prepared by measuring each component separately and pouring it into a flask for mixing. If the water and alcohol are measured by pouring one on top of the other in a cylinder, the volume of the last reagent added will be greater than intended, because these 2 substances react with each other in such a way that the volume is decreased. For example, if 65 mL of alcohol and 25 mL of water are measured separately, and mixed in a flask, and remeasured, the total volume is approximately 87 mL. Because phosphates may precipitate in the tissue if the alcohol content is too high, it is critical that the alcohol concentration be no greater than 70% (65% is preferred) when this solution is used in the tissue processor after exposure to 10% neutral-buffered formalin. Tissue may be stored indefinitely in this solution. This is a good fixative solution when the time for fixation in formalin alone is inadequate, because fixation can be speeded up by using 3 stations of formalin alcohol on the processor in place of separate containers of formalin and 70% alcohol. When combined with formalin, the shrinking effect of alcohol is minimized.

Safety is a primary concern in the use of formaldehyde; therefore, many laboratories prefer to use one of the many commercial solutions available. Formaldehyde is considered a carcinogen; however, most research has been on rats, and how those results translate to human exposure has not been determined. An act related to formaldehyde was passed in 1987 (Occupational Safety and Health Administration [OSHA], and for the first time histopathology laboratories were subjected to federal regulation of the use of this chemical. The exposure of employees to formaldehyde must be monitored for an 8-hour period with the permissible exposure limit (PEL) currently set at 0.75 ppm. There is also a short-term exposure limit (STEL) of 2 ppm over a 15-minute period. The third concentration that must be considered is an action level. If an action level of 0.5 ppm averaged over an 8-hour period can be achieved in 2 separate samples taken at least 7 days apart and the STEL is also within limits, then no further monitoring is necessary unless there are changes in the procedure or process. If the action level exceeds 0.5 ppm, remonitoring must be done at the end of 6 months, and if the STEL exceeds the limit, remonitoring must be repeated at the end of a year. Employees must be notified of the results of monitoring within 15 calendar days of the receipt of monitoring results; if the permissible exposure limit has been exceeded, the affected employees must be provided with a written description of the corrective action to be taken. Records for each employee must be kept for the duration of employment plus 30 years.

The act also mandates that a physical monitoring or medical surveillance program be in place if the laboratory action level is above 0.5 ppm. Emergency procedures, including personnel designated and trained to handle an emergency such as a major spill, must also be defined. The hazards must be communicated to anyone who might be handling a container of formaldehyde; all containers must be labeled with an appropriate hazard warning. The act also states that all eye and skin contact with liquids containing greater than 1% formaldehyde shall be prevented by chemical protective clothing impervious to formaldehyde and other personal protective equipment such as goggles and face shields as appropriate to the operation. Protective clothing is certainly appropriate when working in the gross dissection area. Body substance isolation procedures also require protective clothing and, in general, the same protective measures are also effective for formaldehyde. Employee education is one of the most important parts of the total program and the education must be documented. Although the Environmental Protection Agency does not currently regulate the disposal of formaldehyde, many municipalities do require that formaldehyde be collected and disposed of as hazardous waste.

GLUTARALDEHYDE



Glutaraldehyde is also an aldehyde, but it differs from formaldehyde in that it is a dialdehyde, with an aldehyde group on each end of the molecule. It acts very much like formaldehyde in cross-linking proteins; however, the extra aldehyde group is not involved in most cross-linking reactions. It is left free to react in any method using Schiff reagent for the detection of aldehydes. Therefore, techniques using Schiff reagent, such as the periodic acid-Schiff (PAS) stain, cannot be used on glutaraldehyde-fixed tissue because false-positive results will be obtained. Glutaraldehyde fixes at the rate at which it penetrates, but it penetrates slowly and poorly. Because it fixes as it penetrates, the penetration into the deeper part of the tissue is probably impeded; therefore, gross tissue sections must be thin.

Glutaraldehyde is most frequently used for the fixation of specimens for electron microscopy because it preserves ultrastructure the best of any of the aldehydes. It tends to overharden tissue, so fixation should not be prolonged. Usually 2 hours or less in glutaraldehyde is recommended for fixation, and then the tissue should be transferred into a buffer solution for holding. Tissue should remain in the buffer solution until processing is begun. The concentration of the glutaraldehyde solutions employed may vary from 2% to 4%, and various buffer systems may be used. Because of its poor penetration and overhardening properties, glutaraldehyde has not found wide acceptance as a fixative for light microscopy.

Glutaraldehyde is an unstable substance that breaks down on exposure to oxygen, with a resultant drop in pH. For the preparation of fixative solution for use in electron microscopy, small vials of glutaraldehyde that were sealed under inert nitrogen should be used, and the reagent should be prepared just before use. Any solutions used for light microscopy must be stored in the refrigerator, and after 2 to 4 hours of exposure to the fixative solution, any remaining wet tissue must be stored in buffer solution.

For electron microscopy, glutaraldehyde is commonly used in cacodylate- or phosphate-buffered solutions. The phosphate-buffered solution may be prepared as follows:

Phosphate-Buffered Glutaraldehyde ($NaH_2PO_4 \cdot H_2O$)

($NaH_2PO_4 \cdot H_2O$)	1.9 g
Sodium hydroxide	0.43 g
Distilled water	100 mL
Mix well and add:	
Glutaraldehyde, 25% solution	18 mL

The solution should be made just before use. The buffer can be prepared as a stock solution, and the glutaraldehyde added as needed for use. The pH should be 7.2 to 7.3; adjust if necessary.

Glutaraldehyde is not yet considered a hazard that should be regulated by OSHA, but it is not totally without associated hazards. Because it reacts very much like formaldehyde, it should be handled the same way. Glutaraldehyde is a sensitizer, and can cause irritation to the respiratory tract, digestive tract, and skin. The Threshold Limit Value (TLV) is 0.05 ppm according to the American Conference of Industrial Hygienists (ACGIH). OSHA currently does not have a required exposure limit. Glutaraldehyde is incompatible with oxidizers and alkalis. Dapson and Dapson [1995] consider glutaraldehyde more toxic than formaldehyde, and recommend using the same protective measures for each. Cacodylate-buffered solutions are very toxic because of the arsenic content.

GLYOXAL ($C_2H_2O_2$)

Glyoxal is the smallest dialdehyde and is typically supplied as a 40% aqueous solution. According to Dapson [2007], it has largely replaced formaldehyde in the textile industry for imparting wrinkle resistance and permanent press creases, and it is widely used for other industrial applications. Most of the industrial applications rely on the ability of glyoxal to cross-link. It has replaced formaldehyde in some histopathology laboratories because it is much less toxic than formaldehyde; glyoxal fixatives also are extremely rapid. According to Dapson [2004] surgical specimens are fixed after only 4 to 6 hours exposure, and biopsy specimens can be processed after only 45 minutes of fixation. Glyoxal fixative solutions are supplied commercially as either alcohol- or water-based solutions. Dapson [2007] states that whereas formaldehyde forms adducts with proteins and carbohydrates indiscriminately and subsequently cross-links them, glyoxal reacts with oxygen-containing end groups (carbohydrates) at one pH range and amines (proteins) over a different pH range. Cross-linking occurs only under specific conditions and can be slowed so that it will not occur during normal fixation times. Some of the artifacts seen with formaldehyde fixation, such as smudgy nuclei and distorted staining, are not seen with glyoxal fixation. With prolonged storage of tissue in glyoxal fixatives there is a slight reduction of staining, but this can usually be compensated for with prolonged staining times. Most special stains are satisfactory after glyoxal fixation. Dapson [2007] states that although glyoxal has 2 aldehyde groups with 1 probably left free during fixation, no problems are encountered with the PAS reaction. Some iron may be leached by the acidity of the fixative. The staining of *Helicobacter pylori* is unsatisfactory. Erythrocytes are lysed and granules of eosinophils are dissolved. Umlas and Tulecke [2004] reported that although H&E morphology of glyoxal-fixed tissues was equivalent, detection of microcalcifications in glyoxal-fixed breast biopsy specimens was hampered by loss of basophilia, and the diminished estrogen receptor detection in glyoxal-fixed breast tissue made antigen retrieval necessary.

Glyoxal does not give off fumes, but it has a serious health hazard rating (See a description of ratings in chapter 4, "Safety," p89). It can cause skin irritation and has a threshold limit value (TLV) of 0.1 mg/m^3 (ACGIH); OSHA currently has no requirements for exposure limits. It can be disposed of in the regular sink drain in most localities.

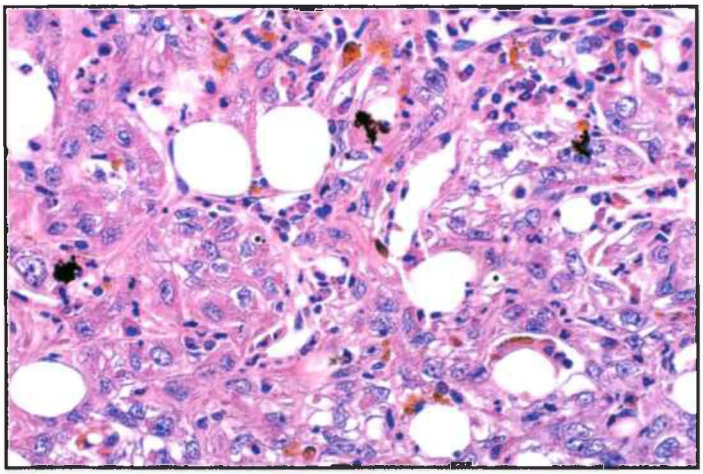
MERCURIC CHLORIDE ($HgCl_2$)

Currently few laboratories are using this chemical in fixative solutions because of its extreme toxicity, but it is included here because of its history, because a few laboratories cling to the morphologic characteristics produced by mercury fixation, and because some of the modifications of the compound fixatives are based on a substitution for this component.

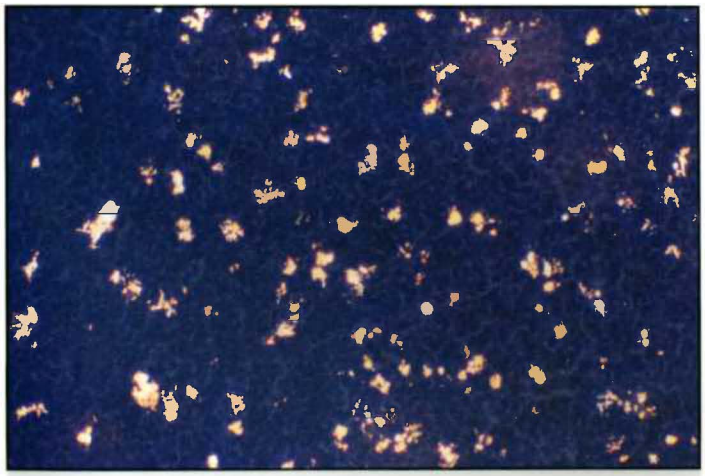
In the older literature, mercuric chloride is referred to as corrosive sublimate or occasionally as bichloride of mercury. It is a very corrosive chemical and all contact with metallic objects must be avoided if possible. Immediately after use with mercury-fixed tissue, instruments should be washed thoroughly. Mercury is not used alone, but is used in compound fixatives because it is a very powerful protein coagulant and enhances staining by leaving the tissue very receptive to dyes. Its presence in tissue prevents freezing, so frozen sections are difficult to prepare [Culling 1985]. Mercuric salts penetrate poorly, will produce shrinkage or will allow shrinkage in the subsequent processing steps, and can harden excessively with prolonged exposure. It is an additive fixative, reacting in acidic solutions with the sulfhydryl groups of the amino acid cysteine to form cross-links between protein chains, and it will react with amino groups in some of the more acidic solutions.

A fixation pigment produced by mercury is one that cannot be prevented but can be removed. Unless the pigment is removed, it appears as either a crystalline or amorphous brown precipitate lying on top of the stained section **[i1.15]**. Like formalin pigment, mercury pigment will also polarize light **[i1.16]**. This extrinsic artifact can be removed by treating the microscopic section with iodine followed by sodium thiosulfate. The iodine oxidizes mercury to mercuric iodide, which is soluble, and sodium thiosulfate removes the excess iodide from the section.

Mercury is a very toxic compound capable of affecting the central nervous system, and may cause acute nephritis when taken into the body in small amounts. Systemic mercury poisoning is possible by skin absorption. Mercury is considered a hazardous substance by the federal government; fixative solutions containing this chemical may not be disposed of in the sanitary sewer system (eg, sink) but must be collected for appropriate disposal. What is frequently forgotten is that many reagents following mercury solutions, or



[il.15] Mercury pigment has been deposited in, but not removed from, this Zenker-fixed, H&E-stained section. Note that the nuclei are not well stained, a phenomenon that frequently occurs with Zenker fixation. Treatment with iodine, omitted in this section, may increase nuclear staining, but the slides also may require additional time in hematoxylin.



[il.16] A Zenker-fixed section of spleen examined by polarized light. The pigment has not been removed on this section. Both mercury and formalin pigments rotate the plane of polarized light and cannot be separated using this method of examination.

those used to remove mercury pigment, are also contaminated and should be collected. Careful records must be maintained of the use and disposal of solutions containing mercury. Hazardous substances must be tracked from "cradle to grave." The amount of chemical remaining in the laboratory plus the amount discarded must equal the amount received. Because of the hazards associated with the use of mercuric fixatives, other metals have been tried as substitutes, but only zinc has found any acceptance.

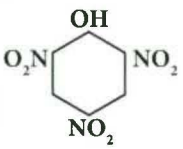
OSMIUM TETROXIDE (OsO_4)

Osmium tetroxide is not used frequently for fixation in the histopathology laboratory. Its primary use is in the fixation of specimens for electron microscopy. Even though specimens for electron microscopy are fixed primarily in an aldehyde solution, they are postfixed in osmium tetroxide to ensure preservation of the lipids. Osmium tetroxide chemically combines with lipids, making them insoluble, 93% of the lipids can be extracted after formaldehyde fixation, but only 7% can be extracted after fixation with osmium tetroxide [Ashworth 1966]. Cell membranes have phospholipids as a major component and will become electron-dense after fixation in osmium tetroxide.

Osmium also may be used to fix small amounts of fat so that the fat will be maintained in sections during paraffin processing. Osmium solutions penetrate only a few cell layers, so the sections must be extremely thin. Osmium tetroxide is a noncoagulant fixative and it is also additive fixative, but the reactions involved with proteins are not yet understood; it is also a noncoagulant fixative. After fixation with osmium tetroxide, the cell cytoplasm has little affinity for the anionic (acid) dyes but will readily accept cationic (basic) dyes.

Osmium tetroxide is a very expensive reagent that is very hazardous because it vaporizes readily; the vapor itself readily fixes the nasal mucosa or the conjunctiva of the eye. The OSHA time-weighted average (TWA) is 0.002 ppm osmium, so contact with the vapor must be avoided. It must be used in a hood, and extreme care must be exercised in electron microscopy if the tissue specimen is to be minced after being in an osmium solution.

PICRIC ACID



Picric acid is unique in that it is the only substance used in histotechnology both as a fixative ingredient and as a stain. The name is derived from the Greek word for bitter. Picric acid is a coagulant fixative, and it is a very strong coagulant of nucleoprotein but leaves DNA soluble. It is a sufficiently strong acid to hydrolyze nucleic acid. If stains for DNA and RNA are anticipated, any fixative containing picric acid should be avoided. It probably is an additive fixative, but the way in which picric acid reacts with proteins is not totally understood. It is not a fixative of lipids or carbohydrates, but Lison [1958] recommends picric acid as a constituent of fixatives for glycogen. Picric acid will decalcify tissue containing small calcium deposits, such as breast tissue. This reagent leaves tissue very receptive to acid (anionic) dyes (eg, eosin) and gives tissue a very good soft consistency; however, it either causes extreme shrinkage or allows extreme shrinkage to occur in the subsequent processing steps. The only fixative reagent allowing greater final shrinkage than picric acid is ethanol.

Picric acid must be washed out of tissue before processing. In the past, it was thought that protein coagulants formed by picric acid were water-soluble and so washing was traditionally carried out with 50% alcohol. Protein coagulants do not appear to be soluble [Baker 1958], but because there does not appear to be any advantage to washing with water and excess picric acid is more readily removed by ethanol, washing with 50% alcohol is still recommended. Picric acid is a fairly acidic solution, therefore it is sometimes washed out with alcohol to which lithium carbonate has been added as a neutralizer. Neutralization of the excess picric acid is an excellent step, because if picric acid remains in the tissue when it is embedded, the staining characteristics of the tissue will change over time. Eventually the staining results will be extremely poor. Luna [1992] states that if the picric acid is not completely removed

from the tissue, the result will be the eventual distortion or obliteration of almost all cellular structures.

The major safety consideration with picric acid is its hazard as an explosive compound. Until displaced by trinitrotoluene (TNT), a related compound, it was used by the military as an explosive; its potassium, sodium, and ammonium salts are still used as explosives. Picric acid, as purchased, may appear to be a dry compound, but it is not. It contains about 10% moisture and is safe as long as it stays moist; for this reason the jar should be tightly capped at all times. In fact, all reagents should be tightly capped, because many of them will absorb water or lose water through evaporation; neither of these phenomena is desirable. If for any reason you suspect that the moisture content of the picric acid has dropped below 10%, open the bottle and add distilled water so that the reagent looks like damp sand. It is not critical how much water is added, because most reagents and staining techniques use a saturated solution of picric acid and the extra water is not important.

POTASSIUM DICHROMATE ($K_2Cr_2O_7$)

Potassium dichromate is rarely used alone for fixation. It is a noncoagulant unless used in an acid solution; then it will act like chromic acid, which is a coagulant. This changeover occurs at a pH of approximately 3.4 to 3.8. Chromium will attach to some lipids, rendering them insoluble, but it does not preserve lipids to the degree that osmium tetroxide does. It preserves mitochondria by rendering the lipid component of the membranes insoluble in alcohol, but it readily dissolves DNA. Potassium dichromate-fixed tissue is soft, but shrinks more after processing for paraffin embedding than tissue fixed in most of the other fixatives. Chromium reacts with both carboxyl (–COOH) and hydroxyl (–OH) groups, and breaks some internal protein links. It increases the number of reactive basic groups ($-NH_2$) that are present, resulting in an increased tissue affinity for eosin.

Chromate solutions can yield another of the fixation pigments. This pigment's formation is very easily prevented; however, once it has formed it is considered insoluble by most authors [Baker 1958; Sheehan 1980]. Culling et al state that the pigment may be removed by treating the sections with 1% hydrochloric acid in 70% alcohol for 30 minutes; [Bancroft 1982] state that while the pigment cannot be removed completely, it is reduced by treating with an acidic alcohol solution. I have tried unsuccessfully to obtain the pigment; therefore, the method of removal remains untested. The pigment may be formed when tissue is taken from a chromate-containing solution directly into an alcoholic solution. Alcohol reduces the chromic compounds to insoluble chromium suboxides; therefore, excess chromates should be removed from the tissue by prolonged washing with running water before processing.

Chromium is a highly toxic chemical substance, both by inhalation of the dust and by ingestion. It is considered a carcinogen and is corrosive to skin and mucous membranes. Chromium must be tracked and collected for disposal in the same way as mercury.

ZINC SALTS ($ZnSO_4$)

In the last decade or so, zinc sulfate has found acceptance as a replacement for mercury because it does not have the associated hazards and preserves tissue antigenicity, often making retrieval procedures unnecessary. In 1947, Russell proposed that an equimolar amount of zinc chloride could be substituted for mercuric chloride in Zenker solution, but this did not find widespread favor at that time. Most laboratorians wished to get away from the hazards associated with mercury or find a fixative that would not decrease antigenicity; therefore, other metals were tried as substitutes, with zinc holding the most promise. Zinc sulfate, in combination with formaldehyde, has been used for postfixation on the open-type tissue processors by the Mayo Clinic [Banks 1985]. Lynn et al described the use of unbuffered alcoholic zinc formalin prepared with zinc chloride. Dapson and Dapson [1995], state that zinc chloride is more corrosive than zinc sulfate, so care must be taken when using zinc chloride formalin on automatic processors: its use is disallowed by most equipment manufacturers because it will damage metal valves and other parts of the instrument.

According to Dapson [1995], many things may cause the precipitation of zinc, such as carbonates present in tap water; phosphates from buffered formalin; pH-altering influences from the tissue; heat, pressure, and vacuum from the processor; and alcohol. This can be a problem, especially with some formulations that tend to precipitate zinc in the first dehydrating alcohol step of processing; such precipitation will occur in alcohol concentrations as low as 70%. In such cases, a precipitate also forms in the tissue, making microtomy difficult, and the precipitate can clog processor lines. Any precipitate in the processor can be removed by rinsing with a dilute acetic acid solution (5%-20%).

Today, zinc salts have found use not only in combination with formaldehyde for routine fixation, but as a substitute for mercury in the B-5 solution commonly used for the fixation of lymph node and bone marrow tissues. Bonds et al reported that acetic acid-zinc formalin was a safe alternative to B-5 and gave staining and morphologic detail comparable to B-5. Tissues fixed in the acetic acid-zinc formalin also achieved equivalent or superior antigen preservation for immunohistochemical studies.

Wester et al reported that immunoreactivity in paraffin-embedded tissue was superior in tissues fixed in buffered zinc formalin compared with that seen in tissue fixed in neutral-buffered formalin. Citing the detrimental effect on DNA and RNA quality as a major drawback to fixation with neutral buffered formalin, they found a significantly higher DNA yield in the tissue fixed in zinc formalin; this significantly impacts analysis of genes and transcripts in complex tissues. The preservation of protein immunoreactivity was improved; 7 of 9 antibodies did not require pretreatment with the tissue fixed in buffered zinc formalin. They also noted that zinc formalin induced more shrinkage in tissues than neutral-buffered formalin.

According to Dapson [1993], it appears that zinc ions hold macromolecules in their native conformation via coordinate bonds. This prevents the damaging cross-linkages that formaldehyde alone creates. The result is enhanced preservation of antigenicity, rare need for antigen retrieval, and the possibility of greater dilution

of antibodies. Dapson [2004] states that zinc formalin fixes more rapidly than formaldehyde alone; specimens fixed in zinc formalin for a few hours are comparable with those fixed in formaldehyde for 30 hours. According to Dapson [2004], zinc ions can also undo some of the deleterious effects of prior formaldehyde fixation, at least with some antigens some of the time; how this is accomplished is not totally understood. Some antigen activity also can be recovered by backing up tissue that has been formalin-fixed and embedded in paraffin, refixing with zinc formalin, and reprocessing; or by simply treating the deparaffinized and hydrated formalin-fixed slide with a solution of aqueous or alcoholic zinc formalin.

Zinc sulfate carries only a moderate health risk. Inhalation can cause irritation to the respiratory tract and the salts may hydrolyze into acid if swallowed. Ingestion of 10 g has been reported to cause a fatality. It is a skin and eye irritant. No airborne exposure limits have been established; however, when it is combined with formaldehyde, all safety regulations regarding formalin solutions must be followed. Zinc chloride is much more of a hazard, rated as a severe health risk. It is corrosive and will cause burns to any area of contact. It is harmful if swallowed, inhaled, or comes into contact with the skin or eyes. The PEL established by OSHA is 1 mg/m^3 (TWA) as fume.

Other Fixative Ingredients

Many other reagents have been investigated over the past few years, whether to improve fixation for some special technique or to decrease the hazards involved in handling many of the older fixatives. Some other reagents that have been investigated for use in fixative solutions are the carbodiimides, diisocyanates, diazonium compounds, tannic acid, cationic surfactants (detergents), diazolidinyl urea, bronopol, and bis-carbonyl compounds. Caution must be used when changing to some of the newer proprietary fixatives, with special caution regarding their claims of greater safety. While some of these reagents form cross-links with certain tissue groups, some are preservatives and not true fixatives. Dapson and Dapson [1995] warn that if a fixative works, it cannot be completely safe, because if it fixes specimens it will also fix your skin and corneas. They further state that if the solution does not endanger your skin and eyes, then it is not a true fixative; it is simply stabilizing the tissue against decomposition. Baker says that in addition to acting like a preservative, a fixative modifies various tissue constituents in such a way that they will retain their form as much as possible if subjected to treatment that would damage them in their natural state. He further states that fixation is a forward-looking process, existing only in relation to subsequent events. Many of the fixatives marketed today are proprietary, so that we do not know their composition, and there are no data on long-term toxicity or preservation of tissue either in wet tissue storage or in the block. When changing fixatives, one must remember that each fixative will create its own set of artifacts in the tissue specimen to which the pathologist must adapt, and frequently the processing and staining procedures also must be adjusted. **[t1.1]** summarizes the major characteristics of various fixative ingredients.

Compound or Combined Fixatives

Other than with formaldehyde, glutaraldehyde, and glyoxal, most of the fixative solutions are combined in such a way that the disadvantage of one component will be counterbalanced by an advantage (or even a disadvantage) of another. For example, the swelling caused by acetic acid is a disadvantage that can be counteracted by the shrinking effect of picric acid, a disadvantage if picric acid is used alone.

As stated earlier, each fixative creates its own set of artifacts in tissue. We do not see in fixed tissue what we would see if the tissue were still living. Morphologic preservation by formaldehyde and osmium tetroxide is probably the most lifelike. We become accustomed to looking at the artifacts created by one fixative, thus changing to a very different fixative can be momentarily confusing, or at least frustrating, to the pathologist. Different fixatives also present different sectioning and staining problems to histopathology laboratory personnel. The following more commonly used compound fixatives will be discussed:

1. B-5
2. Bouin
3. Gendre
4. Hollande
5. Zenker and Helly
6. Orth
7. Zamboni
8. Zinc formalin

B-5 FIXATIVE

Stock Solution

Mercuric chloride	12 g
Sodium acetate (anhydrous)	2.5 g
Distilled water	200 mL

Working Solution

B-5 stock solution	20 mL
Formaldehyde	2 mL

Prepare immediately before use

[t1.1] Characteristics of the Various Fixative Ingredients [Kiernan 1999, Baker 1958, Dapson 2008]

Characteristics	Ethanol, Methanol Acetone	Acetic Acid	Formaldehyde, Paraformaldehyde	Glyoxal	Glutaraldehyde	Mercuric Chloride	Potassium Dichromate pH <3.5	Potassium Dichromate pH >3.5	Osmium Tetroxide	Picric Acid	Zinc Salts
Reaction with proteins	coagulant, nonadditive	nil	noncoagulant additive	additive; forms *N*-hydroxymethyl adducts with 2 carbon atoms	coagulant additive	coagulant additive	coagulant additive, acts like chromic acid	noncoagulant additive	noncoagulant additive	coagulant additive	coagulant additive
Reaction with nucleic acids	nil	coagulant of nucleoprotein and DNA	nil below 45°C	additive; forms *N*-hydroxymethyl adducts with 2 carbon atoms	coagulant	coagulant	coagulant	dissolves DNA, precipitates nucleoprotein	noncoagulant	precipitates nucleo-protein, leaves DNA soluble and partly hydrolyzed	unknown
Reaction with lipids	some extraction	nil	preserves, but with gradual loss	nil	preserves, but with gradual loss	"unmasks" some lipids	oxidizes unsaturated fatty acids	attaches to some, makes them insoluble	reacts with and adds to double bond in lipids, makes them insoluble	nil	unknown
Reaction with carbohydrates	nil, glycon is insoluble	nil	nil	nil	nil	nil	oxidizes to aldehydes	nil	uncertain	nil	nil
Rate of penetration	rapid	rapid	rapid, but slow cross-linking	rapid, little cross-linking	slow, but cross-linking rapidly	rapid	slow	fairly rapid	very slow	very slow	slow
Enzyme activity	preserves some if cold	unknown	preserves some if cold and brief	inferior to formaldehyde	more inhibition than with formaldehyde	inhibits	inhibits	inhibits	inhibits	inhibits	inhibits
Electron microscopy (ultrastructural preservation)	poor	poor	good with Millonig or paraformaldehyde, usually postfixed with osmium	unknown	excellent, usually postfixed with osmium	organelles preserved	poor, causes considerable distortion	poor, causes some distortion	excellent	poor, causes distortion	poor
Special uses	used when urates are to be demonstrated, also good for glycogen, cytology fixative	used in mixtures for fixation and preservation of nucleoprotein	electron microscopy (Millonig and paraformaldehyde), some enzyme histochemistry	antigen retrieval not needed except if arginine is in epitope	electron microscopy	none	none	preserves chromaffin granules (used in Orth solution for fixation of pheochromo-cytomas)	primary use in electron microscopy, or for fat demonstration in paraffin sections	mordant for trichrome procedures	preserves immuno-reactivity, gives good morphological preservation
Special comments	overhardens tissue, shrinks tissue markedly, ***usually used alone***	swells tissue markedly, ***not used alone***	permits more special stains than any other fixative, ***frequently used alone***	chromatin and membranes preserved with excellent clarity, stains for *Helicobacter pylori* unsatisfactory	may give false-positive Schiff reactions, ***usually used alone***	promotes staining, produces an artifactual pigment, ***not used alone***	can produce an artifactual pigment, ***not used alone***	can produce an artifactual pigment tissue, ***not used alone***	penetrates only a few cell layers in depth, ***usually used alone***	should not be used with Feulgen reaction, ***not used alone***	can precipitate in the processor

This fixative found wide acceptance for hematopoietic and lymphoreticular tissue, because of its ability to demonstrate beautiful nuclear detail **[i1.17]**, compared with formalin **[i1.18]**. Excess fixative does not need to removed from the tissue by washing, but sections must be treated for removal of mercury pigment, with a solution of iodine followed by sodium thiosulfate. The sodium acetate added to the solution raises the pH to 5.8 to 6.0, and formalin pigment may or may not be obtained. Tissue cannot remain in this solution indefinitely; after fixation, wet tissue must be placed in a storage solution, most frequently 70% alcohol. B-5 fixation gives excellent results with many special stains and is an excellent fixative for many tissue antigens to be demonstrated on paraffin-embedded tissue.

Because of the hazards associated with the use of mercury, commercial preparation of solutions similar to this are available but substitute zinc for the mercury. The morphologic features seen with these substitutes are similar to those seen with B-5.

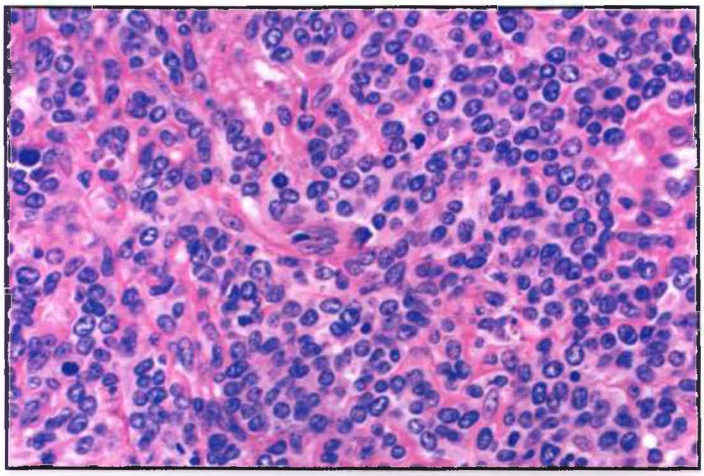
If B-5 is used, then all precautions applicable to formaldehyde and mercury apply to this reagent, including collection of waste solution and tracking of the mercury "from cradle to grave."

Bouin Solution

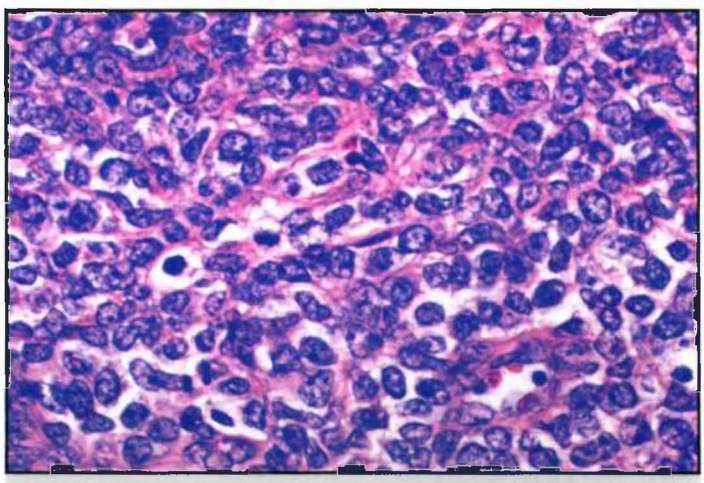
Picric acid, saturated aqueous solution (1.2%)	750 mL
Formaldehyde, 37% to 40%	250 mL
Acetic acid, glacial	50 mL

This fixative lyses RBCs because of its acetic acid content. Iron and small calcium deposits are usually dissolved, and formalin pigment may be obtained. This solution is excellent for tissue that is to be trichrome-stained and for preserving structure with soft and delicate textures. The swelling effect of acetic acid is balanced by the shrinking effect of picric acid, and the hardening effect of formaldehyde is counteracted by the soft fixation of picric acid. The basophilic cytoplasm caused by formalin is offset by the picric acid, resulting in brilliant nuclear and cytoplasmic staining with H&E. Paraffin blocks of Bouin-fixed tissue will section easily. The yellow color must be removed by washing, traditionally done with 50% to 70% alcohol, or 70% alcohol saturated with lithium carbonate. If excess picric acid is left in embedded tissue, the staining will deteriorate over time. Remaining wet tissue should be stored in 70% to 80% alcohol, because tissue cannot be held in Bouin fixative indefinitely. The maximum fixation time in this solution should be less than 24 hours; however, Kiernan states that tissue stored in Bouin solution for several months is sometimes still usable.

Bouin fixative is excellent for use on biopsy specimens of the gastrointestinal tract because the nuclei are much crisper and better stained than with 10% neutral-buffered formalin. Tissue of the endocrine system is well-fixed and many antibodies react well with tissue fixated in this solution. Bouin solution may be used as a routine fixative, but it cannot be used for the preservation of tissue that must be examined ultrastructurally (with electron microscopy) or in which nucleic acids must be demonstrated. Because Bouin solution contains formaldehyde, all regulations governing the use of formaldehyde are applicable.



[i1.17] This is a section from a lymphoma following B-5 fixation. Note the crisp chromatin patterns of the various types of nuclei. This is the type of fixation desired by the zinc formalin substitutes for B-5.



[i1.18] This section was taken from the same tumor represented in **[i1.17]** and fixed in 10% neutral-buffered formalin. Note the difference in nuclear fixation.

Gendre Solution

Alcohol, 95% saturated with picric acid	800 mL
Formaldehyde, 37% to 40%	150 mL
Glacial acetic acid	50 mL

This alcoholic Bouin solution is excellent for the preservation of some carbohydrates, especially glycogen. As with Bouin solution, the excess picric acid should be removed, by washing with 80% alcohol. Because Gendre solution contains formaldehyde, all regulations governing the use of formaldehyde are applicable.

Hollande Solution

Copper acetate	25 g
Picric acid	40 g
Formaldehyde, 37% to 40%	100 mL
Acetic acid	15 mL
Distilled water	1,000 mL

Dissolve each chemical successively in the distilled water without heat.

This modification of Bouin solution is stable and will decalcify small specimens of bone. It has been widely used as a fixative for biopsy specimens of the gastrointestinal tract. Hollande-fixed tissue can be stained successfully with most special stains. The cupric acetate present in the solution stabilizes RBC membranes and the granules of eosinophils and endocrine cells, so that the lysis that occurs is less than that seen with Bouin solution.

The fixative must be washed out before the specimen is placed in a phosphate-buffered formalin solution on the tissue processor; salts present in the solution will form an insoluble phosphate precipitate. This solution is moderately toxic if ingested, and repeated exposure may cause dermatitis. Because Hollande solution contains formaldehyde, all regulations governing the use of formaldehyde apply to this solution.

ZENKER AND HELLY (ZENKER-FORMOL) SOLUTIONS

Because of the associated hazards, the use of these solutions should be discontinued; however, for historical reasons and because they are still used in some laboratories, Zenker and Helly solutions are included in this text. They are considered together because the stock solution is the same, and the solutions differ only in the addition of acetic acid to one and formaldehyde to the other.

Zenker and Helly Stock Solution

Mercuric chloride	50 g
Potassium dichromate	25 g
Sodium sulfate (optional)	10 g
Distilled water	1,000 mL

Zenker Working Solution

Zenker-Helly stock solution	95 mL
Acetic acid, glacial	5 mL

This solution is stable and may be prepared in large quantities if desired. Earlier it was considered unstable because the acetic acid contained impurities that were reducing agents, but this is not true today [Lillie 1976].

Helly Working Solution

Zenker-Helly stock solution	95 mL
Formaldehyde, 37% to 40%	5 mL

Because formaldehyde is a reducing agent, this is not a stable solution. The formaldehyde must be added immediately before use, or the solution will darken and become turbid on standing, indicating that it is not usable.

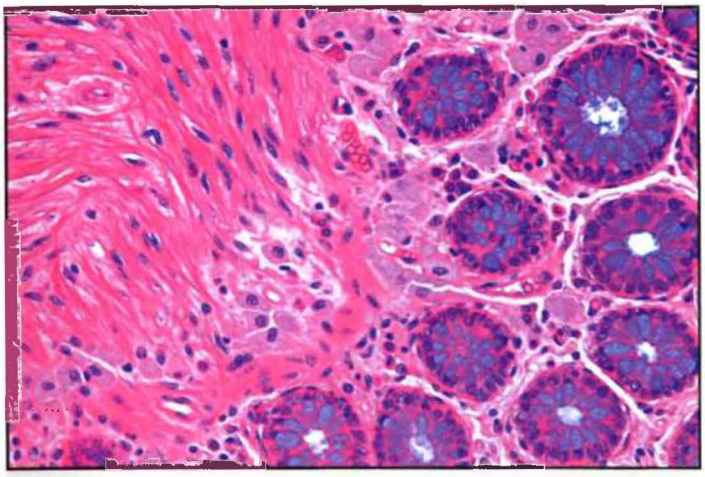
The tissues must be treated for mercury pigment after immersion in either of these fixatives. If excess fixative is not removed by washing with water, reduction of potassium dichromate by the dehydrating alcohol may cause the formation of chrome pigment. Formalin pigment also may be obtained with Helly solution.

Zenker solution will lyse erythrocytes because of its acetic acid content. Helly solution will preserve the erythrocytes, but the presence of acetic acid makes Zenker solution the better nuclear fixative. Zenker solution also has been used to fix and decalcify needle biopsy specimens of bone marrow, but it can dissolve iron. Mallory considered Zenker solution the best of all fixatives, but because of the mercury present in both of the fixatives, a less hazardous fixative should be substituted.

Most staining is satisfactory after fixation in these solutions, with Zenker solution recommended if the Mallory phosphotungstic acid-hematoxylin stain is to be applied. The exception is silver staining; many of the silver techniques are unsatisfactory after fixation in either Helly or Zenker solution.

Tissue specimens may not remain in these solutions indefinitely and the fixation time must be controlled. In general, the maximum time in either of these solutions should not exceed 24 hours, or the tissue becomes overhardened and nuclear basophilia is decreased. After fixation, tissue should be washed very well in running water, and any remaining wet tissue should be stored in 70% to 80% alcohol. Because these fixatives decrease nuclear basophilia and increase cytoplasmic acidophilia, staining time with hematoxylin may need to be increased and that with eosin decreased **[i1.19]**.

Although these solutions are still commercially available, they are very toxic. They have the various hazards associated with mercury, potassium dichromate, and formaldehyde (Helly solution); they must be collected and disposed of in accordance with all hazardous waste regulations. Zenker and Helly solutions can be fatal if inhaled, ingested, or absorbed through the skin. Target organs are the kidneys, central nervous system, and liver. These reagents are also capable of causing cancer in humans and should be used only under a chemical fume hood and with the appropriate protective equipment.



[il.19] A section of gastrointestinal tract fixed in Zenker solution. Compare the cytoplasmic staining in this image with that in **[il.10, p10]** which was fixed in formalin. Mercuric salts bind with sulfhydryl groups in acidic solutions, and chromic salts react with the carboxyl group, leaving the amino groups free to bind eosin; therefore, the cytoplasm is much more acidophilic when fixed in Zenker solution than when fixed in formalin. Formalin reacts with the amino groups in cytoplasmic proteins, thus leaving fewer groups available for binding eosin.

Orth Solution

Potassium dichromate	2.5 g
Sodium sulfate	1 g
Distilled water	100 mL
Just before use, add	
Formaldehyde, 37% to 40%	10 mL

The content of both potassium dichromate and formaldehyde in this solution means that the safety regulations appropriate to each must be followed.

Zamboni Solution (Buffered Picric Acid-Formaldehyde, or PAF)

Paraformaldehyde	20 g
Picric acid, saturated aqueous (double-filtered)	150 mL

Heat to 60°C to dissociate the paraformaldehyde. Add 2.52% aqueous sodium hydroxide dropwise to alkalinize the solution. Filter the solution and allow it to cool. Dilute the solution to 1,000 mL with phosphate buffer prepared as follows:

$NaH_2PO_4 \cdot H_2O$	3.31 g
Na_2HPO_4 (anhydrous)	17.88 g
Distilled water	1,000 mL

Zamboni solution should have a final pH of 7.3. Adjust if necessary.

This fixative is very stable and although not widely used, it is a good general purpose fixative. The fixation time is not as critical as it is with Bouin solution. Zamboni solution allows secondary fixation with osmium, and because it is easy to use and preserves the morphologic characteristics accurately, it is preferred by some institutions as the primary fixative for electron microscopy. Because of its formaldehyde content, all regulations appropriate to formaldehyde apply to this solution.

ZINC FORMALIN SOLUTIONS

Dapson [1993] stated that as immunohistopathology gained in prominence, zinc formalin solutions might replace neutral-buffered formalin as the universal fixative. The introduction and widespread use of antigen recovery systems has prevented this from happening. However, the use of zinc formalin solutions has increased. Antigenicity is not lost with long-term storage of wet tissue in zinc sulfate formalin solutions. Cross-linking is prevented by zinc formalin, and many macromolecules are retained in near native conformation [Dapson 1993].

Today, several varieties of zinc formalin are available, with most supplied commercially and with proprietary formulation. Several formulas for solutions that can be prepared in the laboratory are given below.

Aqueous Zinc Formalin (original formula)

Zinc sulfate, heptahydrate	10 g
Formaldehyde, 37% to 40%	100 mL
Distilled water	900 mL

Zinc is not very soluble in the 70% alcohol used in the first processor dehydration station; therefore, this solution tends to precipitate in processors. The zinc also precipitates inside the tissue specimens, causing microtomy difficulties. This difficulty discouraged some of the early users of this solution.

Unbuffered Aqueous Zinc Formalin

Zinc sulfate	20 g
Distilled water	900 mL
Stir until dissolved and add:	
Formaldehyde, 37% to 40%	100 mL

Formalin pigment (acid hematin) can be produced by this fixative. If zinc formalin is to be followed by a neutral fixative such as phosphate-buffered formalin, the tissue must be washed between reagents to prevent a precipitate from forming on the tissue. A minimum of 4 to 6 hours should be allowed for fixation of biopsy tissues and 6 to 8 hours for most other tissues.

Alcoholic Zinc Chloride Formalin [Lynn 1994]

Zinc chloride	4.5 g
Distilled or deionized water	1,000 mL
Stir until dissolved and add:	
Isopropyl alcohol, 99%	2,000 mL
Formaldehyde, 37% to 40%	400 mL

This solution was recommended as a postfixative solution, following fixation with neutral-buffered formalin. Antigenicity is enhanced, and nuclear detail is improved over formalin fixation. Zinc chloride is a corrosive compound, but at this very dilute concentration should not harm the processor, as found by Lynn [1994] over several years; however, use in the processor may void the warranty.

According to Dapson [1993], alcoholic zinc formalin solutions fix about 1.5 times faster than aqueous solutions. Alcoholic solutions are recommended if 6 to 8 hours cannot be allotted for fixation, and these solutions are also better for fatty tissues. Because of the toxic effects of alcoholic zinc chloride formalin, alcoholic zinc sulfate formalin solutions are recommended and are available commercially.

Proprietary unbuffered solutions are currently available that will not precipitate in 70% alcohol and can be used in all processors. Buffered zinc formalin solutions are also available, but must be selected carefully because some of the formulations precipitate badly in processors. Bonds et al [2005] performed a blinded prospective study to find a safe, mercury-free alternative to B-5, and found that acetic zinc formalin-fixed tissue gave results equal to that seen with tissue fixed with B-5; this included immunohistochemical results. All reagents in the study were commercial, and the reader is referred to this study for their complete results.

Zinc sulfate is only a moderate health risk. Inhalation can cause irritation to the respiratory tract and the salts may hydrolyze into acid if swallowed. Ingestion of 10 g has been reported to cause a fatality. It is a skin and eye irritant. No airborne exposure limits have been established; however, when it is combined with formaldehyde, all safety regulations regarding formalin solutions must be followed. Zinc chloride is much more of a hazard, rated as a severe health risk. It is corrosive and will cause burns to any area of contact. It is harmful if it is swallowed or inhaled, or comes into contact with the skin or eyes. The PEL established by OSHA is 1 mg/m^3 (TWA) as fume.

Nonaqueous Fixatives

The nonaqueous fixative ingredients are primarily acetone and either ethyl or methyl alcohol. These compounds are nonadditive, coagulating fixatives. The nonaqueous fixatives are very flammable and must be stored in fireproof cabinets. These reagents are used only when the desired tissue components are destroyed or dissolved by the aqueous fixatives, because these reagents tend to overharden tissue drastically.

ACETONE

Acetone is a nonadditive protein coagulant historically used when the demonstration of enzymes, especially acid and alkaline phosphatase, was indicated on tissue to be processed for paraffin embedding. Fixation in acetone was done rapidly at refrigerator temperature, and most of the dehydration was accomplished at the same time; thus, the total fixation and processing time was greatly decreased. It is also used as a fixative for brain tissue when subsequent staining techniques for rabies diagnosis are needed. Acetone is frequently used on frozen sections of tissue to be stained for cell surface antigens by immunohistochemical techniques. It is a very rapid-acting fixative, but it causes extreme shrinkage, distortion, and overhardening, and it is recommended only for preservation of special tissue components.

Acetone has an OSHA TWA of 1,000 ppm and a National Institute for Occupational Safety and Health TWA of 250 ppm. It is a narcotic in high concentrations, and skin contact can lead to defatting and dermatitis. It is moderately toxic on ingestion, and is not considered a serious health hazard in normal use in the histology laboratory [Dapson 1995]. It is highly flammable, with a flash point of 4°C.

ALCOHOL

Both ethyl and methyl alcohols are used for fixation. Methyl alcohol is used frequently as a fixative for touch preparations and blood smears, and ethyl alcohol is used to preserve water-soluble tissue components. Two of these water-soluble tissue components are glycogen and the urate crystals that are deposited in gout. Older literature specifies that absolute alcohol is required as a fixative for glycogen; however, much of the glycogen is trapped in tissue by formaldehyde fixation, so absolute alcohol is rarely used for this purpose today. Alcohol is a nonadditive protein precipitant that acts by breaking hydrogen and ionic bonds. This is followed by the removal of bound water. Any chemical groups that had formed the hydrogen and ionic bonds are free to react with any subsequent reagents such as mordants or stains.

Ethyl alcohol preserves most pigments, dissolves fat, and overhardens and shrinks the tissue. The many government restrictions on the use of pure ethyl alcohol present a major drawback to its use. Although alcoholic formalin (described under "Formalin") is not totally nonaqueous, it is included here as primarily a nonaqueous fixative. Alcoholic formalin fixes tissue, begins dehydration, preserves glycogen very well, and penetrates quickly. When combined with formalin, the shrinkage effect of the alcohol is minimized.

The TWA is 1,000 ppm for ethyl alcohol and 200 ppm for methyl alcohol. Although both are toxic by ingestion, methyl is much more toxic than ethyl alcohol. Ingestion of methanol may result in blindness and death. Both are flammable liquids.

Carnoy Solution

Absolute ethyl alcohol	60 mL
Chloroform	30 mL
Acetic acid, glacial	10 mL

Erythrocytes are lysed by this fixative; it is sometimes used in cytology for this purpose. Carnoy solution is rapid-acting, preserves glycogen, and exhibits good nuclear preservation, but causes excessive shrinkage and hardening. Fixation should not be prolonged beyond 4 hours. This fixative should be used only as indicated for the preservation of special tissue components lost through routine fixation. Tissues should be processed through 95% alcohol, absolute alcohol, and xylene as usual, or the processing procedure can be started with absolute alcohol if desired.

Repeated or prolonged exposure can produce damage to the central nervous system, liver, kidneys, and eyes. Chloroform is a suspected carcinogen. It should be used in a fume hood.

Methacarn solution substitutes methyl alcohol for the ethyl alcohol in Carnoy solution, and it hardens and shrinks tissue less than Carnoy fixative. Vacca recommends methacarn solution over Carnoy fixative.

Clarke Fluid

Absolute alcohol	300 mL
Glacial acetic acid	100 mL

Mix just before use. This is one of the oldest fixatives and is excellent for subsequent paraffin embedding. According to Kiernan [1999], Clarke fluid always gets a high score for microanatomical preservation in comparison with other fixatives used in light microscopy.

Refer to alcohol and glacial acetic acid for safety information.

Transport Solutions

If unfixed tissue is to be held for only a brief period or transported only a short distance, it is best to place the specimen on saline-dampened (excess saline squeezed out) gauze, enclose it in a tightly closed plastic container, and then place it on ice. If the unfixed tissue is to be held for several days or transported over a long distance, then Michel transport medium is recommended. Michel medium, however, is not recommended for muscle biopsies, but is used routinely for kidney biopsies that are to be mailed. This solution is commercially available and may be prepared as follows [Michel 1972; Elias 1982]:

Michel Transport Medium

Anhydrous citric acid (FW 192.3)	4.803 g
Ammonium sulfate (FW 132.14)	412.3 g
N-ethylmaleimide (FW 125.13)	625 mg (0.625 g)
Magnesium sulfate (FW 120.37)	1.23 g
Distilled water	to 1 L

It is important to maintain the pH of the transport medium at 7.0 to 7.2 because a lower pH can cause variable results. The *N*-ethylmaleimide minimizes proteolytic activity, and the ammonium sulfate fixes tissue-bound immunoglobulins [Elias 1990]. Before freezing the specimen with isopentane chilled with liquid nitrogen or some other freezing method, the specimen should be washed with mild agitation in three 8-minute changes of phosphate-buffered saline (PBS) containing 10% sucrose. Rinsing with PBS containing sucrose is preferred by Elias [2003] over citrate buffer rinses, because sectioning is facilitated. The PBS and PBS-sucrose are prepared as follows:

PBS Buffer Stock Solution (also used in immunohistochemistry)

Potassium phosphate, dibasic (K_2HPO_4)	188 g
Sodium phosphate, monobasic (NaH_2PO_4)	33 g
Sodium chloride	180 g

First dissolve the potassium phosphate, dibasic, in approximately 800 mL of distilled water in a 1-L beaker using heat and a magnetic stirrer. Add the sodium phosphate, monobasic, and sodium chloride. Dissolve the salts completely and dilute to 1 L. Adjust pH to 7.4 if necessary, and store at room temperature.

PBS-10% Sucrose Solution

Stock PBS solution	4 mL
Distilled water	96 mL
Sucrose	10 g

According to Elias et al [2003], tissue for immune complex deposit studies can be stored in the 10% PBS-sucrose solution for approximately 2 weeks without affecting subsequent immunofluorescence or immunohistochemical studies. However, autofluorescence may be increased with prolonged exposure to Michel transport medium [Elias 1990].

Removal of Fixation Pigments

Formalin pigment resists extraction by most strong acids, water, alcohol, or acetone. Before staining with any desired technique, both formalin and malarial pigments (see chapter 11, "Pigments, Minerals, and Cytoplasmic Granules," p254) may be removed by treating deparaffinized and hydrated microscopic sections with one of the following solutions:

1. Absolute alcohol saturated with picric acid for 10 minutes to 3 hours. After treatment, wash sections well with water.

2. 70% alcohol (100 mL) containing 3 mL ammonium hydroxide for 30 minutes to 3 hours. After treatment, wash the sections well, rinse in 1% acetic acid, and wash again. Sodium or potassium hydroxide may be substituted for ammonium hydroxide.

Mercury pigment is removed as follows:

1. Treat the deparaffinized and hydrated microscopic sections with Gram or Lugol iodine for 10 minutes.
2. Wash the sections briefly in running water.
3. Place the sections in a 5% solution of sodium thiosulfate (hypo) for 3 minutes.
4. Wash the sections for 10 minutes, and stain as desired.

Lugol Iodine Solution

Iodine	1 g
Potassium iodide	2 g
Distilled water	100 mL

Place the iodine and the potassium iodide in approximately 20 mL of distilled water and mix until dissolved. Add the remaining water and mix well.

Troubleshooting Fixation Problems

The major problems encountered with fixation are because of delayed or incomplete fixation.

AUTOLYSIS

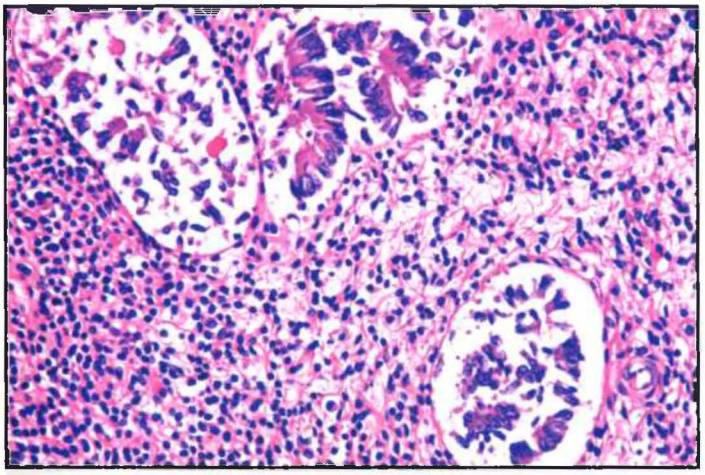
Autolysis is caused by delayed fixation. H&E-stained sections may show a loss or total disappearance of nuclear chromatin. Some cells may disappear, such as epithelial cells in intestinal specimens (see **[i1.5, p5]**), or there may be cell shrinkage with artifactual space around the cells. This artifact usually cannot be prevented on autopsy tissue. However, it can be prevented on surgical specimens by:

- Placing specimens in fixative solution as soon as possible, ensuring that the volume is 15 to 20 times that of the tissue.
- Opening uterus specimens upon receipt so that the fixative can immediately come into contact with the endometrium **[i1.20]**, **[i1.21]**.
- Opening, and pinning open gastrointestinal tract specimens upon receipt so that the fixative can immediately come into contact with the mucosal surface **[i1.22]**, **[i1.23]**.
- Slicing any organ resection (eg, spleen, kidney), breast, or large tissue specimens into thin slices and placing in a large volume of fixative.
- Bisecting lymph nodes if appropriate and placing in fixative **[i1.24]**, **[i1.25]**, **[i1.26]**.

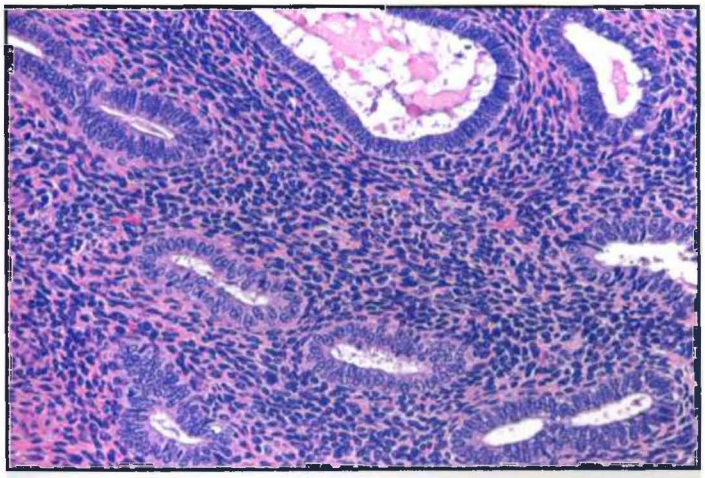
INCOMPLETE FIXATION

Because of the rapid turnaround time deemed necessary, frequently specimens are not allowed enough time in the fixative solution to fix completely before beginning dehydration. The result may be separation of tissue components on the flotation bath during microtomy and poor tissue morphology. The nuclei may be smudgy with no chromatin pattern defined **[i1.27]**, or nuclear bubbling may be seen **[i1.28]**. If tissue is not well-fixed when processing is begun, fixation continues in the alcohol, and the center of the tissue will often be more eosinophilic than the periphery. If signs of incomplete fixation are noted on H&E-stained sections, then the following should be done:

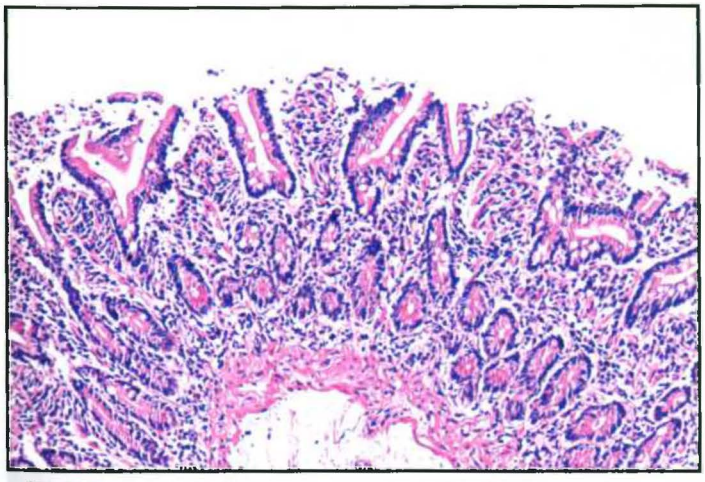
- Increase the time allowed in fixative solution. Some problems may be noted even after 24 hours' fixation in 10% neutral-buffered formalin.
- Change to another fixative, such as zinc formalin, which still requires several hours of fixation time for complete fixation. Glyoxal should also be considered because it is an extremely rapid fixative.
- Place formalin alcohol in the first 3 stages of the processing cycle. This will decrease fixation time, and also begin dehydration.
- Ensure that the grossed sections are thin enough for good reagent penetration, and that the amount of fixative is 15 to 20 times that of the tissue.
- Ensure that the formalin solution is not depleted because of overuse; change the solutions frequently.
- Do not pack cassettes tightly in the processing holders.
- Use agitation of cassettes in fixative holding solutions during or following grossing.



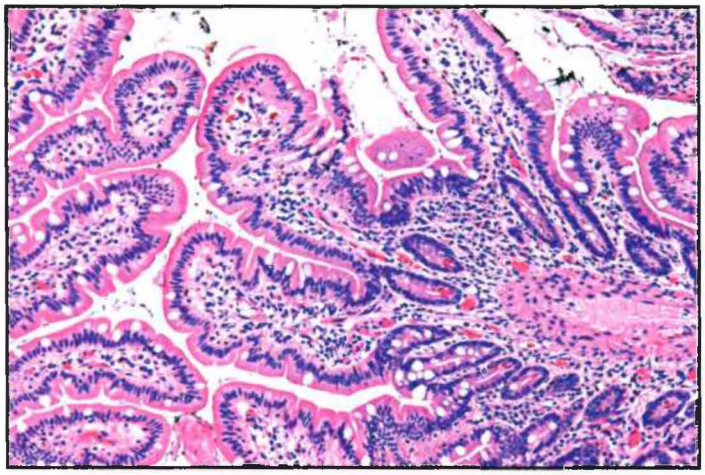
[il.20] The endometrium shows the results of delayed fixation in this section of uterus. Uterine specimens should be opened as soon as possible and immersed in a large amount of fixative so that proper fixation of the endometrium will occur. Compare with the well-fixed section of uterus shown in **[il.21]**



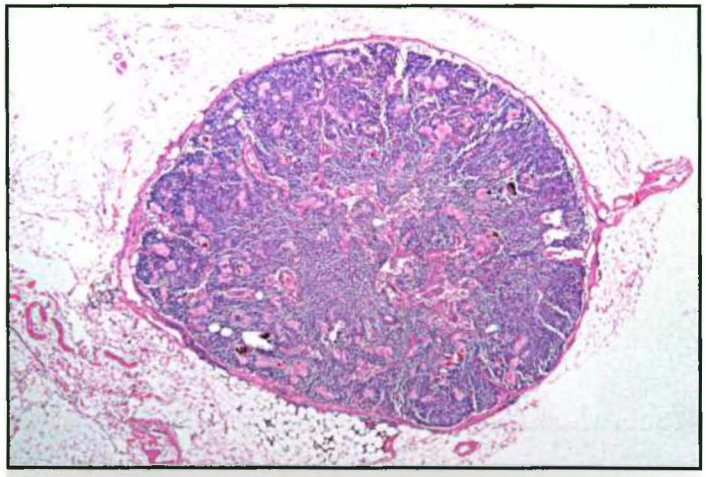
[il.21] The endometrium in this section of uterus demonstrates proper fixation.



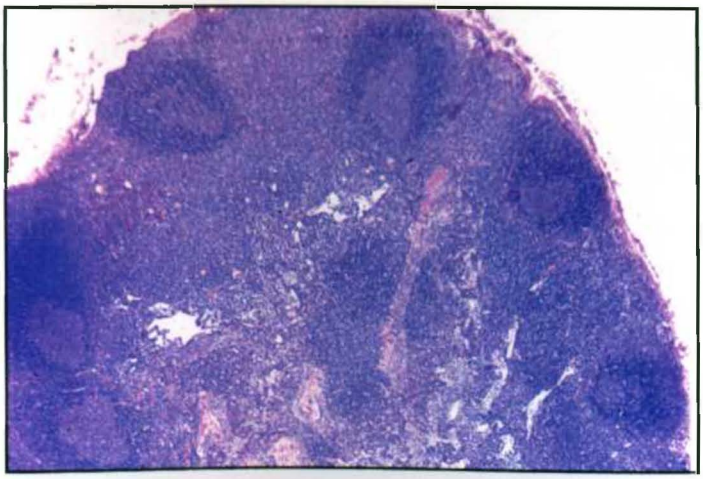
[il.22] A section of small intestine that shows the effects of delayed fixation and incomplete fixation. Much of the surface epithelium is gone, and there is also cell shrinkage. Gastrointestinal tract specimens should be opened as soon as possible, pinned out on a piece of cork, covered with fixative, and allowed an adequate amount of time for complete fixation to occur. Compare with the well-fixed section of small intestine shown in **[il.23]**



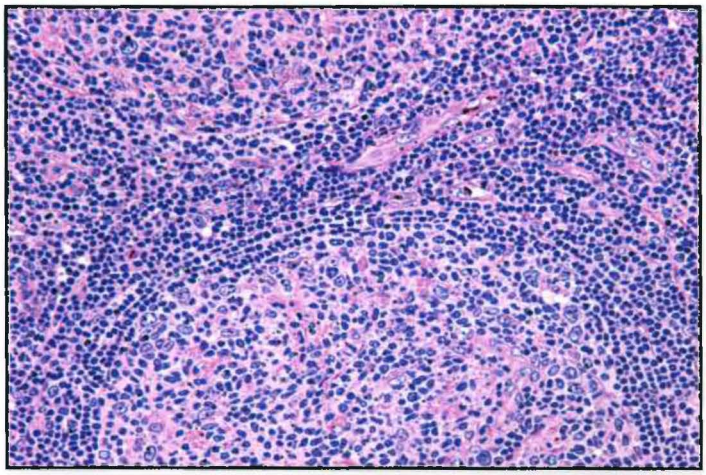
[il.23] A section of small intestine that has been properly and completely fixed. Also see **[il.4, p5]** for a well-fixed section of small intestine, and **[il.5, p5]** for a section that shows total autolysis.



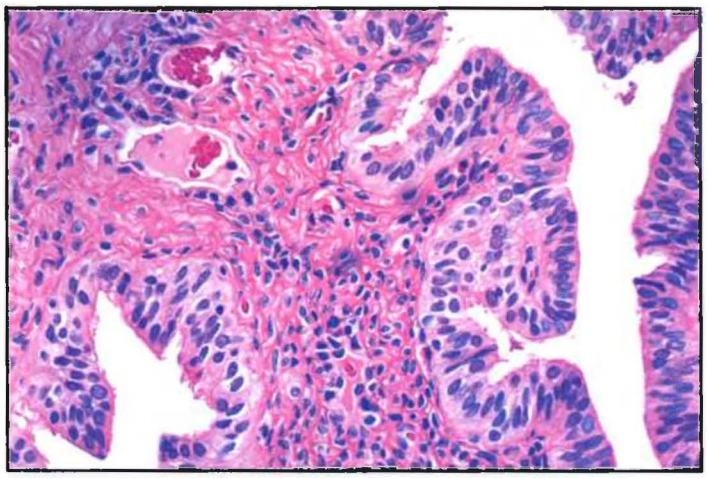
[il.24] A complete cross-section of a small lymph that shows incomplete fixation. Even small nodes should be bisected and allowed to fix well before processing. The cellular detail shown with an H&E on a well-fixed node can be seen in **[il.25]** and **[il.26]**.



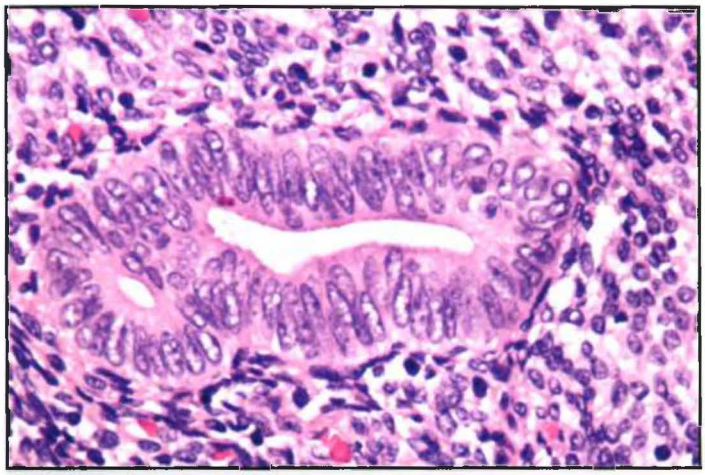
[il.25] A section of a portion of a lymph node that is well fixed. The germinal centers are well preserved, and portions of several trabeculae and medullary sinuses can also be seen.



[il.26] Parts of 2 germinal centers of a lymph node are very well preserved in this section of a well-fixed node.



[il.27] The results of incomplete fixation are apparent in the smudgy nuclei of this H&E-stained section of fallopian tube.



[il.28] Incomplete fixation has led to the nuclear bubbling artifact in this section. [Image courtesy of Lott R, Birmingham, AL].

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LEARNING ACTIVITIES

1. **Fix sections of spleen, liver, and kidney in each of the following:**

   a. 10% neutral-buffered formalin

   b. 10% aqueous formalin

   c. Bouin solution

   d. Zinc formalin

   e. Absolute alcohol

   f. Carnoy solution

   g. Any other fixative that might be in the laboratory

2. **Process the sections appropriately, washing if necessary, and cut 2 sections of each block at 3 to 4 µm. Stain 1 section of each with the routine H&E stain and examine microscopically. Answer the following questions:**

   a. Is there a fixation pigment in any of the sections? If so, remove the pigment in the duplicate section and stain with the routine H&E.

   b. Do all sections stain alike, or do some staining times need to be adjusted? If so, adjust on the duplicate section.

3. **Save the blocks for procedures learned in remaining chapters of this book.**

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CHAPTER 2

Processing

OBJECTIVES

On completing this chapter, the student should be able to do the following:

1. Define:
   a. dehydration
   b. clearing
   c. infiltration
   d. embedding
   e. universal solvent
   f. Carbowax
   g. cross-section
   h. decalcification
   i. ion exchange
   j. chelating agent
   k. miscible
2. List the properties and actions of the following; indicate whether each property or action is an advantage or a disadvantage:

   Dehydrating Agents

   a. alcohol (ethyl, methyl, butyl, isopropyl)
   b. dioxane
   c. acetone
   d. tetrahydrofuran

   Clearing Agents

   e. xylene
   f. toluene
   g. benzene
   h. acetone
   i. chloroform
   j. cedarwood oil
   k. tetrahydrofuran
   l. dioxane
   m. limonene derivatives
   n. aliphatic hydrocarbons
3. Identify any special precautions (eg, time of use, safety) that must be considered when using any of the reagents listed in objective 2
4. Identify why a concentration of ethanol no higher than 65% should be used after fixation with phosphate-buffered formaldehyde
5. State why xylene should be used after clearing with cedarwood oil
6. Identify 3 universal solvents
7. Compare xylene, limonene derivatives, and the aliphatic hydrocarbons as to physical features, disposal options, and health effects
8. State the possible effects on H&E stains if water is present in the clearing agent on the tissue processor
9. State how each of the following methods is similar to paraffin and how each differs (eg, dehydration, clearing):
   a. water-soluble wax (Carbowax)
   b. celloidin
   c. glycol methacrylate
   d. epoxy resin
   e. agar and gelatin
10. State the appropriate processing method or medium for:
    a. electron microscopy
    b. enzyme histochemistry
    c. undecalcified bone
    d. fat demonstration
11. Identify the advantages and disadvantages of paraffin compared with other available embedding media
12. Relate the melting point of paraffin to hardness, section thickness, and ribboning quality
13. Identify the problem encountered when:
    a. the infiltrating paraffin overheats
    b. the embedding paraffin overheats
    c. dehydration is inadequate
    d. dehydration is excessive
    e. clearing is inadequate
    f. clearing is excessive
14. Outline paraffin processing schedules, and describe how an increase or decrease in the time that the specimen remains in each solution will affect the quality of processing
15. Describe the microwave processing procedure and identify the differences between the microwave procedure and the procedure used on an enclosed processor
16. Identify 2 means of decreasing processing time when using paraffin
17. Identify at least 3 paraffin-processing errors and describe the method of correction for each error
18. Identify at least 2 quality-control procedures used in processing tissues for paraffin embedding
19. Relate rapid chilling of paraffin to crystal size
20. State why paraffin wax of small crystalline structure is important
21. Describe the proper orientation when embedding:
    a. skin
    b. tubular structures
    c. structures with a wall (eg, gallbladder)
    d. bone
22. State at least two ways that specimen orientation can be indicated during gross dissection to ensure proper embedding

OBJECTIVES continued

23. Briefly describe the following methods of decalcification:
    a. simple acid
    b. ion-exchange resin
    c. electrolytic
    d. chelation
24. List the advantages and disadvantages of each of the decalcification methods listed
25. Describe the methods for determining the endpoint of decalcification
26. Identify methods for increasing the rate of the decalcification reaction
27. State the effects of overdecalcification and underdecalcification on calcified tissue sections
28. State the effects of decalcification on fixed and unfixed tissue
29. Describe a method of handling paraffin tissue when areas of calcification are discovered during microtomy
30. Identify 3 methods of freezing tissue in preparation for cutting frozen sections, and indicate which procedure should be used for skeletal muscle enzyme studies

Processing is usually considered to include dehydration, clearing, and infiltration. This chapter will also discuss embedding, decalcification, and frozen sections as special areas of processing. Both the fixative and the processing method should be chosen either before or immediately after removal of tissue at surgery or autopsy. The choice of processing method may influence the choice of fixative. For example, when frozen sections are indicated, the tissue is not usually fixed; however, when processing for routine studies, the choice of fixative depends primarily on the preference of the pathologist.

Dehydration

Dehydration means the removal of water, a process necessary to prepare the tissue for embedding in a nonaqueous medium such as paraffin, celloidin, and some plastics. These embedding media will not infiltrate tissue that contains water. The free, rather than the molecularly bound, water in the tissue must be removed before attempting infiltration with the embedding medium. However, with excessive dehydration, bound water may be removed in a nonadditive reaction. This results in hard and brittle tissue that is difficult to section. If dehydration is incomplete, the clearing agent will not act properly, and soft, mushy blocks will be the result **[i2.1]**. Incomplete dehydration accounts for the vast majority of processing problems. The dehydrating agents act to remove water in 2 ways. Some reagents are hydrophilic (water-loving) and attract water from the tissue, whereas other reagents dehydrate by repeated dilution of the aqueous tissue fluids.

ALCOHOLS

Most dehydrating reagents are alcohols. Most of the absolute alcohols contain some water, frequently as much as 1% or 2% [Humason 1979]. If it is necessary to verify that the water content does not exceed 2%, Humason suggests adding a few milliliters of alcohol to a few milliliters of xylene or toluene. A persistent turbidity in the solution indicates the presence of more than 2% water.

Because small colorless biopsy specimens are often difficult to identify at the embedding station, eosin or phloxine frequently is added to the absolute alcohol to dye the tissue a light pink. An indicating dessicant, Drierite (WA Hammond Drierite Co, Xenia, OH), has also been used both to indicate completeness of dehydration and to dye tissue for identification during the embedding step [Carson 1970]. Both eosin and Drierite can be used with ethanol but not with isopropanol. Eosin is insoluble in isopropyl alcohol, and the water content of isopropyl alcohol also renders the Drierite ineffective. Drierite cannot be used on the enclosed processors, so eosin or phloxine should be added to the alcohols on these processors. Because tissue is normally stained with eosin and/or phloxine, these dyes are safe and will not interfere with later routine staining. Mercurochrome definitely should not be used; it contains mercury, is a hazard, and will contaminate the processing reagents.

[i2.1] The bone in this block was not dehydrated or cleared sufficiently, so infiltration was poor. The white area in the center of the block is soft, indicating that this area was not well infiltrated with paraffin and it will not be sectioned.

■ *Ethyl Alcohol (Ethanol)*

Ethyl alcohol is a clear, colorless, flammable liquid. Because it is drinking alcohol, ethyl alcohol is strictly controlled by the federal government, and troublesome recordkeeping is required if the alcohol is purchased tax-free. Ethyl alcohol is reliable and fast acting, and is probably the best of the dehydrants. It is hydrophilic and therefore mixes with water, as well as with many organic solvents, in all proportions. If time permits, ethyl alcohol should be used in a sequence of solutions that gradually increase in concentration; this gradual increase probably reduces some of the tissue shrinkage that occurs in the dehydration process. To save time, the dehydration process is frequently begun with 95% ethanol followed by absolute ethanol; however, it is a better practice to begin with 1 solution of 60% to 65% alcohol, followed by 2 changes each of 95% and absolute alcohol. When phosphate-buffered formaldehyde solutions are used for fixation, phosphate salts will precipitate in the tissue if the concentration of the initial alcohol solution is greater than 70%; these precipitated phosphate salts will cause difficulty in microtomy [Dapson 1988]. Long periods of dehydration in absolute alcohol should be avoided because this causes excessive shrinkage and hardening of the tissue sections.

If laboratory personnel do not wish to undertake the recordkeeping demanded when using pure, tax-free ethyl alcohol, then ethanol that has been made unfit for human consumption, usually by adding methanol and/or isopropanol, may be substituted. This product is known as *denatured alcohol* or *reagent alcohol*, and it has a more pronounced odor than pure ethanol. Other additives sometimes found in ethyl alcohol may cause fading of stains over time, so care should be taken when selecting the type of ethyl alcohol used.

Ethyl alcohol, if used alone, is moderately toxic with a permissible exposure limit (PEL) of 1,000 ppm. It is also very flammable. When denatured with methanol or isopropanol, it becomes much more toxic. If disposed of in the sanitary sewer system, it must not exceed a 24% solution; it is important that flammable concentrations do not build up in the plumbing. Recycling is preferred.

■ *Methyl Alcohol (Methanol)*

Methanol is a flammable, clear, colorless reagent with a slightly unpleasant odor. It is rarely used alone for dehydration, but it can be used like ethanol. Its primary use has been for the fixation of blood smears.

Methanol is poisonous; it is broken down to formaldehyde by the liver and acts as formaldehyde on the body. Overexposure can cause blindness and even death. Methanol has a PEL of 200 ppm, and care should be taken to protect the skin from absorption of this solution.

■ *Isopropyl Alcohol (Isopropanol)*

Isopropanol is an excellent substitute for ethanol in processing tissue for paraffin embedding. However, many stains, such as eosin, are insoluble in isopropanol; therefore, it cannot be substituted for ethanol in the preparation of staining solutions. Isopropanol also cannot be used in the celloidin technique, because nitrocellulose is insoluble in it. Isopropanol does not have any government restrictions, is easily obtained, and does not harden or shrink tissue as much as ethanol. It is never totally absolute because it contains about 1% water; therefore, a slight amount of water also remains in the tissue.

Isopropyl alcohol is mildly irritating to the eyes, nose, and throat, and has a PEL of 400 ppm. It is toxic by ingestion. It is a flammable reagent; for drain disposal, keep the concentration below 10%, because it is considered ignitable at higher concentrations.

■ *Butyl Alcohol (Butanol)*

Butanol is good for dehydrating plant and animal material. It has a pronounced odor and low dehydrating power; therefore, long periods are required for dehydration with butanol. It is an excellent dehydrant when slower processing procedures are needed, and butanol causes less shrinkage and hardening than ethanol. The PEL for butanol is 100 ppm.

ACETONE

Acetone is very rapid acting and less expensive than some of the other dehydrants. According to some laboratorians, it causes excessive shrinkage. If acetone is exposed to the air, it will absorb water. Acetone is very volatile, and maintaining proper solution levels can be a problem in open processors.

Acetone is very flammable, with a flash point of –17°C. The Occupational Safety and Health Administration (OSHA) recommends a PEL of 1,000 ppm of acetone, but the National Institute for Occupational Safety and Health (NIOSH) recommends a PEL of 250 ppm.

UNIVERSAL SOLVENTS

The term *universal solvent*, as used in histopathology, denotes those reagents that avoid the use of 2 solutions; universal solvents perform both the dehydrating and clearing steps. Dioxane, tertiary butanol, and tetrahydrofuran are in this group of reagents. Most universal solvents are unsuitable for use with delicate tissue; tissue distortion results from the heavy diffusion currents that occur when tissue is transferred into the universal solvent and again when the tissue is transferred into the paraffin.

■ *Dioxane*

Dioxane produces less shrinkage during dehydration than ethanol. Even after long periods in dioxane, tissue does not show undesirable changes in consistency or in subsequent staining properties. Dioxane is a faster dehydrant than ethanol but must be used in large volumes. It frequently contains water, and if water is left in the tissue, dioxane may allow as much as 50% shrinkage during infiltration. It is expensive, but may be reclaimed for reuse by treating used solutions for 18 to 22 hours with anhydrous calcium chloride or calcium oxide. Because of its toxicity, dioxane is rarely used today.

Dioxane is cumulatively toxic, has a pronounced odor, and must be used in well-ventilated rooms. The PEL recommended by OSHA is 100 ppm, but the PEL recommended by NIOSH is 1 ppm. Dioxane is a flammable liquid and also a suspected carcinogen.

■ *Tertiary Butanol*

Tertiary butanol is odorous, expensive, and tends to solidify at room temperature. Following the use of tertiary butanol, half tertiary butanol and half paraffin must be used for the initial paraffin infiltration of tissue. If desired, this alcohol may be used for dehydration in the staining process, because tertiary butanol mixes with water, ethanol, and xylene in all proportions. Tertiary butanol has a PEL of 100 ppm.

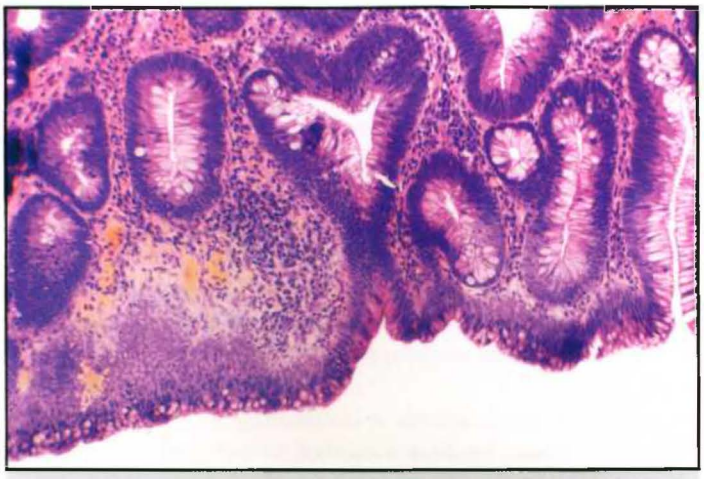
■ *Tetrahydrofuran*

Tetrahydrofuran is miscible with the lower alcohols, water, ether, chloroform, acetone, benzene, toluene, xylene, and melted paraffin. It is much the same as dioxane in properties and use, but unlike dioxane, tetrahydrofuran is not a cumulative toxin. Tetrahydrofuran acts rapidly without causing excessive shrinkage and hardening, and it is the best of the universal solvents. It is very useful in the reprocessing of inadequately dehydrated and cleared tissue. It will not dissolve dyes, but will dissolve mounting media; therefore, it can be used for the dehydration and clearing of stained sections.

Tetrahydrofuran has a rather offensive odor, and may be harmful by inhalation, ingestion, or skin absorption. It has been known to cause conjunctivitis, and skin contact may cause dermatitis. Long-term exposure may cause kidney or liver damage. It is very volatile, has a flash point of –14.5°C, and the lower explosive limit in air is 11.8%. Explosive peroxides also may be formed. Tetrahydrofuran has a PEL of 200 ppm as an 8-hour, time-weighted average, and 250 ppm as a short-term exposure limit (STEL).

Clearing

The process of clearing was originally termed as such because the reagents used for this step have a high index of refraction and will render tissue transparent. Clearing agents must be miscible with both the dehydration agent and the infiltration medium, which most frequently is paraffin. Clearing agents are sometimes referred to as *dealcoholization agents*; their primary purpose is to remove the alcohol used for dehydration and to make the tissue receptive to the infiltration medium. Inadequate clearing will be followed by inadequate infiltration of tissue with the embedding medium. As seen with incomplete dehydration **[i2.1]**, the resulting tissue is soft and mushy. Prolonged periods in many of the clearing agents will produce hard, brittle tissues. Wynnchuk [1993] states that the time of tissue exposure to the hydrocarbon clearing agents is critical, because excessive dealcoholization can cause further denaturation of tissue proteins in the same way that excessive dehydration does. This results in difficulties with microtomy.



[i2.2] Uneven staining, most likely caused by contamination of the clearing and/or infiltration reagents with water. The contamination may be caused by absorption of water by the dehydrating agents in time of high relative humidity, or by condensation of the fixative on the processor lid and then contamination of the succeeding reagents. The problem is worse on small tissues, as in skin, endometrium, and gastrointestinal biopsies [Wynnchuk 1990].

XYLENE

Xylene is the most widely used clearing agent, and although substitutes have been proposed, few have found widespread acceptance. Prolonged treatment with xylene should be avoided, as tissue tends to become overhardened. Fibrous, muscular, central nervous system or cartilaginous tissues are especially affected by the hardening property of this hydrocarbon. Xylene is relatively rapid in displacing alcohol and is miscible with paraffin; however, it is rather intolerant of any water left in the tissue. As with any of the aromatic hydrocarbon clearing agents, tissue that is adequately dehydrated and then cleared with xylene becomes transparent. This property of xylene may be used to determine the adequacy of clearing before embedding the tissue, and corrective action may be taken when indicated. Xylene turns cloudy in the presence of water; if the xylene on the tissue processor is ever noted to be cloudy, the reagent should be changed immediately.

Xylene is a flammable reagent and is considered a hazardous substance, so waste solutions either must be recycled or disposed of in an approved manner. Xylene may not be poured down the sink, because it does not mix with even small quantities of water. Xylene has a PEL of 100 ppm, a short-term exposure limit of 150 ppm, and it should be used with adequate ventilation. It is a neurotoxin with short-term intoxication effects of headaches, dizziness, lack of coordination, mental confusion, and fatigue. Repeated exposure can cause central nervous system damage. It is also a defatting agent, and contact with the skin should be avoided.

TOLUENE

Toluene does not overharden tissue as much as xylene; in fact, tissues may remain in toluene overnight without harm. It is considered by many laboratory workers to be the best of the aromatic hydrocarbon clearing agents, a group of reagents that includes xylene, toluene, and benzene. According to Wynnchuk [1990, 1993], toluene has a greater tolerance for atmospheric water contamination than xylene. She found that much of the universal problem with uneven H&E staining and poor nuclear chromatin patterns **[i2.2]** was the result of incomplete clearing. She related the incomplete clearing to moisture in the clearing agent from such causes as excessive laboratory humidity, or evaporation of the fixative solution with subsequent condensation on the processor pot or chamber lid and then contamination of the succeeding reagents. When the relative humidity is high, hygroscopic substances such as absolute ethanol absorb more water vapor from the atmosphere to reach equilibrium with the environment, so water still remains in the tissue when it reaches the clearing step. With a closed processing system, if there is evaporation and condensation of the fixative, the rise and fall of pressure from the vacuum system during the remainder of the processing cycle will dislodge some of the fixative droplets, and they will fall into the processing chamber or onto the cassettes. Wynnchuk [1994] corrected the problem with excessive humidity by substituting toluene for xylene; the evaporation and condensation of fixative was attributed to a processor problem.

Toluene is flammable and more volatile than xylene, and has a PEL of 50 ppm and a short-term exposure limit of 150 ppm.

BENZENE

Benzene is very fast acting and does not overharden tissue like xylene; however, it hardens muscle, tendon, and uterus more than does toluene. Benzene evaporates rapidly from the paraffin bath; therefore, the paraffin used for infiltration does not require rotation and changing as frequently as other clearing agents. In open processors, the appropriate volume of reagent is difficult to maintain because benzene is very volatile.

Benzene should not be used because it is very toxic, with a PEL of 10 ppm (OSHA) or 0.1 ppm (NIOSH). It is also a carcinogen, primarily affecting blood and bone marrow.

CHLOROFORM

Chloroform leaves tissue less brittle than does xylene. It penetrates slowly, making clearing a longer process. Because chloroform readily absorbs atmospheric moisture, it should be used in tightly covered containers. Chloroform tends to desiccate connective tissue, but even so, it is a better clearing agent than the aromatic hydrocarbons for uterus, muscle, and tendon. It is very volatile, which makes fluid levels difficult to maintain in open processors. Transparency of tissue cannot be used to determine the end point of clearing after immersion in chloroform, because chloroform does not make tissue transparent.

Chloroform is not flammable or combustible, so it presents serious disposal problems because it cannot be incinerated or burned. Heating chloroform solutions may cause the formation of phosgene, a toxic gas. Chloroform has a PEL of 50 ppm, and it is a carcinogen.

ACETONE

Acetone is not mentioned in the literature as a clearing agent; however, because acetone has a very low boiling point (58°C), it will boil off and be replaced by paraffin, provided the paraffin baths are kept at 58°C or higher. Because acetone is boiled off, the paraffin baths do not become contaminated as easily as with the other clearing agents, and the baths do not need to be changed as often. Because acetone also can be used for dehydration, it can be used like the universal solvents. Tissue cleared with acetone will show more shrinkage than those that have been cleared with xylene. The safety considerations for acetone have been described in the section on dehydration.

ESSENTIAL OILS

The essential oils are very expensive and they are used today only for special projects. They are volatile and usually have the characteristic odor of the flavor of the plant from which they are obtained; the odor is usually very strong and can be nauseating if the oil is not used with adequate ventilation. Although the essential oils are volatile, the volatility is not sufficient to allow ready replacement during paraffin infiltration, and if oil remains in the tissue, microtomy is frequently difficult. For this reason, the oil should be removed by an aromatic hydrocarbon clearing agent (eg, xylene or toluene) before infiltration. Essential oils that have been used are clove, origanum, sandalwood, and cedarwood; the best known and most widely used of these was cedarwood, which will clear alcohol-dehydrated tissue quickly and will not cause further tissue shrinkage. Unlike most clearing agents, cedarwood oil will clear tissue after dehydration with 95% alcohol. It hardens and damages tissue less than any other known clearing agent; tissue may remain in cedarwood oil indefinitely without harm.

LIMONENE REAGENTS (XYLENE SUBSTITUTE)

Limonene reagents have become popular during the past decade, and are usually referred to as xylene substitutes because they are used in the place of xylene during processing. Some of these reagents can have an overpowering citrus odor. These limonene reagents tend to harden tissue less than xylene, but they do cause more contamination of the paraffin, making it necessary to change the paraffin more frequently.

Although it was generally regarded as safe when it was introduced, limonene is considered to be an irritant and sensitizer in its concentrated form. Therefore, prolonged or repeated exposure predisposes individuals to allergic reactions [Dapson 1995]. It may cause difficulty in breathing and can also cause headaches; in our laboratory, 1 technician subject to asthma could not tolerate this reagent. PELs are unavailable for inhalation toxicity of the limonene reagents. Even though it is said to be biodegradable, it is not water-soluble, so it cannot be disposed of in the sanitary sewer system. It is against Environmental Protection Agency (EPA) regulations to dispose of substances in the sanitary sewer system that are not miscible with water.

ALIPHATIC HYDROCARBONS (XYLENE SUBSTITUTE)

The newest class of clearing agents is the aliphatic hydrocarbons, generally called *alkanes*. These reagents are low in reactivity and toxicity, and are from the same family of compounds as propane, butane, petroleum jelly, and paraffin wax. Based on the chain length of the molecule, aliphatic hydrocarbons have 2 subclasses. The light weight (short chain) aliphatics are usually chosen for use in histotechnology because they penetrate tissue faster, remove fat more effectively, and when used in the staining process, allow coverslips to dry in the usual fashion [Dapson 1995]. The major disadvantages of the aliphatics are their intolerance for water and their incompatibility with some mounting media. These reagents may be difficult to use in areas of high humidity because of atmospheric moisture absorbed into the alcohol preceding the clearing agent. In an extensive evaluation of xylene substitutes, Wynnchuk [1994] compared several proprietary limonene and aliphatic hydrocarbon clearing agents with xylene and toluene in a blind study. She found that the aliphatic hydrocarbon scores were comparable with or better than xylene, but that some proprietary aliphatic hydrocarbon reagent scores were better than others. The reader is referred to her article for a complete evaluation based on brand name. The aliphatic hydrocarbons can be used on all tissue processors and automated stainers; however, they are not recommended for use on automatic coverslipping instruments. A comparison of the attributes of xylene, limonene, and the aliphatic hydrocarbons is shown in **[t2.1]**.

Because the aliphatic hydrocarbons are less aggressive than xylene, methods of processing and staining become even more critical and adjustments in the procedures are usually necessary. The methods recommended by Dapson [1995] for making the aliphatic hydrocarbons work are shown in **[t2.2]**. The aliphatic hydrocarbons are nonirritating and nonsensitizing, with an 8-hour exposure limit of 300 ppm.

UNIVERSAL SOLVENTS

The universal solvents used for clearing include dioxane tertiary butanol and tetrahydrofuran (see section entitled "Dehydration").

[t2.1] Comparison of attributes of xylene, limonene, and aliphatic hydrocarbons*

Attributes	Xylene	Limonene	Aliphatic hydrocarbons
Health effects			
TLV	100 ppm	Not established	300 ppm
Irritant	Yes	No	No
Sensitizer	No	Yes	No
Neurological effects	Yes	No	No
Physical features			
Fire class	Flammable	Combustible	Combustible†
Evaporation rate	Moderate	Slow	Moderate
Odor	Moderate	Strong	Nearly odorless
Solvent power	Strong	Moderate	Selective
Hardening effects on specimens	Harsh	Moderate	Very gentle
Disposal options			
Recycling	Yes	No	Yes
Licensed hauler	Yes	Yes	Yes
Restrictions on mounting media	No	‡	Several brands not compatible

*Reprinted with permission from [Wynnchuk 1994] and [Dapson 1989].
†One brand in the study was flammable.
‡Be certain that either the limonene-based or the mounting medium contains an antioxidant to prevent fading. Some combinations of brands should be avoided.

OTHER CLEARING AGENTS

Carbon tetrachloride, carbon bisulfide, and aviation gasoline have been used for clearing, but their associated hazards are such that these chemicals have no place in the histopathology laboratory.

[t2.2] Changes associated with a change to the aliphatic hydrocarbons*

Making xylene substitutes work
Use 3 stations of clearant on the tissue processor
Use 3 stations, 3 minutes each, to deparaffinize sections
Rotate alcohols after eosin carefully
Keep anhydrous alcohol dry
Coverslip "upside down"†

*Reprinted with permission from [Dapson 1995].
†Apply the mounting medium to the coverslip and lower the slide at an angle and upside down over the coverslip.

Infiltration

After dehydration and clearing, tissue must be infiltrated with the supporting medium. This medium, generally referred to as an embedding medium, holds the cells and intercellular structures in their proper relationship while thin sections are cut. With paraffin especially, it is important that the tissue be dehydrated and cleared very well, or the tissue will not be infiltrated completely. If the infiltration medium cannot completely replace the clearing agent, then problems will be noted on the H&E-stained section. Complete dehydration and clearing are not as critical with some of the other infiltration media.

PARAFFIN

Paraffin wax remains the most popular medium because large numbers of tissue blocks may be processed in a comparatively short time, serial sections are easily obtained, and routine and most special staining can be done easily. Paraffin is a fairly inert mixture of hydrocarbons produced by the cracking of petroleum. The commercial paraffins marketed for embedding contain various additives such as beeswax (reduces crystal size and increases stickiness and adhesion), rubber (reduces brittleness, increases stickiness, and makes the formation of ribbons during sectioning easier), other waxes (produce smooth texture and smaller crystal size), and plastics (increase hardness and support). These additives also enhance the ability of paraffin compounds to provide support for hard tissues. Ribbons are formed when the first section cut adheres to the microtome knife blade and each subsequent section adheres to the edge of the section immediately preceding it. Each new section cut will push the preceding section away from the knife edge.

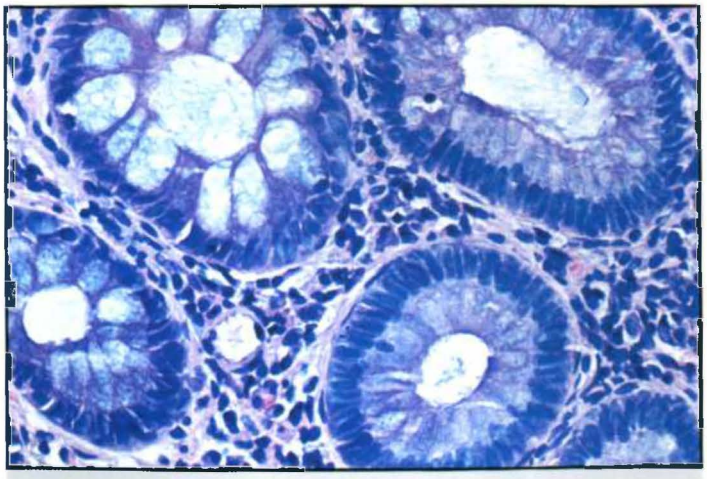
In choosing the paraffin for use, several factors must be considered. In general, the properties of paraffin vary with the melting point. As the melting point increases, the paraffin becomes harder and provides better support for hard tissue. Thus, thinner sections can be obtained, but ribboning becomes more difficult. As the melting point decreases, the wax becomes softer and provides less support for hard tissues. Thin sections also become more difficult to obtain, but ribboning becomes easier. Sheehan and Hrapchak [1980] also relate paraffin hardness to the plastic point, or the lowest temperature at which permanent deformation can occur without fracture. The plastic point varies with the paraffin and is a few degrees below the melting point. Ideally, paraffin should be matched to the hardness of the tissue to be sectioned, the temperature at which sectioning is to be done, and the stain to be used. For most immunohistochemical techniques that can be done on paraffin-embedded tissue, paraffins with lower melting points have become popular because heat may inactivate the antigens.

Paraffin with a melting point of 55°C to 58°C is commonly used for routine work; this seems to be the best compromise for providing good support for most tissues, yet allowing good ribboning and the ability to obtain thin sections.

Tissue should remain in paraffin the shortest time necessary for good infiltration because exposure to prolonged heat causes shrinkage and hardening. The supply of melted paraffin should be kept 2°C to 4°C above the melting point because tissues exposed to overheated paraffin during infiltration will overharden. Overheating the embedding paraffin may change the sectioning quality, but because tissue is not immersed in this paraffin for a long period at the higher temperature, the tissue will not be affected.

Paraffin infiltration is greatly aided by vacuum; however, vacuum and heat should be applied cautiously when processing very small specimens because it frequently results in overhardening. It is difficult to process biopsy specimens and larger specimens such as breast or uterus on the same processing cycle and achieve good results with each. Biopsy specimens should be processed on a much shorter cycle than larger specimens; if they must be processed overnight, they should be put on a separate processor and held in fixative for a much longer time. Large and fatty tissues will not be processed adequately on the shorter cycles. If a schedule that gives good processing of larger specimens is used on biopsy specimens, it will result in hard, friable tissues that are difficult to section or that will contain microtomy artifacts. This overprocessing also leads to staining problems.

Because of the wide variation of processing instrumentation found in histology laboratories, only examples of protocols for overnight processing of large and fatty tissues (protocol 1) and for processing biopsy specimens (protocol 2) are given. Each laboratory will have to formulate its own schedule depending on instrumentation, time constraints, and reagent choice. Because open processors are rarely used today, no protocols for their use will be given. Heat should be used, with rare exception, only in the paraffin infiltration step. Heating of all reagents can cause overprocessing and later problems with staining [i2.3].



[i2.3] Staining problems resulting from improper processing. The hazy blue nuclei are the result of improper use of heat on the processor. With rare exception, only the paraffin should be heated; all other reagents should be at ambient temperature. [Image courtesy of Dapson RW, Anatech Ltd]

■ *Protocol 1*

This is an example of solutions and times for routine overnight processing with a closed system [t2.3].

[t2.3] Solutions and times for routine overnight processing with a closed system

Solution	Time and conditions
Formalin, 10%	2 hours (no heat, vacuum)
Alcoholic formalin (see "Fixation")	1 hour (no heat, no vacuum)
Alcoholic formalin	1 hour (no heat, no vacuum)
Alcohol, 95%	1 hour (vacuum only)
Alcohol, 95%	45 minutes (no heat, no vacuum)
Absolute alcohol	45 minutes (vacuum only)
Absolute alcohol	1 hour (no heat, no vacuum)
Xylene	1 hour (no heat, no vacuum)
Xylene	1 hour (vacuum only)
Paraffin	30 minutes (no vacuum)
Paraffin	1 hour (no vacuum)
Paraffin	1.5 hours (vacuum only)

■ *Protocol 2*

I have found Protocol 2 to be excellent for processing small biopsy specimens (eg, needle biopsies) on which a rapid diagnosis is needed. Vacuum is used on all stations, but heat is used only with the paraffin. The specimens should be allowed to fix at least 30 minutes before being placed on the processor; frequently different fixatives are used so no fixative is included on the processor. This is an example of solutions and times for the rapid processing of biopsy specimens [t2.4].

[t2.4] Solutions and times for rapid processing of biopsy specimens

Solution	Time
Alcohol, 65%	15 minutes
Alcohol, 95%	15 minutes
Alcohol, 95%	15 minutes
Absolute alcohol	15 minutes
Absolute alcohol	15 minutes
Xylene	15 minutes
Xylene	15 minutes
Paraffin	15 minutes
Paraffin	15 minutes
Paraffin	15 minutes

■ *Quality Control of Paraffin Processing*

Adequate reagent volume must be maintained in the processor containers to ensure high-quality tissue sections. The reagents also must be rotated or changed frequently. Hydrometers, which measure specific gravity but also can be calibrated to read the percentage of alcohol directly, may be used to check alcohol for suspected contamination. However, to ensure consistent quality of sections, a routine schedule should be established for the rotation and changing of reagents [**f2.1**]. The frequency of change can be determined in 1 of 2 ways. In a large laboratory where processors are filled to capacity each day, reagents should be changed on a set schedule, but in smaller laboratories where the workload varies day by day, the frequency of change should be determined by the volume of tissue processed. The quality control chart can be taped to the front or side of the processor and checked each day as the reagents are monitored.

Quality control of the paraffin infiltration containers is also very important. Temperatures must be recorded daily, necessary adjustments in the temperature must be made, and any corrective actions must be documented. If not monitored and changed frequently, the paraffin will become contaminated with clearing agent. Paraffin containers should be rotated routinely with frequent changes of fresh paraffin. Where possible, 3 changes of paraffin are recommended for infiltration, and the last change should never have an odor of clearing agent.

■ *Microwave Oven Processing*

Within the last decade, tissue processing with the microwave oven has been introduced into more and more laboratories. Although it is also used for large tissue sections, this technology is especially useful for biopsy tissues. Numerous commercial microwave ovens are available, and for the purpose of processing tissues, only laboratory models should be used. Household microwave ovens are not satisfactory for this purpose, because the temperature must be carefully controlled and the microwave must be vented just like a chemical fume hood.

Tissue should be thoroughly fixed before being placed in the microwave oven, or fixation will be completed by the alcohol and lead to morphologic changes less familiar to the pathologist. If desired, fixation and cross-linking can be speeded up with microwave technology. Only a procedure for processing 1-mm-thick tissues will be described as an example of microwave processing. It uses only 3 reagents (ethyl alcohol, isopropyl alcohol, and paraffin), and requires approximately 45 minutes [Willis 2007].

1. Place fresh tissues in labeled cassettes, and fix for 30 minutes. Place on an agitator or mechanical stirrer to enhance fixation.
2. Rinse cassettes with water to eliminate the possibility of salt precipitation when the cassettes are placed in ethyl alcohol.
3. Place cassettes into the nonstick (Teflon) processing rack. 20 cassettes will fit in 1 rack; leave the top level empty to allow for evaporation. (When processing fewer than 8 cassettes, place at least 8 empty cassettes in the bottom of the rack to regulate the temperature and ensure proper processing.)
4. Place processing rack in plastic container, and fill with 100% ethyl alcohol to rinse off water. Discard alcohol and fill with 400 mL of fresh 100% ethyl alcohol.
5. Place the plastic container in the microwave oven, and place the temperature probe centrally into the container, making sure that the probe does not touch the cassettes.
6. Microwave at a temperature of 67°C for 5 minutes.
7. Remove the plastic container and processing rack from the microwave, remove the rack from the container, and drain on a paper towel.
8. Place the processing rack into the emptied plastic container, and fill with isopropyl alcohol.
9. Place the plastic container in the microwave, insert the temperature probe, and microwave at a temperature of 74°C for 3 minutes.
10. Remove from the microwave, and place the processing rack into an empty container, and fill with paraffin that is at a temperature setting of 60°C. Agitate the rack so that the excess isopropanol will mix with the paraffin. Pour this paraffin into a paraffin pot that is at a temperature of 84°C. Refill the container with fresh 60°C paraffin.
11. Place the container in the microwave, insert the temperature probe, and microwave at a temperature of 65°C for 2 minutes. During microwaving, open the microwave door and agitate the

Month:											**Year:**				
Day 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	

[f2.1] An example of a quality control chart for monitoring reagents on the tissue processor. 1. Place a "U" (used) on each day for each time that tissue, or a "run," is processed. 2. After the processor has been used twice, or two runs have been processed, rotate the containers of fixative, 95% alcohol, absolute alcohol, and xylene. Place a fresh solution last in each type of solution. Indicate rotation with an "R." 3. Paraffins are to be rotated after using three times. Indicate rotation and fresh paraffin with "PC."

rack several times to ensure temperature consistency. Increase the temperature setting to 84°C and microwave for 5 minutes. Any residual isopropyl will be boiled off at this temperature.

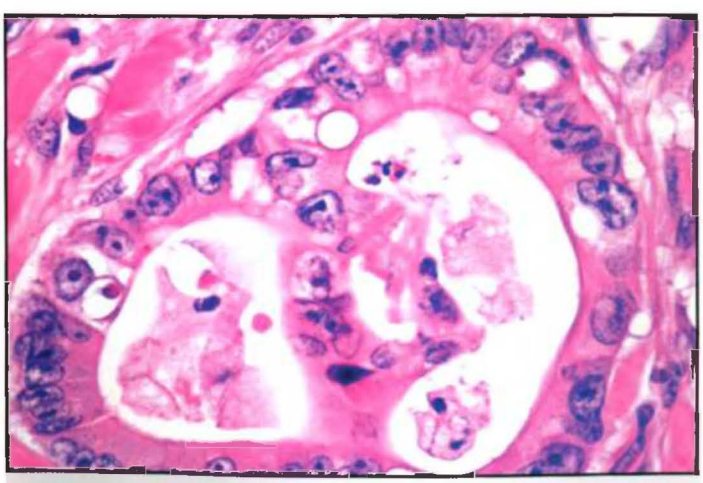
12. Remove the container from the microwave, dump the paraffin in the paraffin pot set at 84°C, remove the processing rack, place the cassettes in the embedding center, and embed tissues.

13. A tissue section processed in a microwave oven is shown in **[i2.4]**. Technical aspects, along with some of the safety considerations, of microwave ovens will be discussed in chapter 3, "Instrumentation," p67.

WATER-SOLUBLE WAXES

Water-soluble waxes, usually referred to as Carbowax (Union Carbide Corp), are solid polyethylene glycols that are water soluble and are used only for special projects. Mixtures of Carbowax 4000 (a hard, dry, flaky compound) and Carbowax 1000 or 1500 (blends of liquid polyethylene glycol and wax) are used. Water-soluble waxes will infiltrate tissue directly from aqueous fixatives; because dehydration and clearing are unnecessary, fat will not be dissolved and may be demonstrated on the embedded tissue sections. However, Carbowax will not infiltrate tissue containing large amounts of fat (adipose tissue), and tissues such as those of the central nervous system require long periods of impregnation. Any well-fixed tissue is suitable for use with this method, and tissue may be embedded after infiltration with melted wax (usually 56°C to 58°C) to the point where the tissue sinks to the bottom of the dish. Usually 3 changes of wax for a total of 3 hours are recommended for infiltration. Some enzymes will remain active and may be demonstrated in sections of tissue embedded in Carbowax. Tissue embedded in water-soluble waxes will remain softer than that embedded in paraffin.

The greatest problem encountered with the water-soluble waxes is in the "floating-out" of sections; sections dissolve on the usual flotation bath, causing diffusion currents, disruption, and disintegration of the tissue. Various flotation solutions have been proposed to combat this difficulty. Examples are the solution developed by Blank and McCarthy [1979], which contains 0.2 g of potassium dichromate and 0.2 g of gelatin in 100 mL of distilled water, and the solution of Jones et al [1959], which contains 100 mL of diethylene glycol, 7 mL of concentrated formaldehyde, 1 mL of Carbowax, and 400 mL of distilled water.



[i2.4] This section from a rectal tumor was fixed in formalin and then microwave processed. [Image courtesy of Willis D, Milestone Medical]

Like paraffin, Carbowax should be cut chilled, although the block must be cooled in a refrigerator because the wax is water-soluble. With experience, 3- to 5-µm sections can be cut easily. In fact, some technicians believe that if the blocks are well chilled and the wax has not become hydrated, thin sections are easier to obtain with Carbowax than with paraffin. The wax is also hygroscopic, so blocks are best stored sealed in polyethylene bags containing a small amount of desiccant.

CELLOIDIN

Celloidin is used as a generic term for any type of nitrocellulose compound used for embedding. Parlodion (Mallinkrodt Chemical Works, Paris, KY) is the compound most frequently used, but this method of embedding is rarely used except in research and neuropathology laboratories. Any fixative may be used before processing.

Processing for celloidin embedding involves dehydration with 95% and absolute ethanol, treatment with a solution of equal parts of absolute alcohol and ether, and then infiltration with celloidin dissolved in equal parts of absolute alcohol and ether. Infiltration with celloidin usually begins with a 2% solution, and the tissues are gradually carried through a graded series of solutions up to 12% to 14% celloidin, which is considered "thick celloidin." This is also the embedding compound. Blocks of tissue are embedded in the 12% to 14% solution, and the celloidin is allowed to thicken by slow evaporation until it achieves the consistency of a gum drop. The blocks are hardened with chloroform and then cut either wet or dry. If the wet method is used, the block, knife, and sections must be kept wet with 80% alcohol. For the dry method, the blocks are infiltrated with cedarwood oil before sectioning and then may be cut dry. The cut sections in either method must be kept wet with 80% alcohol until stained. Staining is tedious, because although there are methods for mounting the sections on slides before staining, most often each section is hand stained and mounted on a slide just before clearing with xylene. Long-term storage of celloidin blocks is a problem because they must be held in 80% alcohol. For further information on the wet and dry celloidin methods, the reader is referred to the *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology* [Luna 1968].

Many neuropathologists believe that celloidin is without equal for processing and embedding tissues of the central nervous system. Because heat is not required in any step of processing, shrinkage and hardening are minimal; however, serial sections and thin sections are difficult to obtain. The processing of large blocks with minimal cellular distortion is possible if one has the extra time this technique requires. The process may require weeks or even months to complete.

This is a hazardous method because both anhydrous ether and nitrocellulose are used, and both of these reagents are explosive. Because of the time required and the associated hazards, celloidin

is rarely, if ever, used in surgical pathology laboratories. If solid strips of celloidin have decomposed to a crumbly powder, the container should not be moved, and a professional hazardous materials team may be needed for disposal.

PLASTICS

■ *Glycol Methacrylate*

Glycol methacrylate (GMA) is an acrylic resin that is miscible with water; however, infiltration is usually performed after dehydration of the tissue with 95% alcohol. GMA is converted to a crystal-clear, hard solid by polymerization. This medium provides an excellent support for very hard tissue, such as undecalcified bone, and it allows 1- to 2-μm sections to be cut. Glass knives must be used to obtain good thin sections. Cellular detail is well preserved and distortion is minimal with GMA; it is especially useful for kidney, bone marrow, and lymph node biopsy specimens. Although fairly large sections can be cut, microtomy is more difficult than with paraffin. The embedding medium usually is not removed from sections, which makes some staining difficult, if not impossible, to perform. Unlike paraffin sections, GMA-embedded tissues are receptive to some enzyme techniques. GMA sections do not adhere well to glass slides, tending to loosen in alcoholic and alkaline solutions. Staining is generally more difficult than with paraffin sections, but the time required can be reduced by the use of the microwave oven.

The chemicals used in this technique are hazardous; they should be used under a hood, and skin contact should be prevented. The catalyst benzoyl peroxide is a strong oxidizer; heat, shock, or contact with other materials should be prevented because they could result in fire or explosive decomposition.

■ *Epoxy Resins*

Epoxy resins require dehydration of the specimen, and unless miscible with ethanol, they also require the use of a transitional fluid. Transitional fluids are synonymous with clearing agents used in the paraffin process, and propylene oxide is used most frequently for this purpose when embedding in an epoxy resin. The most commonly used epoxy resins are Araldite, Epon, and Spurr. The final embedding mixtures contain epoxy resins, hardeners, and catalysts; polymerization is commonly done at approximately 60°C, and a hard block is the result. Because some of these resins cause contact dermatitis and others are toxic, skin contact should be avoided.

These resins are required as embedding media when electron microscopy or ultrastructural examination of tissue is desired, because the very thin sections necessary for good resolution can be obtained only from epoxy resin-embedded material. With properly embedded tissue and a good diamond knife, sections 60- to 90-nm thick can be cut. For light microscopic examination and orientation, 0.5-μm sections, frequently referred to as "thick" sections, are used; these sections are cut with a glass knife. For specific methodology, refer to chapter 14, "Electron Microscopy," p341.

AGAR AND GELATIN

Agar and gelatin can be used to produce a single block of friable tissue or multiple fragments when cutting frozen sections. For gelatin embedding, the tissue is washed overnight, impregnated for 24 hours with a 12.5% solution of gelatin at 37°C, impregnated for 24 hours with a 25% solution at 37°C, and then embedded in 25% gelatin. The block is allowed to solidify, or harden in the refrigerator, and then further hardened by immersion in 5% formalin for 24 hours. Tissue also can be embedded with agar or gelatin and a block formed for processing. This may be done by placing the properly oriented tissue fragments on a glass slide and dropping warm 25% gelatin on top. After allowing cooling, the gelatin "block" can be removed from the slide and placed in fixative; it is processed as usual. This ensures that proper orientation is maintained during processing and embedding.

30% SUCROSE

Perhaps the most satisfactory method for obtaining frozen sections from formalin-fixed, unprocessed tissue is to infiltrate it with an aqueous solution of 30% sucrose before it is frozen. Sucrose is widely regarded as a cryoprotectant, and high-quality frozen sections may be obtained following its use. Because the tissue is already fixed, staining is easily performed using routine H&E stains as well as fat stains. Like other frozen sections on fixed tissue, slides must be dried well to ensure section adhesion.

Troubleshooting Processing

The most common problems encountered in processing are over- or underprocessing of tissue. When tissue is well and completely fixed, the processing problems will be minimized, but tissue today is rarely well fixed when processing is begun.

PRECIPITATE IN THE PROCESSOR CHAMBER AND IN THE TUBING

This is caused when either zinc- or phosphate-buffered formalin is used for fixation and then dehydration is begun with alcohol more concentrated than 70%. It also is caused by the pH of zinc formalin rising above 7.0. The precipitates will also be in the tissue and make microtomy difficult. It can be prevented or corrected by:

- beginning dehydration with 60%-65% alcohol
- ensuring that the pH of the zinc formalin solution remains below 7.0
- removing the precipitate by rinsing the chamber and tubing with a dilute solution of acetic acid (5%-20%)

OVERDEHYDRATION

The result of excessive dehydration will most frequently be seen as microchatter around the edges of the tissue on H&E-stained

sections **[i2.5]**. It is commonly seen on biopsy specimens. Usually too much time has been allowed in the dehydrating solutions. This can be prevented by:

- processing biopsy tissues separately from larger tissues, carefully controlling the time of dehydration
- decreasing the time allowed in the dehydrating solutions

POOR PROCESSING

The results of this problem are most often noted on H&E-stained sections by the poor nuclear staining or even the absence of staining in some nuclei **[i2.2, p35]**. The most frequent cause of this problem is water remaining in tissue when it is placed in clearing agent, resulting in poor clearing and infiltration; however, it can also be caused by improper quality control of the processor. There may be clearing agent in the paraffin, too much heat used during processing, or mechanical problems with the processor. This can be prevented by ensuring complete fixation and then:

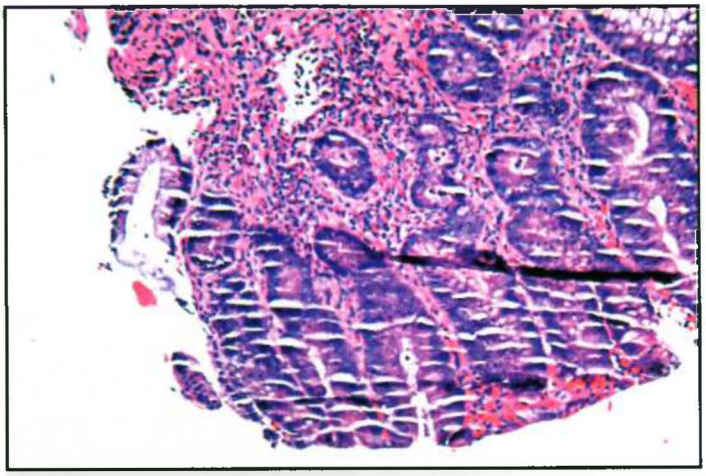
- ensuring that no condensation is occurring in the processor, with either fixative or alcohol droplets dropping onto the tissue
- ensuring that the absolute alcohol has not absorbed water or become diluted
- ensuring that there are no mechanical problems with the processor
- ensuring that a good schedule of reagent rotation is developed and maintained
- using heat only for the paraffin

SPONGE ARTIFACT

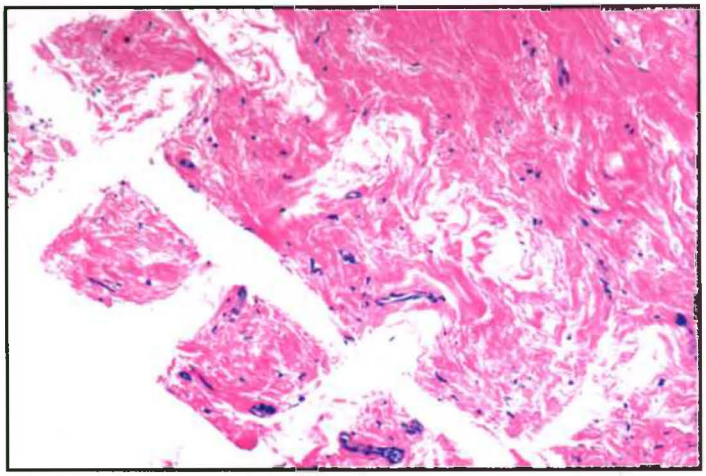
A sponge artifact (cross-hatching as seen in **[i2.6]** or triangular) is created when the tissue is placed between 2 dry sponges for processing. Either a pressure artifact, or an artifact created by trying to separate the tissue from the sponge at the embedding table, will be seen on stained sections. This may be prevented by ensuring that the tissue is placed between sponges that have been presoaked in the fixative.

TISSUE ACCIDENTALLY DESICCATED

Accidental desiccation of tissue rarely happens, because open processors are no longer used in most laboratories. If it does happen and the tissue has been fixed, then tissue morphology usually can be recovered to the extent that a diagnosis can be made. If the tissue has been in paraffin, blot off as much as possible with paper towels, and then place the tissue in a rehydrating solution composed of 50 mL water, 30 mL absolute alcohol, and 20 mL of 5% aqueous solution of sodium carbonate [Zimmerman 1976]. Soak overnight, and then reprocess as usual.



[i2.5] This section has been overdehydrated, causing the chatter seen at the edge of the gastrointestinal biopsy specimen.



[i2.6] The cross-hatching artifact seen at the edge of this section was caused by placing the tissue between dry sponges for processing.

Embedding and Specimen Orientation

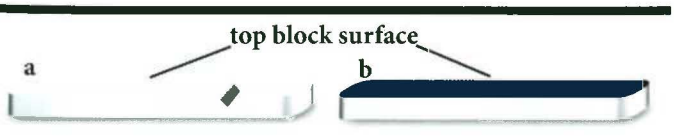
Embedding, also referred to as casting or blocking, involves enclosing the tissue in the infiltration medium used for processing and then allowing the medium to solidify; the method of processing is determined by the embedding medium to be used. Only embedding of tissue processed by the paraffin method is presented in detail in this chapter; embedding of tissue processed by many of the other methods uses the same principles. Commonly the type of paraffin used for infiltration is also the paraffin used for embedding, but this does not have to be the case. The factors involved in the choice of paraffin are discussed thoroughly under infiltration media. One of the most important factors is the choice of melting point and its influence on support and ribboning quality. The higher the melting point, the better the support for hard tissues, and the easier it is to obtain thin sections, but the ribboning becomes more difficult. The opposite is true as the melting point decreases: poorer support is provided for hard tissues, thin sections are more difficult to obtain, but the ribboning becomes easier.

Specimen orientation is the most critical step in embedding, because microtomy may ruin incorrectly oriented tissue with the first section cut. Many sections are large and flat, and so are easily

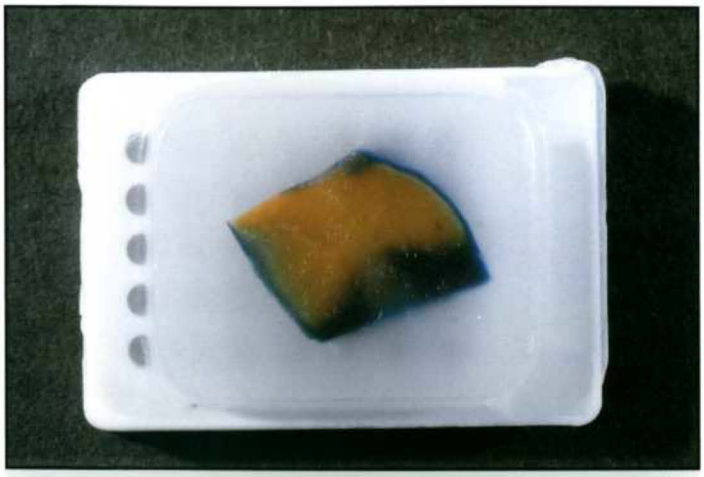
oriented. Usually, on these large, flat sections, the only determination to be made is which surface to embed down (the surface to be cut) in the mold. Normally, we place the side that is down in the processing cassette down in the mold, but if it is critical that the tissue not be flipped over and the wrong side placed down, then the "up" side can be notched lightly with a "V" [**f2.2a**]) or can be marked with India ink or a tattoo ink [**f2.2b**]). Inking is frequently used to indicate margins and to help pathologists with microscopic orientation. Different colored tattoo inks are available, so that 2 or more colors can be used on the same specimen to aid in subsequent identification of important aspects of the specimen.

It is very important that light pressure be applied over the entire specimen during the orientation and initial chilling so that the tissue will be embedded flat; otherwise, a complete section cannot be obtained [**i2.7**]. The orientation of hard tissue, such as bone, can greatly affect the ability to obtain good sections. Such tissues will section more easily if they are embedded diagonally in the mold and not parallel with the mold edges [**i2.8**]. The knife will cut first into a small area of tissue and then will be introduced gradually to a greater surface area instead of hitting the largest surface all at once. In fact microtomy is easier if most tissues are embedded diagonally. Tissues with a wall, such as cysts, gallbladder, and the gastrointestinal tract, must be embedded on edge so that all layers are visible [**i2.9**]. Tubular structures, such as fallopian tubes or the appendix, are embedded in cross-section so that the lumen and all layers of the mucosa, submucosa, and external muscle layers are obvious microscopically [**i2.10**]. Skin should be embedded so that the epidermis, or epithelium, is facing 1 side of the mold (neither turned up nor down). If more than 1 piece of skin is to be embedded in the same block, then the epidermis of all pieces should be faced to the same side of the mold. Multiple small pieces to be embedded in the same block should be carefully arranged in a line parallel to the longer axis of the mold and never just randomly placed in the mold [**i2.11**], [**i2.12**]. The careful alignment of multiple pieces will help ensure that the pathologist does not overlook a piece during microscopic evaluation and that optimum sectioning conditions are also maintained. Tissue should be embedded in the center of the mold with a margin of paraffin around the tissue, but the mold should be selected carefully so that the margin is small. Ribboning may be difficult if the tissue is not properly embedded [**i2.13**].

Because tissue can be transferred easily from 1 block to another at the embedding center, it is good practice to open and embed 1 cassette of tissue at a time. If the process is interrupted, there is no doubt regarding which block is being embedded, and tissue cannot accidentally be transferred to other open cassettes. The embedding forceps should be wiped with gauze between blocks to avoid "forceps metastasis," which occurs when fragments of tissue are



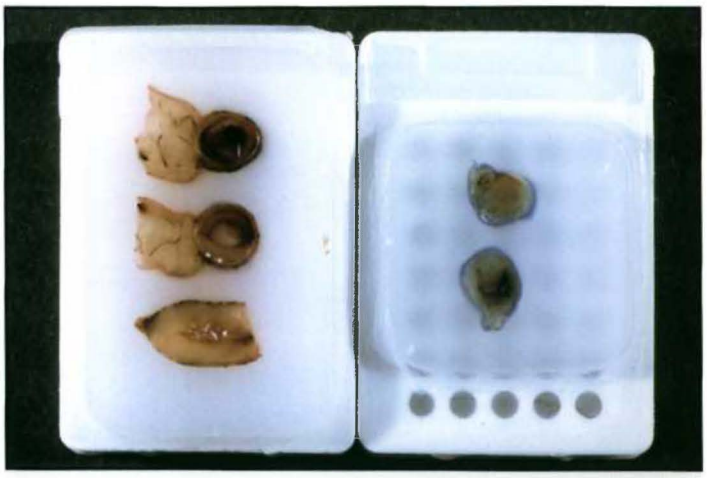
[f2.2] Identification of specimen surfaces for embedding. **a**, notched; **b**, marked with tattoo ink.



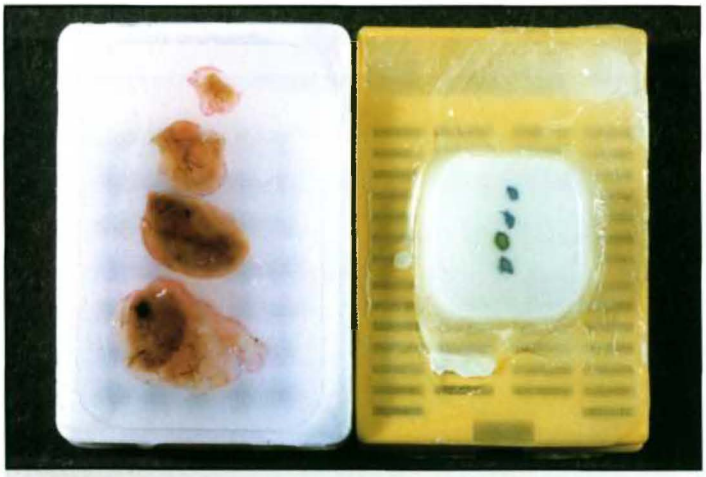
[i2.7] The brain tissue in the block was not embedded flat and the section is incomplete. The darker area at the bottom indicates that this area has not been sectioned. By cutting deeper into the block, upper portions may be lost. The blue color of the tissue is caused by an indicating desiccant in the absolute alcohol.

[i2.8] Microtomy of bone is much easier if the section is embedded diagonally in the block. The knife will contact only a small surface area initially, and better sections will be obtained.

[i2.9] The block contains 2 pieces of small intestine embedded on edge so that all layers will be shown. The sections have been embedded slightly on a diagonal with the mucosal surfaces facing in the same direction (upward).

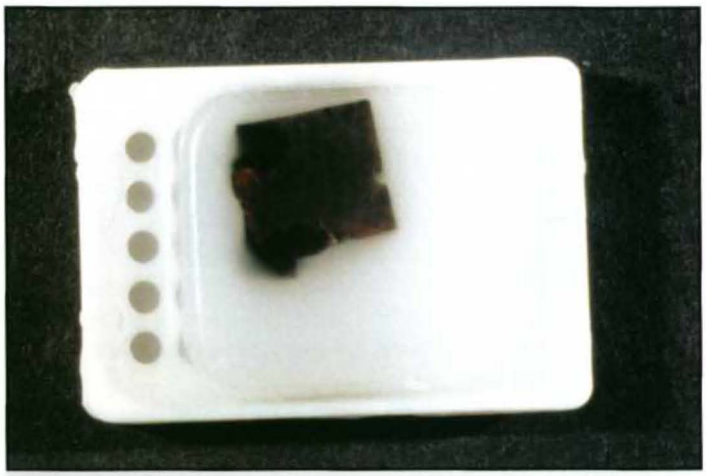


[i2.10] These 2 blocks show the correct embedding of tubular structures. The block on the left contains 3 sections of appendix; the block on the right contains fallopian tubes cut and embedded in cross-section.



[i2.11] Correct embedding of multiple pieces in the same block. The block on the left contains 4 lymph nodes and the block on the right contains 4 specimens from the same gastrointestinal site. The pieces should never be placed randomly in the block, but should be carefully embedded in a line, ensuring that the pathologist does not overlook any of the tissue. The block on the left was processed through absolute alcohol-containing eosin.

[i2.12] This block shows incorrect embedding of multiple pieces. This type of embedding makes sectioning more difficult and increases the chances of the pathologist overlooking 1 of the pieces.



[i2.13] The tissue in this block has not been properly centered. When the paraffin margin is inadequate (top of block), ribbons are more difficult to obtain.

transferred from 1 tissue block to another; this is a serious problem and can lead to a misdiagnosis. After embedding in paraffin, the blocks should be cooled rapidly to obtain the smallest paraffin crystal size possible, because the crystal size affects sectioning quality. A small crystalline structure will allow the wax to fit closely to the embedded tissue. This provides better support for sectioning, whereas a larger crystalline structure provides a poorer support for the tissue.

As part of an embedding quality-control protocol, there should be a record of the number of tissue pieces submitted in each cassette along with any orientation instructions. Many laboratories use "M" (multiple) or "N" (numerous) to record more than 4 or 5 pieces in the same cassette. This information can be recorded using 2 methods. The first is to write it on the side of the cassette, and the second is to include it on an embedding log. Special embedding instructions and the number of pieces should be checked as each block is embedded; the total number of blocks or pieces for each specimen, or case, should be checked against the log at the end of the embedding process. The total number of pieces or slides also should be checked at the end of the staining process; some laboratories also match the block to the slides at this point. Matching the stained and labeled slides to the blocks will aid in determining whether a representative section of the tissue was obtained, whether part of the tissue washed off, and most importantly, whether there is a labeling error.

Troubleshooting Embedding

SOFT MUSHY TISSUE

Despite no apparent problems with processing protocols or solutions, it is obvious at the embedding station that some sections are not adequately processed. They may be processed around the edges but are soft and mushy in the middle **[i2.14]**. The most common cause is that the sections were cut too thick at gross examination

and have been compressed between the top and bottom of the cassette, thus preventing access to the tissue by the processing solutions. If this is caught at the embedding table, the tissue should be sectioned thinner, replaced in the cassette, and then treated using the following protocol:

1. Soak tissues in xylene on a rotator for 20 to 30 minutes to remove residual paraffin.

2. Place cassettes in tetrahydrofuran on the rotator for 30 to 90 minutes to allow the dehydration/clearing process to occur. Visually check to assess transparency.

3. Place sections in xylene for 10 minutes to ensure adequate clearing.

4. Place on tissue processor or in containers of paraffin in an oven and reinfiltrate with 2 to 3 changes of fresh paraffin for 30 to 45 minutes each.

5. Re-embed.

This is a quick and effective way to reclaim inadequately processed blocks that were cut too thick at gross examination. Total time required is 2 to 4 hours, depending on the amount of dehydration and clearing necessary. Tissues can also be backed up on the processor and refixed, but this is a longer process. If refixation is desired, then melt any residual paraffin, place the tissue back in the cassette, and place in the processor. Run the tissue through the purge or cleaning cycle. The tissue is wet with absolute alcohol at this point; it can then be run through 95% and 70% alcohols manually, and placed in formalin for refixation and processing with the next batch of surgical tissues.

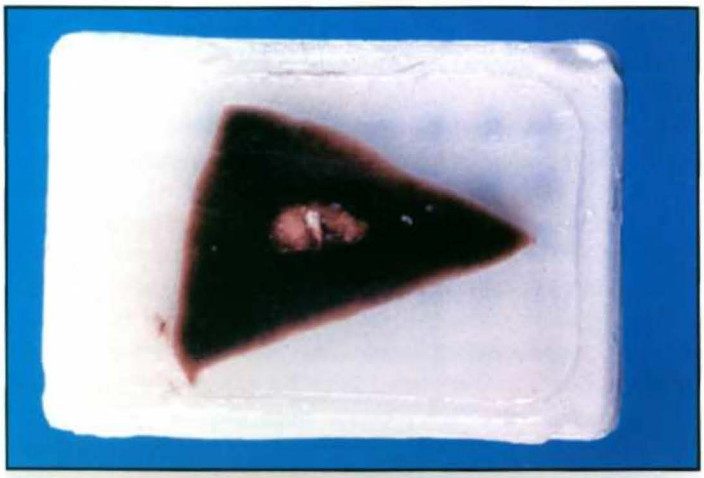
Johnson [2003] described a method for refixation and reprocessing, which involves melting the block, blotting to remove excess paraffin, placing in a cassette, and placing the cassette directly into formalin for reprocessing with the regular overnight run of tissues.

The problem of soft mushy blocks can be prevented by:

- ensuring that the tissues are sectioned thin at the grossing station
- ensuring that the tissues are allowed adequate time for good fixation
- ensuring that the processor has no mechanical problems
- ensuring that a good schedule of processor reagent rotation is developed and maintained

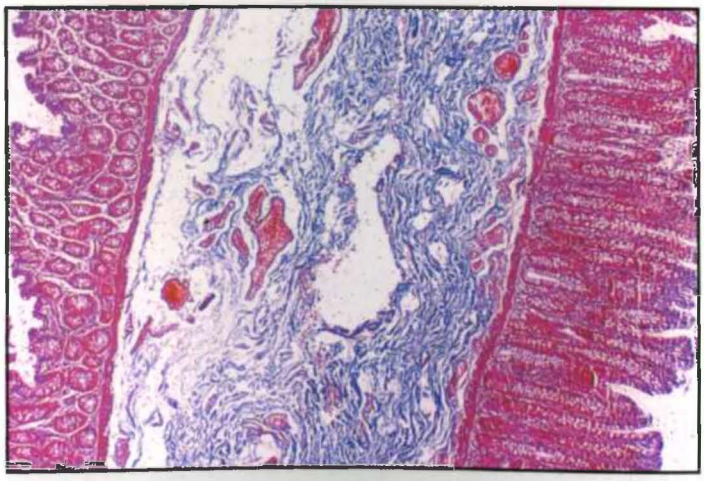
INCORRECT ORIENTATION

Sections may be incorrectly oriented at the embedding station if the correct method is not clearly indicated on an embedding list, on the side of the cassette, or on the tissue [i2.15]. For example, orientation of sections of fallopian tubes is especially difficult,



[i2.14] The center of this block is not completely fixed and processed so it is still soft. This can be caught many times at the embedding table and reprocessing begun.

[i2.15] This section of aorta stained with the Verhoeff-van Gieson stain has been embedded flat and is not on edge as it should be. Marking the orientation on the side of the cassette will help ensure that the specimen is embedded correctly.



[i2.16] Incomplete fixation before grossing this intestinal specimen has allowed it to roll and complete the fixation in that form. Gastrointestinal tract specimens should be opened and pinned open as soon as the specimen comes to the laboratory.

so marking the tissue with ink at the grossing station will help. Incorrect orientations can be prevented by:

- marking the side of the tissue to be embedded facing up with ink at the grossing station
- notching the side of the tissue to be embedded facing up with a shallow "v" at the grossing station
- placing embedding instructions on the side of the cassette and/or on a grossing sheet. These should be referred to for any specimen other than a large flat piece, or tissue such as endometrial curettage, prostatic chips, etc.
- opening and fixing gastrointestinal specimens before grossing, so that they do not roll and make it impossible to see all layers **[i2.16]**

TISSUE CARRYOVER

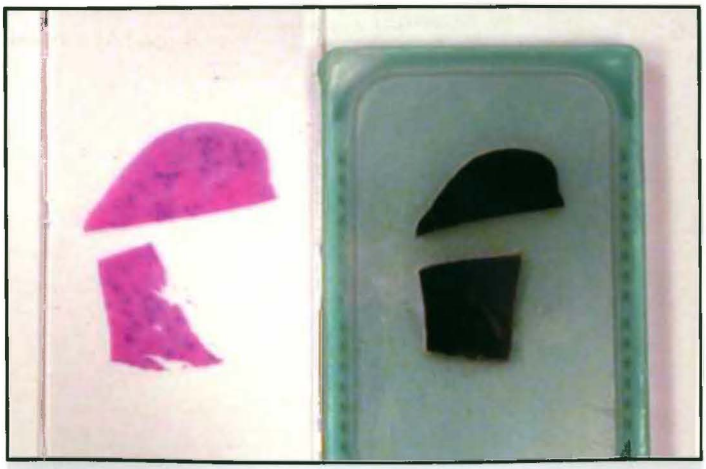
Small pieces or fragments of tissue may be carried from 1 tissue to the next at the embedding table, resulting in cross-contamination. This can be prevented by:

- carefully cleaning forceps used at the embedding table between specimens
- opening only the cassette with the tissue to be embedded. Opening all cassettes at one time and leaving them open while embedding is poor laboratory practice.

TISSUE NOT EMBEDDED AT THE SAME LEVEL

Often tissue is not properly flattened by pressing it down uniformly when it is placed in the embedding mold, or multiple tissues to be placed in the same mold are embedded at different levels **[12.17]**. This makes it impossible to get a good cross section of the tissue(s). This should be prevented by:

- pressing the tissue down uniformly and firmly in the mold



[i2.17] The 2 pieces of tissue were not embedded flat and at the same level in this block. Mild pressure should be placed on tissue so that it will be embedded flat.

- keeping the paraffin molten enough to get all pieces embedded at the same level.
- working very fast when embedding multiple pieces and letting the paraffin chill only enough to get the tissue to adhere to the base of the mold.

PIECES OF TISSUE MISSING FROM THE BLOCK

Small pieces of tissue can be overlooked when embedding. This can be prevented by:

- recording the number of pieces to be embedded on the side of the cassette or on an embedding list if more than 1 piece of tissue is in the cassette
- referring to the number of pieces that should be embedded by checking the side of the cassette or the embedding list
- carefully checking the lid of the cassette before discarding, because tissue frequently clings to the lid
- carefully opening any lens paper or tea bags, and checking for all pieces

Special Techniques in Processing

There are two special techniques that are best described as part of processing: decalcification and frozen sectioning. Decalcification is accomplished after fixation and before dehydration. Frozen sections are most often used in lieu of fixation, processing, and embedding, because either a more rapid diagnosis can be obtained or a particular tissue element to be demonstrated might be adversely affected by routine methodology.

DECALCIFICATION

Tissue containing calcium must undergo calcium removal before embedding unless specific studies requiring undecalcified bone are requested. Because tissues containing calcium cannot be sectioned after paraffin embedding, plastics must be used for studies on undecalcified bone. In diagnostic pathology, most evaluations are made on decalcified sections; undecalcified bone sections are examined primarily for the diagnosis of metabolic bone disease.

Unless a decalcifying agent combined with a fixative is used, tissue must be thoroughly fixed before it is submitted for decalcification, or the tissue morphology can be affected. There are basically only 2 methods; all other methods are modifications of these.

■ *Acid Methods*

Any acid, however well buffered, will have some effect on the stainability of tissue. The stronger the acidity of the solution and the longer the specimen remains in it, the more that subsequent staining will demonstrate injurious effect of the decalcification

process. The most pronounced effect is on nuclear basophilia, and overdecalcification may result in a total lack of nuclear staining; therefore, with many solutions the end point of decalcification must be carefully monitored.

With the acid methods of decalcification, calcium salts dissolve and then ionize. The manner in which these calcium ions are removed from tissue is the basis of the different acid methods. Calcium salts are soluble at a pH of 4.5, and the usual acid decalcifying solutions have a pH between 0.5 and 3.0. If the pH is buffered at 4.5, the calcium will dissolve and some enzymes still can be demonstrated. Most manufacturers of proprietary (commercial) decalcification reagents do not completely disclose the components in their solutions, but most are acid decalcifiers.

Simple acids commonly are used in 5% to 10% solutions. Hydrochloric and nitric acids decalcify fairly rapidly, but the decalcification process must be carefully monitored. Nitric acid can cause serious deterioration of tissue stainability if decalcification is prolonged beyond 48 hours; formic acid is slower acting and affords the user more latitude. It is rare for tissue to lose staining ability after formic acid decalcification even after remaining in the solution for 2 weeks. Mixtures of formaldehyde and formic acid are excellent for simultaneous fixation and decalcification. If hydrochloric acid is used after formaldehyde fixation, the residual formaldehyde should be washed out before placing the specimen in hydrochloric acid. A carcinogen, *bis*-chloromethyl ether, can be formed by a reaction between formaldehyde and hydrochloric acid. With simple acid methods, calcium ions are allowed to migrate out of the tissue into the surrounding solution [**f2.3a**]). The solution around the tissue may become saturated with calcium ions, almost forming a barrier to further decalcification. For this reason, the solution should be changed frequently, and agitation also may be beneficial. Vacuum at the initial stage of decalcification will aid in infiltrating the specimen with decalcifying fluid and will draw off carbon dioxide bubbles that form on specimen surfaces. Suspension of the specimen in an embedding bag will expose all surfaces to the action of decalcifying fluid and will allow any precipitated calcium salts to sink to the bottom of the container. Heat should never be used to speed up decalcification with acid, because heat also increases the effects of the decalcifying fluid on other tissue components, most likely resulting in swelling and maceration.

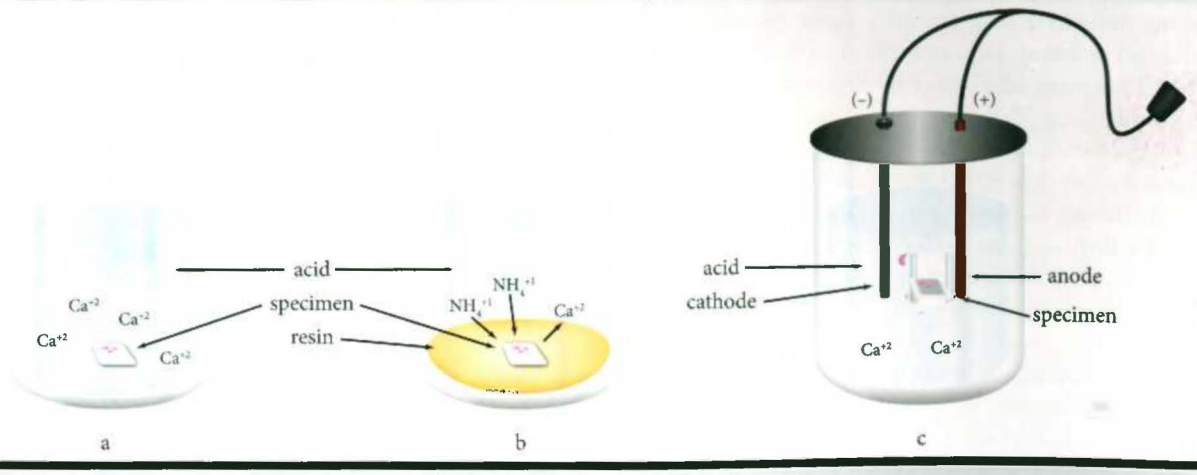
Ion-exchange resins involve the use of formic acid over a layer of an ammoniated salt of a sulfonated resin. Ammonium ions from the resin are exchanged for calcium ions; this keeps the solution free of calcium ions and speeds up the reaction [**f2.3b**]). Staining is excellent after application of this method, and the time tissue remains in the decalcifying reagent is not critical. Because the solution remains relatively free of calcium ions, it does not need to be changed frequently. This is one of the best, if not the best, methods of decalcification.

Electrolytic methods use a mixture of formic and hydrochloric acids placed in an apparatus based on a simple electroplating device. The bone is attached to the anode (positive pole) and a current is passed through the solution. The calcium ions, with their associated positive charge, are rapidly attracted away from the anode and to the cathode (negative pole) [**f2.3c**]. Most bone specimens can be decalcified within 2 to 6 hours, but possibly because of heat generated by this method, there is a strong potential for tissue destruction and a total loss of cellular detail and stainability. Because of the possible loss of stainability and the limited number of specimens that can be processed at any 1 time, this method is rarely used today.

Protective aprons and gloves should be worn when handling the acid used for decalcification. Eye protection should be worn, the reagents should be used under the hood, and the acids should be kept in acid carriers. Remember always to add acid to water when diluting acids—never dilute in the opposite order. For drain disposal, the solution should be neutralized with 1% sodium bicarbonate.

■ *Chelating Agents*

Chelating agents are organic compounds that have the property of binding certain metals. Ethylenediaminetetraacetic acid (EDTA) binds calcium ions. The results obtained are satisfactory if EDTA is used in a solution with a pH between 5.0 and 7.2, but a slightly acidic pH is preferred. Decalcification by chelation is very slow, but many enzyme methods can be used successfully after



[**f2.3**] Acid methods of decalcification. **a**, simple acid; **b**, ion-exchange resin; **c**, electrolytic.

decalcification with EDTA. EDTA can bind only ionized calcium; it acts on the outer layer of the hydroxyapatite crystal, gradually decreasing crystal size by depleting this continuously reforming layer.

Commercial products are available that combine acids and fixatives, or EDTA and acids. Some of these are very good, and they should be used according to the manufacturer's recommendation.

■ *End Point of Decalcification*

This is the most important step in the decalcification process, because underdecalcified tissue will section with difficulty and overdecalcified tissue most likely will stain very poorly. The end point can be determined using 3 basic methods: mechanical or physical, chemical, and radiographic.

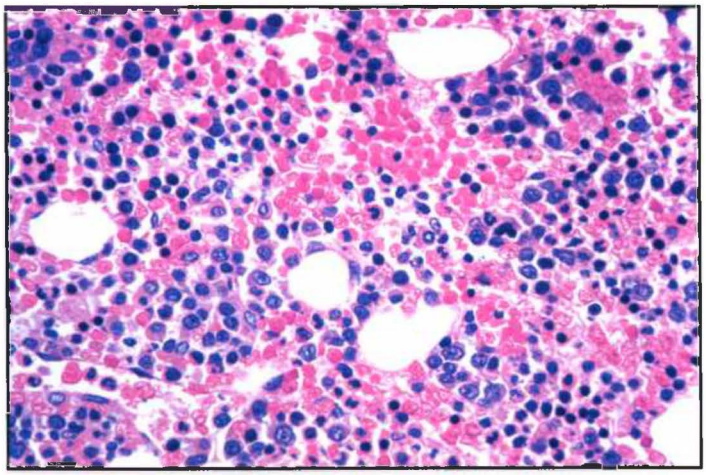
Mechanical methods involve testing the flexibility of the specimen, probing the specimen with a needle or pin, or scraping the section surfaces. This method is the least desirable for determining the end point because of its inaccuracy and the likelihood of creating histologic artifacts.

Chemical methods depend on the precipitation of calcium oxalate when a sample of the used decalcifying fluid is mixed with a solution containing ammonium hydroxide and ammonium oxalate. Approximately 5 mL of the used decalcifying fluid is made neutral to litmus paper with concentrated ammonium hydroxide, and then approximately 5 mL of saturated ammonium oxalate solution is added. The resulting solution is mixed well and allowed to stand for 30 minutes; a persistent turbidity (calcium oxalate) indicates the presence of calcium. The decalcifying fluid must be changed after each chemical check.

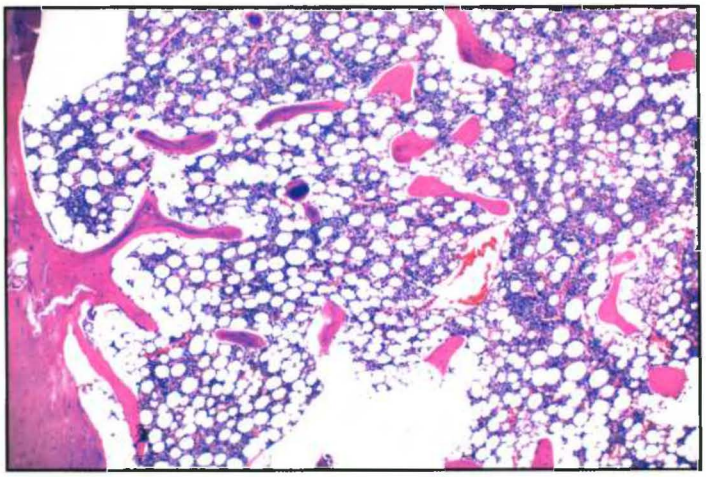
Radiography is the most accurate method of determining the completeness of decalcification, and the small X-ray units available in many histology laboratories are easy to use. This method yields visual evidence that demineralization is complete. Radiographic methods cannot be used with metallic-fixed tissue, such as zinc formalin, Zenker, or B-5 fixed specimens because the metal will render the specimen radiopaque.

After decalcification is complete, the specimen should be washed well with running water to remove any excess acid. In some laboratories, an alkaline solution such as lithium carbonate is used to neutralize any remaining acid before processing the specimen routinely. Following decalcification, some specimens may be trimmed to a size better suited for routine processing. This also removes any bone dust or debris that may have been ground into specimen surfaces during the sawing step. Diamond saws allow the cutting of very thin sections before decalcification without introducing bone dust into the specimen; these thin sections will decalcify much more rapidly.

Decalcification is an area of specimen processing that is sadly lacking in many laboratories, with overdecalcification being the rule rather than the exception. If 1 of the more rapid decalcifying solutions is selected for use, then decalcification should be monitored rigorously, because a decrease in the time required for decalcification becomes unimportant if the end product is an unreadable or nondiagnostic slide. Quality should not be sacrificed in the interest of time, because frequently nothing is gained and all may be lost. Excellent decalcification is demonstrated in **[i2.18]**, **[i2.19]**.



[i2.18] An H&E-stained section of bone that has been properly decalcified. The nuclear basophilia is preserved, and the detail is such that red and white cell lines can be distinguished easily.



[i2.19] Section of bone demonstrating good decalcification and good microtomy. The section is free of artifacts.

If undecalcified bone or tissue containing unsuspected areas of calcification is detected during microtomy, satisfactory sections often may be obtained by surface decalcification. This involves exposing the surface of the blocked tissue by gently rough-cutting the block to expose a complete cross-section of tissue (facing the block) and then treating the exposed surface of tissue with a decalcifying fluid such as 1% hydrochloric acid for 30 to 60 minutes. The block is rinsed well, blotted dry, and gently sectioned. Because the decalcification achieved will be of limited depth, the block must be carefully oriented in the microtome and the first sections obtained must be used.

■ *Undecalcified Bone*

Specific methodology for undecalcified bone is beyond the scope of this discussion. The embedding medium most frequently used for undecalcified bone is GMA. Sections of bone also may

be ground with waterproof sandpaper to a thickness of 75 to 100 μm; these ground sections may be stained and mounted on glass slides for microscopic viewing. Alcohol, buffered formalin, and calcium formalin are the preferred fixatives for the staining techniques usually used on undecalcified bone sections; metallic fixatives interfere with most of the techniques.

TROUBLESHOOTING DECALCIFICATION

The most common problems encountered on decalcified tissues are bone dust, underdecalcification, and overdecalcification.

■ *Bone Dust*

When obtaining sections of bone with the saw commonly used for this process, bone dust is pressed into the surface of the bone [**i2.20**]. This can be prevented or corrected by:

- using a saw with a diamond blade
- trimming the bone surfaces after decalcification but before processing

■ *Underdecalcification*

This may occur because of the necessity of a rapid turnaround time on the specimen or because the laboratory does not have a good procedure for determining the end point of decalcification in place [**i2.21**]. This can be prevented by:

- choosing a decalcifying agent that will allow rapid turnaround times
- developing a good method for detecting the end point of decalcification and rigorously adhering to it
- checking the end point of decalcification with radiography when in doubt

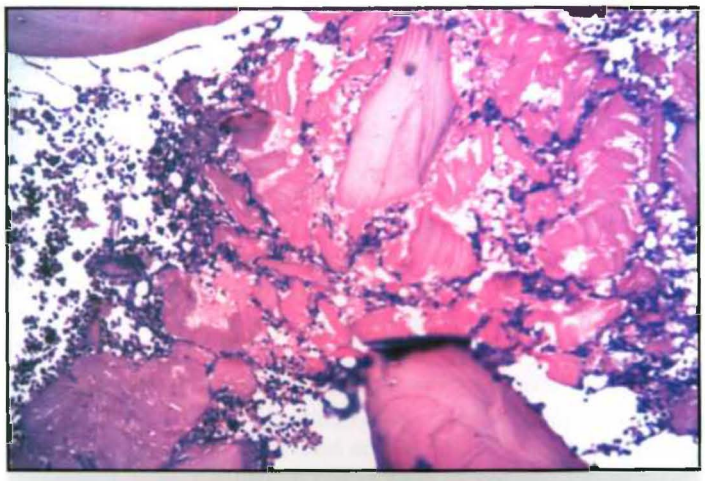
■ *Overdecalcification*

When the end point of decalcification is not carefully checked, overdecalcification may be the result [**i2.22**]. This may be prevented by:

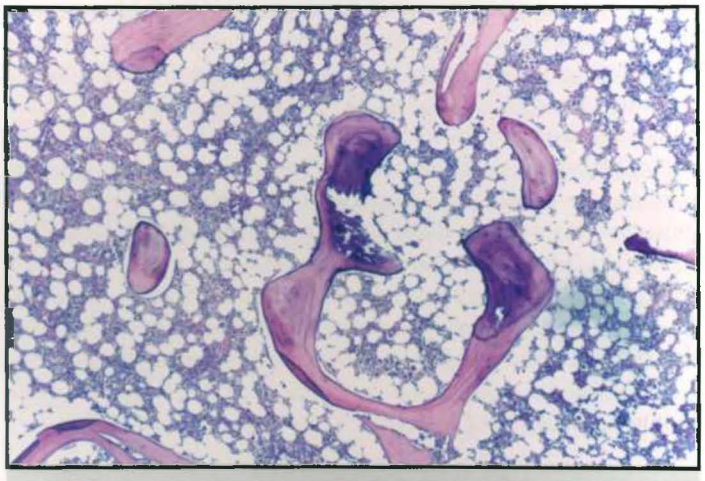
- choosing a decalcification agent that fits the needs of the laboratory
- developing a good method for detecting the end point of decalcification and rigorously adhering to it
- checking the end point of decalcification with radiography when in doubt

FROZEN SECTIONS

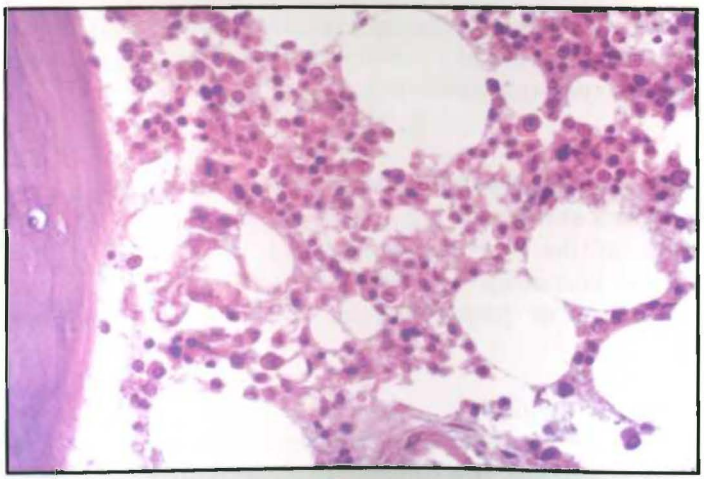
Frozen sectioning is indicated as the method of processing when a rapid diagnosis is desired, or when the staining technique does not work on routinely processed tissue. Frozen sections must be used when fat is to be



[i2.20] Almost the entire slide shows bone dust, with a small amount of bone marrow visible in the upper left corner. Bone dust is an artifact that occurs when bone debris is pressed into the bone surface by the saw. It can be prevented by using a saw with a diamond blade, such as the Buehler Isomet Low-Speed Saw (Buehler Ltd, Evanston, IL) which cuts thin sections without deformation, debris, or burning; or the bone can be cut slightly thick originally, and then the surfaces trimmed after decalcification.



[i2.21] The effects of underdecalcification can be seen in the bony trabeculae in the block. The dark bluish-purple seen in the trabeculae in the center of the photograph is caused by the presence of calcium remaining in the tissue. This makes sectioning difficult and damages the knife edge.



[i2.22] An H&E-stained section of bone that has been overdecalcified. Notice that poor nuclear staining is apparent because of the overdecalcification. The same hematoxylin exposure time was used as in **[i2.18]**

demonstrated, because all fixatives except osmium tetroxide allow fat to be dissolved from the tissue by the reagents used in routine processing. Frozen sections also must be used for many enzyme and immunohistochemical techniques because fixation and the heat involved in processing inactivate most enzymes and many antigens. Immunofluorescence and some lymphocyte surface marker studies are unsatisfactory if the tissue has been fixed and processed routinely. Antigen retrieval and epitope enhancement methods have made it possible to use paraffin-embedded sections for some of the lymphocyte surface markers and other antigens that previously required frozen sections.

Tissue may be frozen in the cryostat, but this process is slow and allows ice crystals to be formed in tissue. When the tissue section is thawed, the ice melts and results in holes in the section **[i2.23]**. While this does not interfere in most tissues on which a rapid diagnosis is needed, if skeletal muscle is frozen in this manner for subsequent enzyme techniques, the holes are of such size that the sections are worthless for diagnostic studies **[i2.24]**. The size and amount of ice crystal formation is directly proportional to the speed of freezing the tissue. Heat extractors have been added to some cryostats, but the best frozen sections are obtained by using isopentane and liquid nitrogen **[i2.25]**. If liquid nitrogen is used alone, gas bubbles tend to surround the tissue and impede freezing, so usually a container of isopentane (2-methylbutane) is lowered into liquid nitrogen. The technique is described more fully in chapter 13, "Enzyme Histochemistry," p313. When the temperature of the isopentane reaches approximately –150°C, the tissue is submerged in the isopentane. If isopentane is not available, the tissue can be dusted with talc to prevent the formation of gas bubbles; then the tissue can be submerged directly into liquid nitrogen. Liquid chlorofluorocarbons (freon), dry ice alone, and dry ice in a slurry with acetone also have been used, and commercial freezing equipment is also available. The technician must decide on the outcome desired and then choose the appropriate method. The use of the cryostat for frozen tissue microtomy and the associated problems are described in chapter 3, "Instrumentation," p64.

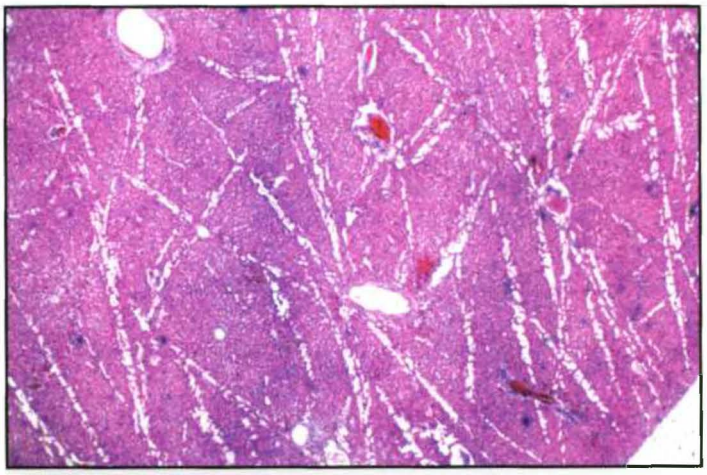
There are hazards associated with frozen section techniques. Isopentane is extremely flammable, some individuals are very susceptible to the cold temperatures, but the greatest hazard is in cutting unfixed tissue. Tuberculosis organisms can become aerosolized, and the practice of using a quick freeze spray adds to the problem. This practice should be prohibited unless working in a cryostat with a negative pressure chamber connected to a high-efficiency particulate air (HEPA) filter.

TROUBLESHOOTING PROCESSING TISSUE FOR FROZEN SECTIONS

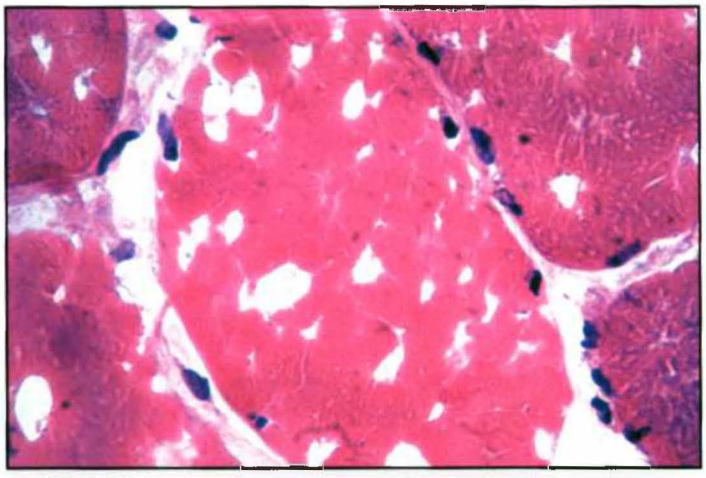
Most of the problems encountered with frozen sections involve microtomy, but there are problems caused by poor processing, or preparation, of the tissue for microtomy.

■ *Freezing Artifact*

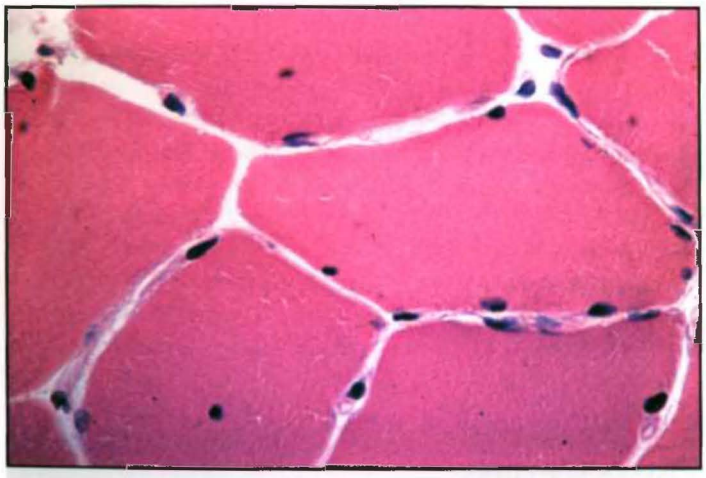
One of the primary problems in obtaining good frozen sections is ice crystal artifact. This is most often caused by improper freezing of tissue **[i2.26]**. For the best preservation of tissue morphology, tissue should be "snap" frozen. Once the tissue has been incorrectly frozen, this artifact cannot be corrected, but it can be prevented by:



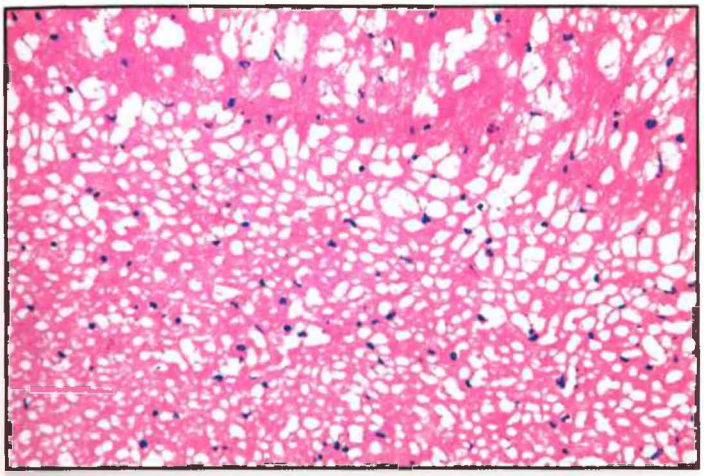
[i2.23] A paraffin section of tissue that was improperly frozen and then improperly thawed before fixation and processing. The morphology is not preserved because of the ice crystals that formed during freezing.



[i2.24] This frozen section of skeletal muscle fibers shows ice crystal artifact, apparent as holes, indicating that the method of freezing was not optimal. The artifact can be much worse than demonstrated, with the crystals displacing or distorting much of the fiber. This artifact can be mistaken for vacuoles that occur in some muscle diseases, and when severe, can make histochemical studies of little value.



[i2.25] Skeletal muscle fibers shown in cross-section. This is an H&E-stained section from a specimen properly frozen in isopentane and liquid nitrogen at a temperature of –150°C.



[i2.26] A brain biopsy specimen was transported in saline to the frozen section area and remained in this solution for about 10 minutes before freezing. The ice crystal artifact is so prominent that the tissue is worthless for making a diagnosis.

- using a heat extractor
- freezing with isopentane chilled to –150°C
- using other methods of rapid freezing
- ensuring that tissue is not immersed in saline before freezing

■ ***Block Loosens from the Chuck while Sectioning***

Occasionally the block will detach from the chuck while cutting frozen sections. This frequently happens because the chuck was too cold when the embedding medium was applied. This can be corrected or prevented by:

- reattaching the tissue block to a clean chuck with additional embedding medium
- avoiding the storage of chucks without embedding medium in the cryostat
- avoiding the storage of chucks with embedding medium in the cryostat overnight during the defrost cycle

■ ***Tissue Not Embedded Flat on the Chuck***

If the tissue is not embedded flat on the chuck, then sectioning will have to be deeper into the block, and some important parts of the tissue may be wasted. This can be prevented by placing the tissue on a slide, surrounding it with embedding medium, and then placing the slide (glass side down) on the freezing bar of the cryostat. As the embedding medium begins to turn white, coat a specimen chuck with embedding medium and invert over the tissue; allow tissue to freeze, and then remove the slide by warming slightly with a finger.

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LEARNING ACTIVITIES

1. **Decalcify a section of bone and stain with H&E. Microscopically examine the section and answer the following questions:**

    a. Is the nuclear detail as good as in the routine H&E-stained sections?

    b. Is there either an absence of, or poor, nuclear staining?

The answer to (a) should be yes and (b) should be no, otherwise the sections may be overdecalcified. Repeat decalcification, adjusting times or selection of reagents, until correct decalcification is obtained.

2. **Process, embed, section, and stain with H&E:**

    a. Gallbladder

    b. Multiple pieces of skin (at least 3 pieces in the block)

    c. Tubular structures, such as fallopian tubes or blood vessel

Microscopically check for correct orientation.

CHAPTER 3

Instrumentation

OBJECTIVES

On completing this chapter, the student should be able to do the following:

1. List the equipment in the histopathology laboratory that employs temperature control and state the proper temperature for each instrument
2. Describe the following types of microscopy and identify indications for their use:
   a. light (compound microscope)
   b. polarizing
   c. phase-contrast
   d. darkfield
   e. fluorescence
   f. electron (scanning and transmission)
3. State the most common objectives used in light microscopy and the approximate magnification of each
4. Describe the method of determining total magnification
5. Define:
   a. clearance angle
   b. bevel angle
   c. wedge angle
   d. resolution
   e. ocular
   f. objective
   g. micrometry
   h. microtomy
   i. microscopy
   j. achromatic
   k. apochromatic
   l. binocular
   m. parfocal
6. List 3 type of microtomes and identify the use of each
7. Describe routine microtome maintenance
8. Identify the different types of microtome blades
9. Identify the various blade angles and their significance
10. Identify at least 6 microtomy problems and the appropriate corrective action for each
11. Identify at least 4 artifacts that occur during section flotation
12. Describe the mechanisms of closed tissue-processing systems
13. Identify critical factors in processing tissue on a short cycle
14. Describe the role of vacuum in processing tissue
15. Correlate maintenance of all laboratory instruments with quality control and/or quality assurance
16. Discuss the principle of pH and the use of a pH meter
17. Describe how to obtain an accurate measurement when using a balance
18. List at least 3 section adhesives and state why the use of an adhesive may be necessary
19. Describe 2 applications in which a microwave oven can be useful
20. Describe the principle of microwave-generated heat
21. Describe the types of automatic stainers used in histopathology
22. Describe the relationship of temperature (blade, chamber, room) to cutting frozen sections
23. Identify problems encountered when using the cryostat and the appropriate corrective action
24. Describe the basic function and benefits of recycling reagents
25. Describe micropipetting techniques for consistency

Microscopes

A microscope is a critical tool for quality control in the histology laboratory and should be readily available for monitoring quality of both routine and special stains. Slides prepared in the laboratory should be reviewed microscopically on a routine basis; the slides should be examined for the quality of processing, sectioning, and staining. As the slides are reviewed, it is also a good time for the beginning student to look at the histologic characteristics of the section and learn to recognize different tissue types. A good histology textbook or atlas should be available for review and study. It is also helpful if a pathologist or a staff technologist reviews the slides with the student, discussing any problems seen on the slide and identifying possible solutions.

LIGHT MICROSCOPE

A magnifying glass (1 lens) is a simple microscope. The light microscope used for the examination of tissue sections combines 2 simple microscopes, or magnifying lens systems; therefore, the light microscope is called a compound microscope. The lens systems found in the compound microscope consist of the objective lenses and the ocular lenses, or eyepieces.

The objectives, located at the lower end of the body tube, provide both image magnification and image resolution. The objectives found on most histopathology microscopes commonly include the following types: scanning lens (×2.5 to ×4 magnification), intermediate lens (×10 to ×20 magnification), high-powered dry lens (×40 to ×45 magnification), and oil immersion lens (×90 to ×100 magnification). Magnification alone is not the aim of the finest microscopes, because it is possible to enlarge an object without revealing any increased detail (empty magnification). The resolving power of the microscope is very important and defined as the ability to reveal fine detail or to discriminate between adjacent details. Resolving power is measured as the least distance between 2 objects at which the objects still can be discerned as 2 separate structures rather than as a single blurred object. With the light microscope, objects usually must be separated by at least 0.2 μm to be seen as 2 separate structures.

When white light enters a lens, it is split (refracted) into the colors of the visible light spectrum. Because colors are refracted at different angles, each has a different point of focus; therefore, an uncorrected lens will give an image surrounded by color fringes. This is known as *chromatic aberration*. Most laboratory microscopes consist of achromatic objectives that are corrected for 2 colors, red and blue. Apochromatic objectives, corrected for 3 colors and also for other lens aberrations, are more expensive and are not necessary for routine use, but ideally they should be used for photomicrography. Objectives should be parfocal: all objectives then will have the focal point in the same plane, and magnification, or objectives, can be changed without the need to refocus. Parfocality is accomplished through a series of adjustments in the microscope, but all oculars must be adjustable for all simultaneous observers to have a parfocal view.

Oculars, or eyepieces, commonly have a ×10 magnification, although ×5 oculars are frequently used on student microscopes, and ×15 oculars are preferred by some microscopists. The total magnification obtained with a microscope is determined by multiplying the magnifications of the ocular and the objective, for example, ×10 oculars and a ×45 objective would give a total magnification of ×450.

The substage is found below, and usually attached to, the stage. The substage can be moved up and down, and on most modern microscopes consists of the condenser and iris diaphragm. The condenser functions primarily to concentrate light on the tissue section. It is important that the condenser be centered accurately and also that it be focused on the same plane as the tissue section. The amount of illumination on the section is regulated by the iris diaphragm and should be varied with the different objectives. The iris diaphragm should be adjusted so that peripheral light rays are blocked, and the light passing through the tissue should be limited so that it just fills the front lens of the objective. This can be determined accurately by removing an eyepiece and looking down the body tube while adjusting the iris diaphragm. When the iris diaphragm is properly adjusted, the light just fills the field. Notice how the iris diaphragm must be readjusted as the objectives are changed.

Occasionally, it is desirable to determine the measurement of a structure observed microscopically. Micrometry, or microscopic measurement, employs a micrometer scale located in 1 eyepiece and a stage micrometer. The stage micrometer contains a millimeter scale engraved in 1/10 and 1/100 graduations. The eyepiece has an engraved arbitrary scale; the value of each division of the eyepiece is calculated for each objective by matching the comparable number of divisions on the stage micrometer. Once this value has been determined, the stage micrometer is no longer needed.

Maintenance of the microscope is very important and should be a part of the routine quality assurance program.

1. Keep the microscope covered when not in use.
2. Clean the lenses frequently with lens paper. Do not use other paper tissue.
3. Remove immersion oil immediately after use.
4. Use xylene on the objectives only as a last resort, and then use it sparingly and remove it immediately.
5. Do not dismantle the objectives.
6. When using immersion oil, be careful that the high-powered dry lens is not dragged through the oil.
7. Reduce the light to a minimum or turn it off when the microscope is not in use.
8. Remove the slides from the stage when the microscope is not in use.
9. Try to always focus upwards, *never* downwards, especially with the higher power objectives.
10. Do not touch the surface of the lens.

POLARIZING MICROSCOPE

The polarizing microscope is finding increased use as a diagnostic tool in histopathology primarily for the identification of crystals such as talc, silica, or urate. It is also used to make the identification of amyloid stained with Congo red more specific.

The polarizing microscope is used to examine tissue for substances exhibiting the phenomena of *double refraction*, *anisotropism*, and *birefringence*, terms that can be defined by the following example. If a dot drawn on a sheet of white paper is viewed through a block of glass laid on top of the paper, only 1 dot can be seen from above; however, if a polished block of crystal such as Iceland spar or calcite replaces the glass, then 2 dots will be seen. A single light ray has been split into 2 rays that emerge from the crystal at different points. The crystal splits the light rays because of its uneven optical density, and the rays are refracted, or bent, to differing degrees [Culling 1985]. This property is described as birefringence (transmitting light unequally in different directions), anisotropism (having unlike properties in different directions), and double refraction.

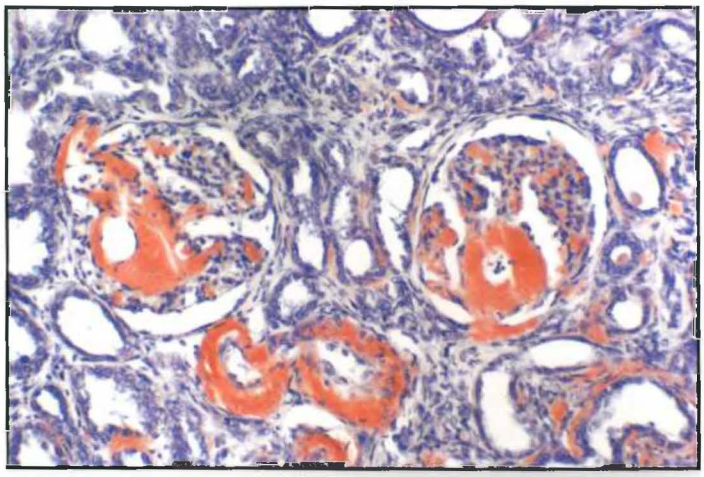
Polarized illumination is achieved by interposing a polarizing device (polarizer) between the light source and the specimen, and inserting a second polarizing filter (analyzer) between the specimen and the eye. Natural light vibrates in many planes, whereas light emerging from the polarizer vibrates in only 1 plane. If the light path of the analyzer is aligned, or parallel, then the light will pass and the field will appear bright. If the analyzer is rotated so that the optical paths are crossed, the light rays are blocked and the field will appear dark. When optical paths are crossed, material having the property of anisotropism or birefringence appears bright against this dark background.

A compound, or light, microscope may be converted easily to a polarizing microscope by placing 1 piece of polarizing film on top of the light source (polarizer) and another on top of the microscopic slide (analyzer). The polarizer is then rotated. Polarizing discs also may be installed in the microscope by installing the polarizer in the substage filter carrier and mounting the analyzer inside or on top of the eyepiece. The eyepiece analyzer or the polarizer is then rotated, and the field varies between bright and dark, with any doubly refractive particles appearing bright against a dark background. Usually the blue filter (if used) should be removed to maximize the birefringence.

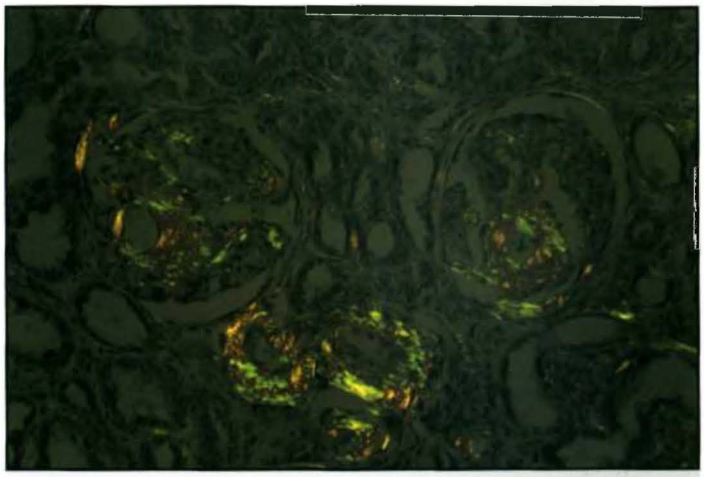
Through the polarizing microscope, amyloid stained with Congo red will exhibit an apple green birefringence; this phenomenon is considered to be specific to amyloid **[i3.1]**, **[i3.2]**. Some normal tissue components, such as collagen, exhibit intrinsic birefringence because of an asymmetric alignment of chemical bonds, ions, or molecules. Foreign substances such as talc may also be identified using polarized light.

PHASE-CONTRAST MICROSCOPE

The phase-contrast microscope is used for the examination of unstained specimens, especially unstained living cells, and allows almost transparent objects to be seen clearly. A standard binocular microscope can be converted to a phase-contrast microscope by replacing the condenser and objectives with special phase



[i3.1] A section of kidney stained with Congo red and viewed with the light microscope. Amyloid is stained orange-red.



[i3.2] The same section of Congo red-stained kidney shown in **[i3.1]** viewed with polarized light. Congo red staining and subsequent apple-green birefringence with polarized light are among the most specific identifying features of amyloid.

equipment, but the performance of converted light microscopes is not equal to that of the specially designed phase-contrast microscope. This type of microscopy is not used in a routine histopathology laboratory.

DARKFIELD MICROSCOPE

Directly transmitted light is excluded, and only scattered or oblique light is used in darkfield microscopy. While most objects that are examined microscopically are transparent, they also have the property of reflecting light rays. If oblique light that does not enter the objective is directed on these objects, the objects will appear self-luminous against a dark background. In darkfield microscopy, objects appear much larger than they are because of their light-scattering properties, and frequently, fine structures are seen much more easily with darkfield than with light microscopy. The preparation to be examined with the darkfield microscope must be thin and free of extraneous refractive material such as air bubbles, oil, or red blood cells. This type of microscopy is used primarily for the study of unstained microorganisms and for silver grains in radioactive staining procedures, and is rarely used in routine histopathology.

FLUORESCENCE MICROSCOPE

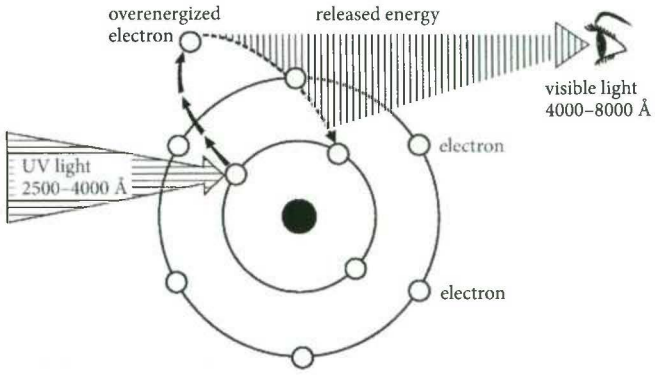
Fluorescence is essentially an optical phenomenon in which light of one wavelength is absorbed by a substance and almost instantly re-emitted as light of a longer wavelength. In fluorescence microscopy, a substance is bombarded with short-wavelength light in the ultraviolet (UV), violet, or blue range, and visible light is emitted **[f3.1]**. Stated another way, short-wavelength light absorbed by an atom or molecule boosts the energy level of electrons to a higher orbit; the return of electrons to the original level of excitation results in the loss of energy, which is expressed as visible light.

Mercury or halogen lamps are the usual light source for critical work; an exciter filter placed between the light source and the specimen transmits light of the desired wavelength but obscures all visible light. A second filter, the barrier filter, is placed in the eyepiece to absorb all UV rays and to allow only visible light rays to pass. The barrier filter protects the eyes from the damaging effects of UV light and reduces nonspecific fluorescence so that the fluorescent object is seen as a bright object against a dark background.

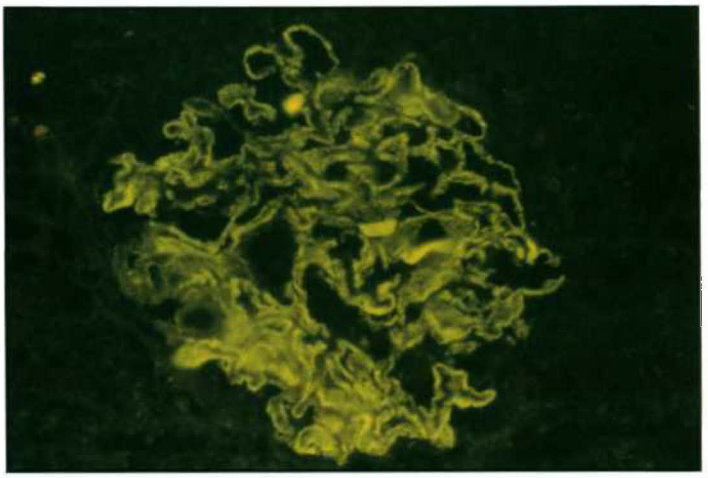
Some compounds and some tissue components, such as collagen fibers, fluoresce naturally. This property of natural fluorescence is known as primary fluorescence, or autofluorescence, and may cause problems for the inexperienced microscopist because some tissue components give the same color autofluorescence as the fluorochrome; this is especially true when using blue light. Substances that are not naturally fluorescent may become fluorescent by interacting with a fluorochrome, ie, a dye that fluoresces if excited by UV light. The filter combinations must be defined for each fluorochrome so that maximum excitation energy will be provided at the optimal wavelength. Fluorescein conjugates used in immunofluorescence work are maximally excited in the blue range, around 495 nm.

In immunofluorescence techniques, one of the components of the reaction (antibody, antigen, or complement) is labeled with a fluorescent dye such as fluorescein isothiocyanate (FITC). In the direct immunofluorescence technique **[i3.3]**, labeled primary antibodies are applied directly to tissue sections to locate and combine with antibodies, complement, or even antigens deposited in tissues such as kidney or skin. Indirect techniques first apply an unlabeled primary antibody to the section. This is followed by an FITC-labeled antibody that is raised in a second animal species against the animal species producing the primary antibody. For example, in the detection of autoantibodies to thyroid components, normal thyroid tissue sections are used; the patient's serum (primary antibody) is applied, and any antithyroid antibodies present combine with the tissue. No color or fluorescence is present at this stage, so an FITC-labeled antibody (second antibody) against human immunoglobulin is applied, and the presence of any antithyroid antibodies in the patient's serum can now be detected with the fluorescence microscope. The second antibody against immunoglobulin G is frequently raised in goats. Indirect techniques are considered to be more sensitive than the direct techniques. Complement immunofluorescence techniques also use more than 1 step.

Acid-fast bacilli and amyloid also can be demonstrated by staining with fluorescent dyes. These are not immunologic techniques and the dye is applied directly to the tissue sections. Auramine-rhodamine is used for the detection of acid-fast bacilli, thioflavin T for amyloid, and thioflavin S for neurofibrillary plaques and tangles.



[f3.1] Diagram illustrating how UV light may excite fluorescence in a molecule. (Reproduced with permission [Culling 1985])

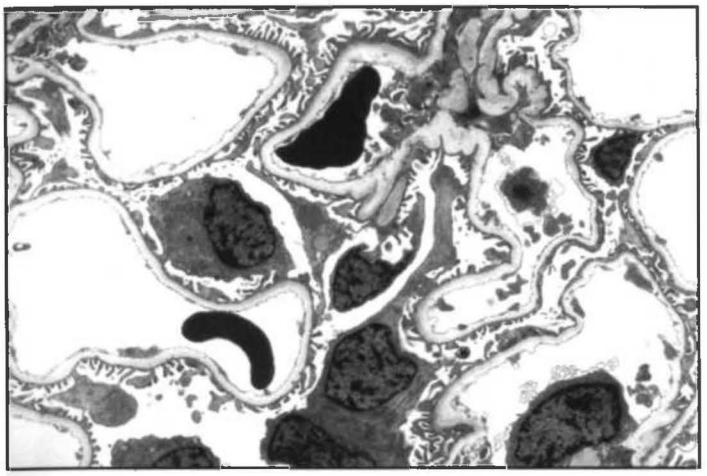


[i3.3] Direct immunofluorescence showing granular staining of the kidney glomerular basement membrane with goat anti-human IgG. The technique requires fresh-frozen cryostat sections of the renal tissue. (Courtesy of Joseph Newman, PhD, Baylor University Medical Center)

Immunofluorescent preparations are subject to fading, which can be controlled to some degree by careful selection of both the mounting medium and the excitation wavelength; however, slides should be viewed as soon as possible after staining and photographs should be taken for a permanent record. Thioflavin S dye is stable and allows the slide to be stored, and the tissue to be reviewed years later. Fluorescence microscopy requires special equipment, skill, and training.

ELECTRON MICROSCOPE

As stated previously with the ordinary light microscope, objects must be at least 0.2 μm apart to be distinguished as separate structures. Crystalline structures no more than 0.35 nm, or 0.00035 μm, apart can be resolved with the electron microscope, but biologic structures are difficult to resolve if they are not separated by at least 2.5 nm. The upper limit of useful magnification with the light microscope is about 1,000 diameters (×); enlargement of photomicrographs above this magnification yields no additional information (empty magnification). The electron microscope has a magnification range from approximately 1,000 to 500,000 diameters, with useful information (resolution) obtained over the entire range. Resolution is dependent upon the wavelength of the energy source used for illumination.



[i3.4] An electron micrograph of a kidney section. Note that only a portion of 1 glomerulus is shown.

The electron microscope obtains extra resolving power by replacing the ordinary light source of a light microscope with an electron gun, that is, an electrified tungsten filament that emits electrons. The extra resolving power results from the fact that an electron beam has a much shorter wavelength than visible light. The electron gun (electron source), the electron beam, and the specimen are all maintained under a vacuum. The electron beam is aimed at the specimen and focused by varying the strength of electromagnetic fields, equivalent to the glass lenses of light microscope. The resulting image is visualized by projection onto a fluorescent screen.

There are 2 types of electron microscopy: transmission and scanning. In transmission electron microscopy, the specimen (typically a very thin section) either transmits electrons (producing electron-lucent, or clear, areas in the image) or deflects electrons (producing electron-dense, or dark, areas in the image), much as light is either transmitted or blocked by a histologic section. A 2-dimensional, black and white image is seen on the fluorescent screen. With transmission electron microscopy, one can appreciate not only the relationship between cells but the ultrastructure of the cell itself. Transmission electron microscopy [**i3.4**] is very useful in the diagnosis of muscle and kidney disease and in tumor identification; however, immunohistochemistry has replaced the electron microscope to a large extent in tumor diagnosis.

In scanning electron microscopy, a dramatic 3-dimensional image results as the electron beam sweeps the surface of the specimen and releases secondary electrons. The highest effective magnification with the scanning electron microscope is much less than that of the transmission electron microscope, but 1 of the great merits of the scanning electron microscope is the great depth of focus. The scanning microscope is used to study surfaces of an object or specimen, and has been used to study cell surface membrane changes in the evolution of malignancy and other pathologic processes. This type of electron microscope is used primarily in research.

Electron microscopes are very expensive and require skilled operators with an extensive knowledge of microscopic anatomy. Because of their limited use and great expense, they are usually found only in larger institutions.

Microtomes

Although several types of microtomes are available for special purposes (eg, retracting for plastic and vibrating for tissues that cannot be frozen or embedded), the rotary, sliding, and clinical freezing microtomes are the types most frequently encountered in histopathology laboratories in the United States. The ultramicrotome used for cutting 0.5-µm plastic sections for light microscopic orientation and 90-nm sections for electron microscopy is a retracting microtome. Today, the rotary microtome is standard in laboratories where routine paraffin and frozen sections are the sole requirements.

ROTARY MICROTOME

A rotary microtome operates with a screw feed or a computerized motor; the block moves up and down, and either the blade holder or the block advances a preset number of micrometers with each revolution of the wheel. This type of microtome is found in most cryostats and is the type most commonly used for sectioning glycol methacrylate- and paraffin-embedded material. Routine maintenance of the rotary microtome consists of the following:

1. Cleaning the microtome thoroughly at the end of each day by carefully removing all accumulated paraffin with a soft brush or soft cloth moistened with xylene and then drying the microtome thoroughly.

2. If the model requires, applying microtome oil or grease to all sliding parts as indicated by the manufacturer. Lubricating should be done on a routine schedule as recommended by the manufacturer.

3. Documenting service, repair, or routine preventive maintenance performed by someone skilled in microtome maintenance. The microtome should be in good working condition for consistency and operator safety.

4. Covering the microtome when it is not in use.

During the last 10 years, microtome designs have offered a broad range of features providing ergonomic benefits. The wheel is typically semi- or completely motorized, giving a smooth wheel rotation. The wheel is lighter, decreasing the resistance needed to turn it and thereby reducing fatigue. The completely automatic microtome has a motor that turns the wheel, allowing both hands to be free to manipulate the ribbon; most have a foot pedal to control starting and stopping the rotations. The motorized wheel controls the rotations through the tissue and reduces thick-thin sections. Some models have an additional device that assists with block alignment, reducing the need for retrimming a previously cut block. Ensuring minimal tissue loss when recutting a previously sectioned block not only preserves precious tissue but saves time. These design innovations have improved both working conditions and section quality.

SLIDING MICROTOME

The block is held stationary on the sliding microtome, and the knife is moved along a horizontal plane past the block face. As the knife is returned to the starting position, it completes each section cycle and a screw feed causes the block to be raised toward the knife at a predetermined thickness. This type of microtome is used for sectioning celloidin and large paraffin blocks; it is not used in routine histopathology. The care of the sliding microtome is the same as that of the rotary microtome.

CLINICAL FREEZING MICROTOME

The clinical freezing microtome has been replaced to a great degree by the cryostat; however, free-floating sections required for some special stains are easier to obtain with this instrument than with the cryostat. For this reason, the microtome will be described. This microtome clamps to the table top and is relatively portable. A chuck with an attached supply of carbon dioxide allows for the freezing of a tissue section placed in a horizontal plane. The knife swings out over the chuck containing the frozen tissue specimen, and as the knife is returned to its starting position, the micrometer screw advances the block upward toward the knife. Sections must be removed from the knife edge and floated in a dish of distilled water. The sections may be mounted on slides from the distilled water and dried before staining, or they may be stained and then mounted at the end of the procedure.

This method of preparing frozen sections is not good for friable tissue. Airborne disease transmission is also more likely to occur with this method, because human tissue may contain possible infection hazards that can be spread by the bursts of carbon dioxide used for freezing.

With all type of microtomes, the micrometer setting is very important. This setting is only approximate and is not an exact determination of section thickness; the actual thickness is influenced by the condition of the microtome and the quality of the blade edge as well as the skill of the microtomist. Microtomy problems will be discussed in the following section on microtome blades. The blade is a major component of the microtome and as such will be addressed separately from the microtome.

MICROTOME BLADES

A sharp microtome blade with an edge free of defects is essential to obtaining good sections. Successful sectioning of poorly processed tissue is often possible with a sharp blade; conversely, nondiagnostic sections may be obtained with a dull blade, even when tissue is optimally processed.

Glass knives are used for cutting sections of plastic-embedded material. Ralph knives, a special conformation of glass, are used to section glycol methacrylate-embedded material and will not be discussed. The use of glass knives for sectioning material embedded in plastic for electron microscopy is discussed in chapter 14, "Electron Microscopy," p341.

There are 2 types of disposable blades, high profile and low profile. The type of microtome blade holder will dictate which of the 2 types is used. Because each microtome brand has a different recommendation of settings for the blade's angle, it is suggested that the technician review the manual for settings and guidelines for the blade.

Disposable blades have replaced steel knives in most laboratories; therefore, knife blades will not be discussed. The disposable blades are special stainless steel razor blades held securely and rigidly in a holder designed for this purpose. These disposable blades have edges superior to knives, and section quality is greatly improved because of the introduction of these blades; however, it has been my experience that not all disposable blades produce sections of equal quality. Because of the safety hazard present, used disposable blades and glass knives should be discarded in a puncture-proof container. Blades or glass knives contaminated with human tissue from use in the cryostat or with paraffin should be placed into a special biohazard "sharps" container that is then incinerated.

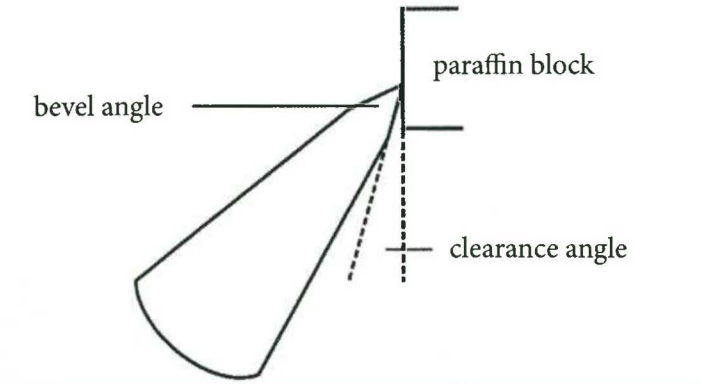
Regardless of the type of material to be sectioned and the type of blade used, section quality is determined by the condition of the edge and the clearance angle more than by any other factors. The clearance angle in relation to the block face is very important when cutting sections [**f3.2**]. This angle routinely is approximately 3° to 8°, but the microtome design can dictate the angle. It is recommended that, upon acquisition of a new microtome, the operational angle be confirmed before use. Errors in properly establishing the clearance angle can lead to many different sectioning problems.

The rate at which sections are cut influences quality, and undue speed invariably yields sections of poor quality. Each type of tissue has an optimum cutting speed that is dependent on the nature of the material, the cutting edge, the angle of the blade, and the thickness of the section desired. A general guide for cutting good paraffin sections is that the microtome drive wheel should be rotated approximately 1 revolution per second. An automated microtome in which the rotation of the wheel is motorized improves section thickness consistency because of controlled speed through the tissue. For most routine hematoxylin and eosin (H&E) staining, the sections should never be more than 1 cell layer thick and should be cut at a 4- to 5-µm setting on the micrometer scale. The thickness of a section can be determined microscopically by focusing up and down through the section. If the section is 4 to 5 µm in thickness, then all nuclei will be in 1 plane of focus; if some nuclei go out of focus as others come into focus, then the section is too thick [**i3.5**]. Tissues such as bone marrow and kidney biopsy specimens routinely may be cut thinner (2 to 3 µm), allowing for better nuclear detail. If the microtome is properly set and thick sections are being produced, the microtome may require service.

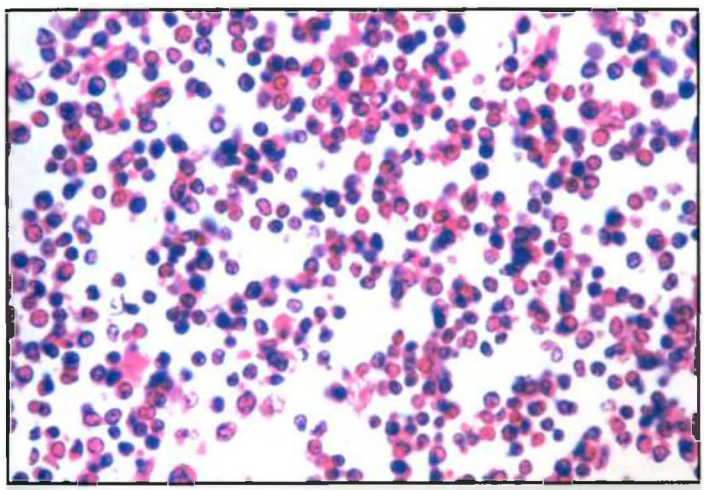
TROUBLESHOOTING MICROTOMY

Many microtomy problems associated with sectioning paraffin, frozen, or celloidin tissue blocks are similar. For example, with each of these techniques, irregular, skipped, or excessively thick and thin sections are usually the result of either too little [**f3.3a**] or too much [**f3.3b**] blade tilt (clearance angle). The problems of irregular, skipped, or thick and thin sections usually can be corrected by adjusting the blade so that the clearance angle between blade and specimen is correct. Grooved, scored, smeared, and deformed sections are frequently produced by a dull edge, and moving the existing blade to an unused surface or replacing with a new blade will usually correct this microtomy problem.

Although regular lengthwise or vertical scratches and splits in the sections usually are caused by a defect in the edge, calcium, bone, or another hard material present in the specimen also can cause this artifact [**i3.6**]. Move the blade to an unused area; if the defect remains in exactly the same area of the new sections, then the problem is in the specimen and not the blade edge [**i3.7**]. Mushy sections result from insufficient dehydration or clearing. While backing up these tissues to the dehydrating agent and then reprocessing may help, the sections rarely will be as good as those from an originally well-processed tissue sample; then additional testing



[f3.2] Bevel and clearance angles related to sectioning.



[i3.5] A section of bone marrow that is too thick. If examined closely, it becomes apparent that some of the cells are in focus while others are not, an indication that the section is more than 5 to 6 μm in thickness.

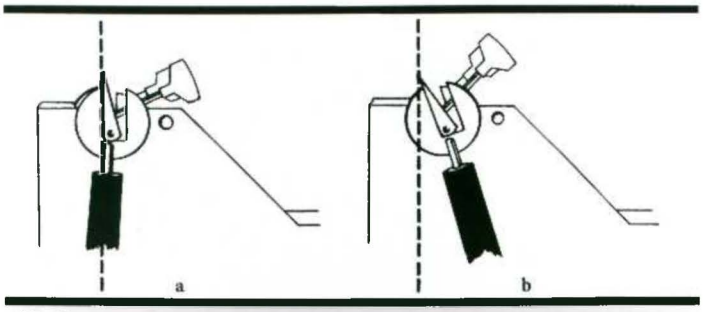
such as molecular studies or immunohistochemistry results may be compromised.

While all of the microtomy problems presented thus far may be encountered with all processing and embedding methods, there are other problems that are commonly encountered with paraffin sectioning.

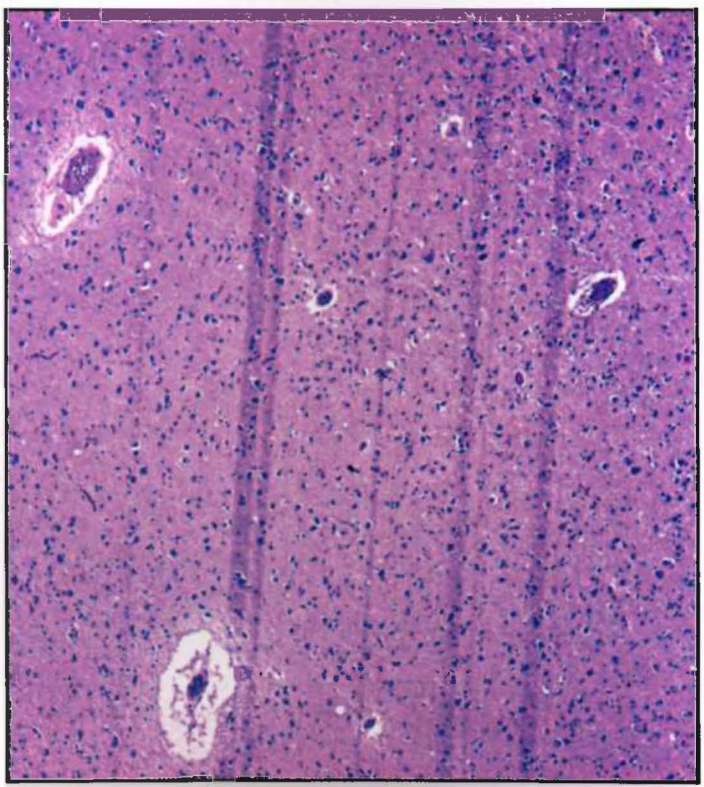
■ *Crooked Ribbons*

Crooked ribbons result when the horizontal edges (top and bottom) of the block are not parallel. They may also be caused if the lower block edge is not parallel to the knife edge when sectioning [i3.8]. Crooked ribbons occur less commonly when the block is not evenly chilled or the hardness of the paraffin varies from 1 side of the block to the other. Imperfections in the blade edge should also be considered as a cause. This artifact can be prevented by ensuring:

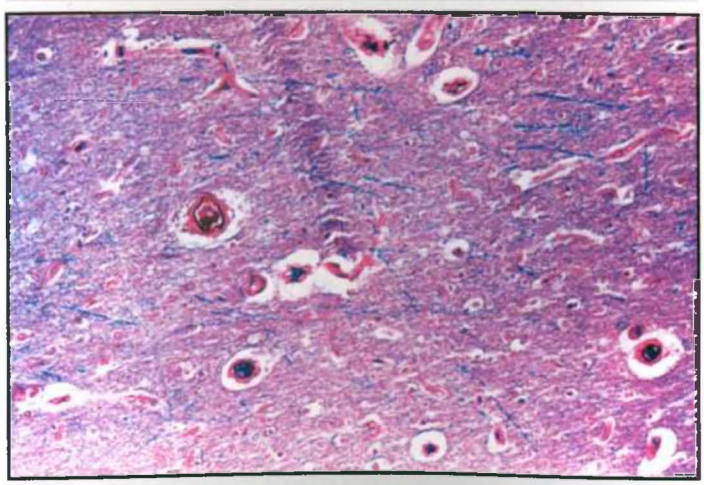
- that the upper and lower block edges are parallel
- that the lower block edge is parallel to the cutting edge of the blade
- that there are no problems with the blade edge
- that the block is evenly chilled



[f3.3] **a** The clearance angle, or blade tilt, is too slight. This most frequently results in sections that are missed or skipped, alternately thick and thin, wrinkled and jammed, or lifted from the blade. **b** The clearance angle, or blade tilt, is too great. This frequently causes chatter, microvibration, washboarding, or undulations in sections and may make it impossible to obtain a ribbon.



[i3.6] A section of central nervous system tissue revealing numerous knife lines caused by defects in the edge of the blade.



[i3.7] A section of central nervous system tissue stained with the Luxol fast blue-periodic acid-Schiff (PAS) technique. Careful observation reveals a knife line beginning in the tissue; this line is caused by the presence of a small focus of calcium in the tissue and not by a defect in the blade edge.

■ *Block Face Unevenly Sectioned*

When the block holder is adjusted so that it is not parallel to the blade, 1 side of the block is exhausted while attempting to get a complete section of the block face [**i3.9**]. This results in uneven sectioning of the block face. When this occurs, it wastes tissue and may cause the loss of important areas of tissue. Uneven sectioning can be prevented by ensuring at the beginning of sectioning that the block holder is adjusted so that the block face and the blade are perfectly parallel.

■ *Holes in the Section*

Holes occur when a block is faced too aggressively [**i3.10**]. Small flecks of tissue are removed from the block, leaving a hole; liver, brain [**i3.11**], and lymph nodes are especially prone to this artifact. Holes seen in sections that are a microtomy artifact will decrease in size as successive ribbons are cut and will finally disappear. Thompson and Luna [1978] attribute this artifact to excessive dehydration [**i3.12**] or improperly processed tissue. Holes may appear in the ribbon if all of the air is not displaced from the tissue during infiltration. This occurs most frequently with lung tissue; it is not a sectioning artifact, and these holes will not disappear with continued ribboning. Holes in sections may be corrected or prevented by:

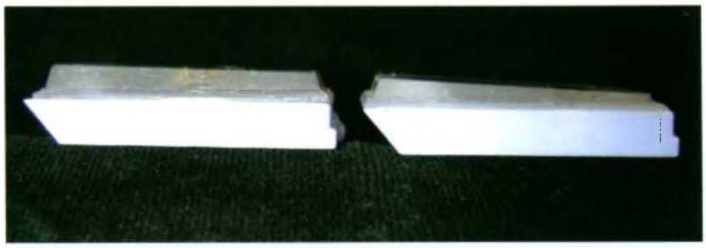
- exposing the tissue, then soaking the block briefly in ice water or with a wet piece of cotton before sectioning
- if there is sufficient tissue in the block, cutting and discarding ribbons until the holes disappear; however, this technique will potentially lose valuable tissue or the area of interest
- facing the block less aggressively, with smaller micrometer advances of the block for each section removed
- facing large autopsy brain sections at 5-μm intervals using a smooth rotation until the tissue is fully exposed
- knowing the type of tissue being sectioned and making modifications as necessary

■ *Failure of Ribbon to Form*

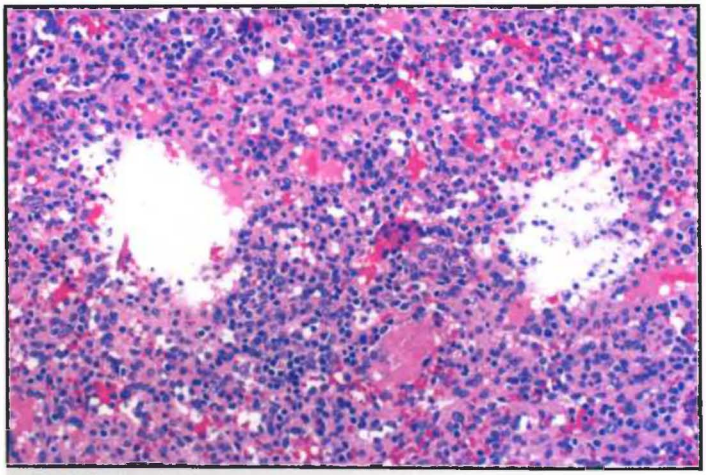
The failure to obtain a ribbon is most commonly caused by a dull blade, but also may result from paraffin that is too sticky (not enough plastic) or too hard (too high a melting point); too much blade tilt; or a room temperature that is too high or low. The formation of a ribbon depends on enough heat being generated by the friction occurring as each section is cut to cause the sections to adhere to each other. If the blade is too cold, it is difficult to generate enough heat for section adherence. This problem may be prevented or corrected by:

- choosing a paraffin with a lower melting point
- decreasing the tilt of the blade (smaller clearance angle)
- changing the room temperature

[i3.8] The block is not parallel to the blade edge. The top and bottom edges of the block should be parallel, and the bottom of the block should be parallel to the blade edge, or crooked ribbons and poor adherence of one section to the next may result.



[i3.9] The entire face of the block was not parallel to the blade, so that ribboning was very uneven, and a major part of one side of the block has been cut away.



[i3.10] Holes in the section occur when the block is faced too aggressively, especially on tissue that has been subjected to excessive dehydration or improper processing.

■ *Lifting of the Section from the Blade as the Block Is Raised*

Lifting of sections from the blade frequently is caused by a dull blade or by too little blade tilt. A warm room or paraffin that is too soft can also cause this artifact. This may be prevented or corrected by:

- increasing the tilt of the knife (greater clearance angle)
- changing to a paraffin that is harder (slightly higher melting point)

If a ribbon cannot be obtained after checking and correcting for any identified problems, single sections usually can be picked up by touching the top or bottom edge of the section with a wet applicator stick held with the long surface of the stick parallel to the section **[i3.13]**, **[i3.14]**. Forceps may also be used to pick up the section.

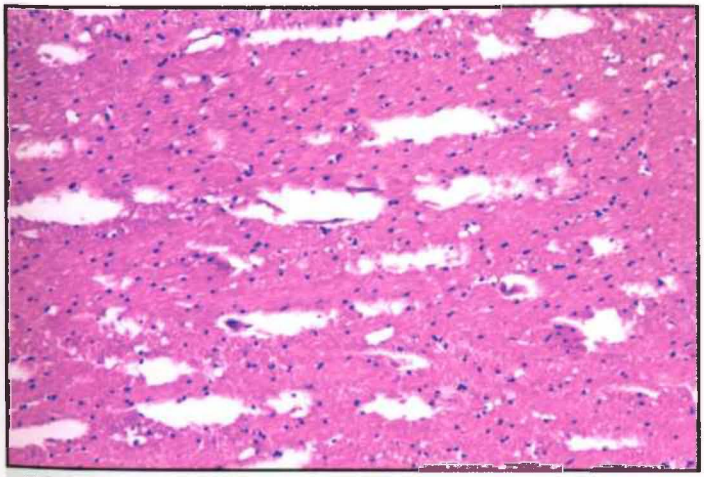
■ ***Washboarding or Undulations in the Section***

This artifact most commonly occurs in very hard tissue such as uterus or in overfixed tissue. It is a macroscopic type of chatter easily seen when the sections are on top of the water bath. It may occur from worn microtome parts that allow too much tolerance in some of the moving parts and from loose clamping of the blade or block. This may be prevented by:

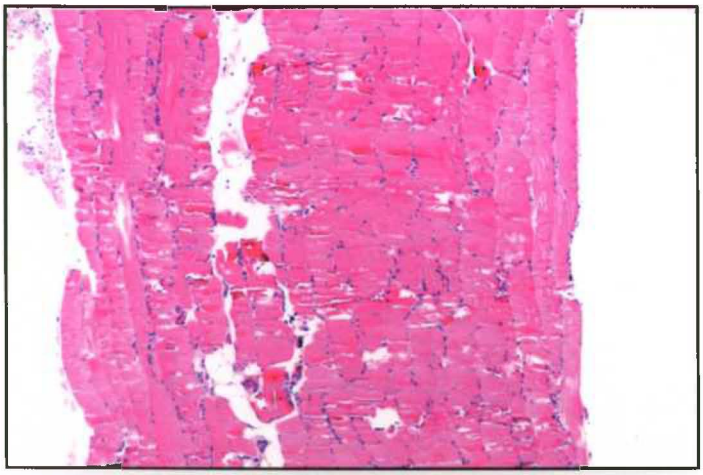
- ensuring that the paraffin is filled on the top of the cassette to provide support for tissue when clamped in the block holder
- ensuring that both the block and blade are tightly clamped in the microtome; however, undulations also may result from a worn microtome part that allows too much tolerance in some of the moving parts **[i3.15]**. A block or blade that is very loosely clamped can cause the blade to chop into the block, resulting in loss of some of the section or pieces of tissue **[i3.16]**
- ensuring that the block holder shaft is not overextended. Microtome models in which the blade is stationary and the block advances or retracts can create washboarding when the shaft is overextended **[i3.17]**, **[i3.18]**
- ensuring that the microtome is in good working order and has routinely scheduled maintenance
- decreasing the blade tilt (smaller clearance angle)

■ ***Chatter, or Microscopic Vibration, in the Section***

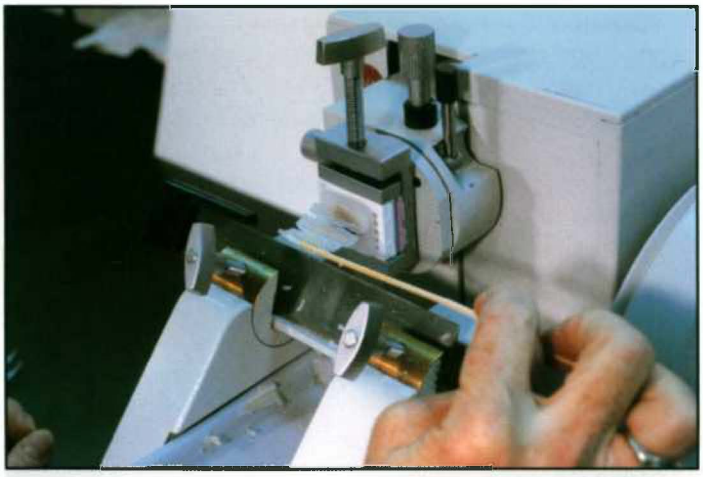
Luna [1988] relates this artifact primarily to overdehydration or a lack of moisture in the tissue. Microscopic chatter **[i3.19]**, **[i3.20]** also can be caused by a dull blade or by too much blade tilt, which



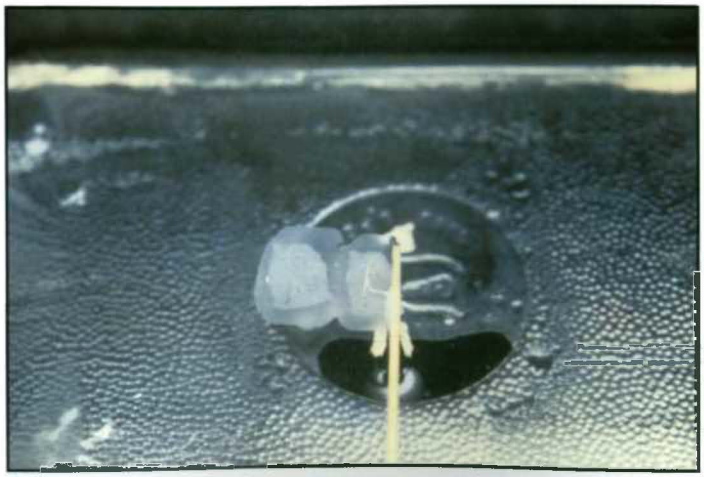
[i3.11] Numerous irregular holes are noted in this section of central nervous system tissue, indicating that the block was faced too aggressively. Brain tissue is frequently subject to this artifact.



[i3.12] The effects of overdehydration are seen in this section of skeletal muscle, with both holes and chatter present.



[i3.13] The sections are lifted from the blade as the block is raised, an artifact frequently caused by a dull blade or a blade tilt that is too small. These individual sections, or short ribbons, can usually be picked up with forceps or a dampened applicator stick, as shown.



[i3.14] The sections seen in **[i3.13]** floated out. The sections were lifted from the block with the aid of an applicator stick. The applicator stick is floating so that the sections did not have to be detached before being placed on the flotation bath.

causes the section to be scraped rather than cut, and by cutting too rapidly. This may be prevented or corrected by:

- ensuring that the processing schedule does not cause overdehydration of tissues; processing different tissues on different schedules
- restoring moisture to tissue by facing the block and soaking it briefly in ice water or placing it face down on an ice tray. A wet laboratory-grade tissue paper (Kimwipe, Kimberly-Clark Corp, Roswell, GA) covering the thumb can also be used to rub the block face with water from the water bath; this slightly warms the block face while rehydrating the tissue. Do not put an ungloved thumb into water bath as it will result in squamous cell contaminant.
- decreasing the tilt of the blade (smaller clearance angle)
- decreasing cutting speed; 1 revolution of the wheel per second is considered a reasonable cutting speed

■ ***Skipped or Varied Thickness of Sections (Thick and Thin Sections)***

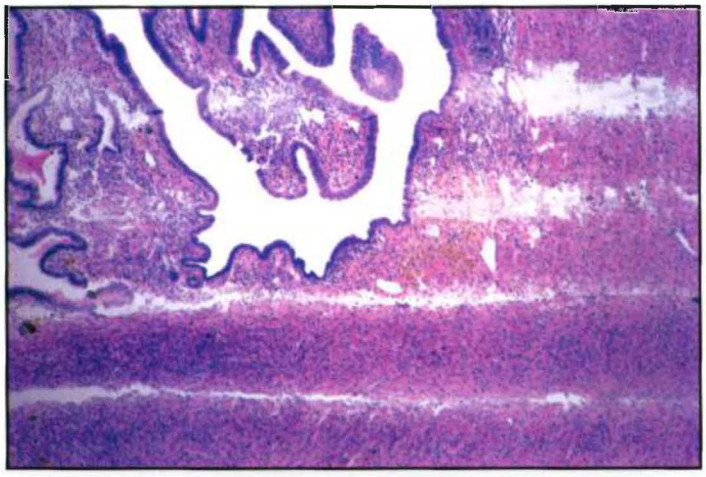
Thick and thin sections may be caused by too little blade tilt, so that the bottom, rather than the top, of the blade facet contacts the block. Compression of the block results, but with continued advance, the blade edge does finally contact the block and a section is cut. This alternate compression and sectioning of the block yields skipped and thick sections. Alternate thick and thin sections can also be caused by loose or worn microtome parts. This may be prevented or corrected by:

- increasing the tilt of the blade (greater clearance angle)
- ensuring that the microtome is in good working order and that routine maintenance is scheduled and documented

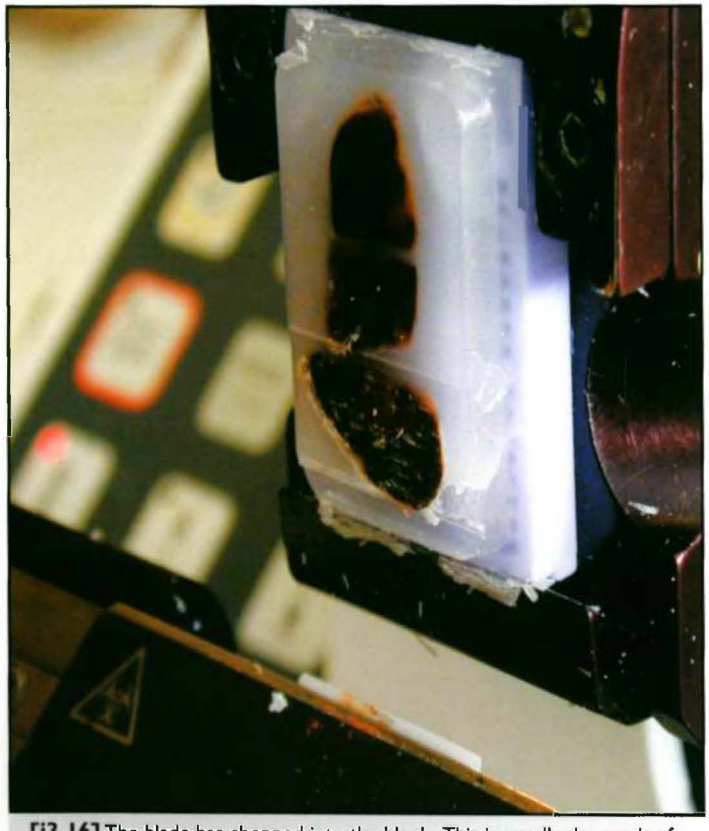
■ ***Compressed, Wrinkled, or Jammed Sections***

A dull blade **[i3.21]**, a blade gummed with paraffin, paraffin sticking in the back side of the holder, too little blade tilt, too-rapid cutting, or a too-warm room will all cause this microtomy artifact. These problems can be prevented or corrected by:

- keeping paraffin from building up on the blade back; the edge should be kept free of paraffin by wiping (up, never down) with gauze slightly dampened with xylene
- ensuring that a sharp blade is in use; changing if necessary
- increasing the tilt of the blade (greater clearance angle)
- decreasing cutting speed; 1 revolution of the wheel per second is considered a reasonable cutting speed



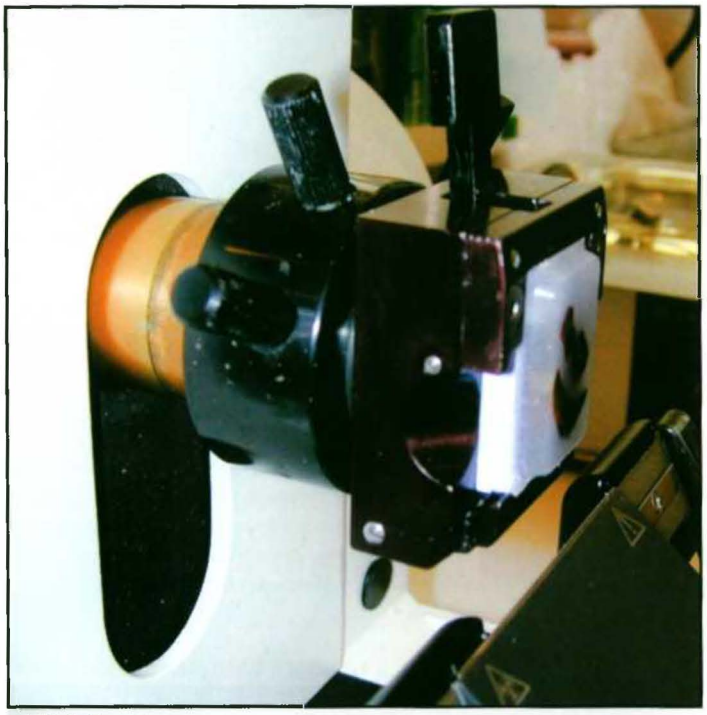
[i3.15] Washboarding or undulations in the section are caused by a major vibration. This artifact usually results from the block or blade not being tightly clamped in the microtome, but may have other causes. Undulations are seen more frequently with very hard tissue such as uterus, or when a microtome has parts that are worn.



[i3.16] The blade has chopped into the block. This is usually the result of the block or blade being inadequately clamped in the microtome. Tissue will be lost in attempting to obtain a complete section of this damaged block.

■ ***Lengthwise Scratches or Splits in the Ribbon***

A defect in the blade edge or a hard particle in the block will cause blade lines or splits in the ribbon **[i3.22]** and **[i3.23]**. If the scratches remain in the same area of the section after the blade is moved, then the defect is in the block; if the defect disappears or moves as the blade is moved, then the defect is in the blade edge. These problems can be prevented or corrected by:

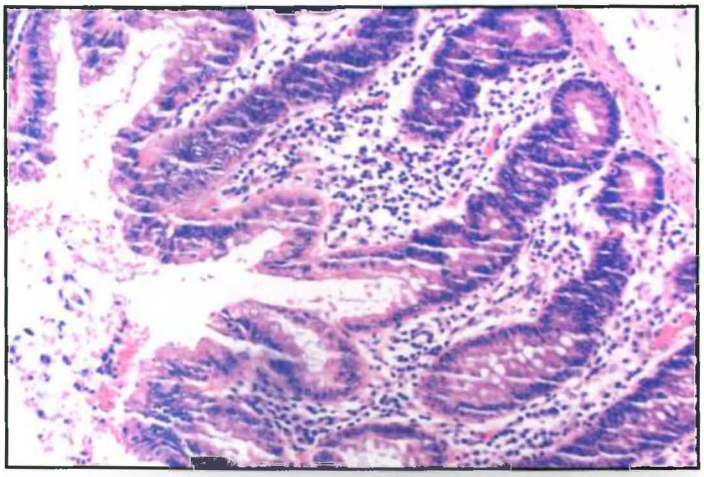


[i3.17] The block holder shaft is overextended. This may cause vibration during sectioning and will result in washboarding or chatter.

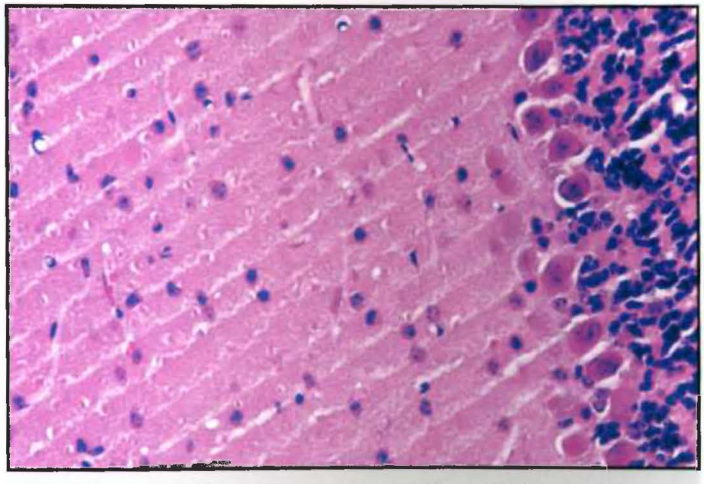
[i3.18] The block holder shaft is correctly positioned.

- avoiding any metal objects or sutures that might damage the blade edge; in extreme cases the tissue may require rotation to reduce the total surface area that will be damaged.

- ensuring that paraffin has not collected in the front or back of the blade edge



[i3.19] Chatter, or microvibration, most often results from overdehydration during processing, but may also result from a dull blade, too much blade tilt, or cutting too rapidly. Soaking the faced block with moistened cotton will help correct this problem if it is caused by excessive dehydration of the tissue.



[i3.20] A section of central nervous system tissue that demonstrates chatter, or microvibration.

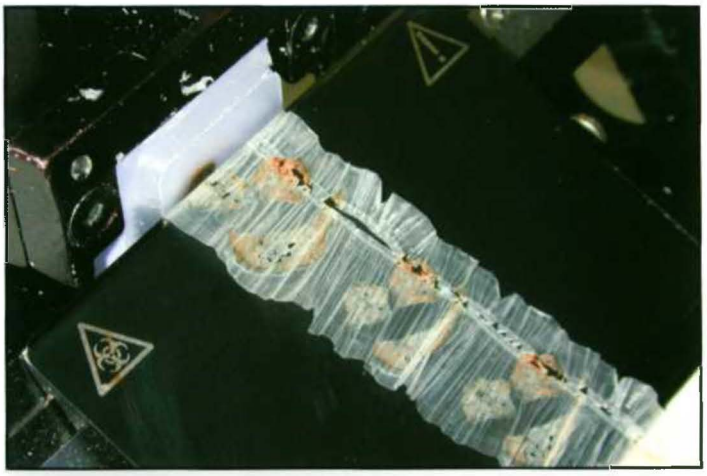
[i3.21] Compressed sections, as seen in this figure, may result from a dull blade, too little blade tilt, paraffin accumulation on the blade, and cutting too rapidly.

- moving to a new area of the blade or replacing with a new blade **[i3.24]**

■ ***Sections Flying and Sticking to Nearby Objects or Other Parts of the Microtome***

This artifact results from static electricity. This problem may be decreased by:

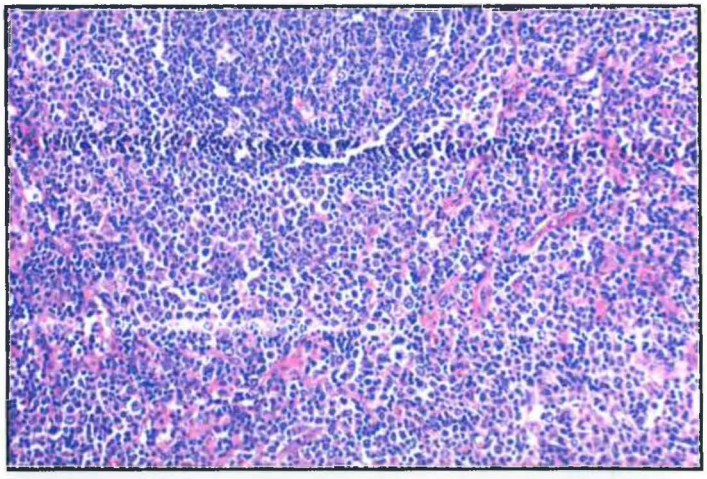
- adding moisture to the air by breathing on the block and blade, using a humidifier near the microtome, or grounding the microtome to a water pipe
- using some method of ionizing the air



[i3.22] The results of a major nick, or defect, in the blade edge can be seen in this paraffin ribbon. This defect possibly resulted from improper handling of the forceps used in sectioning. Any metallic object that is allowed to come into contact with the blade edge can damage the edge and cause this type of artifact.

Cryostat

The cryostat is a refrigerated chamber containing a microtome, usually of the rotary type. It is cooled by a mechanical refrigeration unit. Although a cryostat is easy to operate, practice and skill are needed to obtain good frozen sections. Because sections are picked up directly on the slide, frozen sections that are impossible to obtain with the clinical freezing microtome can be obtained using this instrument. As with paraffin sectioning, the blade must be very sharp and the edge must be free of defects. A high-profile blade is commonly used, and most antiroll devices are designed specifically for these blades **[i3.25]**. As a frozen section is cut, there is a natural tendency for it to curl or roll at the blade edge. This may be reduced by an antiroll plate **[i3.26]**, **[i3.27]** or device; however, improper adjustment of this plate will result in sectioning problems. The tilt of the blade edge is very important, with the optimum tilt considered to be an angle of 30° between the center line of the blade and the block face. The blade should be tilted more for frozen sections than for the paraffin-embedded material; but as with paraffin blocks, too much blade tilt will yield scraped rather than cut sections, and too little blade tilt may yield sections of varying thickness. Basic criteria for the consistent production of good sections are a sharp, properly aligned blade without defects in the edge, a properly adjusted antiroll device, and an optimum sectioning temperature.



[i3.23] A line running horizontally through the upper part of this section was caused by a nick in the blade edge. The horizontal line seen in the lower part was most likely caused by an accumulation of paraffin on the edge of the blade. Note that nicks tear and disrupt sections, but paraffin accumulation does not.

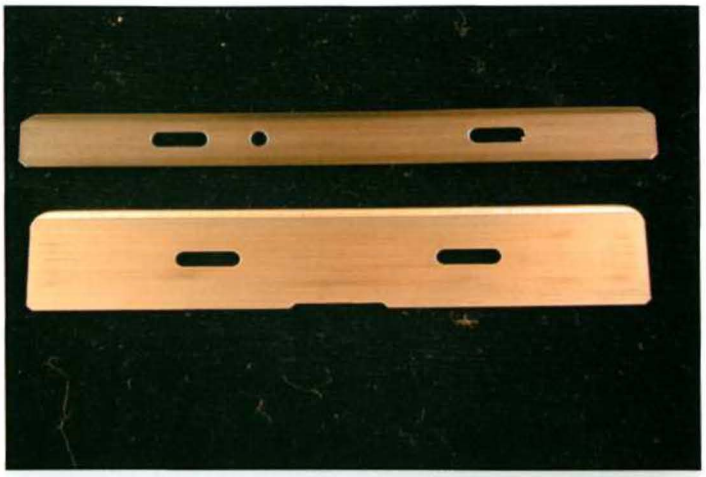
At any specific temperature, each tissue has a firmness directly related to its lipid and water content. Working at the optimum sectioning temperature greatly increases the ability to section any type of tissue. If sections of a particular tissue tend to fragment or split, the temperature may be too low, while if the sections tend to collect at the blade edge, the tissue is probably too warm. Commonly, cryostats are operated at approximately –20°C for most tissues; however, brain, liver, spleen, lymph node, and endometrial scrapings section better at a slightly warmer temperature, while any tissue containing fat requires a much colder temperature. Technology has improved the ability to control cryostat temperatures while the door is open. This is a valuable improvement that increases manipulation time for obtaining a section and decreases the effects of warm air that previously made sectioning a challenge.

[i3.24] An optimal ribbon without any defects is seen in this figure.

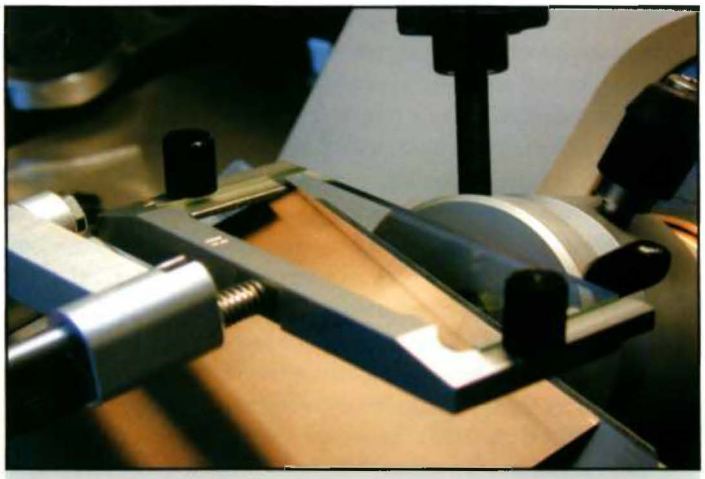
Slow freezing of tissue will allow water present in the tissue to form ice crystals throughout the specimen, with the size of ice crystals proportional to the speed of freezing. The sites of ice crystal formation are seen as artifacts, or holes, in microscopic sections commonly called "freeze thaw artifact." This is particularly disturbing in some tissue such as skeletal muscle. Tissue may be frozen more rapidly by using a heat-extractor attachment, dry ice, hydrochlorofluorocarbon refrigerants (Freon) spray, or a beaker of isopentane suspended in liquid nitrogen. Freon spray is not recommended because of its ability to aerosolize infectious tissue, which can expose the technician to tuberculosis or other highly contagious diseases. Isopentane suspended in liquid nitrogen is the preferred method for freezing skeletal muscle biopsy specimens on which enzyme studies are needed, and reduced artifact is seen if the isopentane is allowed to reach a temperature of –150°C before introducing the specimen. Liquid nitrogen is rarely used alone for freezing because gas bubbles form around the tissue and thus impede freezing.

Following freezing and sectioning, tissue should not be stored unprotected in the cryostat for any length of time, because it will lead to severe dehydration. If a specimen must be stored frozen for any length of time, it should be wrapped carefully to exclude air and then stored in a freezer at –70°C.

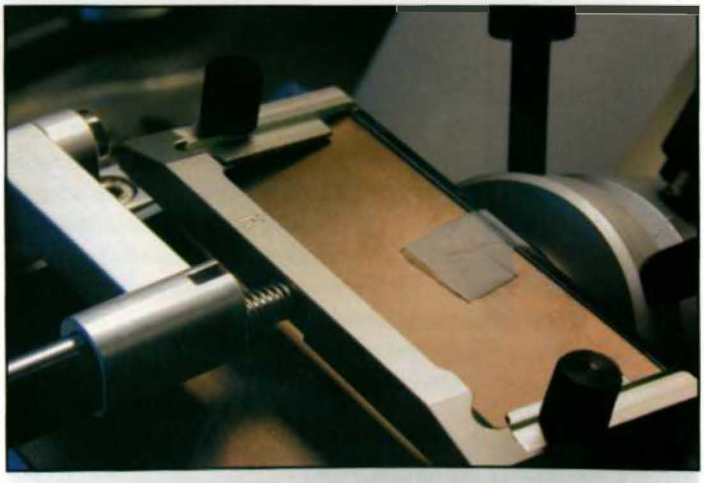
The cryostat must be kept free of the infection hazard posed by debris. Tissue shavings must be picked up frequently with a piece of gauze dampened with alcohol and discarded in a biohazard bag. Manufacturers provide models that reduce exposure risks by assisting with the containment of tissue within the chamber and decontamination by UV light. Some cryostats have a vacuum that assists with pulling the section as it is cut while simultaneously confining the shavings in a biohazard bag. This provides easy disposal of biohazardous tissue and reduces the technician's exposure. The cryostat should be defrosted and cleaned frequently. The microtome must be thoroughly dry before lubricating and recooling, because any remaining water will freeze and make instrument operation difficult. A silicone lubricant that remains fluid at low temperature is usually used. If the cryostat microtome becomes stiff and difficult to operate because of ice crystal formation, it may help to treat the microtome with absolute alcohol followed by lubrication after the alcohol evaporates. The refrigerant coils must be kept free of dust, or the motor may overheat; therefore, coil cleaning should be part of the preventive maintenance program. All routine cleaning and maintenance procedures should be regularly scheduled and should be documented when performed. Daily cleaning should include removal and proper disposal of tissue debris and wiping the chamber with 70% alcohol. The College of American Pathologists (CAP) [2007] recommends that a cryostat in daily use be decontaminated once per week, which requires use of a solution that will decontaminate for tuberculosis, AIDS, and other highly contagious diseases.



[i3.25] High- and low-profile blades are shown; the high-profile blade (bottom) is commonly used for frozen sectioning.



[i3.26] The cryostat anti-roll plate slightly raised from the blade.



[i3.27] The cryostat anti-roll plate properly positioned on the blade, so that the section comes between the blade and the plate.

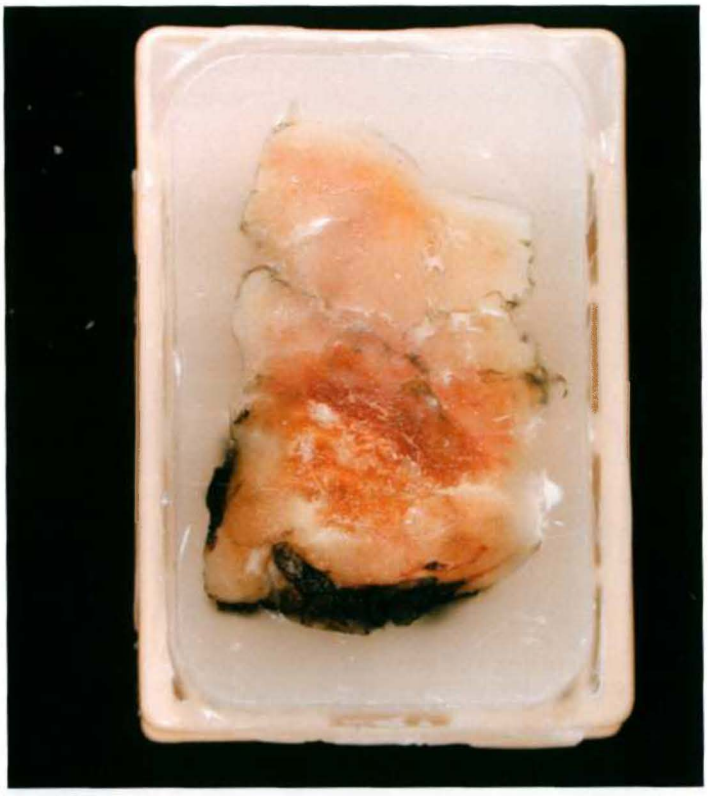
Tissue Processors

CONVENTIONAL PROCESSOR

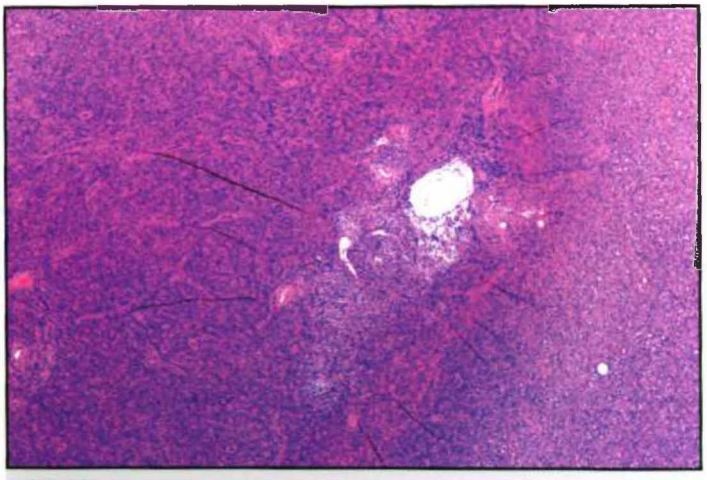
The primary processor in use today is the closed system (fluid transfer), in which the tissue is stationary and fluids are pumped in and out of the pressurized chamber holding the tissue. The closed

system is computerized and provides a digital readout to indicate the instrument status; it is usually equipped with an alarm system to alert personnel to any mechanical problems occurring during the processing cycle. Specific models will perform system checks before processing; this can include checking that all bottles are properly connected and that the vacuum is good. An error alarm allows the technician to make appropriate corrections to ensure a successful run. New models have laboratory information system (LIS) connectivity that sends an alert to vendors to prompt them about possible malfunctions before their occurrence. Closed systems can assist with keeping exposure to toxic vapor to a minimum when they are vented either to the outside air or through a filter. A major advantage of the closed processor is that specimens cannot dry out in the tissue chamber (retort) in the event of a malfunction. Although standardization of reagents for processing is common, a disadvantage is that not all types of reagents can be used on these processors (eg, mercury- or dichromate-containing fixatives and chloroform). Heat and vacuum are available at any station on closed processors. Heat and vacuum can speed up processing but must be used carefully implemented, especially on biopsy specimens. It is difficult to use the same processing program with heat and vacuum to achieve satisfactory processing of fatty tissues, routine tissues, and biopsy specimens (see chapter 2, "Processing"). It is also a mistake to try to process large, fatty, inadequately fixed tissue on a shortened time cycle, because the results will be poor **[i3.28]**, **[i3.29]**. Only very thin, small, well-fixed tissue sections will process well on a short cycle. The routine processors have improved processing times by providing not only vacuum but features like agitation and spinning movements. System improvements that reduce time also include the time in which they pump the reagents in and out; this allows for a longer tissue exposure to each solution.

All processors must be kept clean, and a routine reagent rotation and/or change cycle determined by usage must be established and rigidly adhered to (see chapter 2, "Processing"). The fluid levels must always be higher than the tissue cassettes, and the cassettes should be placed in the baskets or the chamber so that the fluids can circulate freely. Packing cassettes tightly will reduce, and in some cases, prevent reagents from reaching cassettes placed in the center, resulting in poorly or inadequately processed tissue. It is very important to maintain, monitor, and properly rotate the cleaning xylene and alcohol to ensure their effectiveness and to remove residual paraffin and xylene before each processing run. When the cleaning xylene and alcohol are saturated after a cleaning cycle, the retort will contain residual paraffin, and in severe cases the first station solution will have xylene or paraffin contaminants because of insufficient cleaning of the lines. The temperature of the paraffin must be carefully adjusted to no more than 2°C to 4°C above the melting point of the paraffin in use, or the tissue will be brittle and overhardened. The temperature must be monitored and recorded daily, with adjustments made in the paraffin bath temperature when indicated by improper temperature readings.



[i3.28] Fatty tissue has been processed on a shortened cycle, which has resulted in incompletely fixed and poorly processed tissue.



[i3.29] A section of lymph node, not completely fixed when placed in the alcohol on the processor, demonstrates alcohol fixation in the center of the node.

MICROWAVE PROCESSOR

Microwave processor technology has made its way into the clinical laboratory. The basics of how a microwave operates will be described in the "microwave staining oven" section of this chapter. These processors can assist with shorter turnaround times for all tissue types, large or small. The microwave processor offers the laboratory an opportunity to improve workflow without compromising tissue morphology and staining. Some models have the ability to refresh and remove contaminants from reagents, resulting in the ability to reuse reagents, thus reducing operational expense. It is suggested that the biopsy tissue turnaround time can be greatly improved with the microwave processor. As with conventional processing, microwave processed tissue results are dependent upon formalin penetration and appropriate fixation time, and both are successful only when the tissue thickness is controlled and consistent. If fixation time and section

thickness are controlled, the results can be comparable to those of the routine processor. The microwave processor can assist with speeding up formalin fixation for fatty breast tissue, but it should be noted that the effects of microwave processing have not been validated under the American Society of Clinical Oncology-College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer (ASCO/CAP HER 2) guidelines. The microwave has been found to speed up the decalcification time required for bone marrow to approximately 1 hour in an ethylenediaminetetraacetic acid (EDTA) solution; processing can be completed in less than 2 hours after decalcification. The individual laboratory should carefully evaluate the microwave processor and the improvements that it may offer to its processing time and workflow. But the quality of tissue morphology and staining, specifically those for immunohistochemistry or molecular studies, should not be compromised and should be properly validated and documented.

The College of American Pathologists has specific checklist questions regarding the microwave processor, and the technical staff should review those before implementing microwave technology in the laboratory. Basic guidelines are as follows:

1. The microwave processor must be used as recommended by the manufacturer; if the CAP guideline is stricter, then the guideline takes precedence.
2. A household microwave oven may not be used in the laboratory.
3. The microwave processor should be quality controlled for reproducible results in processing. This includes monitoring the instrument for consistency in temperature during processing.
4. If any damage has occurred to the microwave, it should be checked for radiation leakage.
5. Microwavable containers should be used.
6. The microwave processor should be vented to reduce toxic chemical exposure and should be monitored once a year.
7. Review chemical material safety data sheets to ensure that a reagent can be safely used in the microwave processor, and will not cause explosion or damage the microwave.

Stainers and Coverslippers

AUTOMATIC STAINER

Automatic stainers found in the histopathology laboratory are of the following types: linear and robotic. The linear stainers transfer slides from 1 container to the next with the same time allowed in each container. The time in each different solution can be changed only by varying the number of containers holding that particular reagent, or some modules provide smaller buckets to reduce time. This type of stainer has less control over exact staining times in comparison with others. A progressive hematoxylin stain (see chapter 6, "Nuclear and Cytoplasmic Staining," p114) gives more consistency with this stainer. Linear stainers vary in slide capacity but slides may be continuously loaded; there is no need to wait for the previously loaded slides to finish staining.

Robotic stainers are the most flexible, allowing total computerized programming. This technology allows for exact staining times in any given station. Microprocessor technology has brought about a new generation of robotic stainers that allow as many as 11 to 12 baskets of 30 slides each to be stained concurrently. This includes hematoxylin and eosin (H&E) staining as well as special staining and deparaffinization only for slides to be stained immunohistochemically. The robotic stainers reduce chemical exposure by using filters and by the ability to be vented. Each laboratory has different needs that must be kept in mind when evaluating each specific type of stainer before changing to automated staining. The laboratory should monitor the quality of staining daily and have a routine protocol for changing solutions.

MICROWAVE STAINING OVEN

Microwave staining ovens are routinely used for performing special staining. Microwave radiation, a nonionizing radiation, is produced through the interaction of strong magnetic and electrical fields. The microwave electrical field that is produced creates friction as it passes through dipolar molecules (water) and polar side-chains of proteins, causing them to reverse themselves 2.45 billion times per second; this in turn creates heat. These molecules are microwave absorbent, whereas many materials such as plastic wrap, wax paper, and paraffin are microwave transparent. They do not contain any molecules that will be excited by the electric part of the wave. Metals are microwave reflective, neither absorbing nor transmitting microwave energy, and they may cause the energy to bounce from its surface. Metals should not be used in most microwave ovens.

The amount of heat generated by a microwave oven is dependent on the exposure time, solution volume, and oven wattage. Temperature control is very important when using any of the microwave techniques. When the microwave oven is used for fixation (physical fixation), irreversible morphologic damage will result if the temperature is not carefully controlled. It is sometimes useful to place an extra container of water inside the microwave oven. This helps control the amount of electromagnetic waves striking the intended target, and therefore, the amount of heat.

The use of the microwave oven results in a marked time savings in many special staining techniques; however, all stains do not lend themselves to microwave staining. Much less background staining is observed with silver techniques and with some enzyme histochemical staining methods than when the staining is done using conventional methods. Reactions to microwave staining must be monitored very carefully, because the reaction can be completed so quickly that overstaining occurs, especially in some of the silver techniques. Stains are very reproducible provided that the volume of liquid, the time of exposure, and the microwave setting are standardized. Features such as probes that monitor solution temperature and pump air into the solution to reduce

hot spots by creating bubbling improve consistent staining. There are different schools of thought on staining with the microwave oven. Some technologists prefer to use higher power settings, while others believe that the lower power settings are preferable because overstaining is not as likely to occur. Glass Coplin jars can be used with very low power settings, which is an advantage in some cases. Stains using high (or full) and low (minimum) power will be presented in chapters 7-11. It is important to calibrate the oven in use and to try different power variations to determine which is best for your laboratory. Following the development of a special stain using the microwave, the procedure should be validated and documented to be reproducible. This includes volume of solution, type of container, time, power, and wattage necessary to ensure consistency within the laboratory.

Crowder [1995] states that for each setting on the microwave the following procedure should be performed. After the wattage is determined for each setting, the numbers also can be converted into a percentage of power, another way of stating the microwave oven setting to be used. A chart giving the wattage, or percentage of power, for each setting on the microwave should be made for reference. The procedure for determining the wattage follows:

1. Place 1 L of distilled water in a beaker. Record the temperature of the water.
2. Place the beaker of water in the center of the microwave oven.
3. Heat the water for 2 minutes on a given power setting.
4. Remove water from oven, stir, and again record the temperature.
5. Subtract the original temperature from the heated temperature; multiply this number by 35. This will give the wattage of the oven [Login 1993].

Crowder [1995] also states that certain microwave ovens are manufactured specifically for the laboratory with different power wattage settings. In contrast, the wattage in the standard household type of microwave oven does not change as the power settings are changed; instead the microwave emission time is altered.

Some general guidelines on microwave staining follow:

1. Sometimes it is easier to reproduce results if the liquid is heated to the desired temperature and poured over the slides, which are then allowed to stand until the reaction is complete.
2. The microwave oven should be kept very clean by immediately wiping any spills. Placing the staining jar inside a plastic bag will help keep the oven clean, because any boiled-over reagent will be contained inside the plastic bag. Loosely close the bag and be sure that the plastic staining jars are vented by drilling a small hole in their covers. The use of a plastic bag may necessitate recalibration of heating times.
3. Be aware that some fumes are toxic, and heated reagents produce more vapors, increasing the exposure hazard. The microwave oven should be vented or placed in a fume hood.
4. Reagents that are fire or explosion hazards should not be used in the microwave oven.
5. Be very careful when removing heated solutions from the oven because some containers can become very hot.
6. Do not use thin paper or tissue paper of any kind in the microwave oven, because paper can get hot enough to ignite.
7. Power settings vary on different microwave ovens, and even different ovens of the same brand may perform differently. The times given for some of the special stain microwave techniques are a guide only, and adjustments may need to be made. Each oven must be calibrated before use.
8. Solutions heat faster at the top of the staining jar than at the bottom. After exposing the slides to a solution in the microwave oven, the solution should be mixed by dipping the slides up and down, or by using a Pasteur pipette to gently withdraw solution from the bottom of the jar and add it back to the top.
9. If glass staining jars are used, the glass must be heat tempered (eg, Pyrex), because Coplin jars tend to break at the junction of the sides and bottom. Glass also gets hotter than plastic and must be handled carefully.
10. The microwave oven should be checked periodically for radiation leakage.

AUTOMATIC COVERSLIPPER

Automatic coverslipping instruments are becoming standard equipment in high-volume laboratories. These instruments should contain filters or be vented to the outside to reduce hazards associated with the inhalation of xylene during the application of coverslips. The hazards associated with skin absorption, combined with the current shortage of personnel, have greatly popularized the use of these instruments. Coverslipping instruments are designed to take batches of slides directly from an automatic stainer and apply either glass or plastic coverslips in a timely manner, with minimal additional handling or clean-up. Recently, modules were introduced that connect directly to the automated stainers and allow for improved workflow and further reduction of laboratory personnel exposure to chemicals. It is important to adjust the amount of mounting medium that is dispensed during coverslipping to ensure proper long-term storage of slides. When the ratio of xylene to medium is incorrect, or if there is too much xylene in the mounting medium, then high-power focusing does not project crisp cellular detail, and the mounting media will retract from the tissue as drying occurs. The reservoir used to hold the mounting media must be kept tightly sealed to keep moisture out and must be cleaned weekly to reduce the risk of water artifact **[i3.30]**.

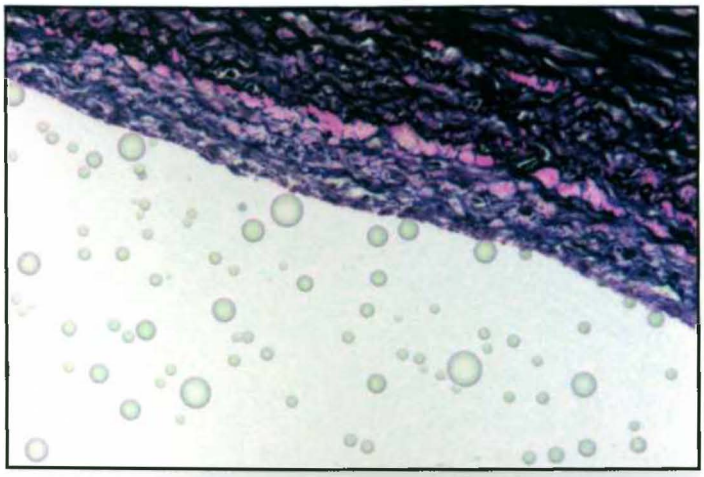
FLOTATION BATHS

Flotation baths are used for floating out paraffin ribbons. While the temperature of the bath depends to a degree on the personal preference of the microtomist, it is usually maintained 5°C to 10°C below the melting point of the paraffin used for embedding; with some of the newer paraffins, the bath is maintained at an even lower temperature. If the bath is too hot or the ribbons are either overstretched or allowed to float for too long, an artifactual separation of tissue components that mimics edema will be seen. "Parched earth" is an artifact that commonly results from improper processing but also may result from a flotation bath that is too hot **[i3.31]**.

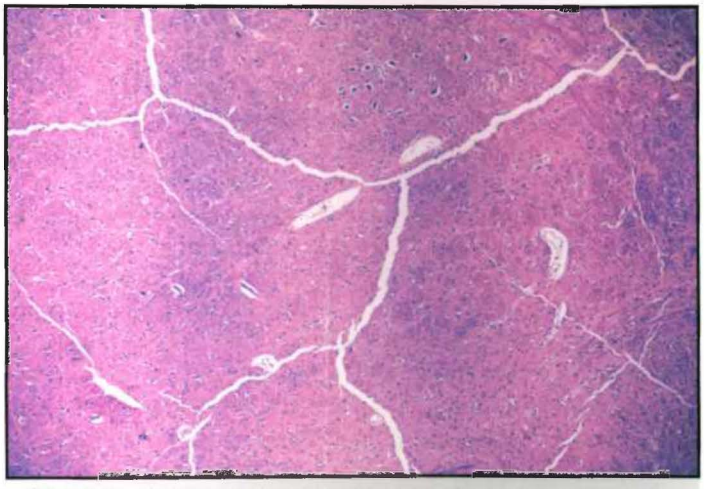
Ribbons should be stretched gently as they are placed on the flotation bath, and then small wrinkles and folds should be quickly but cautiously teased out of the sections. Folds remaining in the section can obscure desired structures that are stained **[i3.32]**. Most air bubbles trapped under the section can be drawn to one side by a blunt dissecting needle that has been bent at an angle to reach under the ribbon. Care should be exercised so that the ribbon is not pierced or torn. Sections may be removed from the blade and transferred to the flotation bath with gloved fingers, forceps, a brush, or a wooden applicator stick **[i3.33]**. The applicator stick attached to one end of the ribbon will aid in stretching the ribbon and will float on the bath so that one does not need to worry about detaching it from the ribbon.

If paraffin sections are picked up on clean, untreated glass slides, the sections are likely to wash off during staining, so some type of adhesive is commonly used. The slides may be coated with albumin, which is a mixture of egg white and glycerin, using a glue derivative (such as a derivative of Elmer's glue, Columbus, OH), or with a poly-L-lysine. Slides also may be treated with aminoalkylsilane or coated with a chromium potassium sulfate alum solution. In most routine work, the adhesive is added to the water bath. Gelatin, agar, and Elmer's glue are all used as water bath additives; the other substances are used to coat the slides. Slides coated with poly-L-lysine are excellent for frozen sections on fixed tissue, some sections to be stained in the microwave, and paraffin sections to be stained with immunoenzyme techniques. Aminoalkylsilane is preferred for sections to be used for in situ hybridization studies. Slides coated with chromium potassium sulfate are effective for frozen sections of fixed tissue and for special stains in which some of the reagents tend to cause loosening of paraffin sections.

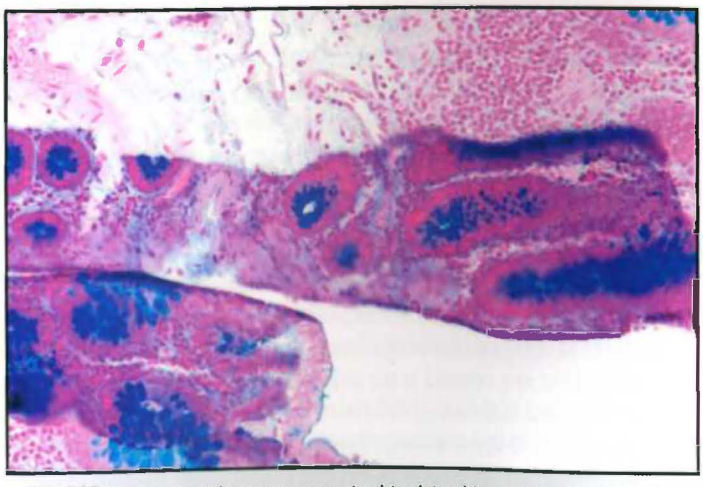
Slides that have a permanent positive, or plus (+), charge are also available. This positive charge attracts frozen and paraffin tissue sections electrostatically to the microscopic slide. No adhesive is needed, background staining is eliminated, and section loss that occurs with some staining procedures is reduced. These slides are excellent for silver stains, immunohistochemical and enzyme histochemical techniques, DNA probes, or other procedures with a marked possibility of section loss. These slides can be fairly expensive, but the laboratory can evaluate the expense of these commercially purchased slides to assess the feasibility of their use in relation to the expense of repeated staining necessary because of washing and tissue loss.



[i3.30] Either this section was not completely dehydrated before mounting with synthetic resin, or the resin contains water.



[i3.31] "Parched earth" may result when tissue that has been improperly processed is floated on the flotation bath, or when the flotation bath is too hot. A parched earth artifact also may occur then the block is chilled with fluorocarbon spray.



[i3.32] Severe wrinkles are present in this alcian blue-stained section of intestinal tract. When this artifact is severe, it can interfere with the proper interpretation of the section.

[i3.33] An applicator stick is useful for picking up ribbons from the blade. The sticks are the least damaging to the blade edge of the various tools used for this purpose. The wet applicator stick is placed under the ribbon and the last section is rolled slightly around the stick. The stick aids in stretching the ribbon as it is placed on the flotation bath, and because it floats, it does not need to be disengaged before the ribbon is placed on the bath.

CHROMIUM POTASSIUM SULFATE-COATED SLIDES [BOYD 1955]

Dissolve 1 g of gelatin in 1 L of distilled water with heat. Cool and add 0.1 g of chromium potassium sulfate. Store the solution in the refrigerator. Dip slides several times in this solution, drain, and dry in a vertical position. Store slides in a slide box.

POLY-L-LYSINE-COATED SLIDES

Dissolve 25 mg of poly-L-lysine in 26 mL of distilled water in a small beaker or flask. Dip an applicator stick in the solution and roll a coating on each slide. Dry overnight at 50°C to 55°C. Store the slides in a slide box; the solution may be stored in the refrigerator.

A 0.1% solution of poly-L-lysine may be obtained commercially. For use, the concentrate is diluted 1:10; slides are immersed in the dilute solution for 5 minutes, drained, and dried overnight at room temperature or for 1 hour at 60°C.

AMINOALKYLSILANE-TREATED SLIDES [RENTROP 1986]

Fill slide racks with slides and soak in clean acetone. Air dry the slides and then soak them for 2 minutes in a well-mixed solution of 250 mL of acetone and 5 mL of 3-aminopropyltriethoxysilane. Rinse the slides well in 2 changes of distilled water, dry at 60°C for 30 minutes, and store in a slide box.

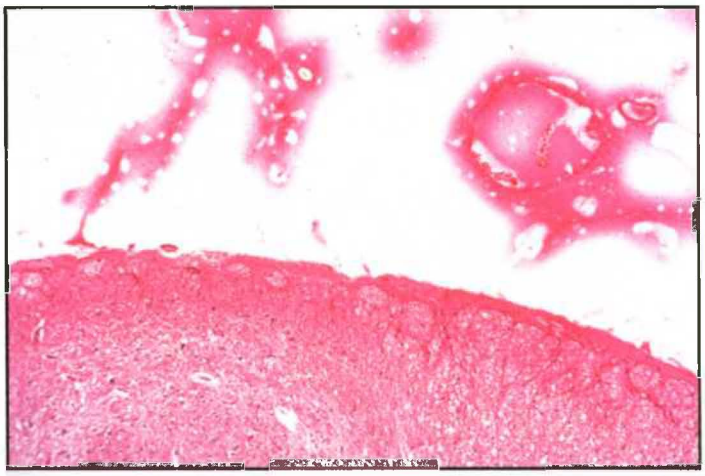
Before some of these newer methods or chemicals for treating slides were available, sections occasionally had to be treated with celloidin to keep them from washing off during staining. Although sections rarely are coated with celloidin today, this method may still be required following errors in processing that have caused extreme tissue dehydration. Sections are deparaffinized with xylene, immersed in absolute alcohol, and then placed for 1 to 2 minutes in 0.5% celloidin dissolved in ether alcohol. Slides are drained briefly and placed in 80% alcohol for 5 minutes to harden the celloidin. After being washed in water, the slides may be stained. Ether is a very hazardous chemical to use because of its explosive properties; therefore, great care must be exercised when celloidin is prepared and used.

An excess of any of the adhesives is undesirable because some will stain and give very messy-appearing sections **[i3.34]**, **[i3.35]**. Visualization of the sections also may be less than optimal. Adding approximately ¼ teaspoon of gelatin to the flotation bath will give good section adherence for routine staining procedures. Distilled water should be used in the flotation bath, which should be filled fresh each morning, cleaned at the end of each day, and left to dry overnight. Wiping the waterbath with acetone after cleaning can improve the cleanliness of the bath and reduce any detergent residue.

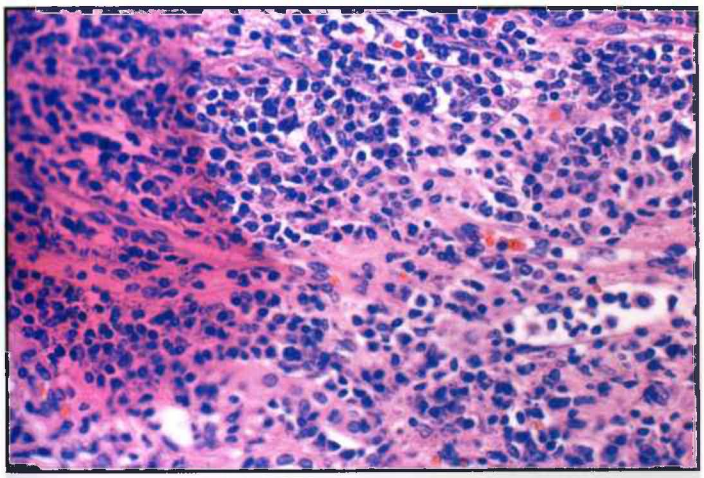
Water and adhesives can be a source of contamination and should be checked carefully for the presence of microorganisms. Millipore-filtered (Millipore Corp, Bedford, MA) deionized water is a good reliable water source for the flotation baths, because even water that been put through a charcoal filter and an ion-exchange column may contain acid-fast organisms [Carson 1985]. Glue has been reported to contain fungi [Fleming 1985], and gelatin has also been reported to contain acid-fast organisms [Carson 1964], so one must always be aware of the possibility of contamination. It is also helpful if enough water to fill the bath is filtered and allowed to stand overnight so that entrapped air is released. Entrapped air may also be released by preheating the water in the microwave. These procedures will decrease the formation of air bubbles under the section **[i3.36]**.

The flotation bath must be kept scrupulously clean during microtomy and should be cleaned after each block is cut by skimming the surface with a lint-free tissue (eg, Kimwipes) or a paper towel to pick up any debris. Debris left in the flotation bath can be picked up on another slide and may lead to a misdiagnosis **[i3.37]**. If a flotation bath becomes contaminated with debris because of unfixed tissue exploding, it is recommended that the bath be dumped, cleaned, and refilled with clean water to eliminate the possibility of contamination to future sections. The microtomist should wear gloves during sectioning to avoid squamous cell contamination to the mounted sections **[i3.38]**. Sources of squamous cells are ungloved hands touching the water in the bath or touching the slide itself.

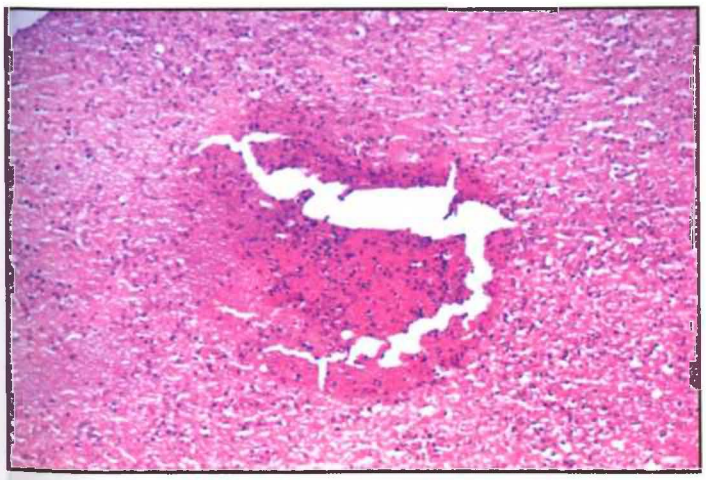
Flat, wrinkle-free sections may be difficult to obtain with some tissues **[i3.39]**, **[i3.40]**, **[i3.41]**; differences in the ease of both sectioning and floating out of the ribbons may also be noted after different methods of fixation. Chemicals that lower the surface tension, such as TritonX-100 or Brij-35 (Sigma-Aldrich, St. Louis, MO), may be added to the flotation bath to help flatten sections. Place an applicator stick in the solution, and then swirl it around in the waterbath. Excess amounts of these reagents will interfere with tissue adhesion to the slide. This technique also assists with slowing down the process of tissue exploding on the waterbath because of poor processing. Other methods, such as using flotation baths at 2 different temperatures or floating the ribbon first in an alcohol solution (5 mL of 95% alcohol and 95 mL of distilled water) and then transferring it to the flotation bath, also have been



[i3.34] Too much egg albumin has been used to coat this slide, and it has stained. It does not interfere with the section on this slide but yields a messy, unattractive slide.



[i3.35] If too much adhesive such as egg albumin is used, it will stain. Notice the heavy eosinophilic area and uneven staining on the left side of this section. This artifact is within the section on this slide, causing a decrease in contrast of the stained elements and making some structures difficult to see.



[i3.36] An air bubble was trapped under the section on the flotation bath and has broken during the staining process. The stain is more intense in this area because the stain had access to both sides of the section.

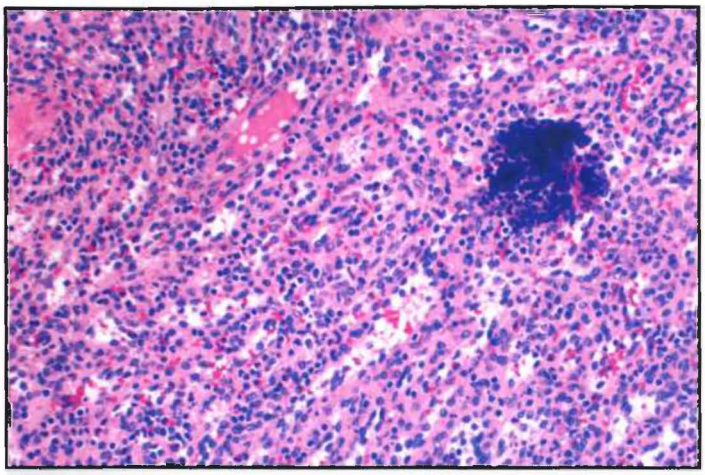
suggested as an aid to obtaining flat, wrinkle-free sections [Sheehan 1980]. In my laboratory, 2 mL of 95% alcohol is added to the water-bath to assist with eliminating wrinkles in biopsy tissue.

DRYERS AND OVENS

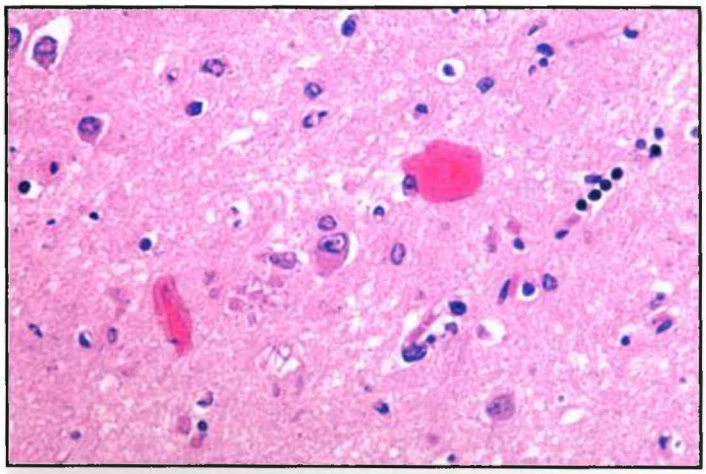
For routine hematoxylin and eosin staining as well as special staining, the tissue slides should be completely dried before beginning the deparaffinization step with xylene. Incomplete drying can cause sections to wash off during staining. Placing the section into an oven, especially one that forces hot air to move within the space provides the most optimal results. This is commonly referred to as a "convection oven," which allows for quicker drying at a lower temperature. A warming plate can be used but all water must be removed from behind the tissue before placing the slide on the plate, or the high temperature may cause artifacts such as nuclear bubbling. Proper technique must be used to ensure that tissue morphology is not compromised. After mounting the tissue onto the slide, never allow water to move back underneath the section, and avoid laying the slide horizontally once tissue is adhered. The tissue must be completely dry before the slides are placed into xylene for deparaffinization; if any water is left, it will not mix with xylene and will cause incomplete deparaffinization. Incomplete removal of paraffin is indicated by white spots that can be seen in tissue as the slides are removed from xylene. This artifact is difficult to correct completely, because these spots may stain more intensely later, but the slides should be treated with absolute alcohol to remove any residual water and then placed back in fresh xylene to remove any remaining paraffin. The slides should then be taken through absolute and 95% alcohols and water, and stained as desired. Note that the container with the water-contaminated xylene should be replaced with a clean dry container and fresh xylene to avoid incomplete deparaffinization of the sections following.

Dryers and ovens are usually maintained at a temperature just above the melting point of paraffin, commonly 60°C, and are used for drying slides. In forced-air slide dryers, drying takes approximately 15 minutes, but it requires about 1 hour in a conventional oven to ensure complete dryness. Immunohistochemistry slides are best without oven drying, but 30 minutes in a forced air dryer at 55°C using positively charged slides works well. High temperatures affect antigens; therefore, slides should never be dried at a temperature higher than 60°C. As previously stated, sections overheated during drying may show artifacts, especially if the sections have not been well-drained before drying **[i3.42]**, **[i3.43]**, **[i3.44]**, **[i3.45]**. Santoianni and Hammami [1990] attribute nuclear bubbling to the presence of water in the nuclei of freshly cut sections that are immediately dried. This is especially pronounced if the slides are dried at temperatures above 60°C. If the need for diagnosis is not urgent, the morphology is maintained more exactly by air drying the sections overnight. This is especially recommended if the stained sections are to be photographed.

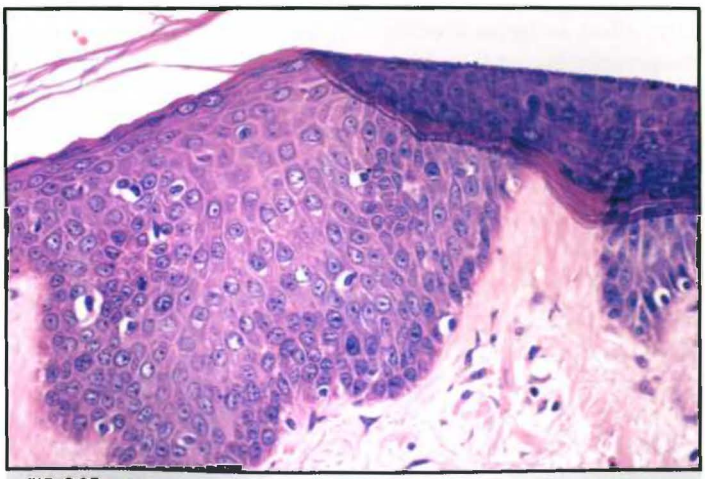
Incubator ovens are maintained at 37°C, or body temperature, and are used for many enzyme reactions, in situ hybridization applications, and some special stains, but are not recommended for slide drying.



[i3.37] Debris from a previous section has been picked up on this slide. In some cases, this can lead to a misdiagnosis and should be prevented by careful, frequent cleaning of the flotation bath with paper towels or Kimwipes, preferably between sectioning of different blocks.



[i3.38] Two squamous cells are seen on this section. Ungloved fingers should never be put in the flotation bath because it may result in desquamation and the cells will be picked up on later sections.



[i3.39] A fold on the edge of a section of skin. This tissue did not adhere completely to the slide and has folded over. This artifact is seen frequently in skin sections.

CIRCULATING WATER BATH

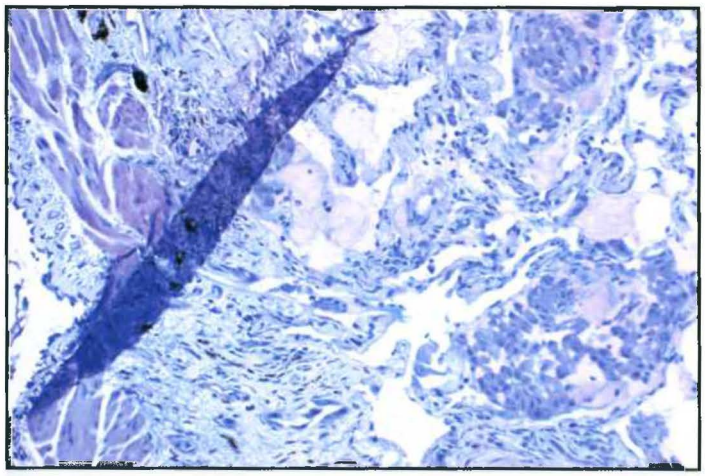
A circulating water bath is a small, deep bath that circulates water to provide better control of a specific temperature with minimal variation. Plastic or glass Coplin jars are covered and placed into the water. The water level should be approximately the same on the outside of the Coplin as the solution level inside the jar to assist with temperature control. The heat from the circulating water brings the solution in the Coplin jar to the desired temperature and holds it at that temperature for a period. The bath works well for a broad range of temperatures and can be used successfully for special stains or mild epitope retrieval techniques. When used with the higher temperatures as needed for heat-induced epitope retrieval, plastic Coplin jars will withstand the temperature better; high temperatures may cause glass Coplin jars to crack. For high temperature applications the laboratorian should use gloves that are water- and heat-resistant to avoid skin burns. If boiling temperatures are needed, ethylene glycol may be added to the water. This chemical gives off noxious fumes when boiled, and the bath should be placed under a chemical fume hood.

FREEZERS AND REFRIGERATORS

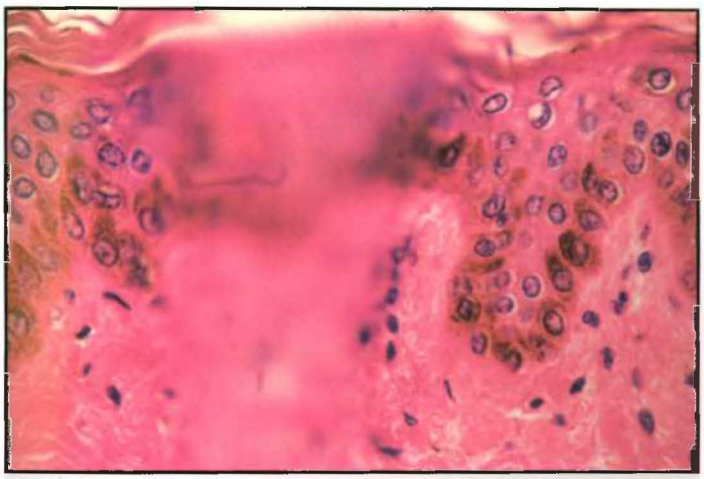
Freezers and refrigerators are used for storing many of the reagents used in histopathology, especially enzyme histochemistry reagents, immunohistochemistry reagents, and buffer solutions. If flammable reagents must be stored at refrigerator or freezer temperatures, then explosive-proof units should be purchased. The refrigerator is usually maintained at approximately 4°C and should never be allowed to reach a temperature above 10°C. The typical laboratory freezer is used at a temperature of –20°C and should not have a defrost cycle if used for reagent or tissue storage because freeze thaw cycles can destroy any reagent or tissue exposed to a repetitive warm temperature. The –20°C freezer is useful to maintain antigenicity in precut immunohistochemistry control slides, thus allowing for long-term storage; –70°C freezers are optional for long-term storage of concentrated antibodies. Undiluted antibodies should be "snap" frozen in liquid nitrogen in a cryogenic vial before being stored at –70°C. Tissue stored in either type of freezer must be well wrapped or dehydration will occur. If tissue becomes desiccated, good frozen sections are impossible to obtain, and staining on improperly stored precut sections may be weak or even give a false-negative reaction. The temperature of laboratory refrigerators and freezers must be monitored routinely, and a daily log must be kept. It is advisable to have an alarm on the freezer to notify personnel when a system failure occurs; a liquid nitrogen backup unit for a –70°C freezer is optimal.

pH METERS

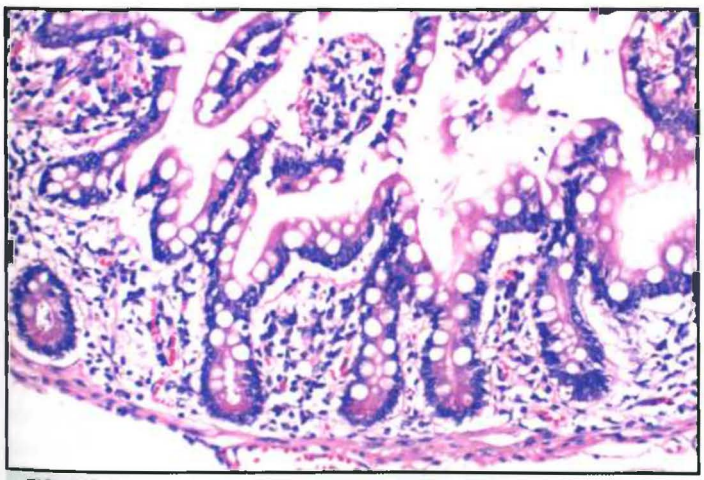
pH meters are used to measure the acidity, or H^+ ion concentration, of a solution. The pH scale goes from 0 to 14, with a pH of 0 indicating a very acid solution and a pH of 14 indicating a very basic solution. To understand a little more about pH, we must look at water. Each molecule of water is composed of 1 oxygen atom and 2 hydrogen atoms. In pure water, most of the water molecules remain intact; however, a very small number of water molecules ionize and react to give hydronium ions, which are acidic, and hydroxyl ions, which are basic.



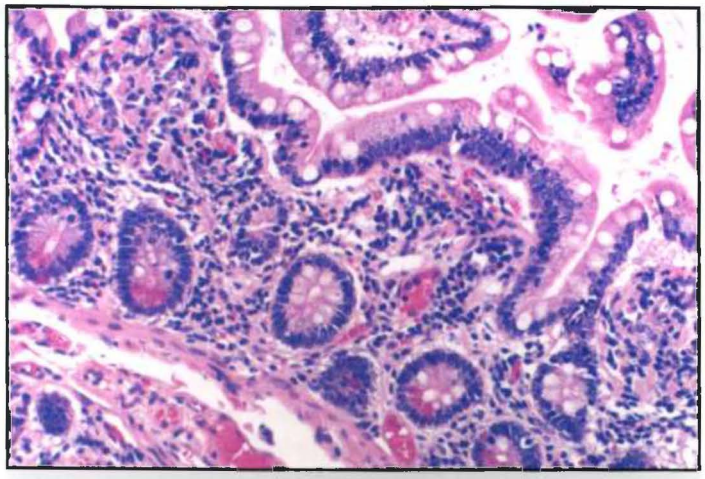
[i3.40] This section was not completely flattened on the flotation bath, which resulted in a wrinkle. The wrinkle in this section of lung, stained with the Kinyoun acid-fast technique, could cause a rare organism to be overlooked, yielding a false-negative report. Sections should be gently stretched and wrinkles teased out as soon as the ribbon is placed on the flotation bath. This must be done carefully or the sections will tear.



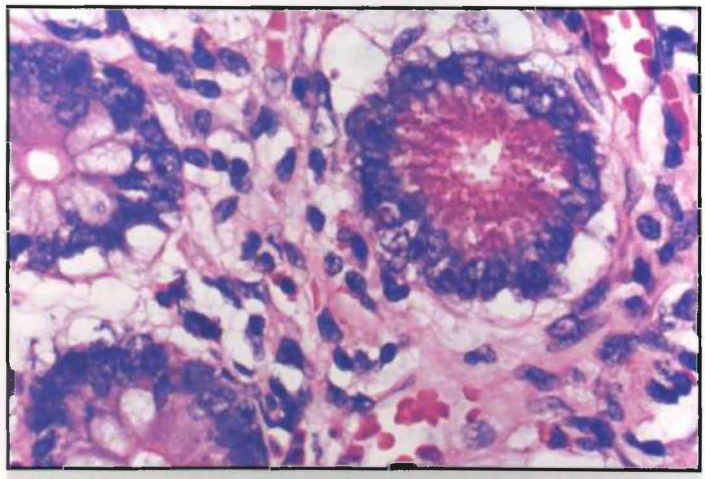
[i3.41] The sections did not flatten completely on the water bath and did not adhere firmly to the microscope slide, producing a wavy section. Letting the section float slightly longer would probably prevent this artifact, which is most frequently seen in skin sections.



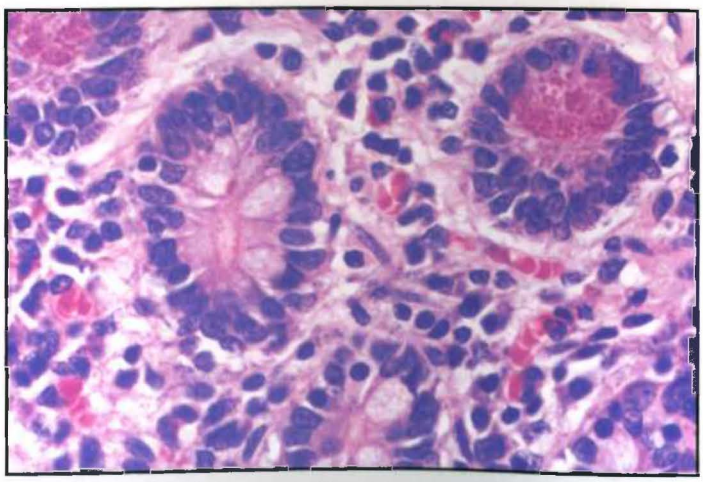
[i3.42] This section of small intestine was dried in a forced air dryer at 80°C for 10 minutes immediately after being picked up from the flotation bath. Note the marked cell shrinkage and the retraction of the lamina propria from the epithelium.



[i3.43] This is a duplicate of the section shown in **[i3.42]**. It was cut at the same time, but allowed to air dry overnight at room temperature before staining.



[i3.44] This is a high-power photomicrograph of the section shown in **[i3.42]**. Note the nuclear bubbling, an artifact usually attributed to formalin fixation. Also note the cell shrinkage and separation of the various tissue components, which leads to the creation of artifactual spaces. This drying artifact was reported by Santoianni and Hammami. When possible, slides should be allowed to drain for several minutes before drying in an oven maintained at no more than 60°C for 1 hour.



[i3.45] Compare this section to that shown in **[i3.44]**. This is a high-power photomicrograph of the air-dried section shown in **[i3.42]**.

When pure water reacts, it produces equal amounts of hydronium and hydroxyl ions and thus is considered neutral **[f3.4a]**.

However, because bare protons or hydrogen ions do not exist in water, the equation is more properly written as shown in **[f3.4b]**.

The addition of acid increases the hydronium ion concentration, and the pH decreases or moves toward a value of 0. If a base such as sodium or ammonium hydroxide is added, the hydroxyl ion concentration increases, and the pH shifts toward 14. The hydronium and hydroxyl ions remain in balance with each other, and an increase in the concentration of hydronium ions causes a proportional decrease in the concentration of hydroxyl ions. Each unit on the pH scale represents a 10-fold change in hydronium or hydroxyl ion concentration, so that a solution at pH 6 is 10 times more concentrated in hydronium ions than a solution at pH 7. The terms *hydronium* and *hydrogen ions* are used interchangeably in many texts.

pH is defined as the negative power (the p in pH) to which the number 10 must be raised to express moles per liter concentration of the solution's hydrogen ions (the H in pH). For water at room temperature, the hydrogen ion concentration is 1×10^{-7} mol/L; therefore, the pH of pure water at room temperature is 7.

The pH meter measures pH with a voltage output in proportion to the active acid concentration. Electrode systems are always composed of 2 electrodes, a sensing electrode and a reference electrode, but for convenience are frequently combined into 1 electrode, a combination electrode. The sensing electrode contains a specially designed surface whose voltage will change as the pH of the solution changes. The reference electrode completes the electrical measuring circuit and gives a stable or unchanging voltage for comparison with the voltage of the sensing electrode.

For use, pH meters are standardized with buffer solutions whose composition has been defined by the US National Bureau of Standards; for the greatest accuracy, the pH meter should be standardized using a standard solution with a value near that of the test solution. For example, when determining the pH of neutral-buffered formalin solution, the pH meter should be calibrated with a pH 7.0 standard, but when preparing the acidulated water for the Warthin-Starry stain, the pH meter should be calibrated with a pH 4.0 standard. Depending on the type of electrode in use, electrode storage may differ. Some electrodes, if not frequently used, are better stored dry with the protective cap on, while other electrodes should be stored in distilled water or in solutions containing potassium chloride. The manufacturer's recommendation on storage and cleaning of electrodes should be followed.

a	H_20	$\Leftrightarrow$	H^+	+	OH^-
	water		hydrogen ion		hydroxyl ion
b	$2H_20$	$\Leftrightarrow$	H_3O^+	+	OH^-
	water		hydronium ion		hydroxyl ion

[f3.4] **a**, Dissociation of water into hydrogen ion and hydroxyl ion. **b**, More properly written equation as hydronium ion as it exists in water.

pH meters are necessary for enzyme histochemistry techniques because most solutions require a specific pH for use. The immunohistochemistry laboratory must verify and document the pH of buffers before use. Phosphate buffers require a specific type of electrode for successful testing. Many laboratories use pH paper for testing buffers but when an exact pH is required, a pH meter is a more reliable source.

BALANCES AND SCALES

Balances or scales are needed for solution preparation; a balance that will weigh 100 to 200 g with a sensitivity of 1 to 2 mg usually is sufficient. More specialized techniques requiring weighing in milligram amounts require an analytical balance. The balance and balance pans must be kept scrupulously clean. The surrounding area should also be kept clean; any spilled chemicals should be carefully wiped up and disposed of. This area of the laboratory and the weighing of dyes and chemicals can be a real safety hazard. The accuracy of an analytic balance should be checked regularly as a part of the preventive maintenance procedure. To properly weigh out a dry chemical, the following steps are recommended:

1. Place an empty weigh boat onto the scale.
2. Clear the weight by performing a step commonly referred to as *taring* or *zeroing* the balance. This ensures that the weight of the boat is not added to the final weight of chemical.
3. Measure the chemical into the weight boat for the desired weight.
4. Carefully remove the weight boat and chemical to continue with reagent preparation.
5. Repeat steps 1 to 4 for each new chemical, wiping down the balance if necessary to avoid contamination.
6. Upon completion, wipe down balance and work area. Cover balance to keep free of dust.
7. The balance should be calibrated annually.

EMBEDDING CENTER

Embedding centers provide a supply of melted paraffin, warm storage for embedding molds, small warming and chilling plates for orientation during embedding, and a larger chilling plate for rapid chilling of the embedded tissue blocks. The embedding center should be kept clean and free of any tissue fragments that could be picked up during embedding. Some centers include magnifying glasses to aid in specimen orientation. Again, the paraffin is kept 2°C to 4°C above the melting point of the paraffin used. If the temperature of the embedding paraffin is allowed to go too high, the nature of the paraffin and the resultant sectioning qualities are affected. The temperature of the paraffin should be monitored and documented daily. High temperatures should be avoided not only for melting the paraffin but also on the surface plate where orientation occurs. High temperatures can destroy the tissue and nuclear morphology if the tissue is laid upon this hot surface. The

tissue should be transferred directly from the cassette to the mold to avoid this artifact.

MICROMETER PIPETTES

Pipettes are routinely used to measure liquids in ranges of 1 to 1000 µL in the immunohistochemistry laboratory. The following 3 ranges would allow for most volumes to be prepared: 0.5-10 µL, 20-100 µL, and 200-1000 µL.

A pipette should never be taken out of the recommended range because this will cause inaccurate draws. If the immunohistochemist is using a pipette for extended periods, ergonomic styling is helpful to reduce the risk of repetitive injury stress. The chamber inside the pipette should be resistant to heat generated from the hand, which can reduce its accuracy.

Good pipette techniques can assist with efficiency. The following are basic guidelines:

1. Always use the pipette in the designated range.
2. Keep pipette straight up and down to improve accuracy of the draw.
3. Do not carry over excess primary or reagent on the outside of the tip to avoid a dilution error.
4. Gently draw and blow reagent 2 to 3 times, and visually check to see if the draw is good before dispensing.
5. Following dispensing, gently draw and blow reagent to clean the tip of any residual antibody left.
6. Keep pipette clean by checking for any residual reagent stains, and wipe clean to avoid contamination to other preparations.
7. Store pipettes in a designated stand, not in a drawer, to avoid damage.
8. Keep pipettes calibrated, checking at least annually.

SOLVENT RECYCLER

Recycling systems once were difficult to maintain and prone to inherent hazards, such as the risk of exposing the user to chemical fumes, as well as dangers from heated glass containers, lifting large volumes of chemical on and off the stand, and handling tubing. The models today are making a positive impact on the laboratory and the environment. These systems can be beneficial to laboratories, generating both small and large volumes of waste. The recycling system works by boiling off the impurities, such as water or other chemicals, separating contaminants from the solvent being recycled, and restoring it to its original composition. These systems can be used for alcohol, xylene, and even xylene substitutes to prepare them for reuse multiple times. Some systems also restore formalin to its original chemical state. With the assistance of computer technology, built-in safety features can closely monitor the system to ensure that it is not exposing the user or surrounding area to unsafe fumes or temperatures. Recycling can be a cost-saving measure for the laboratory by reducing both the expense of reagent purchase and the disposal of used solvents, while having a positive impact on the environment.

Instrument Quality Control

NEW INSTRUMENT VALIDATION

Before using a newly introduced or purchased piece of equipment, it should be validated. The manual should be reviewed for specific instrument requirements, and it should be verified that the proper electrical wattage is available for the operation of the equipment. Some equipment requires dedicated ventilation, plumbing, drainage, a specific room temperature, low humidity, LIS or internet connectivity, and specific computer software. Space requirements (table top or floor) may also be a consideration. Commonly a checklist can be used to ensure that all system requirements are verified before operation. Once the operational requirements are met, the laboratory must validate the results before implementing the equipment for clinical use.

Side-by-side comparisons are the best form of validation. Controls and patient tissue, as appropriate, are handled identically through each step of the process, with the only variable being the "new" instrument; a comparison of the final results are reviewed and approved by the laboratory medical director or designee. It is important to verify an adequate number of controls and patients to ensure consistent results. For example, a processor may require not only a specific number of blocks to be processed but also various tissue types to ensure that all specimens processed meet quality expectations. An immunohistochemical stainer will also require a specific number of side-by-side comparisons using multiple tissue types and/or fixatives. This can be achieved easily by using control slides of tissue processed by the laboratory, which are constructed into multiple tissue blocks (eg, microarray or sausage); this keeps down the expense of validation. The laboratory will be responsible for designing a validation process and procedure suitable for the type of instrumentation and the complexity of the testing performed by the instrument. The design should satisfy both quality expectations and reproducibility of results.

If side-by-side comparisons are not available, the laboratory should define expected results and, in most cases, evaluate the instrument before purchase by having an in-house demonstration or by sending material to the vendor, who then allows the laboratory to assess the quality of the product. I prefer an in-house demonstration, which allows hands-on assessment by the staff as to how the instrument will fit into the laboratory process and also allows identification of any potential challenges.

QUALITY CONTROL PROGRAM

A well-organized quality control program is a must in the histopathology laboratory, and the application of this program to the instrumentation of the laboratory is very important. All

instruments with temperature as an important factor must be monitored, and the temperature recorded daily. If a temperature is found to be out of range, corrective action must be taken and documented. All instruments requiring lubrication should be placed on a periodically scheduled lubrication protocol. All instruments should be kept very clean and should be covered when not in use, if appropriate. Instrument manuals should be kept readily available and the manufacturer's recommendations referred to for both routine care and preventive maintenance; these recommendations should be written into the quality control procedures. Documentation of the performance of all quality control procedures is a requirement for accreditation and regulatory agencies. Examples of forms that might be used to document routine quality control procedures and instrument maintenance history follow **[f3.5]**, **[f3.6]**, **[f3.7]**.

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Equipment Temperature Quality Control Chart

Equipment: **Serial number:** **Acceptable range:** **High:** **Low:**

Year:	January	Februry	March	April	May	June	July	August	September	October	November	December
1												
2												
3												
4												
5												
6												
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27												
28												
29												
30												
31												
Monthly Review												

Corrective action:

[f3.5] Temperature records.

Equipment Maintenance and History

Equipment: Boekel Flotation Bath	**Model:** 145701
Manufacturer: Boekel Scientific	**Purchase Date:** 01/2004
Feasterville, PA 19053	**Purchase Order:** PO543302
Serial No: 01285	**Inventory No:** UT050553

Principle: The flotation bath is used for the flotation of parafin ribbons prior to mounting sections on glass slides. The proper temperature range for the bath is 5°-10° C below the melting point of the paraffin used.

Reference: See operation manual for detailed information on the flotation bath

Routine maintenance: The flotation bath will be monitored daily for temperature. This will be documented on a designated QC sheet for the flotation bath. Unacceptable temperature will be recorded and adjustments will be made at the discretion of the supervisor. If the bath *does not* respond to adjustments, then the lab will seek contracted maintenance to correct the problem.

Procedures:

1. Remove all paraffin with xylene at the end of each day
2. Using hot water, clean glass dish with Liqui-Nox (Alconox, Inc, White Plains, NY)
3. Rinse with deionized water and wipe with acetone
4. To reduce dust particles, store dish upside down
5. Wipe away any water standing in the bottom of the warming unit; this surface should be kept dry

QC Sheet: *Copy follows this page*

Annual electrical check required: Yes

Documentation of contracted maintenance (if applicable)

Date called Tech initial	Problem	Company	Corrective action taken	Date

Note: Keep a copy of all maintenance invoices/bills

[f3.6] Equipment maintenance and history.

Equipment Maintenance and History

Equipment: Cryostat	**Model:** Microm HM505EVP
Manufacturer: Richard Allen Scientific	**Purchase Date:** 05/2003
Kalamazoo, MI	**Purchase Order:** PO730995
Serial No: 25889	**Inventory No:** UT428678

Principle: This instrument is to be used for sectioning frozen tissue, including muscle, renal, and heart.

Reference: See operation manual for detailed information on the Microm HM505EVP.

Routine maintenance: The instrument will be monitored daily for any operational problems and constant chamber temperature. Daily documentation of the temperature will be maintained on the instrument's QC sheet. Technical problems will be recorded and adjustments will be made at the discretion of the supervisor. If the cryostat *does not* respond to adjustments, then the lab will seek contract/outside maintenance to correct the problem. The cryostat should be decontaminated daily by wiping out with alcohol. Complete chamber decontamination, including removal of the microtome, will be at a minimum of every 6 months, depending on the frequency of use and type of tissue being cut. Decontamination should be performed following cutting a highly contagious case to minimize the risk of acquiring infection from blood- or tissue-borne pathogens in unfixed tissue that has been cut on the cryostat. *Note: For examination and readjustment of the microtome, a trained service technician should perform a routine maintenance once every 6 months.*

Procedures:

1. Monitor the chamber temperature, and document it on the daily quality control chart
2. Remove any debris from the chamber, and remove and replace biohazard waste trap if full
3. Wipe the chamber down with 95% alcohol
4. If a suspected contaminated specimen has been sectioned, defrost and decontaminate the chamber
5. Schedule 6-month maintenance for complete removal and decontamination of microtome, or more frequently if used daily
6. Perform annual electrical check

Supplies: Required personal protective equipment: safety glasses, respirator, latex-free gloves, and lab coat

Cleaning supplies: 95% alcohol, gauze, and biohazard bags

QC sheet: *Copy follows this page*

Annual electrical check required: Yes

Documentation of contracted maintenance (if applicable)

Date called Tech initial	Problem	Company	Corrective action taken	Date

Note: Keep a copy of all maintenance invoices/bills

[f3.7] Equipment maintenance and history.

LEARNING ACTIVITIES

1. **Recut the blocks made after reading chapter 1, "Fixation," obtaining the best sections possible, and stain with H&E, removing pigments as indicated. Compare the difficulty of sectioning tissues fixed in different fixatives.**

2. **Cut sections from one of the blocks, changing the clearance angles so that at least 4 different angles are used. Try clearance angles of approximately 1°, 4°, 7°, and 11°. Compare the sectioning ease at the different angles. Stain the sections and note any differences in the microscope quality of the sections.**

3. **Cut sections from 1 of the blocks at different cutting speeds; vary speeds from less than 1 revolution of the wheel per second to approximately 3 to 4 revolutions of the wheel per second. Compare the thickness of the sections. Are the thicker sections obtained with fast or slow cutting speeds? Stain the sections, and compare their microscopic quality.**

CHAPTER 4

Safety

OBJECTIVES

On completing this chapter, the student should be able to do the following:

1. Identify possible chemical, mechanical, and biological hazards in the histopathology laboratory
2. List at least 5 commonly used toxic chemicals, and identify the specific hazard(s) of each
3. List all reagents used in the laboratory for fixation and processing of tissue, and identify the hazards associated with each reagent (see also chapters 1 and 2)
4. Identify the appropriate method of disposal for each of the listed reagents
5. List at least 5 flammable or explosive compounds found in the laboratory
6. Discuss proper storage of chemicals that are fire or explosion hazards
7. Outline measures to be taken for acid or base spills
8. Describe safety measures that should be used because of biological hazards
9. List the 4 types of fire extinguishers and identify the type of fire for which each would be used
10. Define:
    a. time-weighted average (TWA)
    b. permissible exposure limit (PEL)
    c. short-term exposure limit (STEL)
    d. LD_{50}
    e. flash point
    f. hazardous waste
    g. fire triangle
    h. ergonomics
    i. universal precautions
11. Identify the PEL, STEL, and action level of formaldehyde
12. State the action to be taken if monitoring indicates that the TWA of formaldehyde exceeds the allowable limit
13. Identify the purpose of the "Right to Know" law (Hazard Communication Standard)
14. Identify the purpose of the Laboratory Standard (Occupation Exposure to Hazardous Chemicals in Laboratories)
15. State the effect of Creutzfeldt-Jakob disease (CJD) on the brain
16. Outline a method for handling tissue from patients with CJD

Because so many areas of laboratory safety are regulated by various federal, state, county, and city policies, and because policies also may vary depending on the accrediting agency of the institution, safety is a very complex subject. It is difficult to stay up to date on all applicable regulations, unless safety is a primary area of responsibility. This chapter presents only an overview of the aspects of safety that are important to histotechnology personnel, divided into 3 major classes of hazards: biological (infectious), mechanical, and chemical. For a more comprehensive treatment of the subject, the reader is referred to the texts by Davis [2009] and by Dapson and Dapson [1995].

Biological or Infectious Hazards

For a substance to be infectious, it must contain enough pathogens of sufficient virulence to cause an infectious disease when a susceptible host is exposed to it. Since the appearance of acquired immunodeficiency syndrome (AIDS), most institutions have formulated infection control policies using universal precautions or body substance isolation procedures as recommended by the US Occupational Health and Safety Administration (OSHA) [OSHA 1987] and by the Centers for Disease Control and Prevention (CDC) [CDC 1988], and more recently, based on the Bloodborne Pathogen Standard of OSHA [Jensen 1989], which became effective in 1993. This latter standard is intended for the protection of all workers who might come into contact with potentially infectious material, especially specimens from patients who are HIV-positive or who have been infected with the hepatitis B virus. As defined by the CDC [2008], *universal precautions* is an approach to infection control whereby all human blood and certain human body fluids are always treated as if they are infectious for human immunodeficiency virus (HIV), hepatitis B virus (HBV), and other bloodborne pathogens. Universal precautions should be practiced in any environment in which the employee is exposed to blood or other potentially infectious body fluids, such as semen, vaginal secretions, synovial fluid, cerebrospinal fluid, pleural fluid, peritoneal fluid, pericardial fluid, and amniotic fluid. Feces, sputum, vomitis, and other bodily secretions are not included unless they contain visible blood. Compliance methods for OSHA's Bloodborne Pathogen (BBP) plan include the use of universal precautions, engineering controls that isolate or remove the bloodborne pathogen hazards from the workplace, and work practice controls that reduce the likelihood of exposure by altering the manner in which a task is performed. When occupational exposure remains after institution of these controls, personal protective equipment (PPE) shall also be used.

Institutional and laboratory policies are required to have an exposure control plan covering the broad topics of housekeeping, personnel protection, and waste disposal. Employers must conduct an exposure determination identifying employees who may be exposed to blood or other potentially infectious materials and identifying tasks that put the employees at risk. Frozen sectioning, autopsy, surgical gross dissection, and cytology preparatory areas are of primary concern for histopathology personal because fresh tissue or body fluids are handled in those areas. Once the tissue is well-fixed and processed, there is little need for such stringent protection. Appropriate barrier precautions must be used (eg, gowns, gloves, and masks) to prevent skin and mucous membrane exposure. Personnel must be instructed in the proper selection, use, and disposal of the personal protective equipment (eg, disposable gloves). OSHA requires that disposable gloves be replaced when contaminated or damaged; no washing or reusing of gloves is allowed, and hand-washing or antisepsis is required after glove removal. The 2002 revisions to the BBP require a schedule for reviewing the effectiveness of the methods of control, and evaluation of both medical procedures and technological developments that can decrease exposure. The appropriateness and justification for the selection or nonselection of PPE must be documented. The College of American Pathologists (CAP) [2006] accreditation checklist also states that open-toe footwear does not provide adequate protection and should not be worn in the laboratory.

Since the publication of the universal precautions recommendation [CDC 1988], a marked increase in latex sensitivity has been noted. Symptoms can range from contact dermatitis to systemic reactions as severe as anaphylaxis. These allergic reactions are in most part caused by extractable protein remaining in the gloves and also in other latex products; therefore, nonlatex gloves should be used where possible. If latex gloves must be worn, vinyl gloves or cloth liners should be worn underneath the latex gloves. Again, hands should be washed, preferably with soap and water, after removal of gloves.

TUBERCULOSIS EXPOSURE

OSHA currently lacks a regulatory standard on occupational exposure to tuberculosis, but enforces the CDC's 2005 nonregulatory recommendations [CDC 2005] published in "TB infection control guidelines for preventing the transmission of *Mycobacterium tuberculosis* in health care settings." Because of the dramatic increase in the incidence of drug-resistant tuberculosis, OSHA recognizes that continued vigilance is necessary to control the incidence of TB infection in health care settings. The TB infection control program is based on detection, airborne precautions, and treatment. CAP also requires a documented tuberculosis exposure control plan. In addition to an exposure determination at defined intervals for all employees with possible occupational exposure, the plan should include engineering and work practice controls for hazardous procedures that potentially may aerosolize *M tuberculosis*. This includes the handling of unfixed tissues in surgical pathology and autopsy. Policies must be established that address the subjects of ventilation, work practices, personal protective equipment, decontamination, and employee exposure [Dapson 1995]. Respiratory protection equipment must be used when environmental controls and work practices reduce, but do not eliminate, the risk of exposure to *M tuberculosis*.

CRYOGENIC SPRAYS

Cryogenic sprays or procedures that create aerosols are very dangerous because they markedly increase the possibility of exposure to pathogenic organisms. Cryogenic sprays should not be used in the frozen section area or, as has become common in some laboratories, in the microtomy of poorly fixed and processed

specimens. In microtomy, even when a section can be obtained by using the aerosol, the specimen falls apart on the flotation bath, thereby increasing laboratory workers' possible exposure to infectious agents. Better processing procedures and methods of freezing that do not require these sprays need to be developed!

HIV, HEPATITIS C VIRUS (HCV), AND HBV

Personnel expected to have direct contact with body fluids (unfixed tissue) must receive education on precautionary measures, epidemiology, modes of transmission, and prevention of HIV, HCV, and HBV. They should also be educated on the application of "universal precautions" in association with these hazards. Any personnel at risk of HBV should be offered the hepatitis B vaccinations at no cost within 10 working days of initial assignment. Employees who have ongoing contact with patients or blood, and who are at ongoing risk for percutaneous injuries, are to be tested for antibody to HBV surface antigen 1 to 2 months after the completion of the vaccination series. If there is percutaneous, mucous membrane, or abraded skin exposure to these agents, the exposure incidence must be documented and evaluated, and procedures for postexposure follow-up must be in place.

CREUTZFELDT-JAKOB DISEASE (CJD)

Creutzfeldt-Jakob is a prion disease affecting primarily nervous tissue and resulting in a spongiform change in the cortex and subcortical white matter of the brain. Prions are abnormal proteins, with the name "prion" coming from "proteinaceous infectious particle." CJD occurs worldwide, has a long latency period of 20 years or more after infection, affects approximately 1 person in a million, and is rapidly progressive after diagnosis; it is usually fatal within months after diagnosis. Because the prions that cause spongiform encephalopathies are very stable under a variety of conditions, tissues from suspected CJD patients, along with the equipment used, must be handled differently from all other specimens. The prions are resistant to formalin, some organic solvents, enzymes, heat, ionizing radiation, freezing, drying, and autolysis [Brown 1990a]. They are susceptible to autoclaving for 1 hour at 121°C and 2 kg/cm^2 (according to Brown [1990b], CAP recommends raising the temperature to 132°C-134°C), 1N sodium hydroxide for 1 hour, concentrated formic acid for 1 hour, and 5% sodium hypochlorite for 2 hours.

For histologic examination, sections of brain should be fixed in neutral-buffered formalin for at least 48 hours; whole brain specimens should be fixed for 10 to 14 days. Sheppard fixes sections in phenol saturated formalin (approx 15 g/100 mL) for 72 hours. The prion will not be totally inactivated by either method of fixation, so sections should be treated for an additional hour with concentrated formic acid, and then placed back in fresh formalin for 48 hours. The waste formalin should be diluted with an equal amount of 2N sodium hydroxide and allowed to stand for at least 1 hour before disposal. The prion is essentially inactivated by this fixation treatment, so processing and sectioning can be handled in the routine manner.

Steel instruments used for gross dissection should be treated with 1N sodium hydroxide for 1 hour; gowns, gloves, plastic aprons, and other disposables should be incinerated or autoclaved, and the grossing table or board should be disinfected by treating with 1N sodium hydroxide and allowing it to set for at least 1 hour. Instruments and the grossing table or board should be thoroughly washed with soap and water after treatment with sodium hydroxide.

HANDLING TISSUE WASTE

All tissue waste and any materials coming in contact with tissue should be disposed of in biohazard bags; sharp objects should be discarded in special "sharps" containers. Unless universal precautions are used, specimen containers must be labeled with a biohazard warning if they contain potentially infectious material (unfixed material). When universal precautions are used, all blocks, slides, and both unfixed and fixed wet tissues must be handled as if potentially infectious [Montgomery 1995; Dapson 1995]; thus, the observance of universal precautions becomes burdensome and impractical. Housekeeping procedures should be developed and rigidly adhered to. A written policy for whether to return specimens to patients should be established, taking into consideration the chemical and biological risks associated with both fixed and unfixed specimens.

While the CDC and Environmental Protection Agency (EPA) have similar guidelines regarding hazardous waste, the CDC serves as an advisory agency and the EPA serves as a regulatory agency. They both recognize 4 types of waste as infectious:

1. microbiologic or culture material
2. pathologic material
3. blood
4. sharp objects

The recommendations of these agencies should be followed unless the state or city regulations are more stringent. Both agencies recommend steam sterilization or incineration of all waste except pathologic waste; only incineration is recommended for pathologic waste. According to the regulations, blood may be disposed of in the sanitary sewer system (eg, sink). To ensure proper handling and treatment, all infectious wastes should be segregated into clearly identified biohazard containers. Personnel involved in the disposal of waste materials should be trained in handling infection or injury hazards presented by the waste and should be instructed in appropriate handling and disposal methods. A method of disinfecting nondisposable equipment such as saws, cryostats, and the gross specimen photography apparatus should be established and rigidly followed.

Mechanical Hazards

Mechanical hazards primarily consist of sharp instruments (eg, microtome blades, razors, scalpels, and needles), glass, and electrical hazards. Sharps are regulated under OSHA's BBP standard, and sharp containers are defined as engineering controls.

According to demographics gathered by the CAP/National Society for Histotechnology Histology Quality Improvement Program [HQIP 2006], <0.5% of laboratories used standard steel microtome knives in 2006. If these knives are still used, caution should be taken at all times when using, cleaning, or sharpening a knife. The exposed ends of the knife should be covered with a knife guard during microtomy, the blade should be disinfected after use, and the knife should always be placed in its box as soon as it is removed from the microtome. The smaller disposable microtome knives, razors, and scalpel blades used in histology today still present a hazard, and extreme care should be taken at all times when using or handling these instruments. Scalpel blades should be removed with a safety device and all used blades should be placed in the sharps container after use. Sharps containers must be color-coded, closable, and puncture resistant. The container must remain upright, be within easy reach, and be at a height that allows employees to observe when it is full and needs replacement. OSHA's 2002 compliance directive mandates that employees representing areas of exposure risk perform an annual evaluation of safer needle devices; a log of injuries by sharps is also required. Glassware should be checked periodically for chips or cracks and should be discarded if defects are found. Vacuum desiccators present a major hazard if a crack develops in the glass. All electrical equipment should be grounded and the condition of the wires should be checked periodically. The effectiveness of the ground should also be checked periodically. All equipment checks should be documented.

ERGONOMICS

The latest hazard in this area to receive marked interest is the design of the workplace itself. Increasing numbers of cumulative trauma disorders, now more commonly known as musculoskeletal disorders (MSDs), such as carpal tunnel syndrome, trigger finger, tendonitis, and thoracic outlet compression syndrome, have been reported and have caused laboratories to address design problems in the workplace. This is known as ergonomics, the science of adapting the working environment to the anatomic, physiologic, and psychological characteristics of personnel to enhance their efficiency and well-being [Montgomery 1995]. The OSHA Ergonomics Standard, designed to prevent crippling repetitive stress injuries became effective January 16, 2001, but was repealed later that same year. However, CAP requires accredited laboratories to have a documented ergonomics program to prevent MSDs in the workplace. Some of the greatest risks factors specific to histology include the use of manual microtomes, repetitive use of forceps, manual coverslipping, and repetitive opening of tissue cassettes. Automation should be introduced where possible; workstations should be evaluated for ergonomic problems, and corrections made; stretching should be done at least every 20 minutes; tasks should be rotated frequently, and employees should be educated about ways to prevent injury when repetitively using equipment or tools.

Some methods for preventing MSDs are as follows [Dapson 2007]:

- When embedding, use forceps that require minimal force to operate (eg, reverse grip that remain closed unless you apply pressure); change hands frequently and do not use the same motion for opening cassettes; take frequent breaks or alternate with another technologist.
- For computer keyboard operation, keep the wrist in a neutral position above the keyboard, keep fingers curved, and use a gentle touch on the keys.
- For nonautomated microtomy, use the entire arm to make complete revolutions of the handwheel; do not rock the handwheel; keep arms and elbows close to the body; keep shoulders down and neck relaxed; stretch often or take breaks. The chair must be at the correct height so that good relaxed posture can be maintained, and the flotation bath should be at a comfortable distance from the microtome.
- When manually coverslipping, take mini-breaks; alternate duties; do stretching exercises for wrists and fingers.
- When changing solutions on the processor, use proper lifting and bending techniques; use the whole hand or both hands when carrying containers.
- Automate microtomy, staining, and coverslipping if possible.

Chemical Hazards

Chemicals can present physical (fire, explosive, oxidizer) or health hazards. The "Right to Know" law is of the utmost importance with regard to these categories of hazards. The OSHA Hazard Communication Standard [1987] became effective on May 20, 1988. This standard, targeted for industry, ensures that workers are informed about hazards associated with the chemicals in their workplaces so that they can protect themselves [Davis 2009]. The second important standard is the Occupational Exposure to Hazardous Chemicals in the Laboratories, 29 CFR 1910.1450, also known as the Laboratory Standard. This standard requires a plan (or "Chemical Hygiene Plan") for implementing practices to minimize exposure to hazardous chemicals. These standards require that employers communicate to employees the potential dangers of chemicals with which they work, and train the employee in the proper use of safeguards for handling those chemicals. OSHA will cite institutions that fail to comply with these regulations, and heavy fines also may be imposed. The Laboratory Standard does not apply to all laboratories, but where it applies, it supersedes the Hazard Communication Standard, 29 CFR 1910.1200. There is no option of choosing between the 2 standards. If the Laboratory Standard applies to an area, it must be implemented. The Laboratory Standard applies to laboratories meeting the following criteria:

1. Chemical manipulations are carried out on a laboratory scale, meaning that work with chemicals can be easily and safely manipulated by 1 person.
2. Multiple chemical procedures or chemicals are used.

3. Protective laboratory practices and equipment are available and in common use to minimize the potential for employee exposure to hazardous chemicals.

4. The procedures involved are not part of a production process whose function is to produce commercial quantities of materials, nor do the procedures in any way simulate a production process.

Specifically, employers must scrupulously adhere to the following conditions:

1. Employees must prepare an inventory of all hazardous chemicals in the facility, and obtain copies of the Material Safety Data Sheets (MSDSs) for these chemicals from the supplier or manufacturer. During the work shift, the MSDSs must be accessible to all employees. The standard states that suppliers and distributors of hazardous substances must send MSDSs with their first shipments. If such documents are not sent, then the employer must show that there was a good faith effort (such as writing a letter of request) to obtain the MSDS.

2. Employers shall ensure that labels on all incoming containers of hazardous chemicals are not removed or defaced. CAP requires labeling of all containers of hazardous chemicals with the identity of the chemical and appropriate hazard warnings (eg, carcinogen, corrosive, explosive). This applies to all commercially prepared reagents, laboratory-prepared reagents, stationary and permanent containers, temporary containers used for transport, and chemical waste containers.

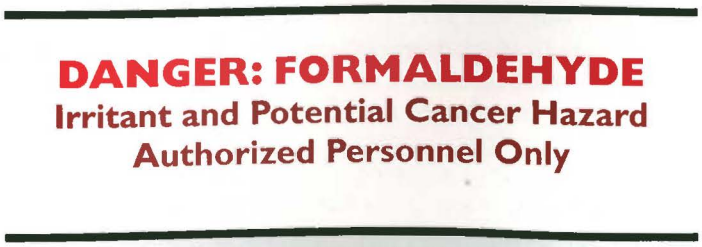
3. Employers must inform employees that they have a right to know the hazards associated with their jobs.

4. Employers must establish a training program in which employees who work with hazardous substances are trained to read and interpret chemical labels and MSDSs, to use protective clothing and equipment that may be required when working with such chemicals, and to properly dispose of hazardous chemicals. Employees should be trained or oriented before their initial job assignments and then at least annually thereafter. This training should be carefully documented and maintained in the employee's file for the duration of employment plus 30 years.

5. Employers must develop a chemical hygiene plan that includes standard operational procedures to protect employees from the health hazards of chemicals and keep exposures below specified limits. The plan should also address particularly hazardous materials that require special consideration.

6. Employers must establish a means to inform nonemployees or personnel from other departments of the hazards present in the workplace. This usually means posting signs such as "Biohazard: Authorized Personnel Only" or "Caution: Microwave Oven in Use." If personnel from other areas take solutions or specimen containers from the laboratory, the appropriate hazard warnings must be on the containers.

OSHA defines *hazardous chemicals* as chemicals that may cause acute or chronic health effects in exposed employees. The term *health hazard* includes corrosives, irritants, sensitizers, toxins, and carcinogens. Hazardous chemicals gain entry to the body by ingestion, absorption, or inhalation. Accidental ingestion of chemicals should not occur, because eating, drinking, smoking, and pipetting by mouth are prohibited in the laboratory. Gloves should be selected and worn to protect against absorption of chemicals in accordance with OSHA's PPE and Hand Protections Policy (1910.132 and 1910.138) [OSHA 1987]. Inhalation is a major consideration in histopathology laboratories, and engineering controls, such as a ventilation hood, are required to prevent or minimize the escape of air contaminants into the laboratory. The Formaldehyde Standard (29CFR1910.1048) was passed in 1992, and revisions for occupational exposure to formaldehyde became effective in 1993. This standard mandates permissible exposure limits (PELs) as the maximum safe concentration levels of exposure to formaldehyde. The PELs for an 8-hour shift are measured as either time-weighted average (TWA) or action level. The maximum allowable TWA for formaldehyde is 0.75 ppm, representing the average concentration, after measuring or monitoring, for an 8-hour exposure. The short term exposure limit (STEL) is used to measure exposure for a short period (15 minutes) only 4 times throughout the day, with at least 1 hour between exposures. The maximum allowable STEL is 2.0 ppm over a 15-minute period. Each employee or job classification must be monitored initially for exposure to identify all employees who are exposed to formaldehyde at or above the "action level" or STEL. If the initial monitoring shows a TWA >0.5 ppm (action level), monitoring must be repeated every 6 months. If monitoring reveals employees at or above the STEL, monitoring must be repeated at least once a year under "worst case conditions." If the TWA is ≤0.5 ppm and also within the STEL limits, then monitoring does not have to be repeated, and a medical surveillance program does not have to be established unless there is a change in procedures or conditions that might change the exposure, or unless an employee exhibits signs or symptoms of exposure. Employers may discontinue exposure monitoring if results from 2 consecutive samplings, ≥7 days apart, indicate exposure below the STEL and action levels. Monitoring results must be provided to the employees within 15 days, either by individual distribution or by posting. A medical surveillance program is required for all employees exposed to formaldehyde at or exceeding the action level or STEL, those workers exhibiting signs or symptoms of exposure, and those employees exposed during an emergency situation. The employer shall establish regulated areas in which the concentration of airborne formaldehyde exceeds either the TWA or STEL and post all entrances and access ways with signs bearing the following information shown in **[f4.1]**.



[f4.1] Example of warning sign to be posted at all access points to areas where concentration of airborne formaldehyde exceeds the TWA or STEL.

Employers shall institute engineering and work practice controls to reduce and maintain employee exposure to formaldehyde at or below the TWA and the STEL. Whenever engineering controls and work practice controls cannot reduce employee exposure to the PEL or below it, then approved respirators can be used to satisfy the exposure requirements of this standard. A written hazard communication program must be developed and available to the employees. Employers are required to provide training to all employees assigned to workplaces where there is exposure to formaldehyde at or above 0.1 ppm. For a more comprehensive discussion of the Formaldehyde Standard, either the text by Montgomery [1995] or by Dapson and Dapson [1995] should be consulted.

The text by Dapson and Dapson [1995] and the NIOSH *Pocket Guide to Chemical Hazards* [1990] are handy references for the laboratory; together they provide synonyms, exposure limits, health hazards, physical hazards, handling precautions, incompatibilities, monitoring methods, skin protection, first aid, spill procedures, recommended respirators, disposal, target organ effects, and the EPA number of chemicals commonly used in histology.

PARTICULARLY HAZARDOUS SUBSTANCES (REPRODUCTIVE TOXINS, SELECT CARCINOGENS, AND SUBSTANCES WITH A HIGH DEGREE OF ACUTE TOXICITY)

Reproductive toxins comprise chemicals that affect the reproductive capabilities, including chromosomal damage (mutations) and effects on fetuses (teratogenesis). According to CAP, every chemical that is used in the laboratory must be evaluated for carcinogenic potential, reproductive toxicity, and acute toxicity. Results of those evaluations must be documented, and the policy and procedure manual must define specific handling requirements for these chemicals according to OSHA directives.

In defining the toxic dose of a substance, different terms may be encountered in the literature. The *toxic dose low* is defined as the lowest dose of a substance that will produce any toxic effect in humans when introduced by any route other than inhalation; substances that are toxic by inhalation are reported as *toxic concentration low.* The *lethal dose low* is usually reported for a substance and is defined as the lowest dose reported to have caused death in humans, or as the lowest single killing dose reported for animals. The LD_{50} is the calculated dose of a chemical substance expected to cause the death of 50% of an experimental animal population, as determined by exposure to the substance by any route other than inhalation. Examples of toxic chemicals are the hydrocarbons (eg, xylene and toluene), formaldehyde, and some metallic compounds (eg, mercury, chromium, and silver). *Designated area* indicates an area that may be used for work with *select carcinogens*, reproductive toxins, or substances with a high degree of acute toxicity. A designated area may be the entire laboratory, an area of a laboratory, or a device such as a laboratory hood.

CARCINOGENS

Carcinogens are substances that cause or greatly increase the risk of malignant disease. OSHA defines *select carcinogens* under the Laboratory Standard as any substance that meets 1 of the following criteria [OSHA 1987]:

1. It is regulated by OSHA as a carcinogen.
2. It is listed as "known to be carcinogen" by the National Toxicology Program (NPT).
3. It is listed by the International Agency for Research on Cancer (IARC) under group 1, 2A or 2B.

Bis-chloromethyl ether, formed by the reaction between hydrochloric acid and formaldehyde, induces lung cancer; chloroform, chromic acid, pararosaniline, and benzidine-based dyes are among other probable carcinogens identified in histopathology. Formaldehyde has been identified as a potential human carcinogen and is regulated under the formaldehyde standard.

CORROSIVE SUBSTANCES

For disposal purposes, corrosive hazards are defined officially as those substances that, by direct contact, will corrode SAE 1020 steel at a rate >6.25 mm/year at 55°C, or more simply, as substances that are capable of destroying mild steel under certain defined conditions. The acids are considered corrosive substances by this definition. Contact with most metallic surfaces should be avoided with all substances that pose a physical corrosive threat. As health hazards, corrosive substances are defined as those substances that will cause injury to the skin and eyes by direct contact or severe damage to the tissues of the respiratory and alimentary tracts when inhaled or ingested. The effects of corrosive chemicals lead to disruption of cell membranes, coagulation of proteins, and death of essential cellular components.

FIRE AND EXPLOSION HAZARDS

Although liquid organic compounds are most commonly thought of as fire hazards, certain chemicals such as dry picric acid, benzoyl peroxide, and ammoniacal silver solutions can be explosion hazards.

Fire is defined as the rapid oxidation of a fuel in the presence of an ignition source, with liberation of heat and light [Moya 1980]. This sequence of events is called the fire triangle. Air, or oxygen, and fuels are mixed closely everywhere in the environment, but fires will not occur unless an ignition source is present; the 3 elements necessary for a fire are present in the laboratory. Fires are classified into 4 groups (classes A, B, C, and D) [**t4.1**].

Fire extinguishers are classified and rated in 4 groups corresponding to the class of fire that they are able to extinguish. Class A includes water-based, foam or loaded-stream, and multipurpose dry extinguishers. Class B includes carbon dioxide, dry chemical, foam and loaded-stream, bromotrifluoromethane (Halon 1301), and bromochlorodifluoromethane (Halon 1211) extinguishers. Class C includes the Halon, carbon dioxide, and dry chemical extinguishers. Class D extinguishers contain dry powder media that will not react or combine adversely with the burning materials. Class D fires are unlikely in the

histopathology laboratory. ABC extinguishers (also called all-purpose or tri-class) are preferred, and an extinguisher must be no more than 50 feet away from flammable or combustible liquids [Dapson 2007]. Documentation must verify that all personnel have been instructed in the correct use of extinguishers.

Health hazards may be created by the use of automatic fire extinguishing systems because of the concentration of the extinguishing agent or the toxic products of decomposition that may result from exposure of the extinguishing agent to a fire or hot surfaces. OSHA limits the Halon and carbon dioxide concentrations released by fixed extinguishing systems when employees are exposed for only a short period. In areas in which employees would normally remain after the automatic discharge of the extinguishing agent, OSHA prohibits the use of Halon 1211 and carbon dioxide. The OSHA [OSHA 1987] and National Fire Protection Association (NFPA) ratings [NFPA 1980] for flammable and combustible materials are as shown in **[t4.2]**.

The flash point of a liquid is the lowest temperature at which sufficient vapors are produced to form an ignitable mixture with air near the surface of the liquid or within the container used. It is the vapors, rather than the liquid itself, that contribute to fire or explosion. Each flammable liquid has a vapor concentration range in air below which the vapor-air mixture is too lean to burn or explode and above which the mixture is too rich to burn. Personnel who work with flammable solvents should be aware of the important flammability characteristics of each solvent in use, and should be familiar with, and follow, the manufacturer's precautions provided on the labels. Many of the flammable liquids also are toxic, so personnel must be aware of this additional hazard.

The hydrocarbons and alcohols used in histopathology are all fire hazards. The flash points of some common laboratory solvents are shown in **[t4.3]**.

Most fires can be avoided if all laboratory personnel follow good safety practices. NFPA and OSHA require that solvents be stored in fire safety cabinets and safety cans. Each laboratory should have an emergency plan that clearly defines actions that both the employer and the employee must take in a life- or injury-threatening emergency. An employee education program is necessary and should designate specific personnel to be responsible for each part of any emergency plan.

HAZARDOUS CHEMICAL SPILLS AND STORAGE

Laboratory personnel must be trained and prepared to handle spills. If floor drains are present, spill dikes should be available to prevent hazardous chemicals from entering the sewer system. Gloves, aprons, and eye protection should be used in the event of a small spill. Spill kits should be available in the laboratory with absorbent pads for the solvents, commercial neutralizers for the acids and alkalis, and cleaning utensils such as scoops, sponges, and bags. It is useful to remember that a 1-gallon spill will cover a 20-square-foot area [Montgomery 1989] and that all materials used for cleaning up a spill should be treated as hazardous waste. If a major spill occurs, the area should be evacuated, and a specially trained and equipped clean-up team called in case respirators are needed.

[t4.1] Classification of fires

Class	Description
Class A	Fires involving ordinary combustible materials such as wood, plastics, paper, and textiles. Class A fires can be extinguished with water or water-based solutions.
Class B	Fires involving flammable liquids and gases, requiring that oxygen be blocked from the fuel in order to be extinguished.
Class C	Electrical fires that must be extinguished with nonconductive media.
Class D	Fires of combustible and reactive elements, such as metallic sodium, potassium, magnesium, and lithium. Class D fires are difficult to control and extinguish because spreading and explosion can occur easily.

[t4.2] Classification of flammable and combustible materials

Class	Description
Class I	Flammable liquids (flash point <100°F) with 3 subclasses
Class IA	Flash point<73°F, boiling point <100°F
Class IB	Flash point <73°F, boiling point ≥100°F
Class 1C	Flash point ≥73°F, boiling point ≥100°F
Class II	Combustible liquid with flash point ≥100°F and <140°F
Class III	Flash point ≥140°F with 2 subclasses
Class IIIA	Flash point ≥140°F and <200°F
Class IIIB	Flash point ≥200°F

[t4.3] Flash points of common laboratory solvents

Solvent	Flash point, °C
Toluene	4.4
Xylene	27.2
Isopentane	<–51
Benzene	–11
Methanol	11.1
Ethanol	12.8
Isopropanol	11.7

When a chemical is spilled on an individual, immediate first aid treatment consists of washing with large amounts of water. If a large area of the body is affected, a safety shower must be used and contaminated clothing should be removed. If only a small area of the skin is contaminated, that area should be flushed with cold water and washed with a mild detergent or soap and water. Do not try to neutralize any acid or base spilled on a body surface. If a chemical is splashed in the eyes, wash for 15 minutes using an eyewash fountain and then seek medical attention if necessary. Although some laboratories have prohibited wearing contact lenses in the laboratory, various policies and regulations now agree that contact lenses may be worn in the laboratory without posing an additional hazard to the wearer. However, it must be remembered that contact lenses are not protective devices, and when eye hazards are present, appropriate eye protection must be worn. If appropriate protective devices are not used, in the event of a chemical splash contact lenses may trap fluid between the lens and the cornea, possibly increasing damage to the eye. In cases of solvent vapor exposure, the lens may adhere to the cornea and be impossible to remove. Flat, closed shoes must be worn to prevent injury to the feet from a chemical spill or a falling object.

CHEMICAL STORAGE

Proper storage of chemicals is very important. Flammable liquids must be stored in approved containers. CAP and NFPA define the maximum working volume of flammable and combustible solvents of class I, II, and III (flash points <200°F) allowed outside a storage cabinet as 1 gallon per 100 ft^2, and that stored in safety cans and safety cabinets as 2 gallons per 100 ft^2. Flammable or toxic liquids should be stored in glass containers only if the purity of the substance requires glass, or if the substance is likely to react with other types of containers. Flammable or toxic liquids should never be stored higher than eye level, because of the possibility of injury to the face and eyes if the container breaks or falls. Flammable liquids that require refrigeration must be placed in a clearly identified flammable refrigerator; these reagents should never be stored in a regular refrigerator. Safety cans must be used for transfer and storage of flammable liquids, acid carriers should be used to transport acids, and corrosives should be stored in dry, well-ventilated areas away from sunlight. If possible, corrosives should be stored separately; if this is not possible, they should be stored away from flammables, toxic substances, oxidizers, and compressed gases. Picric acid must be kept damp; if it appears that the stock picric acid may be desiccating and may contain <10% water, distilled water should be added. When heating a solution of picric acid in a glass Coplin jar, as in mordanting for the Masson stain, the jar should be placed inside another container (secondary containment) for protection in case of breakage. Ammoniacal silver solutions can decompose over time and become a shock-sensitive explosive; old solutions should not be kept in the laboratory. Stock reagent bottles should be well cleaned before new solutions are prepared in the same container.

HAZARDOUS CHEMICAL DISPOSAL

The EPA regulates hazardous waste. Facilities are mandated to follow specific guidelines for handling hazardous waste depending on its designated category as Conditionally Exempt Small Quantity Generator (CESQG), Small Quantity Generator (SQG), or Large Quantity Generator (LQG). CAP-accredited laboratories must have documented policies and procedures for the adequate disposal of hazardous waste which are in compliance with local, state, and federal EPA regulations. The EPA is working with health care facilities to reduce hazardous waste, and CAP also recommends a defined program to minimize the hazardous waste generated or to reduce the degree of hazard with:

1. acquisition constraints (eg, purchase reagents in small quantities)
2. process changes (eg, substitute less hazardous reagents for the more hazardous ones; adopt methods that use smaller quantities)
3. recovery (eg, silver)
4. recycling (eg, distillation and reuse of xylene/xylene substitutes, alcohol, and formalin solutions)
5. redistribution (eg, relocate surplus or unwanted chemicals to laboratories that can use them

Many chemicals may be disposed of down the drain. Any chemical that is flammable or that will not mix with water may not be poured down the drain. Alcohol, provided that the concentration is no higher than 24%, may be poured down the drain. Strong acids and bases must be neutralized or diluted to a pH between 3 and 11 with water, and then poured down the drain at a rate not to exceed 50 mL/min; the water should be running. Compounds containing mercury, chromium, or silver may not be disposed of in the sanitary sewer system. Copper and zinc compounds are not considered hazardous. The EPA defines a waste as hazardous in 40 CFR 261 if it is listed (P-list, U-list) or demonstrates 1 of 4 characteristics **[t4.4]**.

[t4.4] Characteristics of hazardous materials [EPA 2000]

Characteristic	Description
Ignitability	Liquids and ignitable waste, other than aqueous solutions containing <24% alcohol by volume, that have a flash point <60°C
Corrosivity	Aqueous solutions that have a pH≤2, or >12.5
Reactivity	Substances that react violently with water Substances that are unstable Substances that are explosive Substances that contain cyanide or sulfide that generates toxic gas when exposed to a pH in the range of 2 to 12.5
Toxicity	Waste is subject to the toxicity characteristic leaching procedure (TCLP); if contaminants are above the regulated concentrations, they are assigned an EPA hazardous waste code Waste is analyzed for arsenic, barium, cadmium, chromium, lead, mercury, pyridine, selenium, and silver

Substances that cannot be poured down the drain must be collected in appropriate containers, and the containers must be selected carefully. In general, metal containers should be used for noncorrosive, flammable organic compounds, plastic containers for inorganic compounds and weak acid or bases, and glass containers for corrosives and strong acids or bases. Collection containers should be labeled as hazardous waste with the name of the chemical, the starting and final dates of collection, and the approximate composition of the chemicals on the label. For example, the label on the collection container for Zenker and Helly fixatives should state that the solution contains 5% mercuric chloride and 2.5% potassium dichromate.

Hazard Identification

Under the Right-to-Know legislation, appropriate labels must appear on both the chemical containers received from the manufacturer and any reagents prepared in the laboratory. Appropriate signs indicating hazards specific to the area must be posted, and the MSDSs must be readily available for all chemicals used in the laboratory. Unfortunately, manufacturers have not adopted a standard method of labeling the chemicals or a standard format for the MSDS, so one must learn to search out the information presented in a variety of ways.

The NFPA [1980] has a coded system of labeling that will enable firefighters to identify the hazards of a substance quickly. This consists of 4 small, blank, color-coded diamonds on 1 label **[f4.2]**. The sections of each diamond carry the following information: The left blue diamond signifies the health section, the top red diamond provides the flammability rating, the right yellow diamond cites the reactivity, and the bottom white diamond contains special symbols indicating properties and categories not explained by the other sections. The rating system within each section is numbered from 0 to 4, ranging from the least dangerous to extremely dangerous **[t4.5]**. This rating can be obtained from the MSDS or from the labels on the dry chemicals. Some accrediting agencies require the NFPA labels on chemical containers, but these labels are for emergency personnel responding to a fire or spill, and are not intended as the sole reference for general use in the laboratory [Dapson 2007]. For general use in the laboratory, labels should contain:

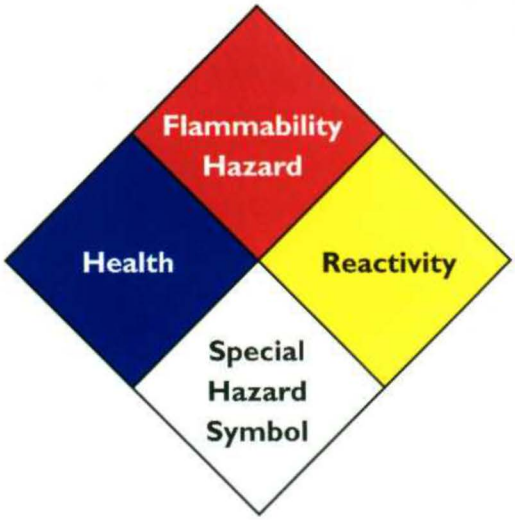
[t4.5] NFPA hazard rating system

Rating	Description
0	No hazard
1	Slight hazard
2	Moderate hazard
3	Severe hazard
4	Extreme hazard

1. contents that must correspond with the MSDS
2. target organ of a hazardous chemical (can be words or symbol; if symbol, a chart or key must be readily available to employees)
3. manufacturer or distributor along with contact information (if chemical falls under the Food and Drug Administration jurisdiction)
3. date of receipt, date of preparation, and/or date placed in service
4. expiration date
5. precautionary label if the chemical is hazardous

OSHA requires that the actual word(s) describing the hazard (eg, "flammable") be placed on the container [OSHA 1987], and Dapson and Dapson [1995] state that, at the moment you need to know about a hazard, there should be no room for error in interpretation. In addition to the word(s) describing the particular hazard, symbols are frequently used for easy recognition and emphasis. Four of the common symbols that might be encountered are shown in **[f4.3]**.

When biopsy specimens are sent to other areas of the hospital, an appropriate warning label must be on the bottle containing the specimen. Regulations suggest the following warning label for formaldehyde: "CAUTION! Contains and releases formaldehyde. Toxic by inhalation and if swallowed. Irritating to the eyes, respiratory system, and skin. May cause sensitization by inhalation or skin contact. Risk of serious damage to eyes. May cause cancer. Repeated or prolonged exposure creates risk."



[f4.2] National Fire Protection Association (NFPA) chemical hazard label for use on chemical reagents. Use the NFPA 0 to 4 rating system (4 = extreme; 3 = severe; 2 = moderate; 1 = slight; 0 = none) to number the appropriate color-coded hazard area. The white area can be used for coding protective gear or for special handling instructions. These labels are for the use of emergency response personnel and are not intended to be the sole hazard warning system in the laboratory.

[f4.3] Some of the symbols used to denote a particular hazard. **a** indicates a flammable hazard, **b** radiation hazard, **c** biohazard, and **d** poison.

General Safety Practices

1. Never pipette by mouth
2. Do not apply cosmetics and lip balm in the technical work areas
3. Do not manipulate contact lenses in the technical work areas
4. When diluting acids, always pour the acid into the water, never the reverse
5. Wash hands frequently; hands should always be washed immediately after the removal of gloves
6. If you are not sure of the reagent properties, handle it as if it were a hazardous chemical
7. Discard (in the appropriate manner) all unused, out-of-date chemicals
8. Examine all procedures for possible replacement of hazardous reagents by less hazardous substitutes
9. If the use of a hazardous chemical cannot be eliminated or replaced, the use and amount of stock should be minimized

In summary, responsibilities are as follows [Montgomery 1995]:

EMPLOYEES:

1. Participate in safety training programs
2. Know location of and how to use emergency equipment
3. Be alert to unsafe conditions, actions, and chemicals
4. Avoid hazards by following accepted procedures
5. Request information regarding unknown chemical hazards
6. Think, act, and encourage safety until it becomes a habit

SUPERVISORS:

1. Establish documented policies and programs as mandated by regulatory and accrediting agencies relative to the specific laboratory
2. Provide safety training programs as mandated by regulatory and accrediting agencies relative to the specific laboratory
3. Provide MSDS data on all chemicals and reagents used
4. Keep records of training on all exposed personnel (initial and annual safety training)
5. Keep records, names, Social Security numbers, and addresses of all exposed personnel and the nature of exposure (these records must be kept for the duration of employment plus 30 years)
6. Keep an inventory of all chemicals, dyes, and reagents used in the laboratory
7. Provide alert, enthusiastic, and sincere interest in personnel safety
8. Be sure that all personnel know that they have the right to know!

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LEARNING ACTIVITIES

1. **Locate the MSDSs in the laboratory, read the ones for hematoxylin and eosin, and answer the following questions:**

   a. Were the MSDSs readily accessible?

   b. Did you learn any new information about either hematoxylin or eosin?

2. **Locate the fire extinguisher in the laboratory and answer the following questions:**

   a. What type of extinguisher is it?

   b. What types of fire would it extinguish?

   c. Is it readily available?

3. **Locate spill kits for acid and alkaline spills in the laboratory and read the instructions for use.**

CHAPTER 5

Laboratory Mathematics and Solution Preparation

OBJECTIVES

On completing this chapter, the student should be able to do the following:

1. Define:
   a. percentage (weight per volume and volume per volume)
   b. liter
   c. meter
   d. gram
   e. milli-
   f. deci-
   g. centi-
   h. micro-, micron
   i. nano-
   j. kilo-
   k. buffer
   l. solute
   m. solvent
   n. meniscus
   o. molar solution
   p. normal solution
2. Calculate amounts of solvent and solute needed for any given percent solution
3. Calculate percentage when given the amounts of solvent and solute used in the solution
4. Calculate dilutions when preparing working solutions from stock solutions
5. Calculate the amount of solute needed to prepare normal and molar solutions
6. Convert figures within the metric system (eg, milligrams to centigrams) and between the US and metric systems (eg, pounds to grams, inches to meters)
7. Calculate and apply the gravimetric factor to compensate for dye concentration differences when preparing staining solutions
8. Convert temperatures from Fahrenheit to Celsius and from Celsius to Fahrenheit

Percentage Solutions

Percentage refers to parts in 100; for example, 10% is 10 parts in a total of 100 parts. In histotechnology, we use solutions in which the percentage of concentrations may be expressed as weight per volume or volume per volume. When diluting liquids, the required volume of the stock solution is diluted to 100 mL with the appropriate solvent (the substance in which something is dissolved). If the solute (the substance being dissolved) is a solid, the desired amount should be placed in a volumetric flask and diluted to 100 mL with the appropriate solvent. This method of diluting gives a solution of definite percentage concentration on a weight-to-volume basis, and although it makes little difference in many instances, the practice of adding 100 mL of solvent to the desired weight of the solid and calling it a percentage solution of definite composition is erroneous because the volume occupied by the solid is not taken into consideration. Weight-per-weight percentages are not used in histotechnology.

Just as a 10% solution may be expressed as the fraction 10/100 or 1/10, it may also be expressed as the ratio 1:10. Students are often in doubt as to whether 1:10 means 1 part plus 10 parts, or 1 part plus 9 parts. The latter is correct and dilution to a total of 11 (1 + 10) parts instead of 10 (1 + 9) parts would change the concentration appreciably.

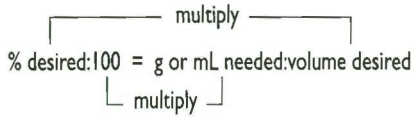
To prepare 100 mL of a 0.9% solution of sodium chloride (NaCl), we would weigh 0.9 g of NaCl (solute) and dissolve it in some distilled water (solvent). When the salt is totally dissolved, enough distilled water is added to make a final volume of 100 mL. When measuring solutions in a cylinder, pipette, or volumetric flask, be sure to hold the calibrated glassware at eye level to read the bottom line of the curved surface of the liquid (the meniscus). Volumetric glassware should be used for solution preparation; calibrated Erlenmeyer flasks or beakers are too inaccurate. The size of calibrated glassware chosen should be equal to or just slightly larger than the volume desired so that the error of measurement will be minimal.

Problems

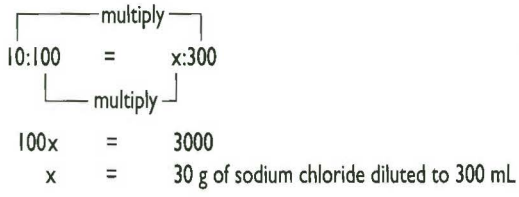
Determine the percentage solution.

1. 0.25 g sodium chloride diluted to 100 mL = ___
2. 6.4 g sodium chloride diluted to 100 mL = ___
3. 4.0 mL hydrochloric acid diluted to 100 mL = ___
4. 0.6 mL nitric acid diluted to 100 mL = ___
5. 20 g of silver nitrate diluted to 100 mL = ___
6. 2.25 g of alcian blue diluted to 200 mL = ___

To prepare a definite amount of a percentage solution, use the following ratio:



Example:



Problems

Calculate the amount of solute needed for the following solutions:

7. 500 mL of 0.55% potassium metabisulfite
8. 500 mL of 5% sodium thiosulfate
9. 1,000 mL of 0.25% light green
10. 500 mL of 3% alcian blue in 1% acetic acid (Note: This must be treated as 2 problems, first preparing the solvent and then determining the amount of alcian blue to add to the solvent.)
11. 300 mL of 0.5% light green in 0.25% acetic acid
12. 250 mL of 1:100 silver nitrate

Problems

Use the preceding formula to calculate percentage or volume.

13. 7.5 mL of ammonium hydroxide diluted to 1,000 = ___ % solution
14. How many milliliters of a 5% solution will 2.5 g make?
15. 0.2 g in 200 mL is equal to what percentage of solution?
16. How many milliliters of a 10% solution will 35 g make?

In the laboratory, it is frequently necessary or easier to make a working solution from an already prepared stock or stronger solution. To make a dilution from a concentrated or stock solution use the following formula:

$$\text{beginning percentage} \times \text{beginning volume} = \text{desired percentage} \times \text{desired volume}$$

(the units must be the same on both sides of the equation)

Example:

Prepare 50 mL of 2% sodium thiosulfate from a 5% solution.

$$5x = 2 \times 50$$

$$5x = 100$$

$$x = \frac{100}{5}$$

= 20 mL of 5% sodium thiosulfate
+ 30 mL of water
50 mL of 2% sodium thiosulfate

For economic reasons, most dilutions of alcohol are made by diluting 95% alcohol instead of 100%. The formula given previously may be used to prepare a definite amount of solution from 95% alcohol, but for routine stock solutions it is easier to dilute to 95% or 950 mL, because then the percentage desired may be changed directly into milliliters if diluting to 95 mL, or 10 times the percentage if diluting to 950 mL. For example, a 70% alcohol solution may be prepared by diluting 70 mL of 95% alcohol up to 95 mL with water, or 700 mL of 95% alcohol up to 950 mL. Because alcohol and water mix in a way that is not exactly additive, the solutions will be slightly less than the indicated percentage, but this is not a problem in routine use. To make an alcohol solution of an exact percentage concentration, both the water and the alcohol must be measured.

Problems

Prepare the following solutions:

17. 50 mL of 2% ferric chloride from a 10% solution

18. 500 mL of 60% alcohol from an 80% solution

19. 950 mL of 70% alcohol from a 95% solution

Determine the correct volume

20. How much water would you add to 100 mL of a 20% solution of formalin to obtain a 10% solution?

Super Problem

21. Prepare 1,000 mL of a 1:5000 solution of light green

Use of the Gravimetric Factor in Solution Preparation

The gravimetric factor is a method of compensating for variances in the actual dye content of dry dyes used in the preparation of staining solutions [Sheehan 1980]. By using this calculation, greater consistency of the staining solutions can be achieved by maintaining the same dye content.

To establish the gravimetric factor use the following formula:

$$\frac{\text{concentration of present dye}}{\text{concentration of new dye}} = \text{gravimetric factor}$$

To use the gravimetric factor, multiply the factor by the amount of dye required to prepare the staining solution. The answer is the amount needed of the new dye powder.

Example:

Prepare 50 mL of 1% acid fuchsin. The present dye has a dye content of 80% and the new dye has a dye content of 65%. Amount of dye needed with the present dye:

$$1:100 = x:50$$

$$100x = 50$$

$$x = 0.5 \text{ g of the present dye}$$

gravimetric factor calculation:

$$\frac{80}{65} = 1.23$$

Amount of the new dye needed:

$0.5 \times 1.23 = 0.615$ or 0.62 g of acid fuchsin diluted to 50 mL

Problems

Prepare the following solutions:

22. 100 mL of 1% alcian blue. The present dye has a dye concentration of 80%, and the new dye has a dye concentration of 86%.

23. 250 mL of 0.5% acid fuchsin. The present dye has a dye concentration of 82%, and the new dye has a dye concentration of 88%.

24. 150 mL of 1.5% alcian blue. The present dye has a dye concentration of 82%, and the new dye has a dye concentration of 94%.

Hydrates

In the preparation of solutions, it is very important to use the exact compound specified and to distinguish between an anhydrous and a hydrated substance. If the compound is a hydrate, that is, it contains water in a close chemical union, be sure that the compound being used contains the number of molecules of water specified in the procedure. For example, there are three calcium sulfate compounds: the anhydrite ($CaSO_4$), with a formula weight of 136.15; plaster of Paris ($CaSO_4 \cdot \frac{1}{2}H_2O$), with a formula weight of 145.15; and gypsum ($CaSO_4 \cdot 2H_2O$), with a formula weight of 172.18. Frequently, a hydrate other than the one specified can be used if the appropriate correction is made in the amount weighed. For example, if a procedure calls for 10 g of anhydrous sodium acetate (formula weight, 82.03) and only the trihydrate (formula weight, 136.08) is available, the calculation would be as follows:

multiply

10:82.03 = x:136.08

multiply

82.03x = 1360.80

$$x = \frac{1360.80}{82.03}$$

x = 16.59 g of the trihydrate should be used in place of 10 g of the anhydrous compound

Normal and Molar Solutions

Molar (M) and normal (N) solutions are rarely used in routine histopathology but are components of buffers and other solutions required by some enzyme histochemical procedures and immunocytochemical techniques. While molar solutions are easy to prepare once the gram molecular weight is known, normal solutions are difficult to prepare without some understanding of chemistry and the valence of elements.

A 1M solution contains 1 g molecular weight of a substance dissolved in enough water to give a final volume of 1 L. The gram molecular weight (formula weight) of a substance is the sum of the combined atomic weights of all atoms in the molecule expressed in grams. For example, the molecular weight of potassium chloride (KCl) is 74.55 (K = 39.10, Cl = 35.45); therefore, a 1M solution contains 74.55 g of KCl diluted to 1 L with distilled water. Most reagents have the formula weight listed on the bottle, but a good handbook of chemistry is an asset to the laboratory.

A 1N solution contains 1 gram-equivalent weight of solute per liter of solution. 1 gram-equivalent weight is that amount of a substance that will combine with or liberate 1 atom, or 1.008 g, of hydrogen. It can also be defined as the molecular weight divided by the number of dissociable or replaceable hydrogen ions. In many instances, the molarity and the normality of a solution are the same, as with hydrochloric acid (HCl) or sodium hydroxide (NaOH); however, in other instances they differ, as with sulfuric acid (H_2SO_4), which has a normality equal to twice the molarity because it has 2 replaceable hydrogen atoms. In other words, 1 molecular weight of sulfuric acid is equal to 98 g and 1 gram-equivalent weight is equal to 49 g, so 98 g of sulfuric acid diluted to 1 L with water is equal to a 1M or 2N solution. The following formulas may be used to determine the weight of a substance needed for a molar solution:

$$\text{weight in grams} = M \times V \times FW$$

where

M = desired molarity

V = desired volume in liters

FW = formula weight (molecular weight), including any water of hydration

Or, for a normal solution:

$$\text{weight in grams} = \frac{N \times V \times FW}{v}$$

where

N = desired normality

V = desired volume in liters

FW = formula weight (molecular weight), including any water of hydration

v = positive valence, or number of dissociable or replaceable hydrogen ions.

Problems

How much of each of the following chemicals is needed to prepare 1,000 mL of both a 1M and a 1N solution (atomic weights: Na = 22.99; Cl = 35.45; Ca = 40.08; S = 32.06; O = 16; H = 1; K = 39.10; C = 12.01; and Al = 26.98)?

25. NaCl

26. $CaCl_2$

27. $CaSO_4$

28. $CaSO_4 \cdot 2H_2O$

29. H_2SO_4

30. K_2CO_3

31. $Al(OH)_3$

Problems

How much solute is needed to prepare the following solutions? (Use the atomic weights given above.)

32. 200 mL of 0.1N H_2SO_4

33. 500 mL of 0.5M CaO

34. 100 mL of 0.2N KCl

35. 200 mL of 0.5N $AlCl_3$

The Metric System

The medical laboratorian must use the metric system of weights and measures, and it may be desirable at times to be able to convert from the US system to the metric system. The major units of the metric system are meter, the measure of length; liter, the measure of volume; and gram, the measure of weight.

Prefixes are used with the basic units to denote subdivisions or multiples [**t5.1**].

One microliter may be written as 1 µL, 1/1,000,000 L, 0.000001 L, or 10^{-6} L; and one milliliter may be written as 1 mL, 1/1000 L, 0.001 L, or 10^{-3} L. Other units may be written in a comparable manner.

In the metric system, cubic centimeters (cc) or milliliters (mL) may be used interchangeably for the expression of volume, although the term "mL" is more frequently used in the United States. In the International System of Units, officially abbreviated SI (Système Internationale) and legally adopted throughout the world, the units of volume depend on the units of length; for example, if the lengths are in meters, the volume will be in cubic meters, or volume = length × length × length. Although liter is not on the SI list of official terms, 1 L is exactly 1,000 cc, and for convenience, the term liter probably will continue to be used in all but the most accurate work, where the official term for liter is cubic decimeter (1 liter = 1 cubic decimeter). At 4°C, 1,000 cc of water weighs approximately 1,000 g. SI units have been adopted as the standard in international medical and scientific literature, so histopathology personnel should be aware of their meanings.

To convert between metric and US systems, common equivalents should be memorized [**t5.2**].

[t5.1] Prefixes used in metric system

Prefix	Denotes
deci- (d)	one-tenth part of
centi- (c)	one-hundredth part of
milli- (m)	one-thousandth part of
micro- (µ)	one-millionth part of (sometimes used as a noun, micron)
nano- (n)	one-billionth part of
kilo- (k)	one thousand times

[t5.2] Unit equivalents between US and metric systems

Unit value	Equivalent
1 lb	454 g
1 m	39.37 in
1 in	2.54 cm
1 L	1.06 qt
1 qt	0.95 L

Conversions may often be calculated by using a ratio, as in the following example:

1 mg = x centigrams (cg)

multiply

1:1,000 = x:100

multiply

Note: 1,000 mg = 1 g, and 100 cg = 1 g, so that both sides are equal

$$1{,}000\,x = 100$$

$$x = \frac{100}{1{,}000} = \frac{1}{10} = 0.1 \text{ cg, or } 1 \text{ mg} = 0.1 \text{ cg}$$

Problems

36. 1 dg = ___ mg

37. 1 cm = ___ mm

38. 1 µL = ___ mL

39. 1 kg = ___ lb

40. 6 in = ___ cm

41. 350 mg = ___ g

42. 5 µm = ___ mm

43. 1 mm = ___ cm

44. 1 mL = ___ cc (careful!)

45. 5 mm = ___ in

46. 1 km = ___ mm

TEMPERATURE CONVERSION

Temperature also is measured on a different scale when the metric system is used. Temperature is reported in degrees Celsius (formerly centigrade) instead of degrees Fahrenheit, and it may be necessary to convert from 1 system to the other. Water freezes at 32°F or 0°C, and boils at 212°F or 100°C. Normal body temperature is 98.6°F or 37°C, the temperature of laboratory incubators and the temperature at which many enzyme reactions are performed. To convert from Fahrenheit to Celsius use the following formulas:

$$°C = \frac{(°F - 32) \times 5}{9}$$

or

$$°C = (°F - 32) \times 0.555$$

To convert from Celsius to Fahrenheit use the following formulas:

$$°F = \frac{°C \times 9}{5} + 32$$

or

$$°F = (°C \times 1.8) + 32$$

Problems

47. Convert –20°C to °F.

48. Convert 4°C to °F.

49. Convert 72°F to °C.

50. Convert 20°F to °C.

Buffers

A buffer is a solution that will maintain a constant pH even after dilution or after the addition of small amounts of acids or bases. Buffers are composed of either a weak acid and a salt of that acid or a weak base and a salt of that base. Examples are the combinations of acetic acid and sodium acetate, boric acid and sodium borate, or potassium monobasic phosphate and sodium dibasic phosphate. Although there are formulas for the preparation of buffers for any given pH, they are beyond the needs of most histopathology laboratories and are not presented. Buffer tables can be found in many of the textbooks on histologic technique.

General Guidelines for Solution Preparation, Use, and Storage

1. Use chemically clean glassware of appropriate type and size.
2. Read the information on the manufacturer's label and the material safety data sheets (MSDSs), and use any precautions indicated when weighing, measuring, or handling the chemical.
3. Label all prepared reagents clearly and immediately. The label should include the name of the reagent, the concentration, the date of preparation, initials of the person preparing the reagent, storage conditions, hazards, and the test or procedure for which the reagent is intended. If the expiration date is known, it should also be included; if not known, the label can read, "Expiration date not determined."
4. Strongly alkaline solutions should not be stored in glass; use polyethylene or polypropylene containers.
5. Keep all solution bottles tightly closed when not in use.
6. Do not pipette directly from stock solutions because of the possibility of introducing contamination; pour reagent into another container for this purpose, and discard any reagent not used.
7. Do not pipette by mouth; use a pipetting aid.
8. Use the appropriate calibrated glassware for measuring liquid chemicals, that is, graduated cylinders and pipettes, *not* flasks and beakers.
9. Mix all solutions very well; use a magnetic stirrer for dissolving dry chemicals, and use an Erlenmeyer flask for mixing liquid reagents. Good mixing can rarely be achieved by using a graduated cylinder.
10. If a magnetic stirrer is not available, to obtain saturated solutions, the solution should be mixed frequently and then allowed to stand overnight before use. Magnetic stirrers are relatively inexpensive, and each laboratory should have one, possibly combined with a hot plate. Every laboratory also should have a pH meter for solution preparation.
11. Examine each solution carefully before use. If the solution shows an unexpected color change or floating debris that might reflect deterioration, contamination, or growth of yeast or molds, the solution should be discarded.
12. Store reagents, especially strong acids, liquid phenol, and other hazardous chemicals, on shelves below eye level.
13. Be very careful when handling reagents to prevent cross-contamination of solution.
14. Use only reagent-grade chemicals for the preparation of solutions.
15. Use dyes that are certified by the Biological Stain Commission.
16. When dyes and chemicals are received in the laboratory, record the following:
    a. date of receipt
    b. date opened

17. The following reagents are among those that should be stored in the refrigerator:

    a. Schiff reagent

    b. Protargol

    c. all solutions containing silver nitrate

    d. buffer solutions

    e. solutions for enzyme histochemistry

    f. osmium tetroxide (seal bottle in a coffee can to prevent discoloration of the inside of the refrigerator).

    g. hydroquinone

    h. glutaraldehyde

    i. aldehyde fuchsin (can be frozen for long-term storage)

    j. paraldehyde (can be frozen for long-term storage)

    k. phosphomolybdic acid

    l. phosphotungstic acid

    m. mucicarmine stock solution

    n. gum mastic, 2.5% (Steiner)

Stability of Solutions

The problem of shelf-life or stability of dry dyes and chemicals and of prepared solutions is one that is constantly faced by personnel in histopathology laboratories. Most of the accrediting agencies require that an expiration date be on the label of all dyes and reagents. Manufacturers now put an expiration date on dry chemicals or on commercially prepared solutions; usually this is much shorter than the true useful shelf-life of the reagent and indicates a minimum length of time that the reagent or dye can be expected to yield maximum results. It is when the solutions are prepared in-house that the question of expiration dates becomes a problem. The best determinant of the condition of all reagents is the result of the procedure as demonstrated on a well-documented positive control slide, but the tabulated information from Churukian [1993] and from Luna [1975] indicating the *minimum* stability of some commonly used staining solutions and other chemicals may be helpful **[t5.3]**. This is presented as a guide only; solutions may vary from the shelf-life indicated because of variations in the temperature of storage and the quality of the distilled water, chemicals, or dyes with which the solutions have been prepared.

References

Churukian CJ [1993] *The Manual of the Special Stains Laboratory of the Department of Pathology and Laboratory Medicine.* 6th ed. Rochester, NY: University of Rochester Medical Center.

Luna LG [1975] Stability of more commonly used special stain solutions. *HistoLogic* 5:68.

Sheehan DC, Hrapchak BB [1980] *Theory and Practice of Histotechnology*, 2nd ed. Columbus, OH: Battelle Press.

[t5.3] Minimum stability of common chemical compounds

Compound	Minimum Stability	Compound	Minimum Stability
Acetic acid, 1% and 3%	6 months	Hematoxylin (Harris)	6 months
Acid alcohol	6 months	Hematoxylin (Mayer)	3 months
Acid fuchsin, 1%	6 months	Hematoxylin (Mallory PTAH)	1 year
Acid fuchsin-picric acid (Verhoeff)	1 month	Hematoxylin, 5% alcoholic	4 months
Alcian blue, 0.5%	2 months	Hematoxylin, Weigert	1 week
Aldehyde fuchsin (Gomori)	2 months	Hydrochloric acid, 1N	1 year
Alizarin red S, 1% (Dahl)	6 months	Lithium carbonate, saturated aqueous	6 months
Aniline blue (Masson)	2 months	Lugol iodine	2 months
Auramine O-rhodamine B (Truant)	3 months	Luxol fast blue (myelin)	1 year
Basic fuchsin, 0.5% (Brown and Brenn)	1 month	Metanil yellow (counterstain)	2 months
Biebrich scarlet-acid fuchsin (Masson)	2 months	Methenamine-silver nitrate, stock (GMS), refrigerate	1 month
Bouin solution	2 months	Methylene blue (Ziehl-Neelsen)	3 months
Carbol-fuchsin (Kinyoun)	1 month	Mucicarmine (Southgate), refrigerate	2 months
Carbol-fuchsin (Ziehl-Neelsen)	2 months	Nuclear-fast red (counterstain)	2 months
Chromic acid, 5% (GMS)	2 months	Oil red O, stock	1 year
Crystal violet (amyloid)	3 months	Oxalic acid, 2% and 5%	2 months
Diastase of malt (PAS with digestion)	1 day	Periodic acid, 0.5% (PAS)	2 months
Eosin Y, alcoholic, stock	4 months	Phosphotungstic acid, 5%	2 months
Eosin Y, alcoholic, working	1 month	Picric acid-acetone (Brown-Brenn)	1 month
Ferric chloride, 2% (Verhoeff)	1 month	Picric acid, saturated aqueous	6 months
Ferric chloride, 10% (Verhoeff)	6 months	Potassium ferrocyanide, 2% (Perl)	1 month
Formalin, 10%	6 months	Potassium metabisulfite, 2%	2 months
Fouchet reagent (Hall)	1 hour	Potassium permanganate, 0.25%	1 week
Giemsa, stock	1 year	Potassium permanganate, 0.5%	2 weeks
Giemsa, working	1 day	Sodium bisulfite, 1% (GMS)	2 months
Gold chloride, 0.2%	1 week	Sodium borate, 5% (GMS)	6 months
Gold chloride, 1%	6 months	Sodium thiosulfate, 2% and 5%	6 months
Gomori trichrome	2 months	Uranyl nitrate, 1.0% (Steiner)	2 months
Gram iodine	1 month	Verhoeff elastic stain	2 hours
Gum mastic, 2.5% (Steiner), refrigerate	6 months	Zenker solution, stock	6 months
		Zenker solution, working	1 month

ANSWERS TO PROBLEMS IN CHAPTER

1. 0.25%
2. 6.4%
3. 4%
4. 0.6%
5. 20%
6. 1.125%
7. 2.75 g potassium metabisulfite
8. 25 g sodium thiosulfate
9. 2.5 g light green
10. 5 mL acetic acid, 495 mL water, 15 g alcian blue
11. 0.75 mL acetic acid, 299.25 mL water, 1.5 g light green
12. 2.5 g silver nitrate
13. 0.75%
14. 50 mL
15. 0.1%
16. 350 mL
17. 10 mL of 10% ferric chloride, 40 mL water
18. 375 mL of 80% alcohol, 125 mL water
19. 700 mL of 95% alcohol, 250 mL water
20. 100 mL of water; a total volume of 200 mL would be obtained
21. 0.2 g of light green
22. 0.93 g of alcian blue
23. 1.16 g of acid fuchsin
24. 1.96 g of alcian blue
25. 58.44 g = 1M; 58.44 g = 1N
26. 110.98 g = 1M; 55.49 g = 1N
27. 136.14 g = 1M; 68.07 g = 1N
28. 172.14 g = 1M; 86.07 g = 1N
29. 98 g = 1M; 49 g = 1N
30. 138.21 g = 1M; 69.11 g = 1N
31. 77.98 g = 1M; 25.99 g = 1N
32. 0.98 g
33. 14.02 g
34. 1.49 g
35. 4.44 g
36. 100 mg
37. 10 mm
38. 0.001 mL
39. 2.2 lb
40. 15.24 cm
41. 0.35 g
42. 0.005 mm
43. 0.1 cm
44. 1 cc
45. 0.195 in
46. 1,000,000 mm
47. −4°F
48. 39.2°F
49. 22.2°C
50. −6.7°C

ANSWERS TO PROBLEMS IN LEARNING ACTIVITIES

1. a. 1.25 g
   b. 500 mL
   c. 10 mL 10% AgNO3, 40 mL H_2O
   d. 190 mL H_2O
2. a. 200 mg
   b. 12.7 cm
   c. 0.01 mL
   d. 4.4 lbs
3. a. −220°F
   b. 21.1°C
4. a. 55.49 g = 0.5M; 27.75 g = 0.5N
   b. 37.28g = 0.5M and 0.5N
   c. 68.07 g = 0.5M; 34.04 g = 0.5N

LEARNING ACTIVITIES

1. **Solve the following problems without referring to the text:**

   a. How much solute is needed to prepare 500 mL of 0.25% light green?

   b. How much solvent is needed to prepare a 0.5% solution from 2.5 gm of acid fuchsin?

   c. Prepare 50 mL of 2% silver nitrate from a 10% solution.

   d. How much water would you add to 10 mL of a 20% solution of silver nitrate to obtain a 1% solution?

2. **Solve the following:**

   a. 2 dg = ___ mg

   b. 5 in = ___ cm

   c. 10 µL = ___ mL

   d. 2 kg = ___ lb

3. **Convert:**

   a. –300°C = ___ °F

   b. 700°F = ___ °C

4. **How much of each of the following chemicals is needed to prepare 1000 mL of 0.5M and of 0.5N solutions (atomic weights: Ca = 40.08, Cl = 35.45, K = 39.10, S = 32.06, O = 16):**

   a. $CaCl_2$

   b. KCl

   c. $CaSO_4$

5. **If you had to refer to the text, go back and study that section carefully, working all problems at the end of the section.**

CHAPTER 6

Nuclear and Cytoplasmic Staining

OBJECTIVES

On completing this chapter, the student should be able to do the following:

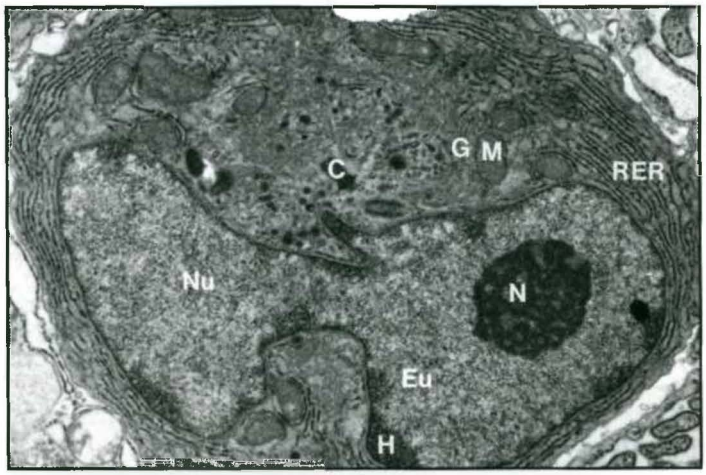
1. Define and give examples of:
   a. absorption
   b. adsorption
   c. mordant
   d. basophilia
   e. acidophilia
   f. ripening
   g. auxochrome
   h. chromophore
   i. polychrome
   j. cationic dye
   k. anionic dye
   l. lake
2. Describe chromatin and differentiate between heterochromatin and euchromatin
3. Describe how cell activity affects the appearance of hematoxylin-eosin (H&E)-stained sections
4. Differentiate between hematin and hematein
5. Differentiate between regressive and progressive staining, and state which of the hematoxylin solutions can be used for each type of staining
6. List 3 methods of differentiation and give examples of each
7. List 4 natural dyes and their sources
8. Identify the ingredients and their purpose in each of the following hematoxylins:
   a. Ehrlich
   b. Delafield
   c. Harris
   d. Mayer
   e. Gill
   f. Weigert
9. List any obvious advantages or disadvantages of each of the hematoxylins listed previously
10. State why iron hematoxylin is the preferred nuclear stain in some staining techniques
11. State how either an excess of acid or an excess of aluminum in hematoxylin solutions can affect nuclear staining
12. Describe the effect of pH on routine H&E staining
13. List 3 factors other than pH that affect staining
14. Describe the effect of overexposure to acidic fixatives or other acidic solutions (eg, decalcification) on staining
15. State how some fixatives such as formalin or Zenker solution affect staining.
16. Identify at least 6 possible sources of error in routine H&E staining, the cause(s), and the appropriate corrective action for each.
17. Identify 3 methods of staining the cytoplasm
18. Identify the 2 types of nucleic acids and give 2 methods of staining the nucleic acids
19. Identify the specific substances stained with the nucleic acid methods
20. Discuss both acid hydrolysis and fixation for the Feulgen reaction
21. List 2 dyes other than hematoxylin that may be used for nuclear staining
22. List 2 methods other than H&E that may be used for staining frozen sections for rapid diagnosis
23. Describe the Romanowsky stains and identify 2 primary uses of these techniques
24. List the 2 major classes of mounting media
25. Identify the advantages and disadvantages of both resins and aqueous mounting media
26. Identify the indications for using aqueous mounting media
27. Describe methods of sealing coverslip edges, and state why coverslip edges may need to be sealed
28. Compare natural and synthetic resinous mounting media
29. Identify 2 common solvents for resinous mounting media
30. Discuss the types and thicknesses of slides and coverslips
31. Identify common coverslipping artifacts
32. State the approximate refractive indexes of aqueous and nonaqueous mounting media, and relate it to the tissue
33. Identify 3 mounting problems and the appropriate corrective action for each

Ultrastructure of the Cell [reproduced with permission: Carson 1979]

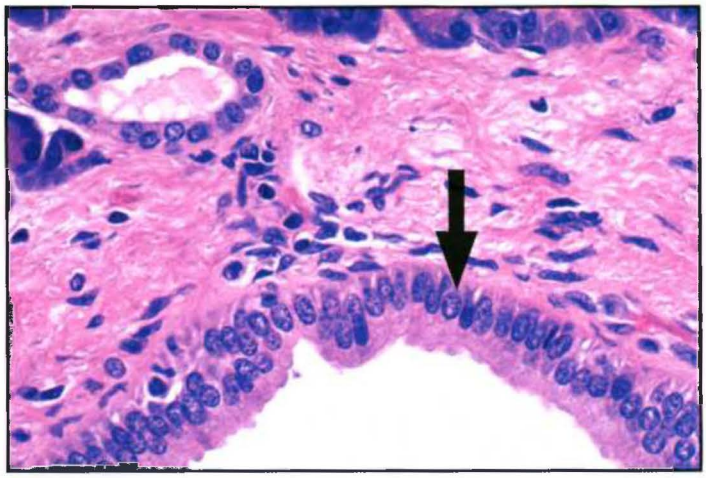
It is appropriate to begin this chapter with a description of the cell so that there can be a better understanding of what is being stained with routine procedures. Although many of the structures that will be described are not visible with the light microscope, it is important that we understand the ultrastructure of the cell, because differences in ultrastructure may change staining characteristics of cells. Any ultrastructural entity that can also be visualized with the light microscope will be illustrated with both light and electron micrographs.

Cells were named by Robert Hooke in 1665 when, with a microscope, he examined the tiny holes in a section of cork. He used the term "cell" because, to him, the thick walls around the holes suggested prison cells. Matthias Schleiden, a German botanist, announced in 1838 that the cell was the basic structural unit of all vegetable matter, and in 1839, Theodore Schwann pointed out that "cells are organisms, and entire animals and plants are aggregates of these organisms arranged according to definite laws." The cell, as we know it today, is a living, moving, and reacting mass of protoplasm; the word *cell* is a general term used for the complex material of which cells are made. A single cell, such as an amoeba, can be self-sufficient and capable of carrying on an independent existence, or cells can be specialized and depend on a highly integrated community life with other cells for their existence.

Cells can be divided into 2 major parts, the nucleus and the cytoplasm. Although these 2 parts are frequently all that can be identified definitively on routine light preparations, many subcellular particles or bodies become visible with the electron microscope **[i6.1]**.



[i6.1] Parts of the cell. C indicates centriole; Eu, euchromatin; G, Golgi; H, heterochromatin; M, mitochondrion; Nu, nucleus; N, nucleolus; and RER, rough endoplasmic reticulum. (Reprinted with permission [Carson 1979])



[i6.2] A row of nuclei (arrow) can be seen in the basal one-third of the simple columnar epithelial cells lining this pancreatic duct. Note that there is an open chromatin pattern in these nuclei, and varying chromatin patterns can be seen in other nuclei present in the section. The stained material of the nucleus is comparable to the heterochromatin seen in the electron micrographs. The nuclear membrane is also well defined in this figure.

THE NUCLEUS

The appearance of the nucleus varies depending on whether the nucleus is resting or undergoing cell division (mitosis). A resting nucleus is more commonly called an interphase nucleus because it is between 2 phases of cell division. Normal tissue will show primarily interphase nuclei that, on H&E-stained sections, appear as discrete, membrane-bound, dark blue to purple (basophilic) masses **[i6.2]**. With the electron microscope, nuclei can be seen to have the following features.

■ *Nuclear Membrane*

The nuclear membrane is an envelope or limiting membrane that can be seen with electron microscopy to be composed of 2 unit membranes separated by a narrow space (perinuclear space). These membranes periodically come together to form a nuclear pore **[i6.3a]**. The nuclear membrane usually is stained a crisp, dark blue with aluminum hematoxylin solutions **[i6.2]**.

■ *Nuclear Pores*

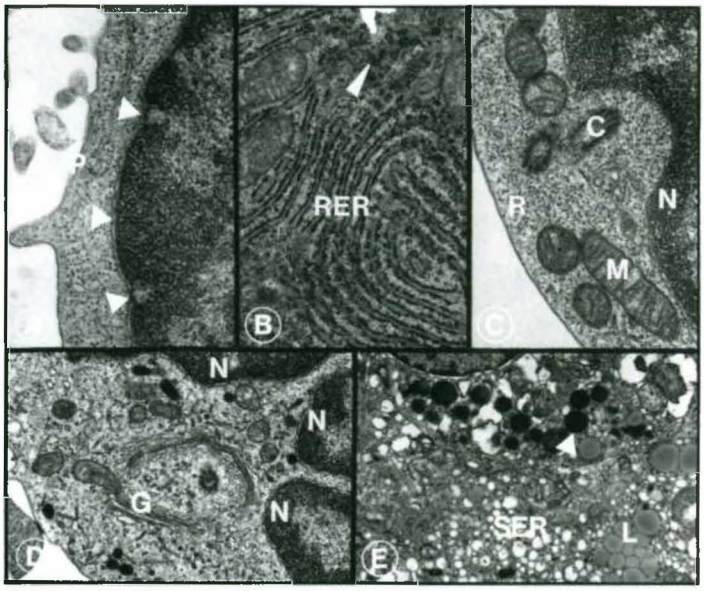
Nuclear pores are small openings or diaphragms in the nuclear membrane that probably are the avenues of communication between the nucleus and cytoplasm of the cell. Nuclear pores can be seen only with the electron microscope **[i6.3a]**.

■ *Nucleolus*

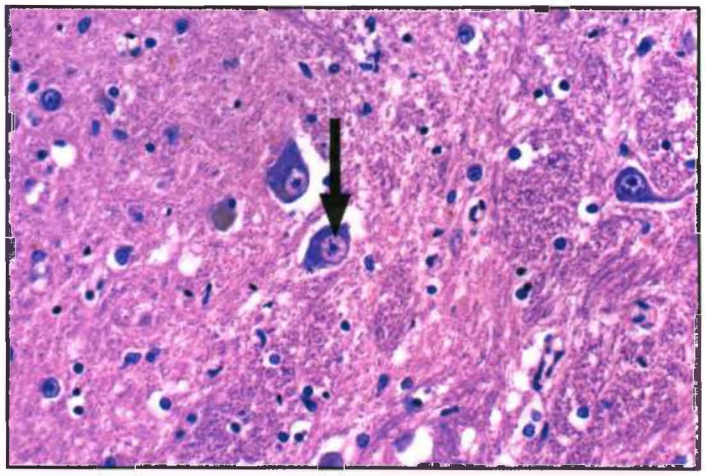
The nucleolus is a dense, rounded, and usually intensely basophilic mass consisting of 80% to 90% protein; however, with a very good H&E stain, the nucleolus will appear acidophilic, or stained with eosin. The nucleolus is shown in an electron micrograph in **[i6.1]** and in the light micrograph in **[i6.4]**. The nucleolus produces most, if not all, of the ribosomal RNA; some very active cells will have several nucleoli within each nucleus.

■ *Chromatin*

Chromatin is used in light microscopy to refer to the stainable substance in the nucleus; however, the nucleus contains both stainable (heterochromatin) and nonstainable (euchromatin) chromatin. Both heterochromatin and euchromatin consist of chromosomes or DNA and attached protein. Heterochromatin contains condensed regions of chromosomes, and is seen as



[i6.3] Ultrastructure of the cell. **A**, The 2 membranes of the nuclear envelope (center arrow) are clearly visible. Nuclear pores (top and bottom arrows) traverse the perinuclear cisterna or space at intervals around the nucleus. The plasmalemma (**P**) is also indicated. **B**, Rough endoplasmic reticulum (**RER**) is extensive in cells synthesizing protein for export from the cell as a secretion. Free polyribosomes or clusters of ribosomes (arrow) are indicative of synthesis of endogenous proteins needed for cell growth and division. **C**, Centrioles (**C**) occur in perpendicularly oriented pairs and most frequently are seen very close to the nucleus (**N**). Free ribosomes (**R**) and mitochondria (**M**) are clearly visualized. **D**, The Golgi complex or apparatus (**G**) plays an important role in the secretory process. **E**, Smooth or agranular endoplasmic reticulum (**SER**) does not have attached ribosomes and has various functions depending on the type of cell in which it is located. Lysosomes (arrow) function as the digestive system of the cell. Lipid (**L**) droplets are frequently seen in the cytoplasm. (Reprinted with permission [Carson 1979])



[i6.4] The nucleolus (arrow) is very prominent in this neuron. It is related to cell activity and may not be seen in some cells. Little chromatin (heterochromatin) is stained, indicating that the cell is very actively engaged in protein synthesis.

intensely basophilic nuclear material with the light microscope **[i6.2]** and as collections of rounded or irregularly shaped dense granules with the electron microscope **[i6.1]**. Genetically active DNA, or the extended portion of chromosomes, is known as euchromatin **[i6.1]** and is not stained with nuclear stains, nor is it visible with the light microscope. The amount of each type of chromatin present is dependent on the activity of the cell. In H&E-stained sections, lymphocytes will exhibit the most intense nuclear staining of all cells because heterochromatin predominates. Because of high activity and the amount of euchromatin present, many neuronal nuclei will exhibit only slight staining with hematoxylin **[i6.4]**. Chromatin patterns can be used effectively to determine when nuclei are overstained or understained. Nuclear staining also may be affected by histones (basic proteins) that are associated with DNA.

THE CYTOPLASM

The amount and appearance of the cell cytoplasm are important criteria for distinguishing different types of cells; it is within the cytoplasm that most of the work ordered by the nucleus is carried out. The labor force for this work consists of organelles, or little organs. While most of these organelles have been observed for many years with the light microscope, a more precise knowledge of the structure and function of organelles has been gained with the electron microscope. Included among the organelles are the following structures.

■ *Plasmalemma*

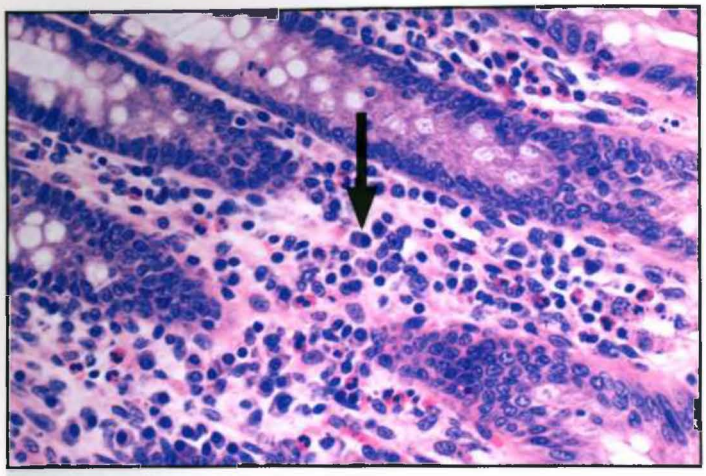
The plasmalemma (plasma membrane) **[i6.3a]** appears in electron micrographs at very high magnification as 2 electron-dense (dark) layers with an intervening electron-lucent (light) layer; therefore, it has been referred to as a trimellar membrane. This same type of membrane is common to all of the membranous cytoplasmic organelles, is a means of compartmentalizing contents or functions, and is known as the unit membrane. The unit membrane consists of a phospholipid bilayer with associated proteins and varies in thickness in different membranous organelles. The plasma membrane is unique because the external surface has a glycoprotein covering called the cell coat or glycocalyx; this asymmetric structure is not found in the other membranous structures. Rarely is the plasma membrane seen on H&E-stained sections, but it is very important in many antigen-antibody reactions.

■ *Mitochondria*

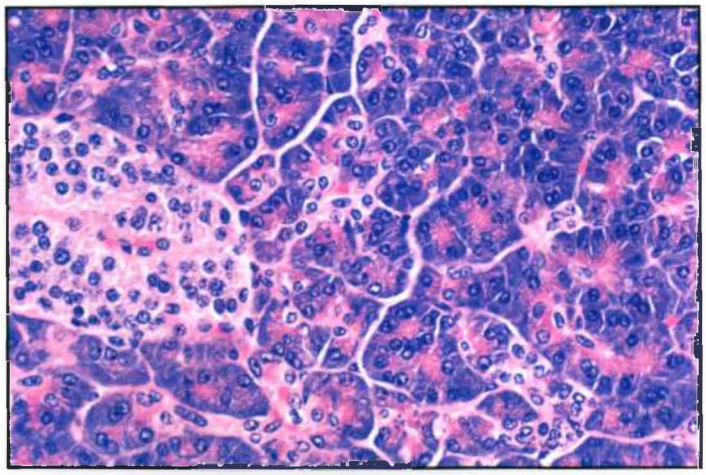
Mitochondria are the energy-producing powerhouses of the cell **[i6.3c]**. These membrane-bound organelles are the site of oxidative reactions by which food energy is made available for the metabolism of the cell. In general, the number of mitochondria in a cell and their internal structure are indicative of the energy necessary to carry out that cell's specific function. Mitochondria are not seen on H&E-stained sections.

■ *Ribosomes*

Ribosomes are the site of protein synthesis **[i6.3c]**. Some ribosomes are attached to the endoplasmic reticulum and are found in greatest abundance in cells that manufacture protein to be released outside the cell. Other ribosomes are free in the cytoplasm, either singly or in clusters, and are concerned primarily with the synthesis of



[i6.5] The basophilia in the cytoplasm of plasma cells (arrow) is the result of rough endoplasmic reticulum, contrasting with the very eosinophilic (acidophilic) granules of the eosinophils. Careful examination of the plasma cells will reveal the area of juxtanuclear "negative" staining at the site of the Golgi apparatus in some of the cells. Eosin has been applied very lightly.



[i6.6] Acinar cells in the exocrine pancreas have very basophilic cytoplasm in the basal part of the cell because of the extensive rough endoplasmic reticulum present. The apical part of the cell becomes very acidophilic because only secretory granules are present. An islet of Langerhans is at left.

protein to be used in the cell. Ribosomes are responsible for the blue tinge or definite blue color seen in the cytoplasm of some cells. With an abundance of free ribosomes, a diffuse cytoplasmic basophilia usually is seen, whereas localized basophilia is usually caused by patches of rough endoplasmic reticulum.

■ *Endoplasmic Reticulum*

Endoplasmic reticulum is a series of membrane-bound channels that are variable in configuration and extent. These channels are pathways for the transportation of secretory products that either are used ultimately by the cell or released externally. There are 2 kinds of endoplasmic reticulum: granular or rough-surfaced **[i6.3b]** and agranular or smooth-surfaced **[i6.3e]**. Granular endoplasmic reticulum is abundant in cells that actively produce secretions to be released outside the cell, and the granular appearance is caused by attached ribosomes; H&E-stained sections show basophilic staining of the cytoplasm of these cells. Examples of this basophilia can be seen in plasma cells **[i6.5]** that produce immunoglobulins and in the basal portion of pancreatic acinar cells, which produce digestive enzymes **[i6.6]**. Some rough endoplasmic reticulum is found in all cells except mature erythrocytes.

■ *Golgi Apparatus*

The Golgi apparatus is the packaging apparatus of the cell. Protein made by ribosomes and delivered by endoplasmic reticulum is concentrated by the Golgi apparatus and then packaged in secretory vesicles for transport to the cell surface **[i6.3d]**. Chemical modification, particularly in regard to carbohydrate content, also takes place in the Golgi apparatus. The Golgi apparatus is usually found close to the nucleus and does not stain with H&E; in very active cells, an unstained (negatively stained) area may contrast with the surrounding basophilic cytoplasm. This unstained area may appear as a nuclear halo and is most apparent in plasma cells **[i6.5]**.

■ *Centriole*

The centriole is responsible for spindle formation in cell division. Centrioles occur in pairs, and commonly there is only 1 pair to a cell; the pair is usually arranged with the long axis of 1 centriole perpendicular to the long axis of the other **[i6.3e]**. Each centriole is a hollow cylinder with the wall composed of 9 evenly spaced bundles of tubules. The basal body of a cilium is identical in structure to a centriole, and a cell produces multiple centrioles in preparation for cilia formation. Centrioles cannot be seen on H&E-stained sections.

■ *Lysosomes*

The lysosomes are part of the defense mechanism of the body. Lysosomes also aid in digestion of food taken into the cell. The lysosome surrounds material to be digested, the membranes fuse, and the contents mix without releasing hydrolytic enzymes into the cytoplasm. By the same process, lysosomes help the cell to rid itself of worn-out parts; thus, lysosomes have been called "suicide bags." Undigestible compounds from this process are retained within vacuoles in the cell cytoplasm and are known as residual bodies. Long-lived or permanent cells such as neurons, cardiac muscle, and hepatocytes accumulate a large collection of residual bodies; these accumulations are referred to as lipofuscin, or wear-and-tear pigment, which appears as a yellow to brown pigment through the light microscope.

The cytoplasm may also contain inclusions such as secretory granules or vesicles (discussed in relation to the Golgi apparatus), pigments, or food such as fat **[i6.3e]** or carbohydrates that have been stored for future use. Pigments are usually classified as endogenous (produced inside the body) and exogenous (produced outside the body and subsequently gaining entrance). Carotene, dusts, and minerals are exogenous pigments; melanin and hemoglobin-breakdown products are endogenous pigments.

Staining Mechanisms

In the past, there was a great deal of concern over whether staining reactions were physical or chemical. Today it is recognized that most reactions involve both physical and chemical factors. The fat stain is an example of a purely physical stain, with the dye absorbed (soaked up) by, and dissolved in, the lipid. However, most stains depend on adsorption of the dye, or the attraction for minute particles from the surrounding solution, by the surface of certain tissue components; the dye is then bound to the tissue primarily by ionic, covalent, or hydrogen bonds.

Ionic or electrostatic bonding occurs when the dye and the substance to be dyed develop different charges and thus become attracted to each other. For example, we can stain the cytoplasm by developing a positive charge on the cytoplasmic proteins and a negative charge on the dye. This type of binding is also referred to as salt linkage.

Hydrogen bonding occurs when covalently bonded hydrogen is attracted to atoms that have a strong electronegative charge. Frequently such bonding occurs between hydrogen and oxygen or hydrogen and nitrogen. Hydrogen bonds are weak and occur naturally in water; they may form between the dye and the water in which it is dissolved. Water also competes for hydrogen bonding sites in tissue, so such bonding is probably not important in most aqueous-based staining reactions [Culling 1995].

Covalent bonding occurs when atoms share electrons. This type of bond is typical of organic chemicals, because carbon, hydrogen, and oxygen commonly form covalent bonds. In the water molecule for example, 1 oxygen atom shares 2 electrons, 1 with each of 2 hydrogen atoms. Each hydrogen atom also shares an electron with the oxygen atom.

Van der Waals forces are caused by the electrostatic attraction of a molecule to the electrons of its neighboring molecules. These are weak physical forces that are effective over only very short distances.

NUCLEAR STAINING

Nuclear staining is not fully understood, but apparently occurs through 2 different mechanisms [Lillie 1976]

1. staining done with the basic (cationic or positively charged) dyes
2. staining done with dyes combined with, or followed by, metal mordants

The first mechanism depends on the presence of the nucleic acids (DNA and RNA) to form dye salt-type unions. The second type of staining occurs in tissues from which the nucleic acids have been removed (eg, decalcified tissue), and also may occur in tissue that is not negatively charged. Some fixatives split off the protein attached to the phosphate groups of the nucleic acids and leave DNA soluble. In this case only basic nucleoprotein remains in the nucleus, yet the nucleus still stains.

Lillie and Fullmer [1976] further suggest that although the term basophilic (base-loving) may be applied properly to acidic (anionic or negatively charged) tissue substances that are readily stained with basic dyes, the term is not proper for the metal-mordant type of staining. The metal-mordant dyes stain many of the same tissue elements as the basic dyes; however, under some circumstances, the metal-mordant dyes stain substances lacking acidic groups, such as myelin and neutral mucopolysaccharides. Lillie and Fullmer [1976] also state that stainability with the aluminum hematoxylins should not be indicated with the term *basophilia*; instead, they propose that a more appropriate term would be *metallophilia* to indicate tissue characteristics that induce staining with metal-mordant complexes.

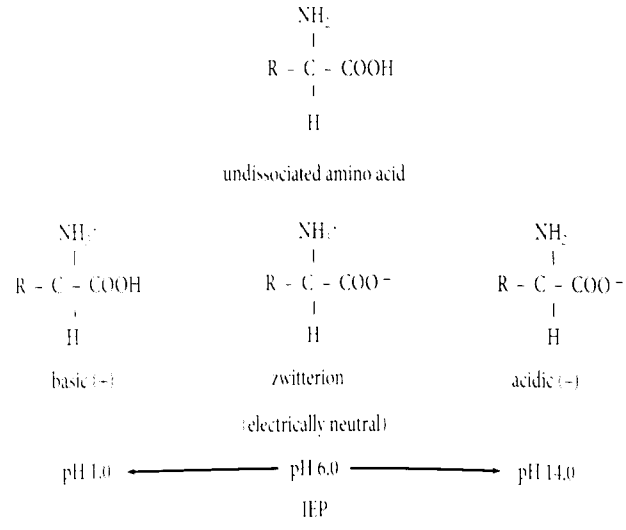
Sheehan and Hrapchak [1980] propose 2 possible binding sites for the hematoxylin-aluminum complex; the phosphate groups of DNA and the histones that are cationic nuclear proteins bound by the phosphate groups. Meloan and Puchtler [1987] state that molecular models show binding by the phosphate groups to be sterically impossible, and agree with Lillie [1965] and Sheehan and Hrapchak [1980] that staining of nuclei with metal-hematoxylins after DNA extraction indicates dye binding by cationic nuclear proteins. Meloan and Puchtler [1987] also propose that during the formation of hemalum, 2 hematein ions form chelates with 1 metallic ion, and because aluminum, iron, and chromium hemateins are anionic (–) chelates, reactions with the cationic components of the nucleus seem probable.

It appears that various forces are involved in the binding of metal-hematein by nuclei, and that a simple explanation is not possible. Ionic and hydrogen bonding as well as van der Waals and hydrophobic forces probably all have some role in the staining of nuclei by aluminum-hematein solutions; a complete understanding of the reaction must await further studies.

CYTOPLASMIC STAINING

Fortunately, the routine staining of nonnuclear elements is understood much better than nuclear staining; nonnuclear staining is primarily caused by proteins or charged groups on the side chains of amino acids constituting the proteins.

Proteins, or polymers (chains) of amino acids, contain a terminal amino ($-NH_2$) group on 1 end and a terminal carboxyl (–COOH) group on the other; in addition, amino acid side chains may have $-NH_2$ or –COOH groups. Because of these 2 groups, proteins may be positively (+) or negatively (–) charged. This charge is pH-dependent, and because proteins can carry either positive or negative charges, they are said to be amphoteric [**f6.1**].



[f6.1] The effect of pH on the amino acids of proteins.

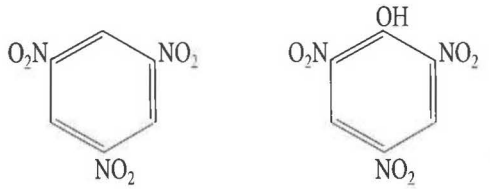
Based on its net charge, a substance migrates in an electrical field; a protein with a net positive charge (more positive than negative charges) migrates to the cathode (–), and a protein with a net negative charge migrates to the anode (+). At the point where the positive and negative charges are equal, there is no migration. This is termed the *isoelectric point* (IEP). To understand the staining of nonnuclear elements, one must understand how pH affects the ionization of proteins and how pH relates to the IEP.

The IEP of proteins is approximately pH 6; below the IEP or below pH 6, the net charge on the nonnuclear proteins is positive and the attraction is for an anionic dye; above the IEP, the net charge is negative and the attraction is for a cationic dye. Substances attracting basic dyes are said to be "basophilic," and substances attracting acid dyes are "acidophilic." This means that if tissue sections are placed in a solution with a pH below 6, the cytoplasmic proteins will develop a predominance of positive charges and will then attract a negatively charged dye such as eosin. Although eosin is negatively charged over a wide pH range, if the pH of the dye solution is dropped too low, the –COO group of eosin recombines with hydrogen and the result is the free acid, uncharged form of eosin. The dye is now uncharged and only nonspecifically stains the tissue. If the eosin solution is not below pH 6, the eosin will not attach to the cytoplasmic proteins because above pH 6, the proteins will have a net negative charge.

The Dyes

When we discuss dyes, we must answer 2 questions: what makes a substance a dye or colored, and why is there an affinity between the dye and the element being dyed. All dyes are organic compounds, and most are coal tar or benzene derivatives. Readjustment of the double bonds results in the formation of a colored compound when 2 hydrogen atoms in the benzene ring are replaced either with oxygen or with another atom or group having 2 valency bonds instead of 1. In the past, formation of the quinoid ring was thought to be responsible for the development of color, but according to Puchtler [1986], chemists have disproved this theory. Benzene would have color if our eyes were sensitive in the UV range, but certain modifications to the benzene ring push the absorption band into the visible spectrum. A group that confers the property of color is called a *chromophore*. The fundamental groups involved in chromophores are C=C, C=O, C=S, C=N, N=N, N=O and NO_2; the more of these that occur in the same compound, the more pronounced the color. Chromophores differ greatly from one another; however, they have 1 common property: they are easily reduced because they all have an unsatisfied affinity for hydrogen. If reduction occurs, the chromophore is destroyed and color is lost. A benzene derivative containing chromophoric groups is called a *chromogen*.

Although compounds containing chromophores (chromogens) are colored, they may or may not be able to act as a dye or combine with the substance to be colored. An ionizing group called an *auxochrome* is required to enable the dye to link firmly to the tissue. The fundamental basic auxochrome is the amino ($-NH_2$) group; aniline contains this group, and many of the dyes are aniline derivatives. The usual acidic group is the sulfonic acid ($-SO_3H$) group, and other acidic auxochromes are the carboxyl (–COOH) and hydroxyl (OH) groups, ionized to produce negative charges ($-SO_3^-$, $-COO^-$, and $-O^-$). The sulfonic group is a weak auxochrome but functions to make a dye water soluble or to change a dye from basic to acidic. Trinitrobenzene and picric acid are examples of chromophores and auxochrome.



Trinitrobenzene (a chromogen) | Picric acid (a dye)

In the above compounds, the $-NO_2$ group is the chromophoric group, and trinitrobenzene possesses 3 chromophores and is yellow, but it is not a dye. Picric acid (trinitrophenol) also possesses the same 3 chromophores but in addition possesses an auxochromic grouping (–OH). Because of the auxochromic group, it is an anionic, or acid, dye.

The application of the terms *acid* and *basic* to dyes can be confusing, because when referring to dyes, these terms have nothing to do with pH. A basic dye is one in which the charge on the dye ion is positive; these dyes are more properly called *cationic* dyes, and the auxochrome is the amino group ($-NH_2$). Acid dyes are those with a negative charge and are more properly referred to as *anionic* dyes. As previously stated, the usual anionic auxochromes are the sulfonic, carboxyl, and hydroxyl groups. Basic, or cationic, dyes most frequently are chloride salts; acidic, or anionic, dyes most frequently are sodium salts. Most dyes remain cationic or anionic throughout the range of acidity or alkalinity within which most staining occurs, usually pH 3 to 9; however, some dyes are amphoteric and are cationic if the pH is below the IEP, and are anionic if the pH is above the IEP. Crystal violet and safranin are typical basic dyes; orange G and picric acid are typical acid dyes; hematein and lithium carminate are amphoteric dyes, with IEPs of about 6.6 and 4.5, respectively [Baker 1958].

Lipid stains are exceptions: they are nonionic compounds incapable of electrolytic dissociation, and although they have color, they are not really dyes. These stains sometimes have been classified as neutral dyes; they are insoluble in water but soluble in some organic solvents, and they color certain tissue components by dissolving in them (physical staining).

Dyes are classified by the chromophore present, but the classifications are beyond the scope of this chapter. Examples of dye classes are triarylmethanes, anthraquinones, xanthenes, and azines. For extensive information on the classification of dyes, the interested reader is referred to the work of Lillie [1965].

Dyes have been used since the dawn of civilization and were all of vegetable or animal origin until the middle of the last century, when natural dyes were almost totally eclipsed by synthetic dye products. Only a few natural dyes have been used in histotechnology to any extent, and today some of these dyes can be even prepared synthetically. The more important natural dyes are carmine, orcein, saffron, and hematoxylin. Carmine is a purified extract of the female tropical cochineal insect; orcein is obtained from lichens, saffron from the pistils of a flowering plant, and hematoxylin from the heartwood of the logwood tree.

FACTORS AFFECTING DYE BINDING

1. The pH of the solution determines whether a dye will bind to certain tissue elements by establishing the appropriate charges on both the tissue element and the dye molecule.

2. An increase in temperature will increase the rate of staining by increasing the diffusion rate of the dye molecules. Swelling of tissue components caused by the increase in temperature is probably an important factor in dye penetration.

3. Dye binding usually increases with an increase in concentration of the dye molecules.

4. Salts other than the dye dissolved in the staining solution can decrease or increase the staining intensity of certain tissue components; probably salt ions and dye ions compete for the same binding sites.

5. By reacting with certain chemical groups in tissue and making those groups unavailable for dye binding, the fixative alters the staining character of the tissue. Some fixatives such as formaldehyde react with the amino (NH_2) group. Because this is the primary group for binding eosin, tissue fixed in formalin will bind less eosin than when fixed in some of the other solutions. Tissue fixed in solutions containing potassium dichromate will tend to take up less hematoxylin and more eosin, because the reaction of potassium dichromate is primarily with the carboxyl (–COOH) and hydroxyl (–OH) groups. Tissue overexposed to acidic fixatives such as Zenker solution, Bouin solution, or unbuffered formalin will lose its basophilic (nuclear) staining properties. In general, formaldehyde, mercuric chloride, and osmium tetroxide increase tissue basophilia or the uptake of cationic or positively charged dyes. Picric acid increases the binding of anionic or negatively charged dyes, and ethyl alcohol is intermediate between these 2 groups.

DIFFERENTIATION

Many dyes, including most of those used in counterstains or cytoplasmic stains, are used progressively, that is, once the desired intensity of color is achieved, the reaction is stopped. Mordant dyes also may be used progressively, but frequently regressive staining is required. *Mordants* are substances or metals that act as a link between dye and tissue. The mordant combines with the dye to form a "lake" that is usually basic in action. In regressive staining, the tissue is overstained and then differentiated, or decolorized, until only the desired element is left stained. Many of the mordant dyes are, or must be, used regressively to achieve differential staining of the desired structure. There are 3 methods of differentiating sections in the histopathology laboratory when mordant dyes are used regressively.

1. Basic, or cationic, dyes are differentiated by weak acid solutions, and acidic, or anionic, dyes are differentiated by weak alkaline solutions. For example, the aluminum hematoxylins can be differentiated with a dilute solution of hydrochloric acid, and eosin can be removed from overstained sections with a dilute solution of ammonium hydroxide. If the differentiating solutions are prepared in alcohol rather than water, better control of differentiation is possible.

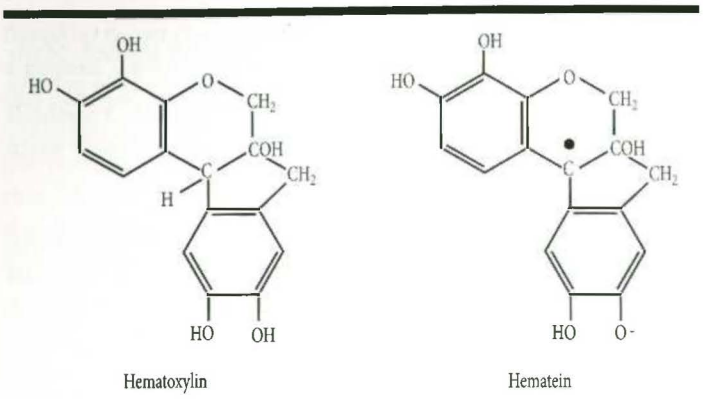
2. Excess mordant will break the tissue-mordant-dye complex. Because the amount of mordant in the differentiating solution is large compared with that bound to tissue, the dye will dissolve from the tissue. The structures that have bound the most dye will be the last to completely lose color. Careful control of the differentiating process will leave the desired structures well stained and the background colorless. Sections stained with regressive iron hematoxylin methods are differentiated with excess mordant. Iron hematoxylin is also the nuclear stain of choice in many special stains that use acidified solutions after nuclear staining. Iron hematoxylin is fairly resistant to decolorization with acid; aluminum hematoxylin is not.

3. Although oxidizers presumably work by oxidizing the dye to a colorless substance, the effect is that of differentiation, because those substances containing the most dye will still remain colored if the oxidation process is stopped at the appropriate time. Potassium permanganate and chromium trioxide have been used as oxidizing differentiators. Potassium ferricyanide is used in the Weil myelin stain as the second differentiating solution.

Sheehan and Hrapchak [1980] list buffers as a fourth method of differentiation, one rarely used in the histopathology laboratory. The best example of this method of differentiation is the staining of blood smears with the Wright stain. The staining solution is buffered to a pH of approximately 6.5; at this pH, the buffer solution will act as a basic differentiator of the eosin and as an acidic differentiator of the methylene blue components of the stain.

THE NUCLEAR DYES

Because the mordant dyes are those used in what we consider routine nuclear staining, those will be described in depth in this section. Hematoxylin, the most widely used nuclear stain, is extracted from logwood (also known as campeachy wood). *Haematoxylon campechianum* is a tree that is indigenous to Central America, but has been scientifically cultivated in Jamaica since 1715. The freshly cut wood is colorless but becomes dark reddish-brown when exposed to atmospheric oxidation; the oxidized dye is *hematein*. As pointed out by Baker [1958], the second "e" in the word "hematein" distinguishes the oxidized dye from the unrelated hematin deposits such as formalin pigment or acid hematin. Hematin is pronounced with 3 syllables and hematein with 4.



[f6.2] Hematoxylin and hematein structures. Two hydrogen atoms are removed during the oxidation of hematoxylin, leaving a negatively charged phenolate-O-group and a coordinately unsatureated carbon atom (dot). [Adapted with permission from Meloan 1987]

Hematoxylin was introduced as a dye in England during the reign of Queen Elizabeth I, but in 1580, it was banned by Parliament because of stubborn opposition from the older school of dyers who felt that it was not colorfast. It was about 100 years later that the true value of hematoxylin was appreciated and the law was repealed [Leggett 1944]. According to Baker [1958], the first report of its use in microtechnique appeared in the 1840s. Synthetic hematoxylin is also available, but most laboratories still use solutions prepared from the natural product.

Hematoxylin is not a dye; hematein, the oxidation product of hematoxylin, is a weak anionic dye. Puchtler [1986], stating that chemists have disproved the traditional quinoid formula of hematein, proposed different structural formulas for hematoxylin and hematein **[f6.2]** from those traditionally found in the literature.

Oxidation of hematoxylin is necessary and may be achieved naturally by exposing the solution to atmospheric oxygen, or by using oxidizing agents such as sodium iodate, mercuric oxide, and potassium permanganate; this oxidation process is also called ripening. Delafield and Ehrlich solutions are naturally ripened, while Harris, Mayer, and Gill solutions are chemically oxidized. Solutions should always contain some unoxidized hematoxylin because the process of ripening continues with atmospheric oxidation, and complete oxidation or overoxidation lead to a breakdown of the solution and the loss of good staining. The rate of oxidation is also influenced by the pH of the solution; hematein forms rapidly in alkaline solutions but more slowly in acidic solutions.

Oxidized hematoxylin (hematein) has little affinity for tissue but becomes a strong dye with a particular affinity for nuclei when combined with a metallic mordant. Traditionally, metal-hematein lakes have been considered basic, or cationic, dyes. Meloan and Puchtler [1987] state that the hematein-metal chelate is anionic, and the misleading hypothesis that metal-hematein chelates are cationic was based on the ability of these chelates to stain nuclei. However, many investigators believe that the metal-hematein lake is cationic and binds with the phosphate group of the nucleic acids. As indicated previously, the staining of nuclei by metal-hematein chelates is very complex and not yet fully understood.

In some solutions of hematoxylin, the oxidizer also serves as the mordant; these solutions, most commonly the iron hematoxylins, are not stable. To achieve stability, the mordant should not oxidize the solution. Ammonium or potassium aluminum sulfate, phosphotungstic acid, and phosphomolybdic acid are in this category of nonoxidizing mordants. The mordant-dye combination is called a *lake*, and the most commonly used hematoxylin lakes are combinations of hematein with either aluminum or iron. The term *alum* is frequently misused in histotechnology; alums are double sulfates such as potassium aluminum sulfate, ammonium aluminum sulfate, ferric ammonium sulfate, or chromium potassium sulfate. The use of terms such as *ferric alum* or *iron alum* should be avoided; students have no idea of the true nature of the compound, and if preparing solutions in the laboratory, they frequently have trouble trying to find the correct dry chemical. The routine nuclear stains should be called *aluminum hematoxylins* or, more properly, *aluminum hemateins*, because aluminum is the mordant. A double sulfate is not required as a mordant; aluminum sulfate alone is used to prepare Gill hematoxylin.

More selective nuclear staining can be achieved by adding either an excess of acid or an excess of aluminum [Vacca 1985]. The H^+ of the acid will combine with weakly acidic groups in the tissue sections and prevent them from taking up hematoxylin; an excess of protons (H^+) in the solution will cause selective binding of the dye to the nucleus, and differentiation becomes unnecessary. Excess aluminum added to aluminum hematoxylin solution will counteract overoxidation by chemical oxidizers. However, too much aluminum or improperly dissolved aluminum salts can precipitate on top of the tissue, giving a crystalline artifact [Thompson 1978]. Formulas for some of the aluminum hematoxylins follow.

Harris Hematoxylin

Hematoxylin	5 g
Absolute ethyl alcohol	50 mL
Ammonium aluminum sulfate	100 g
Distilled water	1,000 mL
Mercuric oxide	2.5 g

The original formula for Harris hematoxylin uses mercuric oxide; today sodium iodate (0.37 g) [Vacca 1985] is used to replace the very toxic mercuric salt. To prepare the solution, dissolve the hematoxylin in alcohol (it may be necessary to add about 20 mL of water to completely dissolve the hematoxylin), and dissolve the ammonium aluminum sulfate in the water with heat. Remove from heat and carefully mix the 2 solutions. Bring to a boil as rapidly as possible; remove from the heat and slowly add the sodium iodate. Reheat to boiling, and boil for 2 to 3 minutes or until the solution becomes dark purple. Remove from heat, and immediately plunge the vessel into a basin of ice. The stain is ready for use as soon as it cools or as soon as a metallic sheen develops on the surface of the solution. Filter just before use, and add glacial acetic acid to give a final concentration of 4% (4 mL of glacial acetic acid for every 96 mL of hematoxylin).

The mordant in this solution is aluminum and the chemical ripening agent (oxidizer) is sodium iodate (mercuric oxide in original formula). Sodium iodate is the oxidizer found in most commercial preparations. According to Meloan and Puchtler [1987], Harris intended that his hematoxylin solution be used acidified and/or diluted as a progressive stain and did not mention differentiation. The use of Harris hematoxylin as a regressive stain was introduced later. Meloan and Puchtler [1987] further state that because there is no definite end point in the differentiation process, the results will be variable, whereas the acidified aluminum hematoxylins will yield consistent staining results, even in the hands of beginners. We find that this solution used progressively for 1 to 3 minutes will give good, consistent nuclear staining. Culling [1978] recommended acidifying the solution with 1% hydrochloric acid to a pH of 1.0 to 1.2 to achieve very selective nuclear staining.

Delafield Hematoxylin

Solution a—saturated ammonium sulfate	
Ammonium aluminum sulfate	180 g
Distilled water	1,000 mL
Dissolve with the aid of heat and allow to cool. When cool, some of the alum will crystallize and settle to the bottom of the flask; the supernatant will be saturated.	
Solution b—hematoxylin	
Hematoxylin	4 g
Alcohol, 95%	25 mL
Dissolve the hematoxylin in the alcohol.	
Mix as follows:	
Solution a (saturated ammonium aluminum sulfate)	400 mL
Solution b (hematoxylin)	25 mL
Expose the solution to sunlight and air in a clear, cotton-plugged bottle for 3 to 4 days, then filter and add:	
Glycerol	100 mL
Ethyl alcohol, 95%	100 mL
Age for 3 to 6 months in light and then test the solution's readiness for use.	

The mordant in Delafield hematoxylin is aluminum, and oxidation occurs naturally with exposure to light and air. Alcohol functions principally as a preservative, and glycerol stabilizes the solution against overoxidation and also aids in preventing rapid evaporation, according to Sheehan and Hrapchak [1980]. These authors suggest that several tests can be used to determine whether the aluminum hematoxylin solutions are ready for use. Good hematoxylin has a winelike smell and a deep purple-red color. When a few drops of the solution are dropped into a container of tap water, a bluish-black color should result; overoxidized and underoxidized solution will be red to red-brown. A drop of aluminum hematoxylin solution placed on filter paper will show a maroon spot with a purple edge if the solution is ready for use; absence of the purple border indicates that the stain is underoxidized and is not ready to be used. Delafield hematoxylin is usually used regressively.

Mayer Hematoxylin

Hematoxylin	1 g
Distilled water	1,000 mL
Sodium iodate	0.2 g
Ammonium or potassium aluminum sulfate	50 g
Citric acid	1 g
Chloral hydrate	50 g
Dissolve the hematoxylin in the water, heat the solution to boiling, and boil for 5 minutes. Remove from heat, and as soon as the boiling stops, add the sodium iodate. Let ripen for 10 minutes. Add the remainder of the reagents in order, making sure each one is dissolved completely before adding the next.	

Aluminum is the mordant, and sodium iodate is the oxidizer in Mayer hematoxylin. Citric acid is added to adjust the pH and, along with the chloral hydrate, helps prevent the scum and precipitates that tend to form in aluminum hematoxylin solutions. Sheehan and Hrapchak [1980] state that this solution will keep for a long time without overripening, and Vacca [1985] agrees; however, Lillie and Fullmer [1976] indicate a shelf-life of approximately 2 to 3 months for Mayer hematoxylin, and we find this to be accurate. This is the recommended solution for use in immunoperoxidase techniques when 3-amino-9-ethylcarbazole is used as a chromogen, because Mayer hematoxylin does not contain alcohol; alcohol will dissolve the reaction product. Routine nuclear staining may be slower with this formula than with some of the other solutions, but it is very difficult to overstain the sections, and very crisp nuclear staining can be achieved. The solution is used progressively.

Ehrlich Hematoxylin

Hematoxylin	2 g
Alcohol, 95%	100 mL
Distilled water	100 mL
Glycerol	100 mL
Ammonium or potassium aluminum sulfate	3 g
Glacial acetic acid	10 mL
Dissolve the hematoxylin in the alcohol, and then add the other ingredients. Expose to the air in a cotton-plugged bottle for 2 weeks or longer to ripen. If desired, 0.4 g of sodium iodate may be added to cause immediate ripening.	

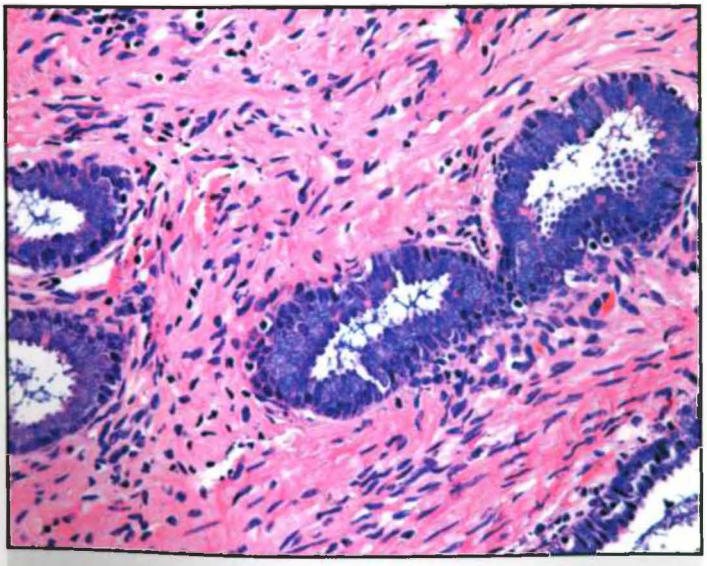
Aluminum is the mordant in Ehrlich hematoxylin, which may be ripened either naturally or chemically. Although this hematoxylin will give a very sharp nuclear stain and may be used progressively, it is more commonly used regressively.

Gill Hematoxylin

Distilled water	730 mL
Ethylene glycol	250 mL
Hematoxylin, anhydrous	2 g
Sodium iodate	0.2 g
Aluminum sulfate, $Al_2(SO_4)_3 \cdot 18H_2O$	17.6 g
Glacial acetic acid	20 mL

Ethylene glycol is an excellent solvent for hematoxylin, and it prevents the formation of surface precipitates. Sodium iodate is the oxidizer, and aluminum is the mordant. If crystalline hematoxylin is used instead of anhydrous, then 2.36 g must be used. The sodium iodate must be weighed accurately to ±0.01 g. The stain can be used immediately, but provides a better intensity if allowed to ripen for 1 week in a 37°C incubator. The solutions used by Gill [1974] have found wide acceptance and are marketed commercially in 3 different strengths, depending on whether the solution is to be used for cytology or histology. Gill II and Gill III are used for staining tissues. Gill III is the most concentrated stain and can be used for staining glycol methacrylate sections. Gill II may be prepared by using the Gill I formula and doubling the amount of hematoxylin and sodium iodate and quadrupling the amount of aluminum sulfate used.

Mucin, especially in goblet cells, will be stained by Gill hematoxylin, but not by the other hematoxylin solutions. This fact may be important in selecting the hematoxylin to be used for the routine H&E stain **[i6.7]**.



[i6.7] A section of cervix stained with Gill hematoxylin. The mucin is stained blue, a characteristic of staining with Gill hematoxylin.

Many other formulas have been proposed for aluminum hematoxylin solutions, but the formulas given above are most commonly used today. However, the user of commercial products should read labels for chemical content carefully because several companies have modified the original formulas. Most hematoxylin solutions are only moderate health risks if mercuric oxide is not included in the preparation; with mercuric oxide-containing solutions, the solutions must be saved and disposed of appropriately. The material safety data sheets also should be requested, and referred to, for each commercial solution.

On standing, most hematoxylin solutions will develop a metallic sheen of oxidized dye, actually aluminum-hematein, and should be filtered before use. If the solutions are not filtered, a blue-black precipitate may be seen on the stained sections. Mayer and Gill hematoxylins are not as prone as other hematoxylins to form the surface sheen. The acetic acid added to some solutions will help prevent the formation of this scum by retarding oxidation. The color of the solution is an indicator of the freshness of the aluminum mordant; bluish solutions are seen with fresh mordant, but the color changes toward red as the solution ages [Sheehan 1980]. Overoxidation, resulting in the formation of oxyhematein, will cause the color to become brown, and nuclei stained with this type of solution will be brown rather than blue.

Although more consistent results are obtained with progressive staining, some laboratories prefer to use a regressive hematoxylin staining procedure. When using hematoxylin regressively, the sections are markedly overstained, and then hematoxylin is removed by a process known as differentiation. Commonly this is done with weak hydrochloric acid solutions prepared with water or alcohol, but also may be done with acetic acid solutions. Although the differentiation of hematoxylin is not completely understood, there probably is competition between the dye-mordant complex and the hydrogen ions of the acid for anionic binding sites in the nucleus. Because of differences in thickness of sections, types of tissue, and individual timing, regressive staining will rarely give consistent staining results. The use of automation has improved the consistency of staining to a great degree.

Scott Solution

Magnesium sulfate ($MgSO_4$)	10 g
or ($MgSO_4 \cdot 7H_2O$)	20 g
Sodium bicarbonate	2 g
Tap water	1,000 mL

After nuclear staining with hematoxylin solutions and differentiation if indicated, the sections are blued. This is done with solutions that are weakly alkaline; dilute lithium carbonate, ammonium hydroxide, or Scott solution are frequently used blueing agents. Running alkaline tap water also can be used. Scott solution is a gentle alkaline substitute for tap water and is prepared as shown above.

The change in pH induced by the blueing agent changes the solubility of the dye lake. The aluminum-hematein complex is red and soluble below pH 5; blueing converts the red, soluble complex to a more desirable blue lake that is insoluble in the usual staining solutions.

Weigert Hematoxylin

Solution a	
Ferric chloride, 29%	4 mL
Distilled water	95 mL
Hydrochloric acid, concentrated	1 mL
Solution b	
Hematoxylin	1 g
Alcohol, 95%	100 mL

*Mix equal parts of solution **a** and solution **b** for use. This solution can be used for 2 or 3 days, but do not try to use it longer.*

Occasionally, iron hematoxylin is used as a nuclear stain. It is not used in the routine H&E stain, but is used as a nuclear stain in many of the nonroutine techniques because it resists decolorization in acidic staining solutions. Ferric chloride is a strong oxidizer, so it serves both as mordant and oxidizer for Weigert hematoxylin, the most widely used nuclear stain employing iron as the mordant.

Celestine Blue

Celestine blue	1 g
Ferric ammonium sulfate	4 g
Distilled water	200 mL

Dissolve the celestine blue in 100 mL of the water, and the ferric ammonium sulfate in the other 100 mL of water. Be sure that the ferric ammonium sulfate is completely dissolved, waiting overnight if necessary; mix the 2 solutions. The solution should be dark blue, and after filtering, it is ready for use.

Celestine blue may be substituted for hematoxylin in the H&E procedure and gives identical results. During the hematoxylin shortage in the 1970s, this dye was widely used as a substitute. For staining, a dye-mordant lake is formed, with iron serving as the mordant. The solution is used progressively for 5 to 30 minutes.

As a substitute in the iron hematoxylin methods, gallein may be used instead of celestine blue. Safranin, nuclear-fast red, methylene blue, thionin, and toluidine blue O also may be used to stain nuclei, but most frequently they are used as counterstains in special staining methods or in the rapid staining of frozen sections.

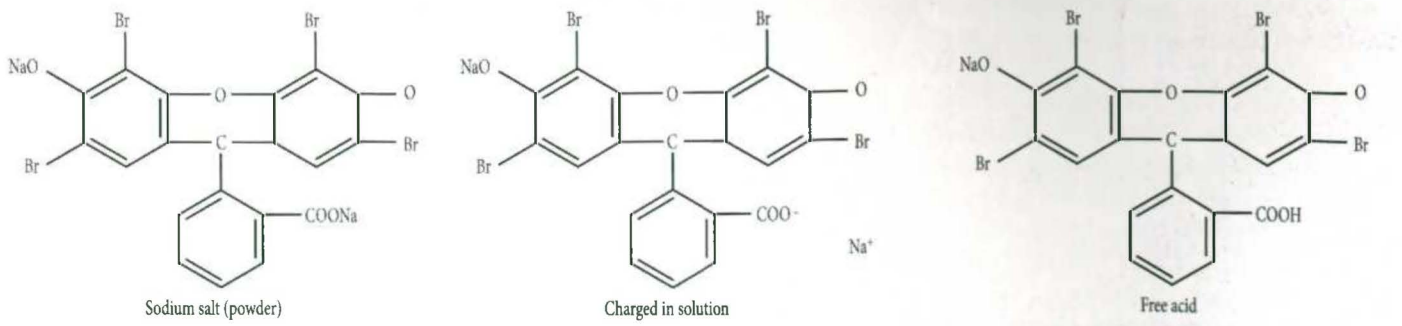
PLASMA STAINS

The plasma stains are most frequently anionic, or negatively charged, dyes that combine with very cationic, or positively charged, tissue groups. The basic amino acids, such as argini ne, histidine, and lysine, are common sites for dye binding.

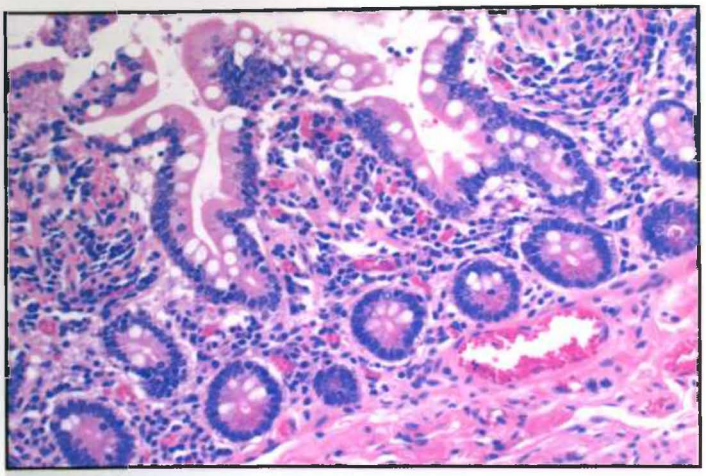
Eosin Counterstain

Eosin Y (1% aqueous solution)	200 mL
Ethyl alcohol, 95%	600 mL
Acetic acid, glacial	4 mL

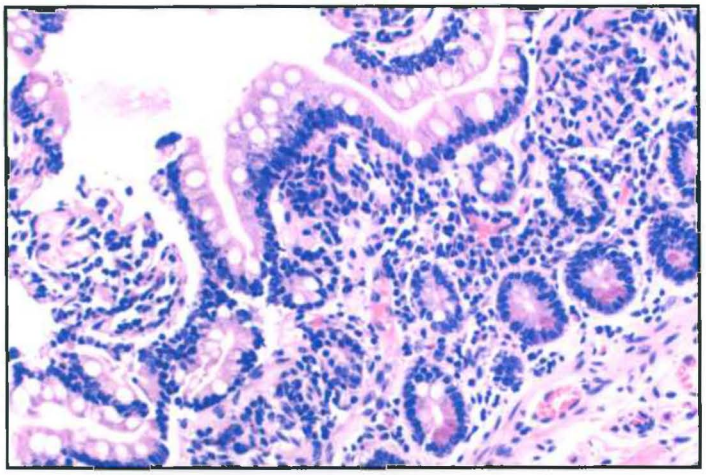
Eosin is the most widely used counterstain in the routine staining of sections. Usually eosin Y is used, but Lillie [1965] preferred eosin B. Eosin is the sodium salt of a color acid; the chromophore is in the anionic (–) part of the molecule. Eosin is fully charged at a pH of 7, but because the IEP of proteins is approximately 6, we must stain below pH 6 to develop a net positive charge on the protein. Below pH 4, the amount of charged dye will be greatly decreased, because the eosin is converted to a free acid at a lower pH **[f6.3]**. This free acid may remain in solution and nonspecifically bind to sections; this attachment is caused by hydrogen bonding, and the sections will appear muddy [Koski 1977]. The best staining with eosin will occur at a pH of approximately 4.6 to 5. Used properly, at least 3 shades of pink can be obtained with eosin alone; erythrocytes, collagen, and the cytoplasm



[f6.3] Formulas for eosin. As the pH of an eosin Y solution decreases, the amount of charged dye also decreases.



[i6.8] A section of small intestine counterstained with eosin-containing acetic acid so that the pH is between 4.6 and 5. Compare this section with that seen in **[i6.9]**, where no acid was added to the eosin solution.



[i6.9] An adjacent section to that seen in **[i6.8]**. No acetic acid was added to the eosin solution, so that the pH was above the isoelectric point of the proteins. The staining time was the same for both sections. Note the marked reduction in uptake of the eosin.

of muscle or epithelial cells should stain different shades or intensities of pink. Erythrocytes should always be the deepest shade of pink; whether the collagen or muscle/epithelial cells are the intermediate shade of pink is determined by choice of fixative, duration of fixation, heat during processing, stain formulation, differentiation step, and the nature of the collagen itself [Dapson 2004]. Regardless of which tissue is the intermediate shade of pink, with good processing, staining, and differentiation, 3 shades should always be seen. The effect of pH on eosin binding is demonstrated in **[i6.8]** and **[i6.9]**.

Eosin-Phloxine B Counterstain

Eosin Y (1% aqueous solution)	100 mL
Phloxine B (1% aqueous solution)	10 mL
Alcohol, 95%	780 mL
Acetic acid, glacial	5 mL

Some laboratories prefer an eosin-phloxine B solution, because the pink shades are more vivid. It is very easy to overstain with this solution and to lose some of the fine differentiation possible with counterstains. This solution also must be acidic to develop the appropriate charge on proteins.

Another counterstain that can be used is phloxine B-safran. The phloxine and safran solutions are used separately, and the counterstaining takes longer. Following the hematoxylin, blueing, and washing steps, the slides are stained in 1.5% aqueous phloxine B for 2 minutes. The slides are then washed in tap water for 5 minutes, dehydrated with 3 changes of absolute alcohol, stained for 5 minutes with 2% safran du Gatinais in absolute alcohol, rinsed with absolute alcohol, cleared with xylene, and mounted with synthetic resin.

H&E Staining

MANUAL PROGRESSIVE STAINING METHOD

The following procedure works very well in a surgical pathology laboratory. The use of Mayer hematoxylin increases the time needed for each basket of slides and can dramatically increase the time required to stain the day's routine surgical slides. For this reason, we prefer Harris hematoxylin with 4 mL of glacial acetic acid added to every 96 mL of hematoxylin. Used progressively, Harris hematoxylin stains rapidly with very reproducible results. We also prefer the more delicate staining given by eosin alone, but if more intense red shades are desired, eosin phloxine can be used. The formulas for the solution are the ones given previously.

■ *Procedure*

1	Xylene, 3 changes	2 minutes each
2	Absolute alcohol	10 dips
3	Alcohol, 95%, 2 changes	10 dips each
4	Tap water	rinse until water runs off evenly*
5	Hematoxylin, Mayer or acidified Harris	15 minutes 1-3 minutes†
6	Tap water, 2 changes	10 dips each*
7	Ammonia water, 0.25% or lithium carbonate, 0.5%	until blue‡
8	Tap water, 2 changes	10 dips each*
9	Eosin or eosin-phloxine	10-20 dips 1-3 minutes
10	Alcohol, 70%	10-15 dips

11	Alcohol, 95%	10-15 dips
12	Absolute alcohol, 3 changes	10-15 dips each
13	Xylene, 3 changes	10-15 dips each

Let slides remain in last container until a coverslip is applied.

*Change water frequently; where 2 changes are indicated, 1 container should be changed after each basket. Rotate the containers so that the clean water is in the second container. Running water may be used in any step requiring rinsing with water, if convenient.

†With a large volume of slides, we find it is best to use a staining time of 1 minute with fresh Harris hematoxylin staining solution, add 30 seconds per day until 3 minutes of staining time is reached, then change to fresh solution.

‡Do not agitate in the ammonia water, because most section loss will occur at this point. The step requires 10-30 seconds, and the solution should be changed when it becomes discolored.

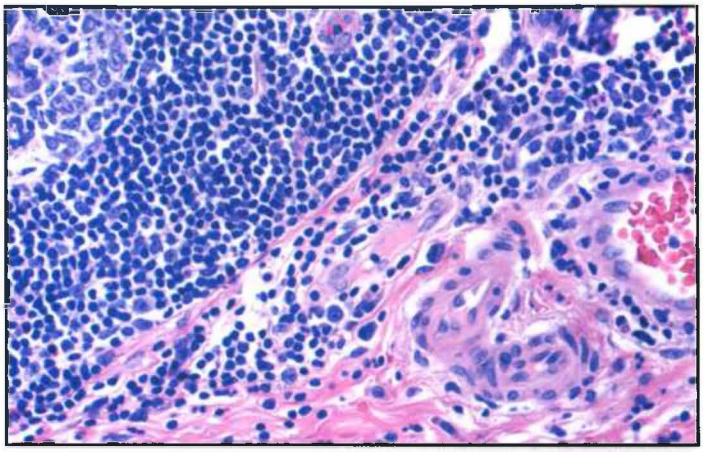
■ ***Results*** **[i6.10], [i6.11], [i6.12]**

• Nuclei	Blue
• Erythrocytes and eosinophilic granules	Bright pink to red
• Cytoplasm and other tissue elements	Various shades of pink

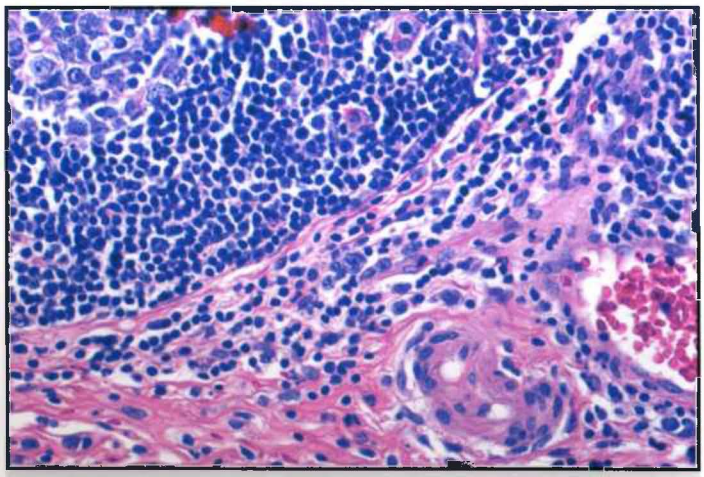
MANUAL REGRESSIVE STAINING METHOD

■ ***Procedure***

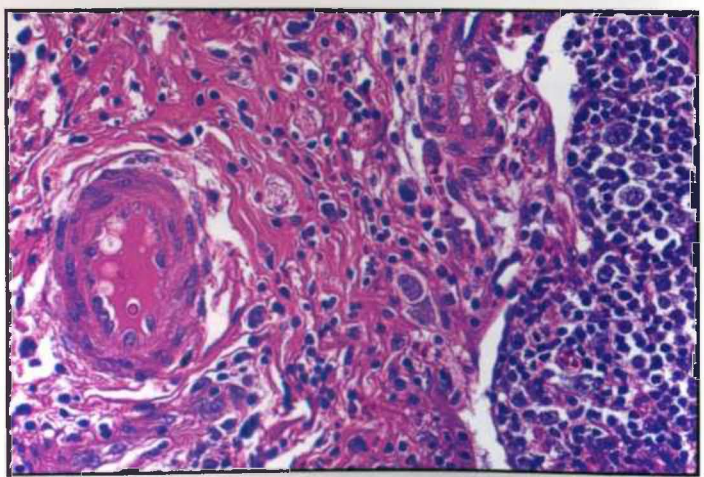
1	Xylene, 3 changes	2 minutes each
2	Absolute alcohol	10 dips
3	Alcohol, 95%, 2 changes	10 dips each
4	Tap water	rinse until water runs off evenly*
5	Hematoxylin, Delafield, Ehrlich, or Harris without acid	10-15 minutes
6	Tap water, 2 changes	10 dips each*
7	Hydrochloric acid 1% in 70% alcohol	5-10 dips
8	Running water	wash well*
9	Ammonia water, 0.25% or lithium carbonate, 0.5%	until blue†
10	Tap water, 2 changes	10 dips each‡
11	Eosin or eosin-phloxine	10-20 dips 1-3 minutes



[i6.10] Part of a lymph nodule and the submucosa can be seen in this section of H&E-stained appendix. The eosin solution did not contain any other dye. The hematoxylin stain shows blue nuclei with various chromatin patterns and well-defined nuclear membranes, and 3 shades of eosin can be seen.



[i6.11] A duplicate of the section shown in **[i6.10]** stained with hematoxylin and counterstained with eosin-phloxine. Again, the hematoxylin stain shows blue nuclei with various chromatin patterns and well-defined nuclear membranes. The very dark nuclei indicate lymphocytes. The eosin-phloxine has been applied so that the nonnuclear elements are still well differentiated.



[i6.12] A duplicate of the sections shown in **[i6.10]** and **[i6.11]** that has been overstained with eosin-phloxine. Note the loss of differentiation between the various nonnuclear elements, and also that the contrast between the nuclei and the cytoplasm is not as good as that in **[i6.10]** and **[i6.11]**. Unless this counterstain is used with care, overstaining occurs and the nuclei also tend to become stained with the phloxine.

12	Alcohol, 70%	10-15 dips
13	Alcohol, 95%	10-15 dips
14	Absolute alcohol, 3 changes	10-15 dips each
15	Xylene, 3 changes	10-15 dips each

Let slides remain in last container until a coverslip is applied.

*Change water frequently; where 2 changes are indicated, 1 container should be changed after each basket. Rotate the containers so that the clean water is in the second container. If convenient, running water may be used in any step that requires rinsing with water.

†Do not agitate in the ammonia water, because most section loss will occur at this point. The step requires 10 to 30 seconds, and the solution should be changed when it becomes discolored.

‡A slide should be checked microscopically at this point until sufficient experience is gained with differentiation; if necessary, return the slides to the hydrochloric acid solution. Running tap water should be used for this step if possible, because all traces of blueing agent should be removed before placing slides in eosin.

■ ***Results* [i6.10], [i6.11], [i6.12]**

• Nuclei	Blue
• Erythrocytes and eosinophilic granules	Bright pink to red
• Cytoplasm and other tissue elements	Pink shades

When using hematoxylin regressively, great care must be taken in the differentiation step so that the nuclei are not overdifferentiated or underdifferentiated; improper nuclear staining may lead to the loss of important diagnostic features. Marked overstaining of the cytoplasm is also undesirable.

AUTOMATED STAINING

The H&E stain can be automated very easily. I had approximately 20 years' experience with a linear stainer, and have found that very satisfactory and more consistent staining can be achieved with automated staining. Linear stainers transfer either individual slides or small groups of slides from 1 container to the next, leaving the slide in each container for the same amount of time. The time that slides are in any given solution can be varied only by varying the number of containers of that solution. Progressive staining is the method of choice for this type of stainer. Robotic stainers are the most flexible, allowing total computerized programming with the ability to return the slides to the same container or solution that was used in a previous procedural step; progressive or regressive hematoxylin staining can be used also.

■ ***Procedure***

The following is an example of a procedure for use on a linear stainer that leaves slides in each solution for 30 seconds.

1	Xylene	6 containers
2	Absolute alcohol	2 containers
3	Alcohol, 95%	1 container
4	Running tap water	2 containers
5	Harris hematoxylin with acetic acid	2 containers
6	Running tap water	3 containers
7	Alcohol, 80%	1 container
8	Eosin	1 container
9	Alcohol, 70%	1 container
10	Alcohol, 95%	1 containers
11	Alcohol, absolute	3 containers
12	Xylene	2 containers and holding tank

The containers must be kept filled as full as possible, and the levels must be checked frequently while the stainer is in use. Filter the hematoxylin daily, and rotate the hematoxylin containers every 2 to 3 days, depending on the volume of slides stained. Change all solutions except the hematoxylin and eosin daily. The eosin should be changed weekly.

■ ***Note on Results of H&E Staining***

Tissue sections from most areas of the body should show blue nuclei with various chromatin patterns along with a crisp nuclear membrane. Lymphocytes will have the densest chromatin, and epithelial cells should show a more open chromatin pattern. As stated under "Plasma Stains," properly stained sections should show 3 shades of eosin staining. If necessary, changing the alcoholic differentiation times after the eosin staining solution, especially in the 70% alcohol, will aid in achieving this.

■ ***Hints to Help Achieve Good H&E Staining***

The following hints are useful for achieving good H&E staining regardless of the method used:

1. Microscopically check a slide from each basket to be sure that proper staining has occurred. If an automated stainer is used, it is useful to stain a control slide before other slides are stained. A section of small intestine provides a good control.

2. Do not allow sections to dry at any point during staining.

3. Keep the solutions covered when not in use to prevent evaporation and to to keep the absolute alcohol from taking up atmospheric moisture. Always make sure that the solutions completely cover the slides. If any precipitate is noted at the top of the hematoxylin container, filter the solution into a clean, dry container.

4. Develop a routine schedule for changing solutions based on the number of slides stained each day.

5. After applying the ammonia water, wash the sections very well with tap water; the pH of the eosin is critical, and if too much ammonia is carried over into the eosin, cytoplasmic staining will be lacking.

6. Do not pass the slides through the dehydrating solutions too quickly, because dehydrating solutions also serve to differentiate. However, remember that the more dilute the alcohol, the more eosin that will be removed.

7. Tissues that have been fixed for longer than normal may require increased staining times. For autopsy tissue, staining times in hematoxylin and eosin may need to be increased by as much as one-third.

8. Staining times also may need to be adjusted according to the fixative used; the time in hematoxylin may need to be increased after fixation in Helly, Zenker, or B-5 fixatives, and the time in eosin will frequently need to be decreased.

9. If using a xylene substitute, the manufacturer's recommendations must be closely followed. With the aliphatic hydrocarbons, the last absolute alcohol must be truly absolute. Some mounting media are not suitable for use with the aliphatic hydrocarbons and limonene-based clearants (see chapter 2, "Processing," p36, for a more complete discussion of xylene substitutes).

10. While running tap water is probably the best for complete rinsing, Dapson and Feldman [1985] caution that some tap water may not be acceptable before or after hematoxylin. Iron, sulfur, and chlorine will produce weak nuclear staining. If the tap water in your area has a noticeable odor or color, deionized or distilled water should be used. Chlorine content, which varies seasonally, will also cause staining variability. Highly alkaline or hard water may serve as an excellent blueing agent but may create dark nuclear or background staining.

11. Remember that what appears to be a staining problem is not always a staining problem. If the problem cannot be identified easily as a staining problem, cut and stain sections from a previous day's workload in which the staining was excellent. If the staining is still excellent on the previous material, then the source of the problem must be in some other area. The duration of fixation, the use of heat during processing, and the possible carryover of formalin or water into the clearing and infiltration reagents are areas that should be examined when an apparent staining problem is proved not to be so.

■ *Restoring Tissue Basophilia*

The loss of tissue basophilia may result from various causes, from leaving the wet tissue too long in Bouin, Zenker, or unbuffered formalin solutions to overdecalcification of bone specimens. The basophilic staining properties of markedly overdecalcified bony tissues cannot be restored, so proper initial decalcification is very important. The following methods are given by Luna [1992] as possible ways of restoring tissue basophilia after overexposure to fixative solutions. Luna also suggest Weigert iron hematoxylin as the best solution for staining nuclear chromatin in specimens overexposed to unbuffered (acid) formalin and /or an acid-decalcifying agent.

METHOD I

1. Place deparaffinized slides from tissue overexposed to Bouin solution in 5% aqueous lithium carbonate solution for 1 hour.

2. Wash in running tap water for 10 minutes, and stain using desired method.

METHOD II

1. Place deparaffinized slides in a 5% aqueous sodium bicarbonate solution for 3 hours (4 hours for tissues overexposed to Zenker solution).

2. Wash in tap water for 5 minutes, and stain using desired method.

METHOD III

1. Place deparaffinized slides in 5% aqueous periodic acid for 30 minutes.

2. Rinse in 3 changes of distilled water, and stain using desired method.

FROZEN SECTION STAINING

1. Cut the frozen section and fix in 37% to 40% formaldehyde for 20 seconds.

2. Rinse the section very well in at least 3 changes of tap water.

3. Stain in Harris hematoxylin with acetic acid for 1 to 1½ minutes.

4. Rinse in 2 changes of tap water.

5. Place slide in 0.25% ammonia water or another blueing agent, and leave until blue.

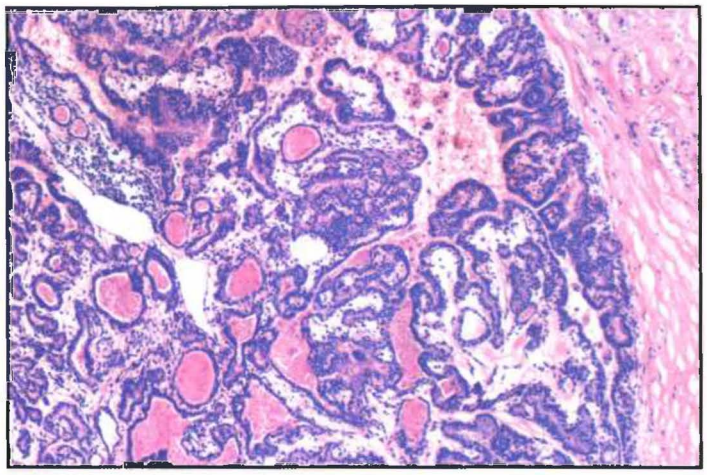
6. Rinse in 2 changes of tap water.

7. Stain in eosin (formula given previously) with 15 to 20 dips or until the desired intensity is achieved.

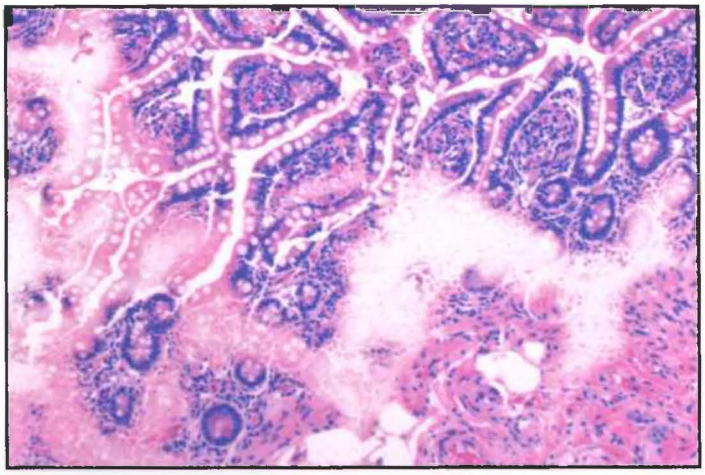
8. Dehydrate with 95% alcohol and absolute alcohol—10 dips in 2 changes of each alcohol.

9. Clear the sections with xylene—10 dips in 3 changes.

10. Mount with synthetic resin.



[i6.13] An H&E-stained frozen section showing a papillary carcinoma of the thyroid. Eosin only was used as the counterstain. Note the excellent quality of both the frozen section and the staining.



[i6.14] The white (unstained) areas in this section are caused by the incomplete removal of paraffin. Stains will not penetrate the tissue in areas in which paraffin remains.

■ *Results* [i6.13]

• Nuclei	Blue
• Cytoplasm and other tissue elements	Pink shades

■ *Notes on Frozen Section Staining*

1. Many laboratories use acetone or alcohol as the fixative for frozen sections; however, concentrated formaldehyde yields morphologic preservation more like that seen in permanent sections. Fixation in alcohol formalin (eg, Penfix, Richard Allan Medical) for 30 seconds is also good.

2. The hematoxylin may need to be changed twice weekly, depending on the number of slides stained, because any formaldehyde carried over into the hematoxylin will act as a reducing agent.

3. This rapid H&E method is preferred by many pathologists to other frozen section stains; it requires about 2 minutes to complete the procedure, and the slide is permanent. Other frequently used methods are those involving metachromatic dyes (toluidine blue O) and polychrome solutions (commercial preparations and polychromed methylene blue). These methods are most often used on unfixed sections, and the slides are not permanent. If dehydrated, cleared, and mounted with synthetic resins, metachromatically stained sections become monochromatically stained.

4. Fix cut sections immediately; do not allow the slides to air-dry or morphologic preservation will be poor.

Troubleshooting the H&E Stain [Carson 1992, 2005]

INCOMPLETE DEPARAFFINIZATION

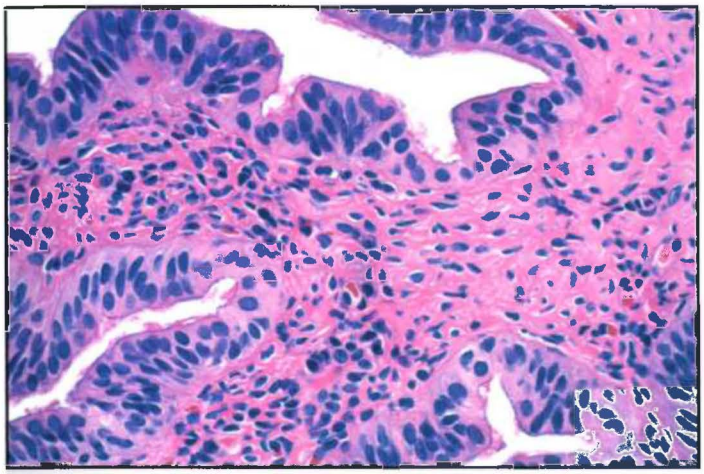
White spots may seen in tissue sections after the deparaffinization step; if the spots are not recognized at this point, spotty or irregular staining will be seen microscopically on the stained section **[i6.14]**. This problem is caused by water left in the tissue, incomplete drying, or not leaving the slides in xylene long enough for complete deparaffinization. To prevent or correct incomplete deparaffinization:

- dry section properly before beginning deparaffinization; if improper drying is the cause, slides can be treated with absolute alcohol to remove the water, and then re-treated with xylene to remove the paraffin; if incomplete drying is severe, the sections may loosen from the slides
- allow sufficient time in xylene for complete deparaffinization; if this is the cause, return to xylene for a longer time
- avoid contaminated xylene; change the solution if necessary
- if the slides have been stained, decolorize and restain

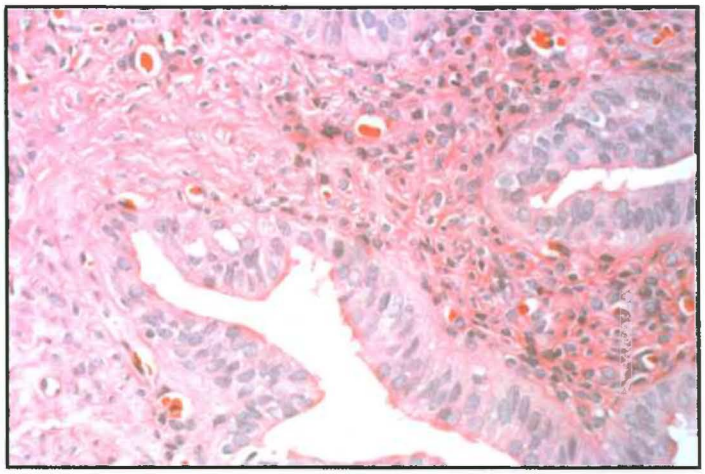
NUCLEAR STAINING IS NOT CRISP

When distinct chromatin patterns cannot be seen in the nuclei, it is sometimes referred to as *smudgy* or *muddy* nuclear staining. The causes are varied, but frequently incomplete fixation is a major contributor to this artifact **[i6.15]**. Other causes are too much heat during processing or drying of the microscopic sections. To ensure crisp nuclear staining:

- fix tissue specimens completely
- dehydrate and clear tissues completely before infiltrating with paraffin



[i6.15] The nuclei are not crisply stained in this section of fallopian tube; rather they are muddy or smudgy. This most likely is the result of incomplete fixation before processing.



[i6.16] The nuclei are too pale in this section of fallopian tube, so the contrast between the nuclei and the cytoplasm is very poor. Good nuclear staining is critical to obtaining a good H&E stain.

- do not use heat on the processor except for the paraffins
- do not leave tissues in melted paraffin for a prolonged period
- dry microscopic slides at the correct temperature (≤70°C) and for the shortest time possible that ensures complete drying

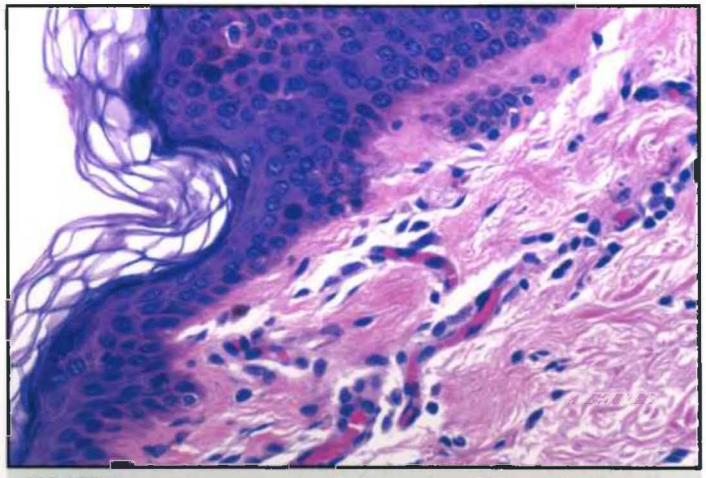
PALE NUCLEAR STAINING

Pale nuclear staining, or hematoxylin that is too light, as shown in **[i6.16]**, can result from:

1. not leaving slides in hematoxylin long enough
2. staining with overoxidized or depleted hematoxylin
3. overdifferentiating the hematoxylin

Pale nuclei in bone sections may result from overdecalcification **[i2.22, p49]**. To prevent or correct pale nuclear staining:

- leave slides in hematoxylin for an adequate length of time
- do not use overoxidized or depleted hematoxylin; change to a fresh solution
- time differentiation step properly for good nuclear definition
- restain section after identifying the source of the problem
- if sections have been fixed with an extremely acidic fixative such as Zenker solution, have been overdecalcified, or have remained in fixative for a long time, the ability to stain the nuclei may be impaired; this possibly can be corrected by increasing the time in hematoxylin or using a method to increase tissue basophilia (see section entitled "Restoring Tissue Basophilia").



[i6.17] The nuclei are overstained in this section of skin, so that nuclear detail is lost. The cytoplasm, especially in the epithelium, contains some hematoxylin staining, and the contrast between the nuclei and cytoplasm is poor.

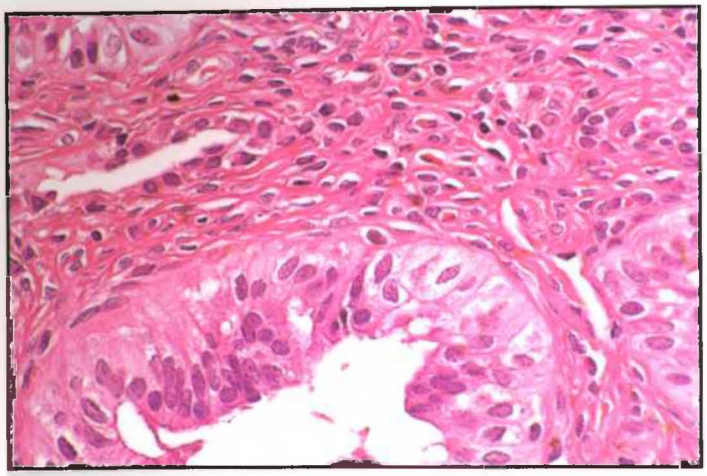
DARK NUCLEAR STAINING

If the nuclei are too darkly stained, as seen in **[i6.17]**, the most likely causes are:

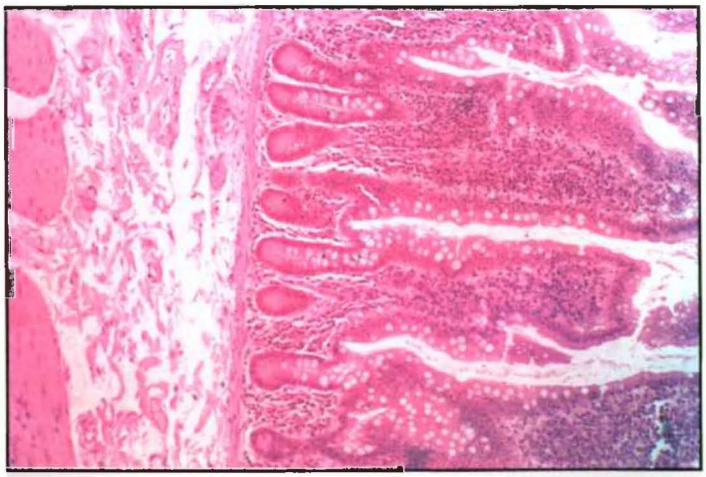
1. sections left too long in hematoxylin
2. sections too thick
3. differentiation step too short

To correct or prevent dark nuclear staining:

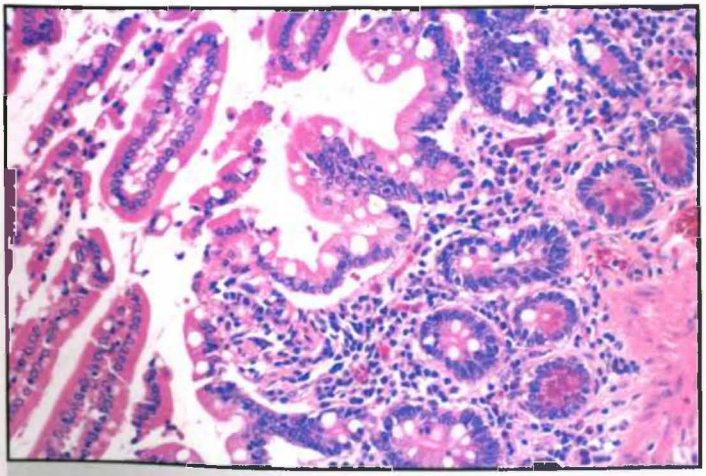
- ensure thin sections; if focusing up and down with the microscope shows more than 1 layer of nuclei, the section is too thick
- if section not too thick, decolorize and restain, making appropriate adjustments in the staining and/or differentiation times
- decrease time sections remain in hematoxylin
- increase time of differentiation



[i6.18] The nuclei are reddish-brown in this H&E-stained section of fallopian tube, indicating that the hematoxylin was not blued sufficiently, or that the hematoxylin is breaking down (overoxidized).



[i6.19] The nuclei are reddish-brown in this H&E-stained section of small intestine, indicating that the hematoxylin was not blued sufficiently, or that the hematoxylin is breaking down (overoxidized).



[i6.20] The eosin staining is much too pale in this intestinal section.

RED OR RED-BROWN NUCLEI

When nuclei are stained red or reddish-brown instead of blue, either the hematoxylin is breaking down or the blueing step was not properly done **[i6.18]**, **[i6.19]**.To prevent or corrected reddish nuclear staining:

- ensure that sections are blued properly; it is not possible to overblue the sections.
- check oxidation status of hematoxylin as given in section following "Delafield hematoxylin"

PALE CYTOPLASMIC STAINING

Pale cytoplasmic staining may result if the pH of the eosin rises above 5; the higher pH may result from carryover of the blueing agent. The sections may be too thin, or they may have been left too long in the dehydrating solutions **[i6.20]**. To prevent or correct pale cytoplasmic staining:

- check eosin solution pH; adjust with acetic acid if necessary
- completely remove blueing reagent before transferring the slides to eosin
- do not allow stained slides to stand in the lower concentrations of alcohols after the eosin; the more water in the alcohol, the more eosin that will be removed
- ensure that sections are not too thin

DARK CYTOPLASMIC STAINING

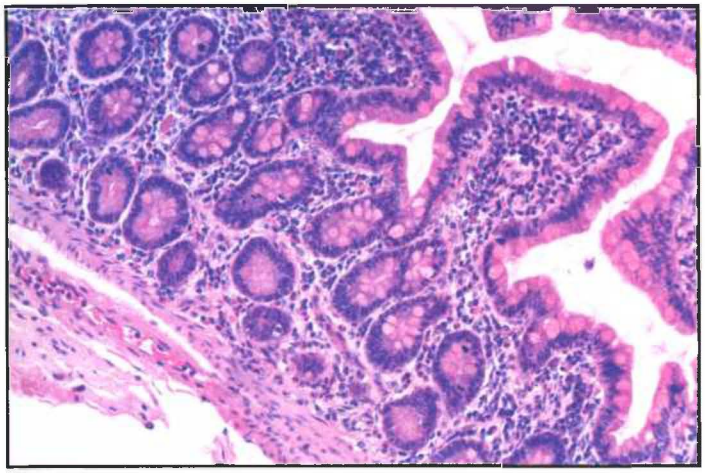
Especially with eosin-phloxine, the cytoplasm may be overstained, and the differentiation may be poor **[i6.21]**. To prevented or correct dark cytoplasmic staining:

- avoid overconcentrated eosin solution, especially if phloxine is present; if necessary, dilute the eosin solution
- do not leave sections in eosin too long
- allow sufficient time in dehydrating solutions, especially 70% alcohol, to allow good eosin differentiation
- check section for proper thickness

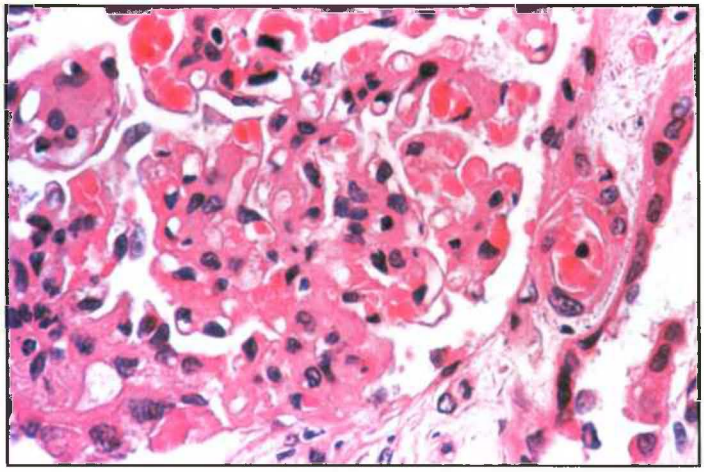
EOSIN NOT PROPERLY DIFFERENTIATED

If properly differentiated, eosin will demonstrate 3 shades of staining. Red blood cells and the granules of eosinophils will be the most intense, and collagen and muscle/epithelial cell cytoplasm should show different intensities of eosin staining (see description in the section on "Plasma Stains"). If 3 shades of eosin are not apparent **[i6.22]**, **[i6.23]**, ensure:

- timely and complete fixation



[i6.21] Marked overstaining with eosin-phloxine has occurred in this intestinal section, so that contrast between the nucleus and cytoplasm is lost, and there is no differentiation of the nonnuclear elements.

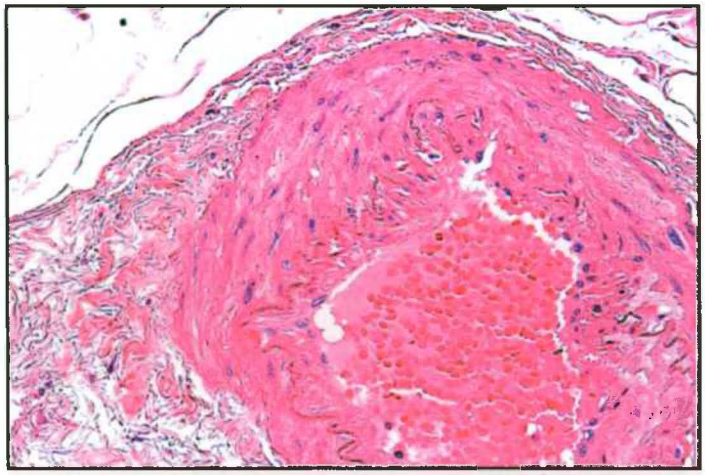


[i6.22] The eosin has not been properly differentiated in this section of kidney, so only 2 shades of eosin are seen, and the contrast between the erythrocytes and connective tissues is less than desired.

- good dehydration and clearing during processing
- eosin-stained section remains in the lower dilutions of alcohol for proper differentiation; adequate time in 70% alcohol will give the best differentiation of the eosin
- eosin is at the correct pH

BLUE-BLACK PRECIPITATE ON TOP OF SECTIONS

A precipitate may be noticed on top of stained sections if the metallic sheen developing on most hematoxylins has been picked up on the slides [**i6.24**]. This can be prevented by filtering the hematoxylin daily before staining.



[i6.23] Eosin differentiation is extremely poor in this section. Erythrocytes, smooth muscle, and collagen are all the same shade of pink.

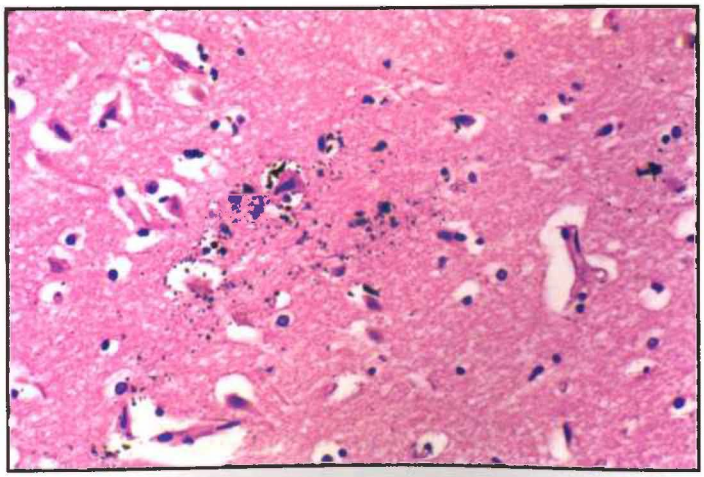
HAZY OR MILKY WATER AND SLIDES

■ ***When Slides Are Placed in Water Following Alcohol During Deparaffinization***

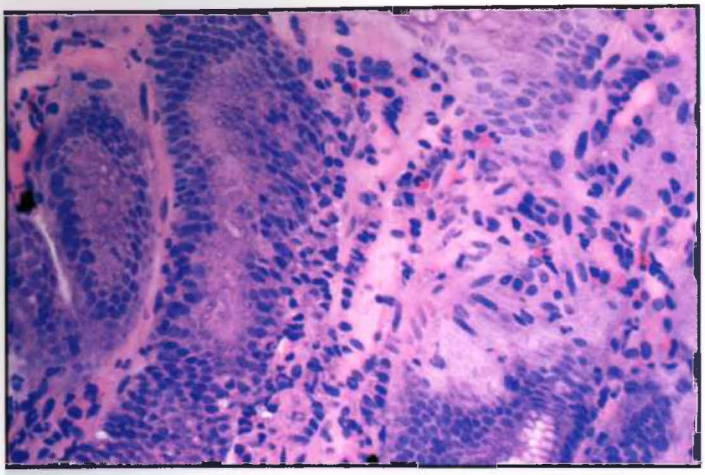
When the slides and water turn milky following the rehydrating alcohols, it indicates the presence of xylene on the slides. This problem can be prevented or corrected by backing the slides up and changing the alcohols, then taking the slides through fresh absolute and 95% alcohols to water. The water should now be clear.

■ ***In Last Xylene Before Applying Cover Glass***

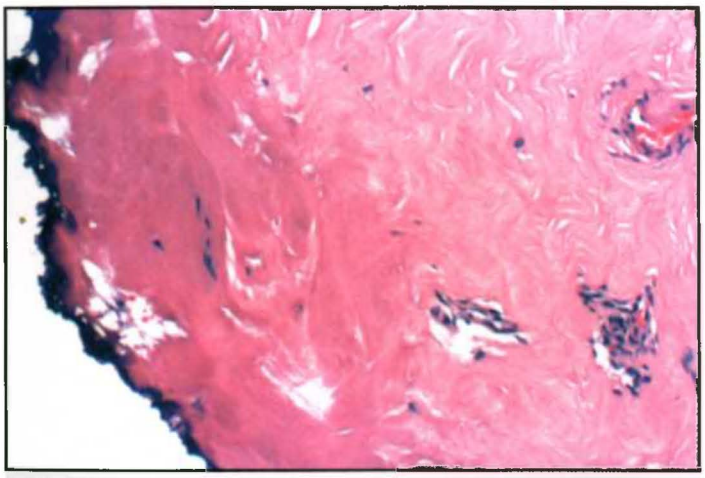
When slides appear hazy or milky in the last xylene used in clearing, it indicates that water is still present on the slides and that dehydration is not complete. This may be corrected or prevented by backing the slides up and changing the alcohol and xylene solutions, then redehydrating and clearing the sections. The slides and xylene should be clear, or transparent, at this point.



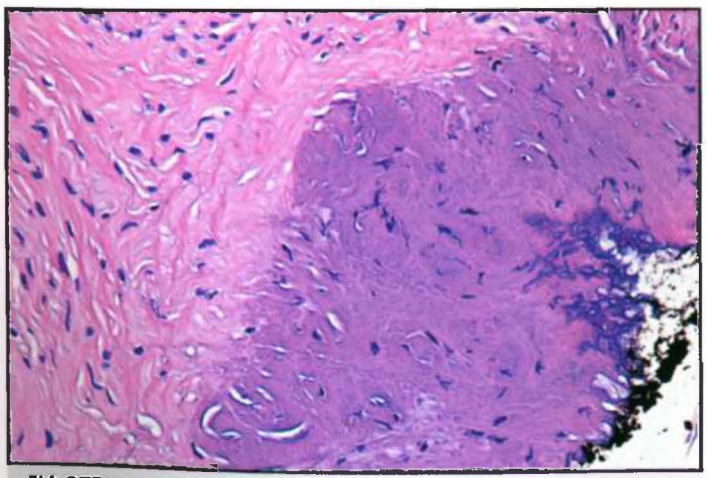
[i6.24] A section of brain stained with unfiltered Harris hematoxylin. Precipitate can be seen in the middle of this section.



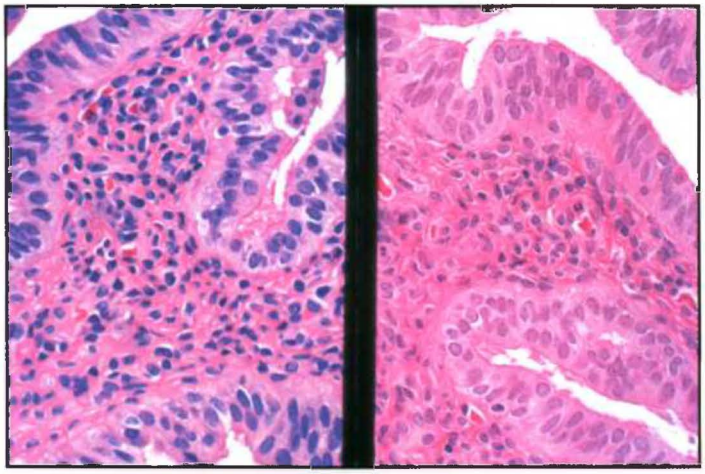
[i6.25] The results of incomplete dehydration during processing can be seen in this image. The nuclear stain is not crisp and also shows some unevenness.



[i6.26] Tissue removed by electrocautery will show a heat artifact. Note the very dark basophilic stain (burned appearance) at the very edge of this tissue, and also note that the cytoplasm shows a denser, more basophilic staining for several millimeters into the tissue.



[i6.27] The basophilic staining at the edge of this tissue is more pronounced than in that seen in **[i6.26]**. This is an artifact resulting from the extreme heat caused by an electrocautery.



[i6.28] Duplicate sections of fallopian tube. The section on the right demonstrates very poor contrast between the nucleus and the cytoplasm; the section on the left demonstrates that the poor contrast is the result of markedly understained nuclei in the section on the right. The cytoplasmic stain is identical. The nuclei are not crisp, probably because of incomplete fixation before processing.

UNEVEN H&E STAINING

Rarely, slides will show uneven H&E staining, varying from small patches to large, diffuse areas in the sections; nuclei may also show poor chromatin detail **[i2.2, p35]**, **[i6.25]**. This may be caused by water or fixative in the infiltrating paraffin, or by contamination of reagents in closed tissue processors because of equipment malfunction or absorption of atmospheric water by the dehydrating alcohols on the open processors [Wynnchuk 1990]. This cannot be corrected, but can be prevented as follows:

- use toluene instead of xylene in areas of high humidity if using open processors; toluene is more water tolerant than xylene
- check equipment for malfunction (see chapter 2, "Processing")

Uneven staining may also be caused if the levels of all reagents are not sufficient to cover the entire slide. Especially with open staining containers, the levels should be checked often.

DARK BASOPHILIC STAINING OF NUCLEI AND CYTOPLASM, ESPECIALLY AROUND TISSUE EDGES

Laser and electrocautery techniques denature macromolecules and produce heat artifact, generally marked by dark basophilic staining in nuclei and cytoplasm **[i6.26]**, **[i6.27]** [Dapson 1990]. There is no remedy for this artifact.

POOR CONTRAST BETWEEN NUCLEUS AND CYTOPLASM

When the nucleus and cytoplasm do not contrast well, the cause is usually poor staining of one or the other, and either:

1. the nucleus is too pale to contrast well with the cytoplasm **[i6.28]**
2. the cytoplasm is overstained and masks the nuclei

3. the nuclear stain is too dark for the cytoplasmic stain, or
4. the cytoplasmic stain is too pale for the nuclear stain

Preventive or corrective steps include:

- determining whether nuclear stain or cytoplasmic stain is inadequate, and then adjusting staining times
- checking pH of staining solutions, and adjusting if necessary
- monitoring water pH daily for standard readings; agricultural run-off or other contaminants may affect water quality and staining
- referring to previous troubleshooting sections for dark and pale nuclear staining, and dark and pale cytoplasmic staining; applying any suggestions as necessary

Nucleic Acid Stains

There are 2 types of nucleic acid, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). DNA is found chiefly in the nucleus, where it is a major constituent of nuclear chromatin; RNA is found in the nucleolus and ribosomes.

Nucleic acids may be detected by reactions that involve the sugars ribose or deoxyribose, the phosphoric acid groups, or the purine and pyramidine bases. Most of the reactions are not practical for use in a routine histopathology laboratory, but 2 methods are commonly used to demonstrate nucleic acid: the Feulgen method for DNA and the methyl green-pyronin method for DNA and RNA.

FEULGEN REACTION [PEARSE 1968, CARSON 1983]

■ *Purpose*
The demonstration of DNA

■ *Principle*
The Feulgen reaction is based on the mild hydrolysis of DNA by hydrochloric acid, which rapidly removes the purine bases (adenine and guanine) but leaves the sugars and phosphates of DNA intact. This hydrolysis generates an aldehyde group that can be demonstrated with Schiff reagent. According to Pearse, 2 reactions occur almost simultaneously. First, there is a rapid removal of the purine bases with formation of aldehyde groups in the remaining uncovered deoxyribose groups; second, there is a progressive removal of histones and apurinic acids. As hydrolysis proceeds, the second reaction will eventually predominate and a negative Feulgen reaction will result; therefore, the time of hydrolysis must be carefully controlled. The molecular structure of RNA is different and hydrolysis with hydrochloric acid does not occur. So RNA is not demonstrated by this method.

■ *Fixative*
Any fixative except Bouin solution

■ *Equipment*
60°C oven, Coplin jars, Erlenmeyer flasks, graduated cylinders, filter paper

■ *Technique*
Cut paraffin sections at 4 µm

■ *Quality Control*
No control section is required because all nuclei will give a positive reaction

■ *Reagents*

Hydrochloric Acid, 1N

Hydrochloric acid, concentrated	83.5 mL
Distilled water	916.5 mL
Slowly add the acid to the water	

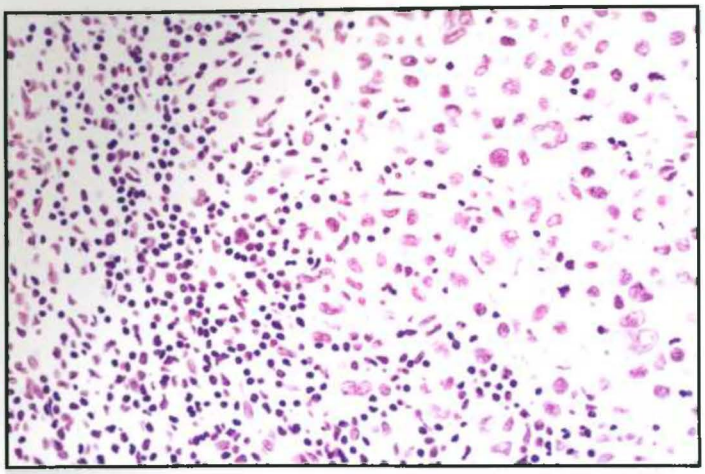
Schiff Reagent (De Tomasi Preparation)

Distilled water	200 mL
Basic fuchsin	1 g
Sodium metabisulfite	1 g
Hydrochloric acid, 1N	20 mL

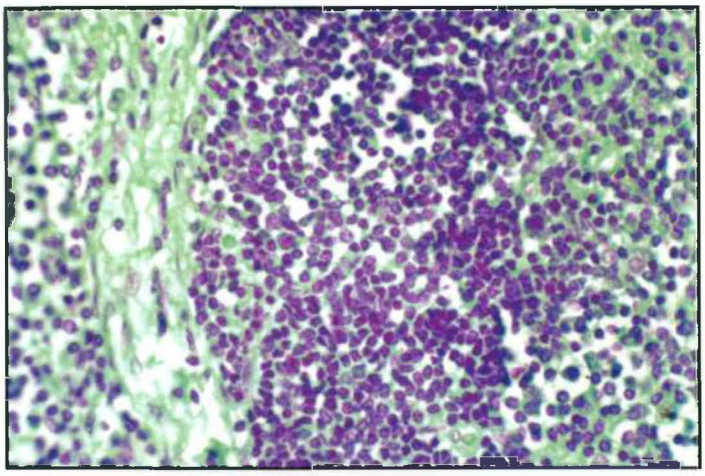
Bring the water to a boil, remove from the heat, add the basic fuchsin, and again bring to a boil. Remove from the heat, cool to 50°C, and filter. Add the 1N hydrochloric acid. Cool completely, and then add the sodium metabisulfite. Allow to stand overnight in the dark; the solution should be light amber. Add 0.5 g of activated charcoal, shake for 1 minute, and filter. The reagent should be colorless. Store the solution in the refrigerator.

Sulfurous Acid

Sodium metabisulfite (anhydrous), 10% aqueous solution	10 mL
Distilled water	180 mL
Hydrochloric acid, 1N	10 mL
Prepare and use under a hood	



[i6.29] The Feulgen reaction demonstrated on a lymph node. The DNA of the nuclei is bright rose-red because of the reaction of aldehydes released by acid hydrolysis with Schiff reagent. No counterstain has been used.



[i6.30] A light green counterstain was applied to this section of lymph node stained with the Feulgen reaction.

■ *Procedure*

1. Deparaffinize sections in xylene, and then hydrate in 2 changes of absolute alcohol, 2 changes of 95% alcohol, and distilled water
2. Rinse slides briefly in cold 1N hydrochloric acid
3. Place the sections in 1N hydrochloric acid, preheated and maintained at 60°C, for the following times:
   a. formalin-fixed sections: 8 to 12 minutes
   b. Zenker-fixed sections: 5 to 8 minutes
   c. Helly-fixed sections: 5 to 8 minutes
   d. Optimum times will have to be determined for other fixatives
4. Rinse sections briefly in cold 1N hydrochloric acid and then in distilled water
5. Stain in Schiff reagent for 1 hour
6. Wash sections briefly
7. Rinse sections in 3 changes of freshly prepared sulfurous acid; perform this step under the hood
8. Wash at least 5 minutes in running tap water
9. Counterstain with 1% aqueous light green if desired
10. Dehydrate, clear, and mount with synthetic resin

■ *Results* **[i6.29], [i6.30]**

• DNA	Reddish purple
• Cytoplasm (if counterstained)	Light green

■ *Technical Notes*

1. The optimum hydrolysis time is fixative dependent, and various times should be tried with each fixative to determine the optimum time needed in your laboratory **[i6.31]**.
2. Bouin solution hydrolyzes the nuclei excessively during fixation; therefore, tissue fixed in Bouin solution is unsatisfactory for use with the Feulgen reaction.
3. If a counterstain is used, it should be applied very lightly because it tends to mask the Feulgen reaction when applied too heavily **[i6.32]**.

METHYL GREEN-PYRONIN Y [POTVIN 1979]

■ *Purpose*

This method for nucleic acid will differentiate between DNA and RNA. Its primary use is to identify plasma cells or immunoblasts in tissues.

■ *Principle*

DNA stains with methyl green, while RNA is colored red with pyronin. Studies suggest that differential staining may be caused by the differing degrees of polymerization of DNA and RNA molecules. Methyl green is bound by more highly polymerized DNA, whereas RNA, a lower polymer, binds pyronin [Sheehan 1980].

■ *Fixative*

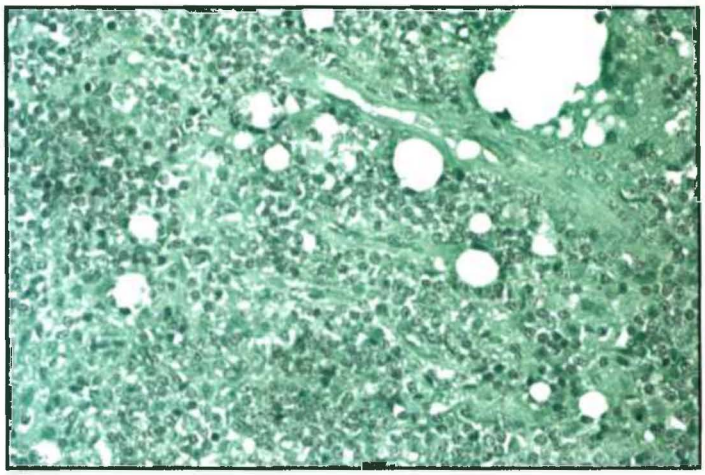
10% neutral-buffered formalin is preferred for this modification; B-5, Helly, or Zenker fixation is satisfactory.

■ *Equipment*

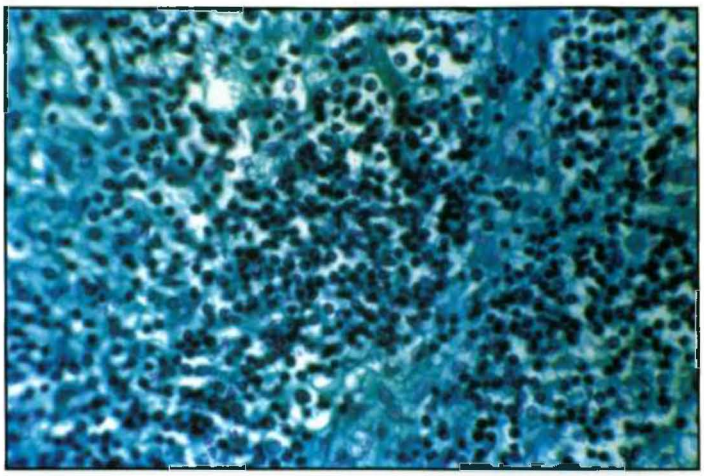
Separatory funnel, ring stand, pH meter, Erlenmeyer flasks, graduated cylinders, staining rack.

■ *Technique*

Cut paraffin sections at 4 µm.



[i6.31] The hydrolysis step in this Feulgen reaction was either overdone or underdone, so that the aldehydes are not demonstrated with Schiff reagent. Compare with **[i6.29]**



[i6.32] Too much light green counterstain has been applied in this section, so that all of the Feulgen reaction has been masked. Compare with **[i6.29]** and **[i6.30]**. This section is also too thick.

■ *Quality Control*

A formalin-fixed, paraffin-embedded section containing many plasma cells should be used.

■ *Reagents*

STOCK ACETATE BUFFER SOLUTIONS

Solution a—0.2M Acetic Acid

Glacial acetic acid	2.3 mL
Distilled water	197.7 mL

Solution b—0.2M Sodium Acetate

Sodium acetate trihydrate	2.72 g
Distilled water	100 mL

Acetate Buffer Solution (Working)

Adjust to pH 4.2 with sodium hydroxide or acetic acid.	
Solution a (0.2M acetic acid)	150 mL
Solution b (0.2M sodium acetate)	50 mL

Methyl Green Staining Solution

Acetate buffer solution (working)	200 mL
Methyl green dye*	1 g

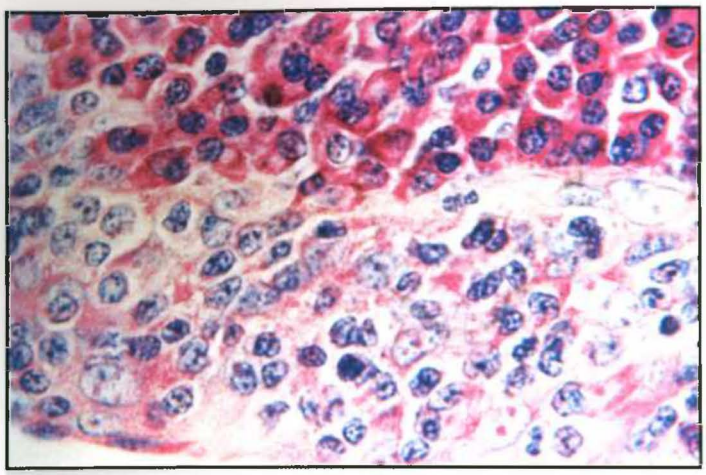
The preparation and purification of the methyl green staining solution is carried out under a chemical hood as follows: Place the solution in a separatory funnel. Add 50 mL of chloroform, and shake. Allow methyl green and chloroform to separate into layers. Discard the chloroform (bottom layer). Repeat until the chloroform is clear and all traces of methyl violet have disappeared (2 to 3 hours). Allow the methyl green staining solution to stand in an open flask overnight so that any residual chloroform evaporates. The solution is stable for several months.

Methyl Green-Pyronin Y Staining Solution

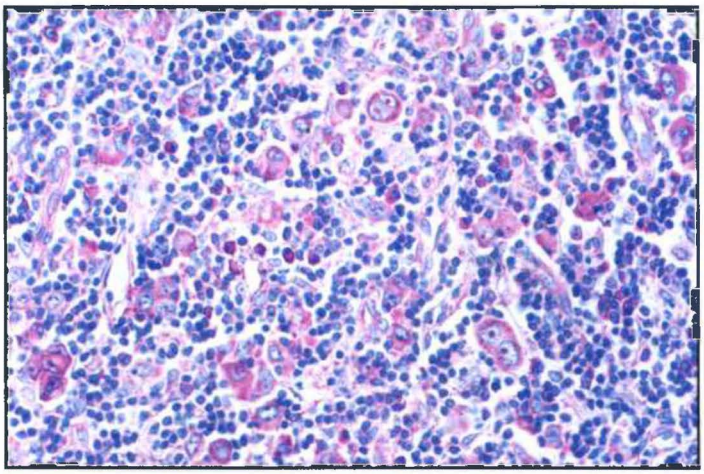
Purified methyl green solution	10 mL
Pyronin Y*	10 mg (0.01 g)
If a more intense green appearance is desired in the nuclei, rather than blue-green, additional purified methyl green solution (up to 5 mL) can be added.	

■ *Procedure*

1. Deparaffinize sections in xylene and then hydrate in 2 changes of absolute alcohol, 2 changes of 95% alcohol, and distilled water.
2. Stain 1 slide at a time by placing the slide on a staining rack. Using a Pasteur pipette, flood the slide with methyl green-pyronin Y solution. Let stand for 5 minutes.
3. Quickly rinse the slide with distilled water.
4. Blot the section completely dry with filter paper.
5. Dip the slide in 2 changes of acetone (15 quick dips).
6. Dip in equal parts of acetone and xylene (15 quick dips).
7. Dip in 2 changes of xylene (15 dips).



[i6.33] With the methyl green-pyronin technique, plasma cell cytoplasm will show an intense rose staining with pyronin (RNA), and the nuclei will show blue to green staining with methyl green (DNA). Note the absence of background staining.



[i6.34] RNA and DNA are differentiated with the methyl green-pyronin technique. Cells containing abundant rough endoplasmic reticulum (contains ribosomal RNA) show marked rose staining of the cytoplasm. The nucleolus (RNA) also stains rose, while the nuclear chromatin (DNA) stains blue to green.

8. Let the slides remain in the last change of xylene for 5 minutes

9. Mount with synthetic resin

■ *Results* **[i6.33], [i6.34]**

• DNA	Green to blue-green
• RNA	Red to rose
• Goblet cells	Mint green
• Background	Pale pink to colorless
• Immunoblast and plasma cell cytoplasm	Intense red
• Nuclei	Green to blue-green

■ *Technical Notes*

1. True methyl green was usually contaminated with crystal violet, hence the need for purification. It is no longer available; instead ethyl green (CI 42590), a much purer dye, is frequently sold under the name methyl green and should be used in this procedure. Chloroform extraction will thus be avoided. Pyronine Y (CI 45005) can be mixed with ethyl green and the stain performed as follows [Penny 2002]:

   a. Dissolve 1 g ethyl green in 100 mL of 0.2 M acetate buffer, pH 4.2, then add 0.1 g pyronine Y.

   b. Stain deparaffinized and hydrated slides individually for 5 minutes, rinse quickly with water, blot dry with filter paper, dehydrated rapidly in 2 changes of acetone, acetone-xylene, and 2 changes of xylene; mount with synthetic resin.

   c. The results should be the same as those given above.

2. Most methyl green-pyronin techniques require Carnoy-fixed tissue for optimum results, but Potvin's methyl green-pyronin procedure works well on formalin-fixed sections of tissue.

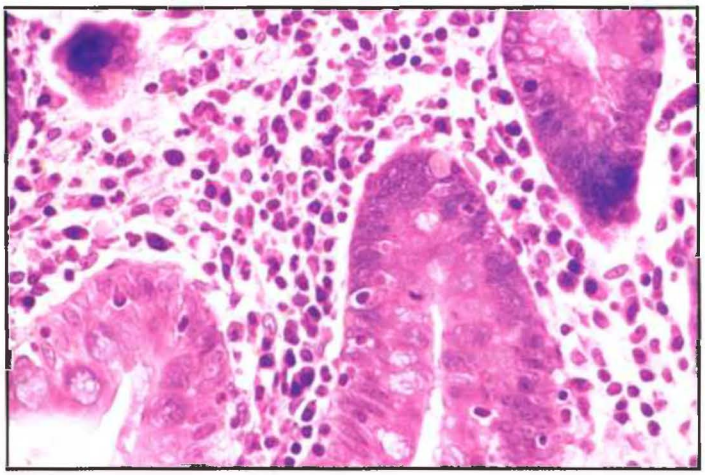
3. Acids and some fixatives may cause a depolymerization of DNA, resulting in a loss of the ability to bind methyl green; when that occurs, the DNA will bind pyronin and differential staining will not occur [Sheehan 1980] **[i6.35].**

4. Pyronin is not specific for RNA; cartilage, osteoid, keratin, eosinophil granules, and mast cell granules will also stain with pyronin, but pyronin cannot compete with methyl green for DNA.

5. The chloroform used to purify the methyl green is toxic, and the contaminated solution must be disposed of as hazardous waste. The OSHA ceiling limit is 50 ppm. No practical glove material is available for laboratory use, and it must be used under a hood. Several preparations of methyl green that do not need purification should be tried as a substitute for the listed reagent.

Polychromatic Stains

Polychromatic stains are used in some laboratories as routine nuclear and cytoplasmic stains for bone marrow biopsy sections because of the range of cytoplasmic staining obtained. A polychromatic stain may be defined as a compound dye or dye mixture that contains components of different colors. Polychroming is a process in which a dye forms other dyes spontaneously. For example, methylene blue solutions that have been prepared for some time, particularly at an alkaline pH, will contain lower homologues, primarily azure A and azure B [Humason 1972]. Romanowsky-type stains, with Giemsa being the most common example used in histopathology,



[i6.35] A section of small intestine that is incorrectly stained. Both RNA and DNA are stained rose-red in this section and no methyl green staining is seen.

are combinations of the basic dye, methylene blue, and the acid dye, eosin. Romanowsky, in 1891, used a combination of 1% aqueous eosin and saturated aqueous methylene blue to stain malarial parasites that had never been stained before. His success started a wealth of research to explain or modify the technique; the research continues today. All mixtures of eosin with methylene blue and allied compounds that give similar colors are commonly referred to as Romanowsky dyes. *Polychrome methylene blue* was the term coined by Unna in 1891 for the compound formed in methylene blue solutions [Marshall 1976], either on standing or on the addition of a dilute alkali. Today, the commercial Wright and Giemsa stains are not prepared by polychroming methylene blue, but by using weighed amounts of the azures, which gives more consistent results.

MAY-GRUNWALD GIEMSA STAIN [LUNA 1968]

■ *Purpose*

To permit differentiation of cells present in hematopoietic tissue. The stain is also used for the demonstration of some microorganisms.

■ *Principle*

The Romansowsky stains, "neutral" dyes combining the basic dye methylene blue and the acid dye eosin, give a wide color range when staining blood smears. This is because of impurities present in the actual dye solution. On standing in solution, particularly at an alkaline pH, methylene blue gives rise to new substances that are metachromatic; however, as indicated earlier, most commercial solutions are prepared with weighed amounts of the azures, and most omit methyl violet.

■ *Fixative*

Zenker or B-5 is preferred; 10% neutral-buffered formalin may be used.

■ *Equipment*

Coplin jars, 60°C oven, Erlenmeyer flasks, graduated cylinders

■ *Technique*

Cut paraffin sections at 3 to 4 µm.

■ *Quality Control*

Spleen sections may be used as controls.

■ *Reagents*

Stock Jenner Solution

Jenner dye	1 g
Methyl alcohol	400 mL

Working Jenner Solution

Stock Jenner solution	25 mL
Distilled water	25 mL

Stock Giemsa Solution

Giemsa powder	1 g
Glycerin	66 mL
Absolute methyl alcohol	66 mL

Mix Giemsa powder and glycerin, and place in a 60°C oven for 30 minutes to 2 hours. Finally, add 66 mL of absolute methyl alcohol.

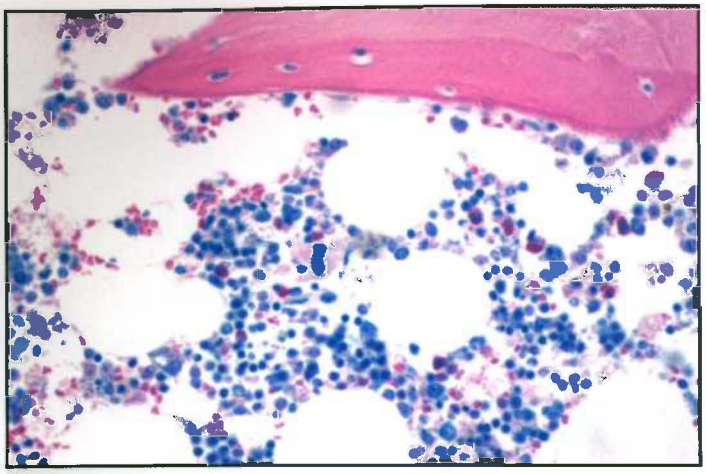
Working Giemsa Solution

Stock Giemsa solution	50 drops
Distilled water	50 mL

Prepare solution at time of use. Do not reuse.

Acetic Water, 1%

Glacial acetic acid	1 mL
Distilled water	99 mL



[i6.36] A section of bone marrow stained using the Giemsa technique. Very dense blue nuclei of red blood cell precursors can be distinguished easily from the more open chromatin patterns of leukocyte precursors. Differential staining of the cytoplasm of the various types of cells is also shown.

■ *Procedure*

1. Deparaffinize and hydrate to distilled water.
2. If the sections are fixed in Zenker or B-5 solution, remove the mercury pigments by placing the slide in Lugol iodine solution for 5 to 10 minutes. Wash in tap water, and then treat with sodium thiosulfate for 5 minutes to remove iodine. Wash well in running water, and rinse in distilled water.
3. Place slides in absolute methyl alcohol for 3 minutes.
4. Place slides in a second change of methyl alcohol for 3 minutes.
5. Stain in working Jenner solution for 5 to 6 minutes.
6. Transfer directly into working Giemsa solution for at least 45 minutes. Carry forward 1 slide at a time.
7. Rinse quickly in distilled water.
8. Differentiate in 1% acetic water, and check microscopically.
9. Rinse in distilled water.
10. Dehydrate, clear, and mount with synthetic resin.

■ *Results* [i6.36]

• Nuclei	Blue
• Cytoplasm of leukocytes	May be shades of pink, gray, or blue, depending on cell type and development
• Bacteria	Blue

■ *Technical Notes*

1. Working solutions are not stable; prepare solution just before use, and discard after use.
2. The pH of the staining solution is critical and ideally should be adjusted for different fixatives. Lillie [1965] adjusts the red-blue balance of the final stain by using buffers to vary the pH of the stain mixture. More acid pH levels give more selective chromatin staining and less cytoplasmic basophilia; less acid pH levels give denser nuclei and increased cytoplasmic basophilia. If staining is poorer than desired, the effect of the pH adjustment should be investigated. Sheehan and Hrapchak [1980] state that the pH should be between 6.4 and 6.9.

Mounting Stained Sections

In rare instances, sections are examined without mounting, but it is more common to apply coverslips to stained sections using some type of mounting medium. There are 2 types of mounting media: aqueous and resinous. Resinous media are preferred unless this type of medium or the dehydrating and clearing agents will cause some change in the staining results.

RESINOUS MEDIA

Natural resins, such as Canada balsam and gum dammar, were used for many years for mounting sections. The natural resins, usually dissolved in xylene, were inherently acidic and caused fading of some stains after the sections were stored for several years. These resins set very slowly, sometimes taking months to harden to nonstickiness, and they also tended to yellow with age. Natural resins have been replaced by synthetic resins that harden quickly, are neutral in reaction, and do not yellow with age. Staining reactions are less affected during prolonged storage when sections are mounted with synthetic resins than when they are mounted with natural resins.

Resinous media consist of solid resins dissolved in an appropriate solvent; the viscosity of the medium should be such that the solution will enter the tissue spaces and flow readily between the slide and cover glass. Air bubbles should be displaced quickly. Most resinous media are dissolved in toluene. Because slides are usually mounted from xylene, xylene should be the solvent for the mounting medium [Sheehan 1980]. Toluene is more volatile than xylene, and thus bubbles are more likely to appear.

The refractive index of the mounting medium is also important. Tissue has an average refractive index of 1.53 to 1.54. As the refractive index of the mounting medium approaches that of the tissue, the tissue becomes more and more transparent; unstained objects may be impossible to discern unless the refractive index of the medium is slightly below or above that of the tissue. When the index of refraction is exactly the same for the medium and the tissue, it may be difficult to locate the tissue if the stain has faded [Lillie 1976]. Most synthetic resins in solution have an index of refraction ranging from 1.51 to 1.55.

All of the resinous mounting media will cause a gradual fading of the blue component of the Romanowsky stains. Heavy mineral oil is the best preservative for this type of stain; however, preparations mounted with oil are messy, and the edges of the coverslips must be sealed.

If dehydration of stained sections is not complete, opaque or cloudy areas may be seen macroscopically, and numerous fine droplets of water may be seen in the section microscopically. This problem is easily corrected with the following steps: Remove the coverslip and any remaining mounting medium with xylene, redehydrate with the appropriate reagent, clear with xylene, and remount with synthetic resin.

AQUEOUS MOUNTING MEDIA

Aqueous mounting media are used when dehydrating and clearing will adversely affect the stain. Lillie and Fullmer [1976] classify the aqueous mounting media used in histology as simple syrups, gum arabic media, and glycerol gelatins. Both gum arabic and glycerol gelatin media cause, or allow, diffusion of basic aniline dyes into the surrounding medium. This can be prevented by adding large amounts of sugar (sucrose, fructose, or D-sorbitol) to the gum Arabic or glycerol gelatin media. The addition of potassium acetate (20% by weight) or one of the sugars (60% by weight) will prevent bleeding of crystal violet stains used for the demonstration of amyloid. The syrups remain wet and sticky in most climates and so serve only as temporary mounting media.

Aqueous mounting media have an index of refraction that differs greatly from that of tissue, usually in the range of 1.41 to 1.43; therefore, the transparency of tissue is not as great as with the synthetic resins, and microscopic evaluation is difficult with a 45× or higher objective. These mounts are not considered permanent; however, some stains mounted with glycerol gelatin remain usable even after several years. The more viscous media may require that the cover glass be sealed around the edges with glue, cement, or fingernail polish.

Commercial mounting media are available for mounting sections from water. ImmuMount (Shandon Inc, Pittsburgh, PA) contains polyvinyl alcohol and can be used for many stains requiring an aqueous mounting medium; it is not satisfactory for immunoperoxidase stains using 9-amino-3-ethylcarbozole. Crystal Mount (Biomeda Corp, Foster City, CA) is an aqueous-based mounting medium designed especially for the permanent preservation of immunoperoxidase- and immunoalkaline phosphatase-stained sections. This medium is applied, allowed to set, and then hardened with heat. After the slides are cool, the sections may be examined microscopically without further treatment, but for long-term storage, coverslips are usually applied using a synthetic resin. The visibility, transparency, and permanence of 9-amino-3-ethylcarbozole-stained sections mounted with Crystal Mount equals that of diaminobenzidine-stained sections mounted with synthetic resins.

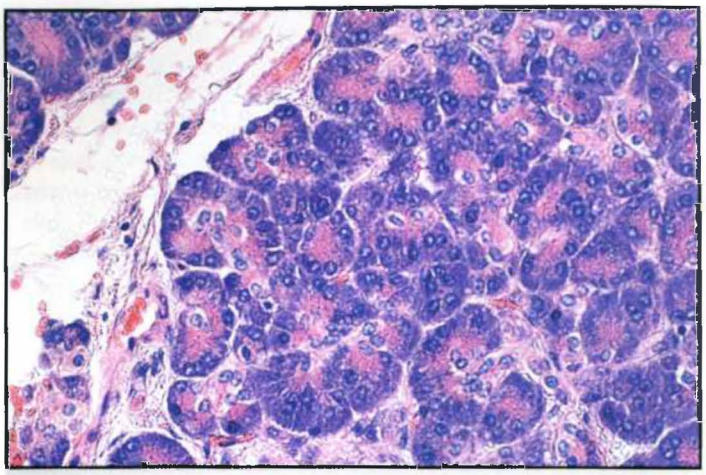
COVERSLIPS

Coverslips are applied to most sections to preserve the stained section of tissue and to allow a better microscopic examination at various magnifications. Slides vary slightly in width and length, depending on whether they are manufactured and sold by the metric (millimeter) or the English (inches) system; slides also vary markedly in thickness. Slide choice is by individual preference. Coverslips are commonly provided in various sizes and thicknesses, denoted by a number that increases as the coverslip becomes thicker. Although the ideal range for photomicrography is stated to be number 1½ (approximately 180 mm thick), number 1 coverslips (approximately 150 mm thick) are used by most laboratories. As the thickness increases, the section transparency is reduced.

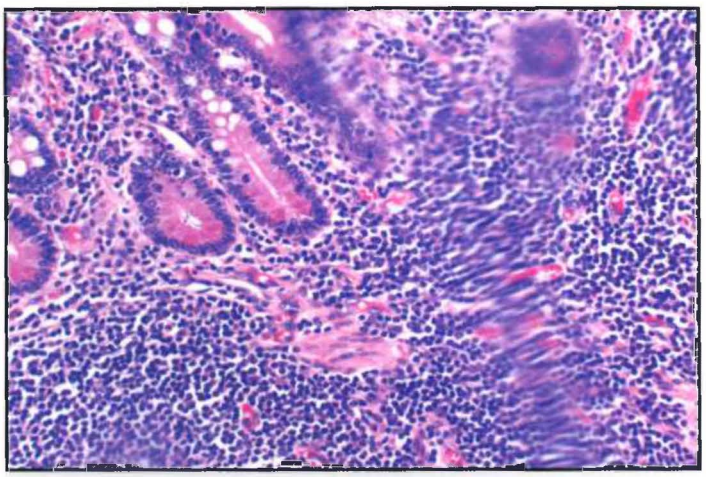
There are various ways of applying the coverslip to the slide, and it really does not matter which method is used as long as the final preparation is neat and air bubbles are not trapped between the tissue and the coverslip. If many air bubbles are present in sections mounted with a synthetic resin, it is best to return the slide to xylene to remove the coverslip, and then remount the section. Air bubbles sometimes can be removed by gently applying pressure to the coverslip with forceps. Do not use this method of removing bubbles (pressure on the coverslip) on fat stains, because the fat may be displaced. Any excess of mounting medium should be removed very carefully from the edges of the coverslip. This can be done by using a brush or gauze dampened either with xylene (for synthetic resins) or water (for aqueous media). Some technicians turn the slide over and blot it on a towel; unless a clean area of the towel is used each time, this technique most often deposits mounting medium on top of the coverslip. This is extremely irritating to the pathologist because it makes focusing on the tissue very difficult. If the mounting medium has been thinned with too much xylene or if the coverslip is warped, the medium may gradually pull back from the edges, this retraction introduces air between the tissue and the coverslip and makes it impossible to evaluate the tissues microscopically.

The mounting medium should not be allowed to become too thick because section transparency then decreases; cloudiness of the section may result from either thickened medium or an excess of medium between the section and the cover glass. The slides also should not be allowed to dry before the coverslip is applied, because an artifact will be produced. The section that has dried may exhibit brown stippling that resembles pigment, or the nucleus may appear as a distinct glossy black structure [Luna 1983]. If a drying artifact is noted, the slides should be placed in xylene to remove the coverslip and the mounting medium. The slides should be rehydrated and placed in running water for 15 to 20 minutes, restained if needed, and dehydrated, cleared, and mounted with synthetic resin.

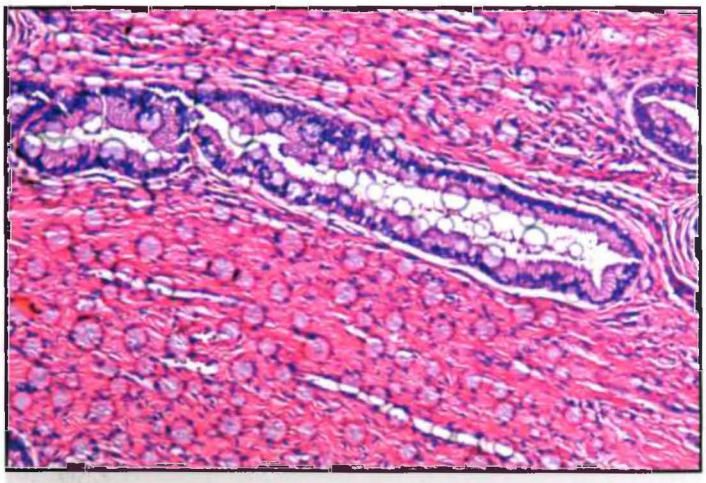
At least 1 commercially available automated coverslipper uses a unique cellulose triacetate film coated on 1 side with a mounting medium that is activated by xylene. Provided the slides are not dry when the film is applied, no drying artifact will be obtained. The film is optically clear, and the mounted slides require no drying time. Thus they may be examined and filed almost immediately after preparation.



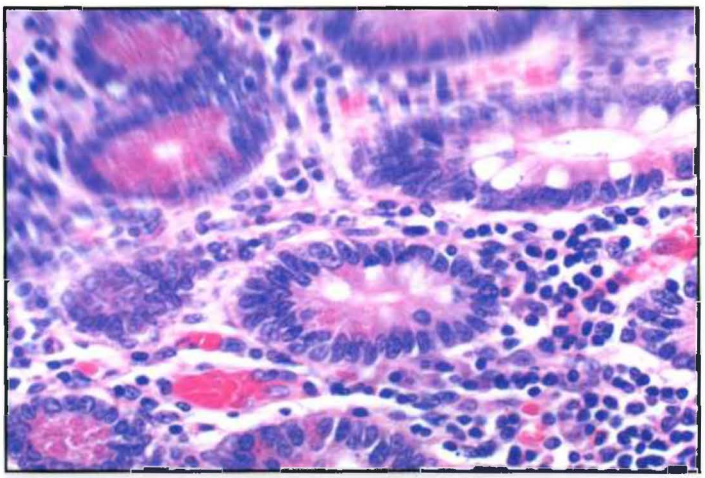
[i6.37] If sections are to be mounted with synthetic resins after staining and are not properly dehydrated, adequate clearing will not occur. The sections will contain droplets of water (note the droplets in the spaces) and may appear cloudy or possible opaque. Compare this figure showing the effects of incomplete dehydration with **[i6.6, p106]**, in which the section of pancreas has been properly dehydrated and cleared.



[i6.39] If synthetic resin gets on top of the coverslip, it is impossible to focus the microscope properly in these areas. The cloudy areas seen in this section are because of mounting medium on the cover glass.



[i6.38] Marked incomplete dehydration is seen in this section, with numerous water droplets seen.



[i6.40] A higher magnification of the section seen in **[i6.39]**.

Troubleshooting Mounted Stained Sections

[Carson 1992, 2005]

WATER BUBBLES NOTED IN MOUNTED SECTIONS

If the sections are not completely dehydrated before clearing in xylene or a xylene substitute, the sections will show microscopic droplets of water beneath the coverslip after mounting. The section also will not have the normal transparency **[i6.37]**, **[i6.38]**. To prevent or correct this problem:

- change all dehydrating and clearing solutions before staining any more sections
- remove coverslip and mounting medium on mounted sections with xylene, and return to fresh absolute alcohol (several changes); after sections are dehydrated, clear with fresh xylene and mount with synthetic resin

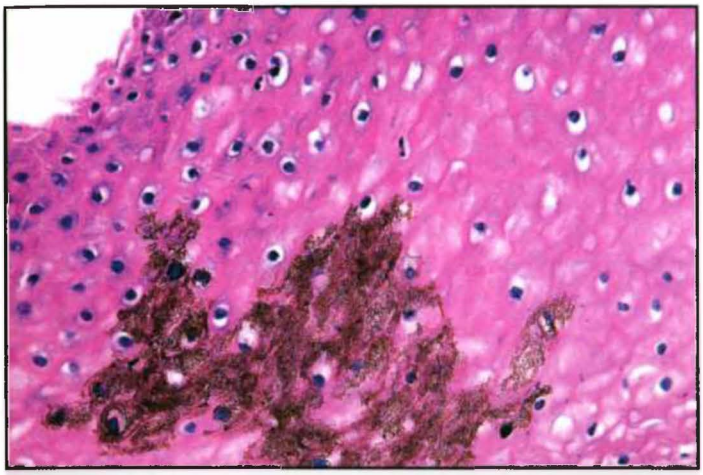
ALL AREAS OF SECTION CANNOT BE BROUGHT INTO FOCUS

Difficulty bringing some areas of the stained section into focus is usually the result of mounting medium on top of the cover glass **[i6.39]**, **[i6.40]**. To prevent or correct this problem:

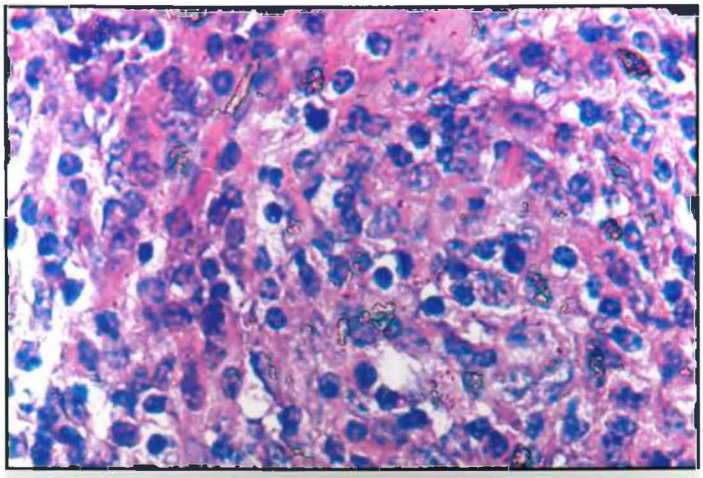
- avoid mounting medium on the top of the cover glass; if necessary, modify or change technique for applying the cover glass
- if section is mounted, remove cover glass and remount section with clean cover glass

CORN-FLAKING ARTIFACT SEEN ON MOUNTED SECTIONS

When sections are allowed to air-dry before mounting; a drying artifact is created in the tissue. This may manifest as



[i6.41] An area of corn-flaking seen in the stratified epithelium in this section indicates partial drying of the section before mounting.



[i6.42] Small bright areas can be seen in this section stained with acid-fast bacilli as a result of drying before mounting.

granular brown stippling resembling pigment or as glossy black nuclei [**i6.41**], [**i6.42**]. To prevent or correct this artifact:

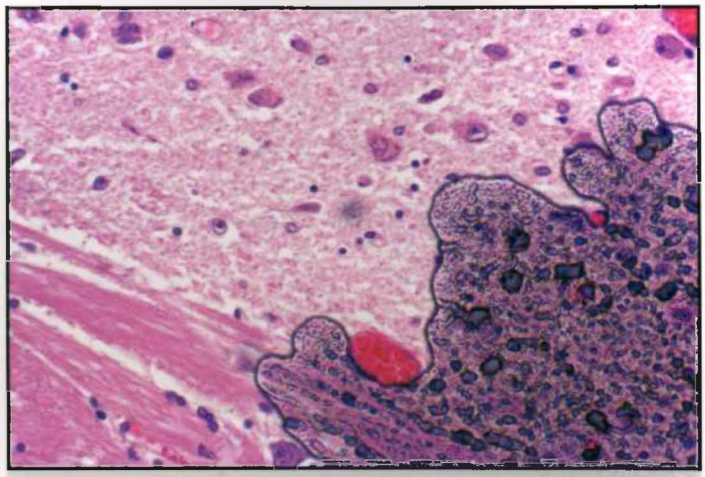
- do not allow slides to dry before mounting
- if sections are mounted, remove the coverslip and mounting medium with xylene; return to water to rehydrate, and then redehydrate and clear; keep the slide wet with xylene before mounting

MOUNTED STAINED SECTIONS ARE NOT AS CRISP AS USUAL WHEN VIEWED MICROSCOPICALLY

Occasionally stained sections will not look as crisp or transparent as usual when viewed microscopically. To troubleshoot loss of crispness, determine if the mounting medium is too thick, thus holding the cover glass too far above the section. If this is the case, remove the cover glass and residual mounting medium with xylene, and remount with fresh mounting medium.

RETRACTED MOUNTING MEDIUM

Mounting medium will retract when a warped cover glass is applied to the section, or when the mounting medium has been thinned by too much xylene [**i6.43**]. To correct this problem, remove the cover glass and apply a new cover glass with fresh mounting medium; if retraction occurs with the fresh mounting medium, then the entire box of cover glasses is suspect. Prevent retracted mounting medium by keeping the container of mounting medium tightly capped when not in use and discarding it, should it become too thick.



[i6.43] When the mounting medium retracts from the edge of the coverslip, as can be seen on the right in this section, and the evaporation involves the tissue, an adequate microscopic examination is impossible. Most frequently, this retraction is caused by a warped coverslip or a mounting medium that has been diluted with too much solvent.

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LEARNING ACTIVITIES

1. **Cut 6 sections each of 4 different tissues and stain with H&E. Use the following variations on one section of each of the four tissues:**

   a. perform regressive hematoxylin staining

   b. perform progressive hematoxylin staining

   c. stain without adding acid to the eosin

   d. do not blue following hematoxylin

   e. do not wash out the blueing agent, but go directly into the eosin (use a small container of eosin that can be discarded)

   f. use the routine H&E procedure

   Microscopically examine all slides carefully and note what difference, if any, was caused by each variation.

2. **Perform the Feulgen and methyl green-pyronin stains on sections of a formalin-fixed lymph node, and the Giemsa stain on a section of formalin-fixed spleen. Microscopically examine each stained section, and compare with the results given in the procedure. If the results are unsatisfactory, analyze the procedural steps for possible sources of error. If a mistake is identified, repeat the stain after correcting the problem and reexamine the slides.**

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CHAPTER 7

Carbohydrates and Amyloid

OBJECTIVES

On completing this chapter, the student should be able to do the following:

1. Classify the following techniques as to substance demonstrated:
   a. periodic acid-Schiff (PAS)
   b. PAS with diastase
   c. Best carmine
   d. Mayer mucicarmine
   e. alcian blue, pH 2.5
   f. alcian blue, pH 1.0
   g. Müller-Mowry colloidal iron
   h. alcian blue, pH 2.5, or colloidal iron, with hyaluronidase
   i. alcian blue-PAS
   j. Congo red
   k. crystal violet
   l. thioflavin T
2. Outline each of the techniques listed in objective 1, considering the following:
   a. most desirable fixative
   b. if another fixative has been used, what can be done
   c. primary reagents and/or dyes and their purpose
   d. results of the stain
   e. appropriate control material
   f. sources of error and appropriate correction
   g. mode of action of reagents
   h. special requirements (eg, chemically clean glassware)
   i. microscope used
3. Identify a technique that makes the Congo red stain more specific for amyloid
4. Identify the type of microscopy used by the thioflavin T technique
5. Define:
   a. carbohydrate
   b. polysaccharide
   c. acid mucopolysaccharides
   d. birefringence
   e. metachromasia
   f. polychromasia
6. Differentiate between *epithelial* and *connective tissue* mucins

Carbohydrates

Carbohydrates (hydrated carbon) are important organic compounds that include sugars, starch, cellulose, and polymers that are mostly linked to protein. Carbohydrates are defined chemically as ketone or aldehyde derivatives of polyhydroxy alcohols, and they may be classified as monosaccharides (mono: 1; saccharide: sugar unit) oligosaccharides (oligo: few, eg, 2-10), or polysaccharides (poly: many). Glucose is the only monosaccharide found in the body in any demonstrable quantity; however, because glucose is extremely soluble in aqueous solution and is of small molecular size, it cannot be demonstrated in tissue sections. For the same reasons, oligosaccharides also cannot be demonstrated in tissue sections. Glycogen, a polymer of glucose **[f7.1]**, is the form in which carbohydrates are stored in humans, with the liver and skeletal muscles serving as the primary storage sites. To meet energy needs of cells, glycogen is readily broken down by the body into glucose. Because this enzymatic breakdown also may occur after death, prompt fixation is important when subsequent glycogen demonstration is required. Glycogen is relatively insoluble in aqueous solutions and thus can be demonstrated in tissue sections.

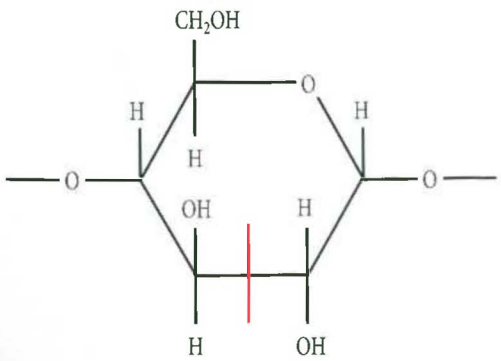
The remainder of the carbohydrates with which we are concerned is conjugated to either protein or lipid, and the histochemistry of these carbohydrates is both complex and confusing because the terminology and classifications are inconsistent. Culling [1974] used a system that placed the naturally occurring polysaccharides in 4 groups based on histochemical differences.

GROUP 1: NEUTRAL POLYSACCHARIDES (NONIONIC HOMOGLYCANS)

1. Glucose-containing: glycogen, starch, cellulose

2. N-acetyl-glucosamine-containing: chitin

This group gives a very positive PAS reaction and a negative reaction with the other frequently used carbohydrate stains (alcian blue, colloidal iron, mucicarmine)



[f7.1] Repeating α-0 glucose unit of glycogen (red line indicates periodate oxidation site).

GROUP II: ACID MUCOPOLYSACCHARIDES (ANIONIC HETEROGLYCANS)

1. Carboxylated (COOH): hyaluronic acid, found in connective tissues and umbilical cord

2. Sulfated (OSO_3H) and carboxylated (COOH)

   - Chondroitin sulfate A (chondroitin-4-sulfate)
   - Chondroitin sulfate C (chondroitin-6-sulfate), found in cartilage, chondrosarcomas, cornea, and blood vessels
   - Chondroitin sulfate B (dermatan sulfate), found principally in skin, also in connective tissue, aorta, and lung
   - Heparin, found in mast cells and the intima of arteries

3. Sulfated only (COOH-free): human aorta and bovine cornea

All polysaccharides in this group are acidic (anionic) and are thought to be attached to protein, even though the word protein does not appear in the name. The acid mucopolysaccharides are the so-called connective tissue mucins and are PAS negative, but stain with alcian blue, colloidal iron, and mucicarmine.

GROUP III: GLYCOPROTEINS (MUCINS, MUCOID, MUCOPROTEIN, MUCOSUBSTANCES)

1. Neutral: ovimucoid (egg white), mucin in stomach, Paneth cell granules

2. Carboxylated (COOH): sialoglycoproteins that contain sialic acid but no sulfate

   - Sialomucins found in submaxillary gland mucin, small intestine mucins, fetal mucins, the upper part of colonic crypts, and human sublingual gland
   - Serum glycoproteins
   - Blood group substances

3. Sulfated (OSO_3H) and carboxylated (COOH): sialoglycoproteins that contain both sialic acid and sulfate, found in colonic mucins of sheep and humans

These are mostly *epithelial mucins*, but some may occur in connective tissue. These glycoproteins are potentially but not necessarily, PAS positive.

GROUP IV: GLYCOLIPIDS

1. Cerebrosides: fatty residue bound to a carbohydrate structure

2. Phosphatides: PAS-positive, noncarbohydrate-containing lipids, including lecithin, cephalin, and sphingomyelin. This compound is included because of PAS positivity.

The term *acid mucosubstances* is sometimes used to include both the acid mucopolysaccharides and the acidic glycoproteins, and although both groups react similarly with many of the histochemical techniques (eg, alcian blue), they may react differently with others (eg, PAS).

Almost invariably, polysaccharides occur in the body as a mixture. The histochemical differentiation between those components with a series of long-chain carbohydrate polymers attached to a small protein core and those with short-chain carbohydrate polymers attached to a large protein core is not possible, but frequently, histological localization permits an educated guess. The information that can be obtained is based on the groups present in carbohydrates that can be histochemically demonstrated. Specifically, these groups are: 1,2 glycol; carboxyl (COOH); and ester sulfate (OSO_3H). Additional information can be obtained with enzyme digestion procedures involving the use of diastase, hyaluronidase, and sialidase. Blocking procedures may also add information and aid in the identification of carbohydrates, but blocking techniques are less frequently used in routine histopathology.

Special Staining Techniques

PAS REACTION [McMANUS 1948, CARSON 1983]

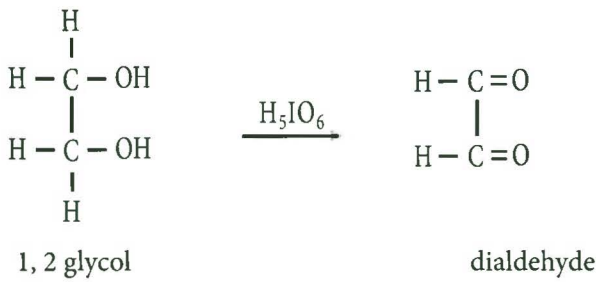
■ ***Purpose***

Demonstration of polysaccharides, neutral mucosubstances, and basement membranes

■ ***Principle***

The reaction is based on the oxidation of certain tissue elements to aldehydes by periodic acid. The most common reactive group is the 1, 2 glycol group, but other groups are also selectively oxidized by periodate [f7.2].

Schiff reagent is prepared by treating basic fuchsin (pararosaniline) with sulfurous acid. Reduction causes the loss of the quinoid structure and masking of the chromophores. A colorless compound, often referred to as *leucofuchsin*, is formed. Following the Schiff reaction, washing in running water causes the loss of the bound sulfurous acid group attached at the central carbon atom, the restoration of the quinoid structure in the dye bound by the aldehyde, and the visualization of the typical Schiff color. Metabisulfite rinses are used to remove excess Schiff reagent and prevent false colorization of the tissue elements because of oxidation of any adsorbed reagent.



1, 2 glycol dialdehyde

[f7.2] Oxidation of glycol to dialdehyde by periodic acid.

■ ***Fixative***

10% neutral-buffered formalin or Bouin solution. Blood smears should be fixed in methyl alcohol for 10 to 15 minutes.

■ ***Equipment***

Hot plate, Coplin jars, balance, Erlenmeyer flasks, graduated cylinders, filter paper

■ ***Technique***

Cut paraffin sections at 4 to 5 µm. Cut kidney sections at 1 to 2 µm.

■ ***Quality Control***

A section of kidney is the most sensitive control. If the procedure is used to demonstrate glycogen (see procedure with diastase digestion, p140), use a section of liver containing glycogen or a section of cervix (include both endocervix and ectocervix).

■ ***Reagents***

Periodic Acid, 0.5% Solution

Periodic acid	2.5 g
Distilled water	500 mL

1N Hydrochloric Acid

Hydrochloric acid, concentrated (specific gravity, 1.19)	83.5 mL
Distilled water	916.5 mL
Add the acid to the water and mix well	

Potassium Metabisulfite, 0.55%

Potassium metabisulfite	2.75 g
Distilled water	500 mL

Schiff Reagent

Distilled water	800 mL
Basic fuchsin	4 g
Sodium metabisulfite	4 g
1N hydrochloric acid	80 mL

Heat water to the boiling point. Remove from flame, add basic fuchsin, and again heat solution to the boiling point. Cool the solution to 50°C, and then filter. Add 80 mL of 1N hydrochloric acid, cool completely, and then add 4 g of sodium metabisulfite. Let the solution stand in the dark overnight; it should turn light amber. Add 2 g of activated charcoal, and shake for 1 minute. Filter the solution, and store in the refrigerator. The solution should be stable for 2 to 4 months.

Test for Quality of Schiff Reagent [Luna 1968]

Place 10 mL of 37% to 40% formaldehyde in a beaker or Erlenmeyer flask. Add a few drops of Schiff reagent. If the solution rapidly turns reddish purple, it is good. If the reaction is delayed and the resultant color is a deep blue-purple, the solution is breaking down.

■ *Conventional Procedure*

1. Deparaffinize and hydrate slides to distilled water.
2. Place sections in 0.5% periodic acid solution for 5 minutes.
3. Wash slides in 3 changes of distilled water.
4. Place sections in Schiff reagent for 15 minutes. Schiff reagent should be allowed to warm to room temperature before use.
5. Wash for 1 minute in each of 2 jars of 0.55% potassium metabisulfite to remove excess stain (optional step, see Technical Note 3, p139).
6. Wash in running tap water for 10 minutes to develop full color.
7. Counterstain ½ minute in Harris hematoxylin with acetic acid (2 mL acetic acid and 48 mL hematoxylin).
8. Wash sections well to blue the hematoxylin.
9. Dehydrate with 95% and absolute alcohols, clear with xylene, and mount with synthetic resin.

■ *Microwave Procedure* [Crowder 1991]

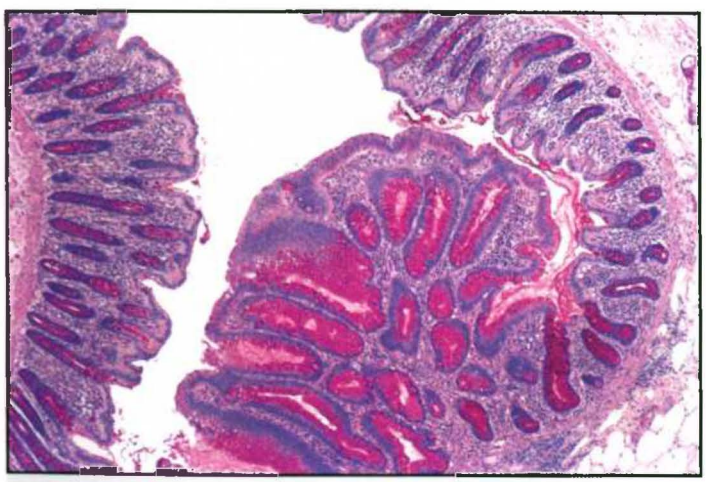
1. Deparaffinize and hydrate slides to distilled water.
2. Place sections in 0.5% periodic acid solution in a plastic Coplin jar, cap loosely with vented cap, and place in the microwave oven. Microwave on high for 15 seconds.
3. Wash slides well in running water.
4. Place sections in Schiff reagent in a plastic Coplin jar, cap loosely with vented cap. Heat on high in the microwave oven for 10 seconds. Remove from the microwave oven, stir solution and let stand for 30 sections. Schiff reagent should be allowed to warm to room temperature before use.
5. Wash slides in running tap water for 5 to 10 minutes.
6. Counterstain ½ minute in Harris hematoxylin with acetic acid (2 mL acetic acid and 48 mL hematoxylin).
7. Rinse well with water.
8. Blue nuclei in 0.75% ammonia water or other blueing reagent.
9. Wash sections well in running water.
10. Dehydrate with 95% and absolute alcohols, clear with xylene, and mount with synthetic resin.

■ *Results*

Glycogen, neutral mucosubstances, certain epithelial sulfomucins and sialomucins, colloid material of the thyroid and pars intermedia of the pituitary, basement membranes, and fungal walls show a positive PAS (bright rose) reaction **[i7.1]**.

■ *Technical Notes*

1. Reid and Culling [1980] state that, contrary to the generally held assumption, Schiff staining after periodate oxidation does not necessarily indicate the presence of carbohydrate residues, and conversely, the absence of staining does not necessarily mean that carbohydrate residues are absent. They concluded that the intensity of staining in the routine PAS reaction is the result of a combination of 4 factors.



[i7.1] A section of colon with a small polyp stained with the PAS reaction and counterstained with hematoxylin (PASH). Neutral polysaccharides, glycoproteins, and, occasionally, carboxylated and sulfated glycoproteins stain positive.

a. number of the available 1, 2 glycol groups

b. reactivity of Schiff reagent with the reaction product

c. structure of the polymer oxidized

d. exact procedural reaction conditions

2. A fast green counterstain may be used instead of hematoxylin when the stain is used for the demonstration of fungi. Stain for 1 minute in a 0.02% solution of fast green containing 1 drop of acetic acid for every 60 mL of staining solution.

3. Although most histotechnologists omit the metabisulfite rinses after Schiff reagent without appreciable difference in the results, some consider the sulfite rinses essential to removing any uncombined leucofuchsin following exposure to the Schiff reagent [Troyer 1980; Lillie 1976; Vacca 1985]. Highly chlorinated water is capable of oxidation, and if the sections are transferred directly to this tap water, any loosely adsorbed Schiff reagent may be reoxidized to basic fuchsin, which may then nonspecifically stain the section.

4. For color development, washing in tap water is very important after the sulfite rinses.

5. Glutaraldehyde is not recommended as a fixative if PAS reactions are to be performed. Glutaraldehyde is a dialdehyde, and 1 aldehyde group may not be involved in protein cross-linking during fixation, but may be left free to react with the Schiff reagent.

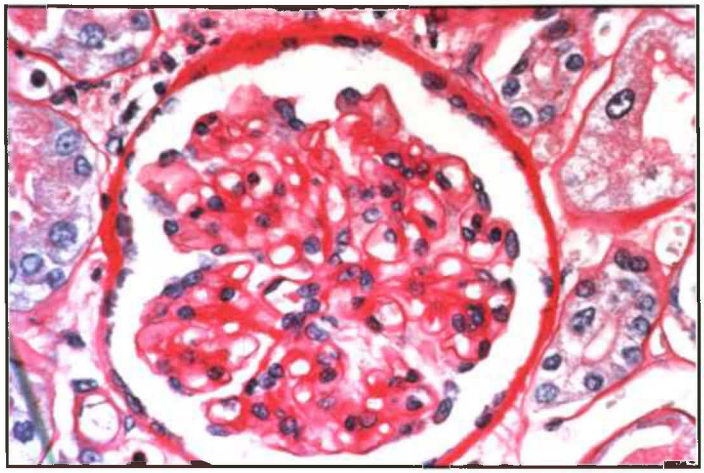
6. To determine if there are any previously reactive aldehyde groups present in the tissue, a control slide should be run through all steps of the procedure except the periodate oxidation step.

7. Chromate-containing fixative may overoxidize reactive groups during fixation, and the resulting reaction with Schiff reagent may be weak.

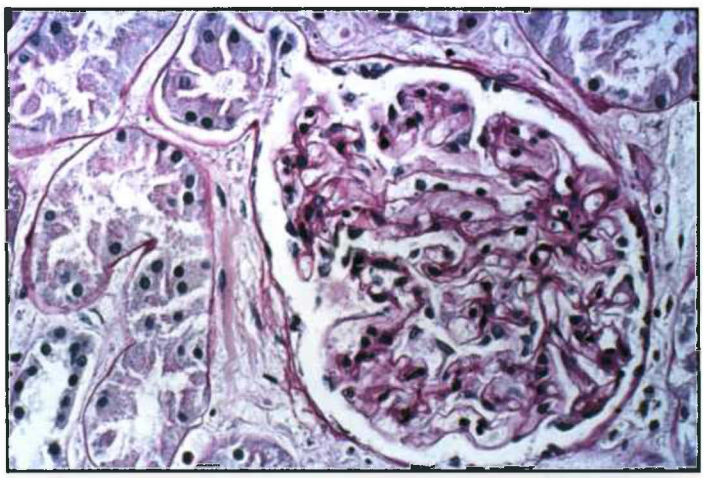
8. Liver containing large amounts of glycogen should not be used as a control for the routine PAS reaction, because the reaction can be weak but still very apparent, and poor or depleted reagent would not be detected. Weak reactions can lead to false-negative results on components that normally do not yield a strong reaction. Reagent problems are more readily apparent if a small section of kidney is used along with the normal control tissue for the intended substance **[i7.2]**, **[i7.3]**.

9. Oxidizing agents other than periodic acid have also been used before the Schiff reagent (eg, chromic acid in the Bauer-Feulgen reaction and potassium permanganate in the Casella reaction), but these other reagents are stronger oxidizers than periodic acid and will oxidize many groups beyond the reactive aldehyde stage.

10. Although Schiff reagent is referred to in most literature as leucofuchsin, this is chemically incorrect. Even through it is colorless, Schiff reagent is not a leuco compound and its properties are very different from true leucofuchsin [Vacca 1985].



[i7.2] The PAS reaction on a section of kidney showing a well-stained glomerular basement membrane. Kidney provides a very sensitive control for the PAS reaction and the quality of the reagents used. A hematoxylin counterstain was used.



[i7.3] A duplicate section of kidney stained with the PASH stain, but here the Schiff reagent was old (ie, overused) so the reaction is very weak. Fresh periodic acid should be used each time, and the Schiff reagent should not be used more than twice.

PAS REACTION WITH DIASTASE DIGESTION [LUNA 1968]

■ *Purpose*

Demonstration of glycogen in tissue sections

■ *Principle*

This is a very sensitive histochemical method for glycogen. Diastase and α-amylase act on glycogen to depolymerize it into smaller sugar units (maltose and glucose) that are washed out of the section. The Schiff reaction has been described in the PAS procedure.

■ *Fixative*

10% neutral-buffered formalin, formalin alcohol, or absolute alcohol

■ *Equipment*
Hot plate, pH meter, Coplin jars, balance, Erlenmeyer flasks, graduated cylinders, filter paper

■ *Technique*
Cut 2 paraffin sections at 4 to 5 µm. Label 1 section "with" and the other section "without."

■ *Quality Control*
2 control sections of liver containing glycogen must be used, 1 labeled "with" and the other labeled "without." Cervix (including both endocervix and ectocervix) is also an excellent control.

■ *Reagents*

Periodic Acid, 0.5% Solution

Periodic acid	2.5 g
Distilled water	500 mL

1N Hydrochloric Acid

Hydrochloric acid, concentrated (specific gravity, 1.19)	83.5 mL
Distilled water	916.5 mL
Add the acid to the water and mix well	

Schiff Reagent

Distilled water	800 mL
Basic fuchsin	4 g
Sodium metabisulfite	4 g
1N Hydrochloric acid	80 mL

Heat water to the boiling point. Remove from flame, add basic fuchsin, and again heat solution to the boiling point. Cool the solution to 50°C, and then filter. Add 80 mL of 1N hydrochloric acid, cool completely, and add 4 g of sodium metabisulfite. Let the solution stand in the dark overnight; it should turn light amber. Add 2 g of activated charcoal, and shake for 1 minute. Filter the solution, and store in the refrigerator. The solution should be stable for 2 to 4 months.

Test for Quality of Schiff Reagent [Luna 1968]

See the PAS procedure, p138

Potassium Metabisulfite, 0.55% Solution

Potassium metabisulfite	2.75 g
Distilled water	500 mL

Malt Diastase Solution

Diastase of malt	0.1 g
Phosphate buffer, pH 6	100 mL

Phosphate Buffer, pH 6

Sodium chloride	8 g
Sodium phosphate, monobasic	1.97 g
Anhydrous disodium phosphate (dibasic)	0.28 g
Distilled water	1,000 mL
Adjust pH to 6 if necessary; store in refrigerator	

■ *Procedure*

1. Deparaffinize and hydrate slides to distilled water.
2. Place the sections labeled "with" in diastase solution preheated to 37°C for 1 hour. Hold the sections labeled "without" in distilled water.
3. Wash in running water 5 minutes.
4. Place all sections ("with" and "without") in 0.5% periodic acid solution for 5 minutes.
5. Wash in 3 changes of distilled water.
6. Place in Schiff reagent for 15 minutes.
7. Wash for 1 minute in each of 2 jars of 0.55% potassium metabisulfite to remove excess stain.
8. Wash in running tap water for 10 minutes to develop full color.
9. Counterstain ½ minute in Harris hematoxylin with acetic acid (2 mL acetic acid and 48 mL hematoxylin).
10. Wash well in running water to blue the hematoxylin.
11. Dehydrate with 2 changes each of 95% and absolute alcohol, clear with xylene, and mount with synthetic resin.

■ *Results*

Glycogen will stain bright rose red on the section labeled "without" [i7.4] and will be absent from the section labeled "with" [i7.5].

■ *Technical Notes*

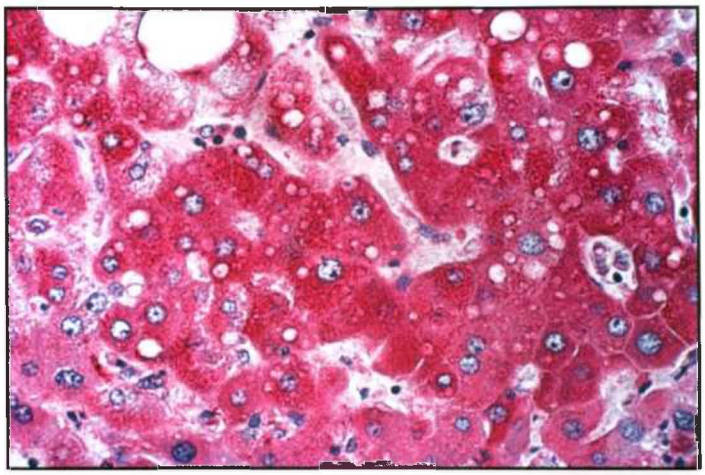
1. Malt diastase, containing both α- and β-amylase, is commonly used for digestion but tends to loosen the sections and does not always completely digest the glycogen. For this reason, as well as the decreased digestion time, many histotechnologists prefer to use human saliva, which contains only α-amylase. If preferred, digest with saliva for 20 minutes at room temperature.

2. If malt diastase is used, it is important not to heat the solution beyond 40°C when digesting sections, because the enzyme activity can be destroyed at higher temperatures; however, insufficient heat may not allow adequate enzyme activity for complete digestion of the glycogen.

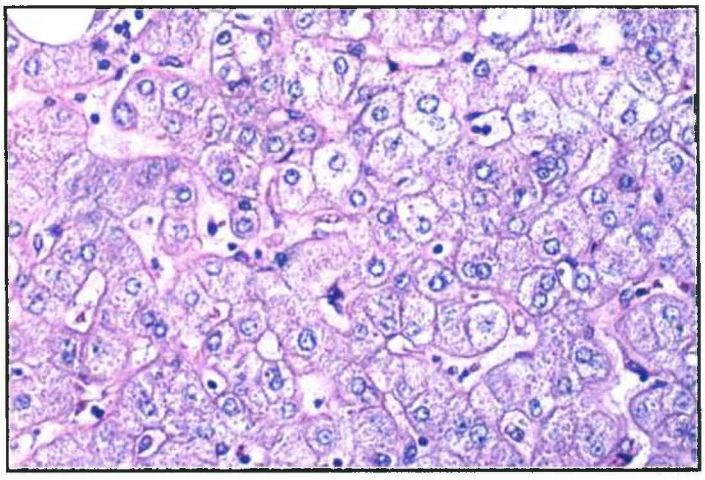
3. Tyler found that digestion with type II-A α-amylase (2% solution in distilled water for 15 minutes at 56°C or 30 minutes at room temperature) derived from the *Bacillus* species gave results far superior to those obtained with malt diastase and comparable to those obtained with human saliva.

4. Glycogen fixed in picric acid-containing fixatives may be more resistant to diastase digestion than when digestion follows other fixatives [Sheehan 1980].

5. Cervix, with both endocervix and ectocervix, provides an excellent control for glycogen. Both the stratified squamous epithelium of the ectocervix (glycogen) and the glands of the endocervix (mucin) will show a positive Schiff reaction on the slides "without" digestion, whereas on those "with" digestion, the stratified squamous epithelium will be negative while the glands of the endocervix remain positive.



[i7.4] A section from a fatty liver stained with a PASH stain. The section was not digested.



[i7.5] A duplicate of the section in **[i7.4]** stained with a PASH stain after diastase digestion.

BEST CARMINE [SHEEHAN 1980; LUNA 1992]

■ *Purpose*

Demonstration of glycogen

■ *Principle*

The Best carmine technique for glycogen is not as specific as the PAS method with and without diastase digestion, and according to Sheehan and Hrapchak [1980], it does not demonstrate as much glycogen. It is an empirical method, but hydrogen bonding is thought by some to play a role in the staining; at the high pH of the staining solution (pH 9-11) the phenolic groups of the dye ionize to the anion O^- that can bind to the glycol groups of glycogen by hydrogen bonding. Others have proposed that possibly carmine does not react with the glycogen itself, but with a basic compound associated with glycogen [Vacca 1985].

■ *Fixative*

Absolute alcohol is preferred; Carnoy and Bouin solution may also be used.

■ *Equipment*

Hot plate, Coplin jars, balance, Erlenmeyer flasks, graduated cylinders, filter paper

■ *Technique*

Cut paraffin sections at 4 to 5 µm.

■ *Quality Control*

A section of liver should be used.

■ *Reagents*

Carmine Stock Solution

Carmine	2 g
Potassium carbonate	1 g
Potassium chloride	5 g
Distilled water	60 mL

Boil solution gently and cautiously for several minutes. Cool completely, and add 20 mL of concentrated ammonium hydroxide. Store in refrigerator.

Working Carmine Solution

Stock carmine solution	10 mL
28% Ammonium hydroxide	15 mL
Methyl alcohol	15 mL
Mix well; use this solution only once	

Differentiating Solution

Absolute ethyl alcohol	20 mL
Methyl alcohol	10 mL
Distilled water	25 mL

■ *Procedure*

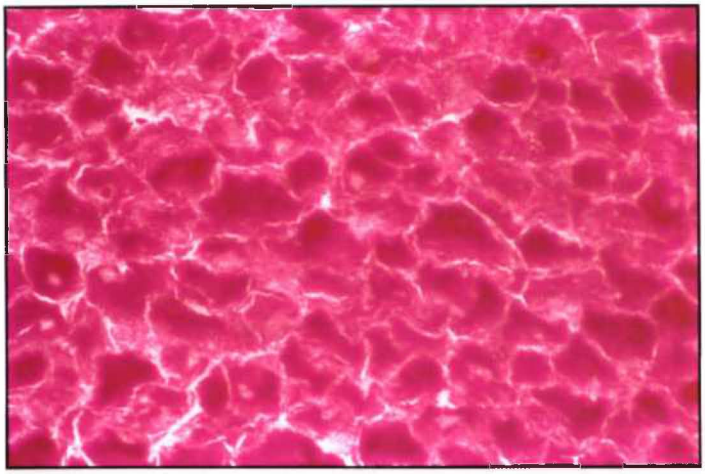
1. Deparaffinize slides, and hydrate as usual.
2. Stain nuclei with Harris hematoxylin for 5 minutes or Mayer hematoxylin for 15 minutes.
3. Wash in running water for 15 minutes.
4. Place slides in working carmine solution for 30 minutes.
5. Immerse in differentiating solution for 30 seconds.
6. Rinse quickly in 80% alcohol, or place immediately into 95% alcohol.
7. Dehydrate with 3 changes of 95% alcohol and 3 changes of absolute alcohol.
8. Clear in xylene, and mount with synthetic resin.

■ *Results* [i7.6]

- Glycogen — Pink to red
- Nuclei — Blue

■ *Technical Notes*

1. Vacca [1985] states that because the PAS reaction is more pleasant to handle than the ammoniacal solutions of the Best carmine method, is more specific, and is chemically well understood, it is the method of choice for glycogen.
2. Autopsy liver sections are frequently depleted of glycogen. If possible, use a surgically removed liver for obtaining control sections.



[i7.6] Sections of liver from a patient with von Gierke disease (a glycogen storage disease) stained with the Best carmine technique.

3. Bancroft and Cook [1994] state that the precipitation of the dye on sections, which commonly occurs during staining, is the result of evaporation of the ammonia in the carmine solutions. For this reason, the working solution should be filtered and used in a closed container. The ammonia serves partially as a solvent for the carmine and to help maintain the high pH.
4. Concentrated ammonium hydroxide is a severe eye and respiratory irritant. It is also corrosive. The short-term exposure limit set by the Occupational Safety and Health Administration is 50 ppm. The carmine solution should be prepared and used in a chemical hood.

MAYER MUCICARMINE [MALLORY 1942; PAYNE 1981]

■ *Purpose*

Staining of "epithelial" mucin in tissue sections

■ *Principle*

In the past, this was thought to be primarily an empirical stain; however, the specificity of the mucicarmine stain was compared with 8 other techniques for mucin by Laurén and Sorvari [1969], and the staining pattern was comparable to that of alcian blue. It stains carboxylated and sulfated mucins, but not neutral mucins. Aluminum is believed to form a chelation complex with the carmine; the resulting compound has a net positive charge and attaches to the acid groups of mucin.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

1,000-mL Erlenmeyer flask, 50-mL Pyrex test tube, long-handled test tube holder, stainless steel spatulas, glass stirring rod, 100-mL graduated cylinder, boiling water bath, balance, Coplin jars, filter paper

■ *Technique*
Cut paraffin sections at 4 to 5 µm.

■ *Quality Control*
Section of unautolyzed colon, small intestine, or appendix

■ *Reagents*

Mucicarmine Stock Solution [Payne 1981]

Using the materials listed, follow the procedure exactly; small variations can be critical to the results obtained from the staining solution.

Carmine, alum lake	4.25 g
Aluminum hydroxide	4.45 g
Ethyl alcohol, 50%	375 mL
Ethyl alcohol, 25%	25 mL
Aluminum chloride, anhydrous	2.05 g
Use a hood for the preparation of this reagent	

Thoroughly mix the dry carmine and aluminum hydroxide in the 50-mL test tube. Add the 25 mL of 25% ethyl alcohol to the test tube, and stir thoroughly with a glass stirring rod until as much of the dry mixture as possible is in solution. Using the test tube holder, warm the solution by lowering the tube intermittently into the boiling water bath, stirring continuously with the glass rod. Warming should last no longer than 1 minute. Do not allow solution to boil or be contaminated with the boiling water. Using the premeasured 375 mL of 50% alcohol, rinse the entire contents of the test tube into the 1,000-mL flask, stirring with the glass rod each time so that the mucicarmine mixture is removed from both the rod and the inside of the test tube. Using the dry stainless steel spatula, slowly and gradually add the aluminum chloride to the solution in the flask, swirling after each addition. Do not breathe the hydrochloric acid vapors given off! After adding all of the aluminum chloride, immediately place the flask into the boiling water bath and watch closely for signs of boiling inside the flask. Boil for exactly 2½ minutes. Promptly remove from the water bath and allow to cool. Seal the cooled flask of solution with Parafilm (American National Can, Neenah, WI), and refrigerate for 24 hours. Remove from the refrigerator, and allow to reach room temperature, agitating periodically. Filter once with standard laboratory filter paper to obtain stock solution. Store in the refrigerator.

Mucicarmine Working Solution

Mucicarmine stock solution	10 mL
Distilled water	40 mL
Prepare just before use	

Weigert Iron Hematoxylin

Solution a	
Hematoxylin	10 g
Alcohol, 95%	1,000 mL
Solution b	
Distilled water	475 mL
Hydrochloric acid, concentrated	5 mL
Ferric chloride, 29% solution	20 mL
Working Solution	
Mix equal parts of solutions **a** and **b**.	
This solution may be used for 2 or 3 days	

Metanil Yellow, 0.25% Solution

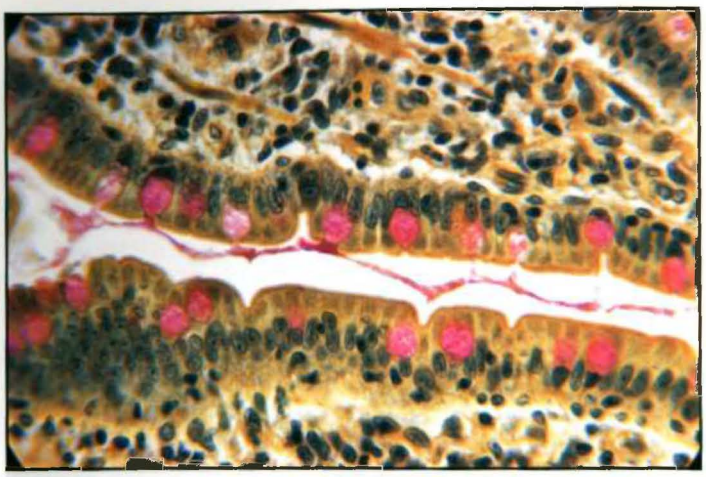
Metanil yellow	0.25 g
Distilled water	100 mL
Glacial acetic acid	0.25 g

■ *Procedure*

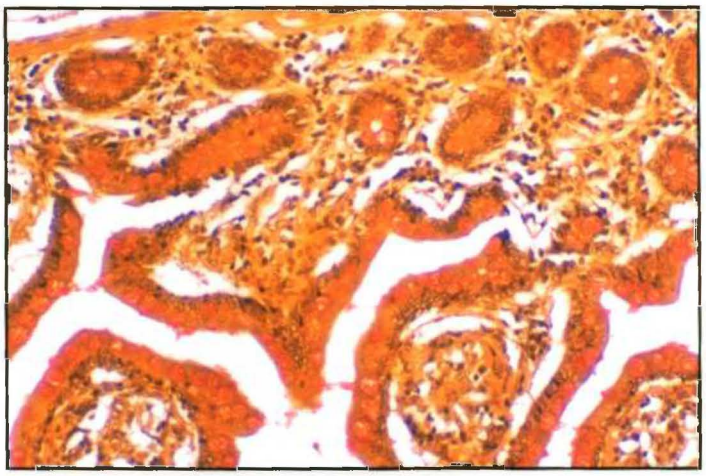
1. Deparaffinize sections, and hydrate to distilled water.
2. Stain in Weigert iron hematoxylin solution for 7 minutes.
3. Wash in running water for 10 minutes.
4. Stain in working mucicarmine solution for 60 minutes.
5. Rinse quickly, and remove excess water before the next step.
6. Stain in metanil yellow solution for 30 seconds to 1 minute.
7. Dehydrate with three changes of 95% alcohol and three changes of absolute alcohol.
8. Clear in xylene, and mount with synthetic resin.

■ *Results* [i7.7]

• Mucin	Deep rose to red
• Capsule of *Cryptococcus*	Deep rose to red
• Nuclei	Black
• Other tissue elements	Blue or yellow



[i7.7] A section of small intestine stained with Mayer mucicarmine. At this high magnification, the bright red goblet cells in the epithelium can be identified easily among the predominant absorptive columnar cells (Reprinted with permission from [Carson 1984])



[i7.8] This section of small intestine has been overstained with metanil yellow, masking the mucin. The section was also dried at too high a temperature immediately after mounting on the slide, which caused the lamina propria to retract from the epithelium.

■ *Technical Notes*

1. Carminophilic properties will be obscured if sections are overstained with either hematoxylin or metanil yellow [i7.8].

2. If Gill hematoxylin is used as the nuclear stain, the mucin may have a bluish cast.

3. The stock mucicarmine solution will gradually deteriorate, even with refrigeration, so solutions that are several months old may need to be discarded and fresh solution prepared; carefully monitor the control for decreased staining.

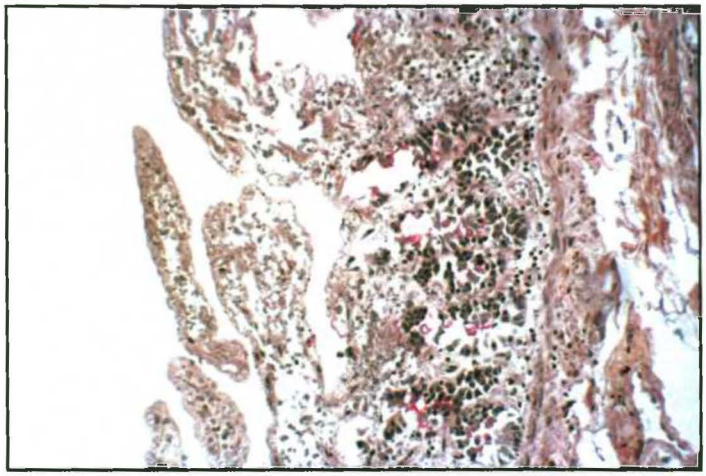
4. It is important that autolyzed tissue not be used as control material [i7.9].

5. Bancroft and Stevens state that while the mucicarmine technique is useful for the demonstration of *Cryptococcus neoformans* [i7.10]; otherwise, the technique's place in the contemporary histochemical repertoire is questionable. They suggest that the combined alcian blue-PAS technique will establish the presence or absence of mucins with more certainty and also provide more information.

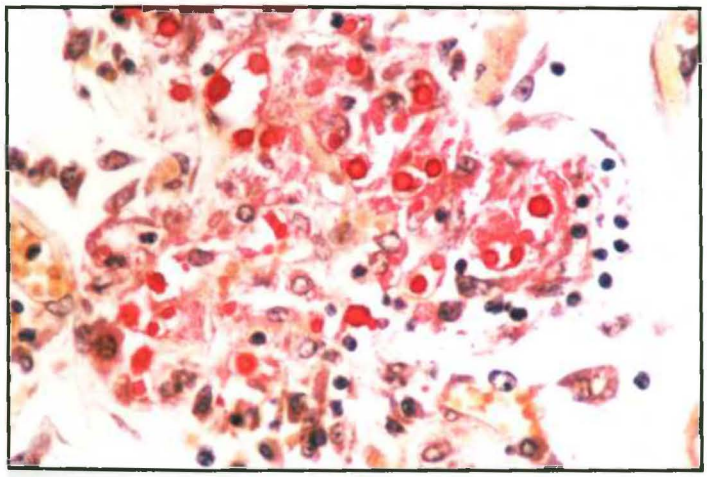
6. *Mucin* is a term used to describe the intracellular secretions of various cells, and although these secretions appear to be microscopically similar, they differ slightly in composition. Culling lists the following properties of mucin:

   - staining with basic dyes
   - metachromatic
   - precipitated by acetic acid (except gastric mucin)
   - soluble in alkaline solutions

7. Note that mucicarmine stock solution should be prepared under a hood; anhydrous aluminum chloride reacts with atmospheric moisture and water to give off vapors of hydrogen chloride.



[i7.9] An autolyzed section of small intestine has been stained with Mayer mucicarmine. No goblet cells can be positively identified in this section; autolyzed tissue does not provide a good control for this stain.



[i7.10] The *Cryptococcus neoformans* organisms are well demonstrated in this section after staining with Mayer mucicarmine. [Reprinted with permission from Test your knowledge. *J Histotechnol* 1987;10:15]

ALCIAN BLUE, pH 2.5 [LUNA 1968; CARSON 1983]

■ *Purpose*
Demonstration of acid mucopolysaccharides

■ *Principle*
Alcian blue is a copper phthalocyanin basic dye that is water soluble and colored blue because of its copper content. When used in a 3% acetic acid solution (pH 2.5), alcian blue stains both sulfated and carboxylated acid mucopolysaccharides and sulfated and carboxylated sialomucins (glycoproteins). Alcian blue is believed to form salt linkages with the acid groups of acid mucopolysaccharides.

■ *Fixative*
10% neutral-buffered formalin or Bouin solution

■ *Equipment*
Coplin jars, pH meter, Erlenmeyer flasks, graduated cylinders, filter paper

■ *Technique*
Cut paraffin sections at 4 to 5 µm.

■ *Quality Control*
A section of unautolyzed small intestine, appendix, or colon should be used as a positive control.

■ *Reagents*

Acetic Acid, 3% Solution

Glacial acetic acid	15 mL
Distilled water	485 mL

Alcian Blue, 1% Solution

Alcian blue-8GX	5 g
Acetic acid, 3% solution	500 mL
Adjust the pH to 2.5, filter, and add a few crystals of thymol	

Nuclear-Fast Red (Kernechtrot) Solution

Nuclear-fast red (Kernechtrot)	0.5 g
Aluminum sulfate	25 g
Distilled water	500 mL

Dissolve the aluminum sulfate in the distilled water, and then add the nuclear-fast red. Heat the solution until the nuclear-fast red has dissolved. Cool, filter, and add a few grains of thymol as a preservative.

■ *Procedure*

1. Deparaffinize and hydrate sections to distilled water.
2. Place slides in 3% acetic acid solution for 3 minutes
3. Place slides in alcian blue solution for 30 minutes at room temperature or for 15 minutes in a 37°C water bath.
4. Rinse sections briefly in 3% acetic acid solution to remove excess alcian blue.
5. Wash slides in running tap water for 10 minutes.
6. Rinse in distilled water.
7. Counterstain in nuclear-fast red solution for 5 minutes.
8. Wash in running tap water for at least 1 minute.
9. Dehydrate in 2 changes each of 95% alcohol and absolute alcohol, and clear in xylene.
10. Mount with synthetic resin.

■ *Results* **[i7.11]**

• Weakly acidic sulfated mucosubstances	Dark blue
• Hyaluronic acid	Dark blue
• Sialomucins	Dark blue
• Background	Pink to red
• Nuclei	Red

■ *Technical Notes*

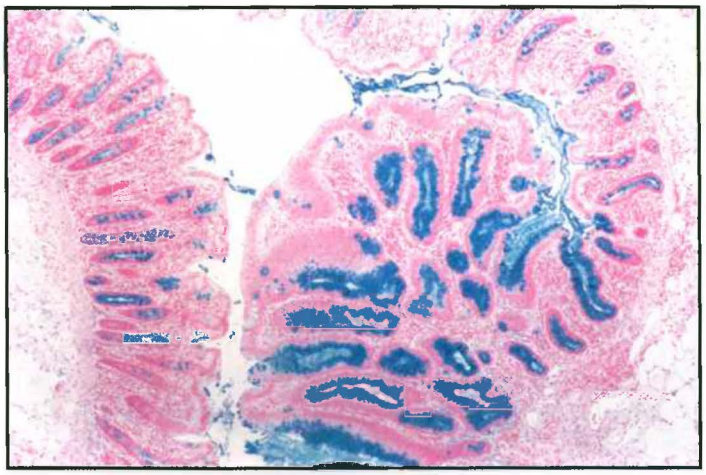
1. According to Mowry [1991], different lots of alcian blue vary in their staining efficacies. Alcian blue certified by the Biological Stain Commission should be used.
2. The meaning of the alcian blue suffixes, eg, -8GN, -8GS, and -8GX is obscure. The most recent, and available since about 1959, is alcian blue-8GX [Mowry 1991].
3. The concentration of the alcian blue solution and the duration of staining may need to be varied with different dye lots.
4. Rinsing sections with acid before the alcian blue solution will protect a solution that is used repeatedly from pH changes because of the introduction of water. Rinsing with acid after the alcian blue solution will help prevent nonspecific staining. [Mowry 1991].

5. Churukian [1993] reported that the alcian blue-pyridine variant is a superior alternative to alcian blue-8GX, as seen when retesting stored samples from previously certified alcian blue-8GX showed a decrease in dye content and poorer dye solubility than when originally tested. The retested alcian blue-pyridine variant did not show these changes.

6. Complete hydration of the sections during the deparaffinization and hydration steps is very important for alcian blue staining, because some alcianophilic structures hydrate slowly; if not completely hydrated, the result will be weak staining.

7. Prolonged staining in the alcian blue solution may cause nuclear staining.

8. If the slides are not washed well with water after the nuclear-fast red, clouding will result when the slides are placed in the alcohols.



[i7.11] A duplicate of the section shown in **[i7.1]** but stained with alcian blue, pH 2.5. Both carboxylated and sulfated acid mucopolysaccharides and glycoproteins (sialomucins) stain at this pH.

ALCIAN BLUE, pH 1.0 [SHEEHAN 1980; CARSON 1983]

■ ***Purpose***

Demonstration of sulfated mucosubstances

■ ***Principle***

When used in a 0.1N hydrochloric acid solution (pH 1.0), alcian blue stains only sulfated acid mucopolysaccharides and sulfated sialomucins (glycoproteins). Acid mucopolysaccharides and sialomucins that are carboxylated only will not be stained.

■ ***Fixative***

10% neutral-buffered formalin or Bouin solution

■ ***Equipment***

Coplin jars, pH meter, Erlenmeyer flasks, graduated cylinders, filter paper

■ ***Technique***

Cut paraffin sections at 4 to 5 µm.

■ ***Quality Control***

A section of unautolyzed small intestine, appendix, or colon should be used as a positive control.

■ ***Reagents***

0.1N Hydrochloric Acid Solution

Hydrochloric acid, concentrated	8.2 mL
Distilled water	991.8 mL

1% Alcian Blue Solution, pH 1.0

Alcian blue-8GX	3 g
Hydrochloric acid, 0.1N	300 mL

Nuclear-Fast Red Solution

See alcian blue, pH 2.5, p145

■ ***Procedure***

1. Deparaffinize and hydrate sections to distilled water.
2. Rinse sections briefly in 0.1N hydrochloric acid.
3. Stain in 1% alcian blue in 0.1N hydrochloric acid for 30 minutes at room temperature or for 15 minutes in a 37°C water bath. Filter solution back into stock bottle.
4. Rinse sections briefly in 0.1N hydrochloric acid.
5. Blot sections dry with fine filter paper. Do not wash in water, because this can change the pH and cause nonspecific staining to occur.
6. Counterstain with nuclear-fast red solution for 5 minutes.
7. Wash well in distilled water.
8. Dehydrate in 2 changes each of 95% and absolute alcohol, and clear in xylene.
9. Mount with synthetic resin.

■ *Results* [i7.12]

• Sulfated mucosubstances	Pale blue
• Background	Pink to red
• Nuclei	Red

■ *Technical Notes*

See alcian blue, pH 2.5, p145

ALCIAN BLUE WITH HYALURONIDASE [LUNA 1968; CARSON 1983]

■ *Purpose*

Differentiation of epithelial and connective tissue mucins

■ *Principle*

The alcian blue reaction is outlined in the previous procedure. Staining will disappear or be dramatically reduced when tissue sections containing hyaluronic acid, chondroitin sulfate A, or chondroitin sulfate C ("connective tissue" mucin) are digested with testicular hyaluronidase. Glycoproteins ("epithelial" mucins) will not be affected.

■ *Fixative*

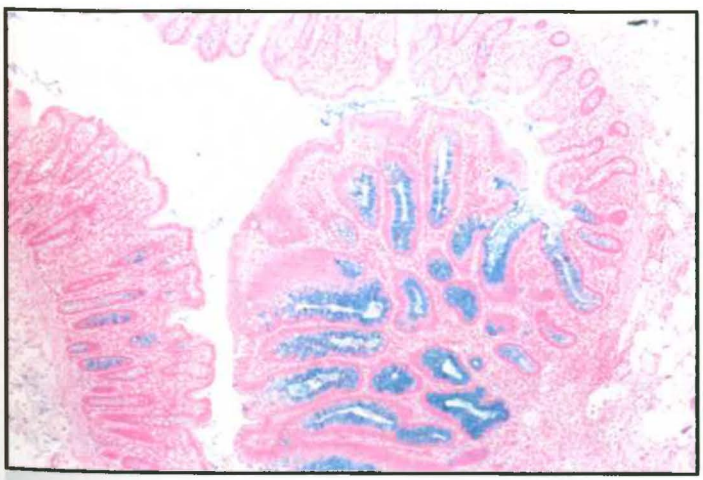
10% neutral-buffered formalin is preferred

■ *Equipment*

Hot plate, Coplin jars, Erlenmeyer flasks, graduated cylinders, filter paper

■ *Technique*

Cut 2 paraffin sections at 4 to 5 µm.



[i7.12] A duplicate of the sections shown in **[i7.1]**, **[i7.11]**, but stained with alcian blue at pH 1. Only sulfated acid mucopolysaccharides and glycoproteins (sialomucins) will stain at this pH.

■ *Quality Control*

2 sections of umbilical cord should be used as a control ("with" and "without"). A section of small bowel, appendix, or colon may be used as a second control to demonstrate epithelial mucins.

■ *Reagents*

0.1M Potassium Phosphate, Monobasic

Potassium phosphate, monobasic (KH_2PO_4)	13.61 g
Distilled water	1,000 mL
Store in the refrigerator.	

0.1M Sodium Phosphate, Dibasic

Sodium phosphate, dibasic (Na_2HPO_4)	14.20 g
Distilled water	1,000 mL
Store in the refrigerator	

Buffer Solution, pH 6.0

Potassium phosphate, monobasic, 0.1M solution	94 mL
Sodium phosphate, dibasic, 0.1M solution	6 mL
Adjust pH to 6.0 if necessary	

Hyaluronidase Digestion Solution

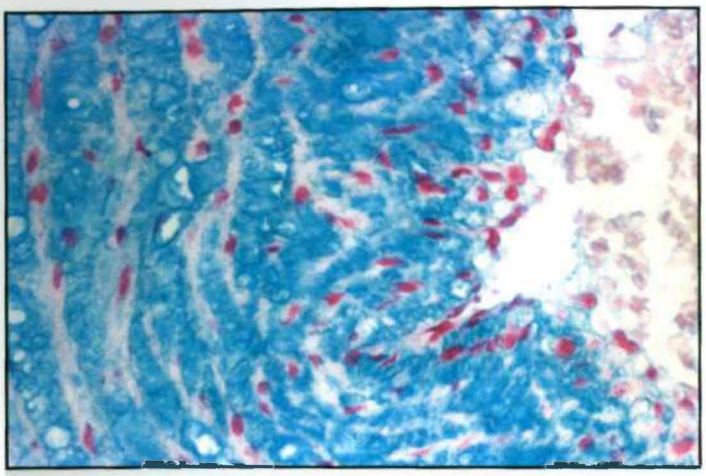
Testicular hyaluronidase	0.025 g (25 mg)
Buffer solution	50 mL
Mix just before use	

Alcian Blue Staining Solution

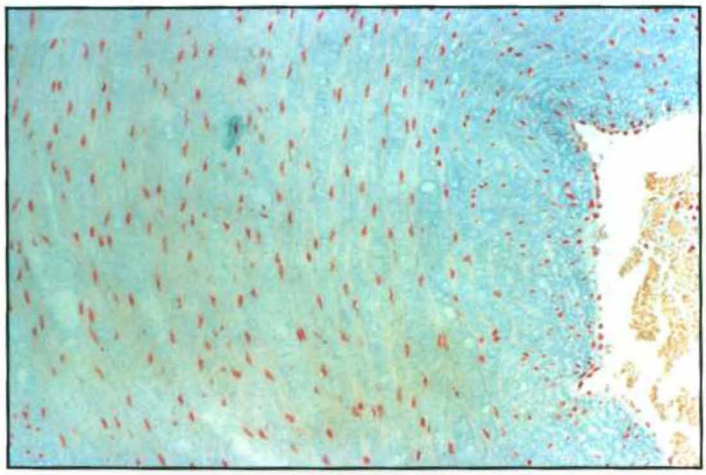
Alcian blue-8GX	1 g
Acetic acid, 3% solution	100 mL
Adjust the pH to 2.5, filter, and add a few crystals of thymol	

Nuclear-Fast Red Solution

See alcian blue, pH 2.5, p145



[i7.13] A section of umbilical cord stained with alcian blue, pH 2.5, without digestion.



[i7.14] A duplicate of the section shown in **[i7.13]** stained with alcian blue, pH 2.5, following testicular hyaluronidase digestion.

■ *Procedure*

1. Prepare buffer and digestion solutions. Preheat 1 Coplin jar of each at 37°C for 1 hour.
2. Deparaffinize and hydrate sections to distilled water.
3. Place sections from each block and control labeled "with digestion" in preheated digestion solution. Place duplicate sections labeled "without digestion" in preheated buffer solution. Incubate at 37°C for 2 hours.
4. Wash both sets of slides in running water for 5 minutes. Combine slides.
5. Place slides in 3% acetic acid solution for 3 minutes.
6. Place slides in alcian blue solution for 30 minutes.
7. Wash slides in running tap water for 10 minutes.
8. Rinse in distilled water.
9. Counterstain in nuclear-fast red solution for 5 minutes.
10. Wash in running tap water for at least 1 minute.
11. Dehydrate in 2 changes each of 95% alcohol and absolute alcohol, and clear in xylene.

■ *Results*

- Without digestion **[i7.13]**, acid mucopolysaccharides and sialomucins: Deep blue
- With digestion **[i7.14]**, mucosubstances containing hyaluronic acid and chondroitin sulfates A and C: Marked loss of staining

ALCIAN BLUE-PAS-HEMATOXYLIN [LUNA 1968; CARSON 1983]

■ *Purpose*

Differentiation between neutral and acidic mucosubstances; this procedure is used in many laboratories today for the detection of intestinal metaplasia

■ *Principle*

Acidic mucosubstances are stained with the alcian blue technique and neutral mucosubstances are stained by the PAS reaction.

■ *Fixative*

10% neutral-buffered formalin or Zenker solution

■ *Equipment*

Hot plate, Coplin jars, Erlenmeyer flasks, graduated cylinders, filter paper.

■ *Technique*

Cut paraffin sections of routine tissue at 4 to 5 µm. Sections of kidney should be cut at 2 to 3 µm.

■ *Quality Control*

Use a kidney or a mucin control, depending on the diagnostic tissue to be stained. A section of cervix containing both endocervix and ectocervix also provides a good control.

■ *Reagents*

Acetic Acid, 3% Solution

Glacial acetic acid	3 mL
Distilled water	100 mL

Alcian Blue, pH 2.5

Alcian blue	5 g
Acetic acid, 3% solution	500 mL

Periodic Acid, 0.5% Solution

Periodic acid	2.5 g
Distilled water	500 mL

Stock Reducing Rinse

Sodium metabisulfite	10 g
Distilled water	100 mL

Working Reducing Rinse

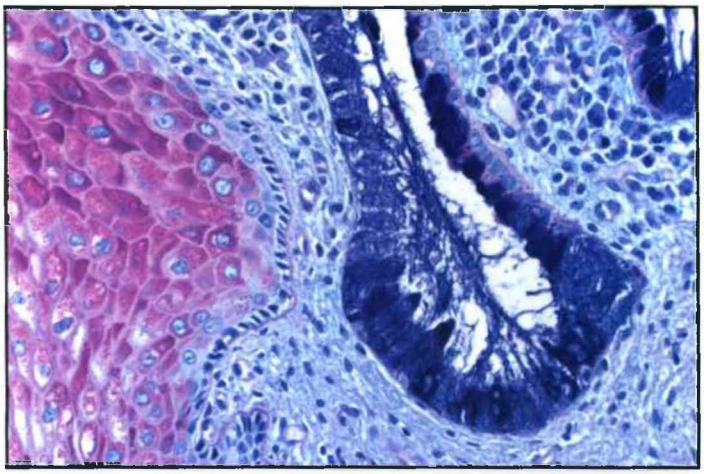
Stock reducing rinse	2.5 mL
Distilled water	50 mL
Prepare just before use	

Schiff Reagent

See the PAS procedure, p138

■ ***Procedure***

1. Deparaffinize sections, and bring to water as usual.
2. Place sections in 3% acetic acid for 1 minute.
3. Stain sections in alcian blue for 30 minutes.
4. Wash sections in running tap water, then rinse in distilled water.
5. Place sections in 0.5% periodic acid for 10 minutes.
6. Wash slides in running tap water for 5 minutes, then rinse in distilled water.
7. Place slides in Schiff reagent for 10 minutes.
8. Place slides in reducing rinse for 5 minutes.
9. Wash in running tap water for 10 minutes.



[i7.15] A section of cervix stained with the alcian blue-PAS technique. Glycogen present in the stratified squamous epithelium of the ectocervix is stained rose; staining of the mucin-secreting gland of the endocervix varies from dark blue to purple, indicating a mixture of neutral and acidic mucins. The section has been counterstained with hematoxylin.

10. Stain sections with Harris hematoxylin containing acetic acid (48 mL hematoxylin/2 mL glacial acetic acid) for ½ to 1 minute. *This step is optional.*
11. Dehydrate in 2 changes each of 95% and absolute alcohols, clear in xylene, and mount with synthetic resin.

■ ***Results*** **[i7.15]**

• Exclusively acid mucosubstances	Blue
• Neutral polysaccharides	Magenta
• Certain substances will be colored by both PAS and alcian blue	Purple

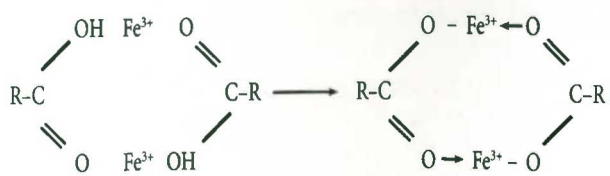
MÜLLER-MOWRY COLLOIDAL IRON [MOWRY 1958]

■ ***Purpose***

Demonstration of carboxylated and sulfated mucopolysaccharides and glycoproteins

■ ***Principle***

Colloidal ferric ions are, at a low pH, absorbed principally by carboxylated and sulfated mucosubstances **[f7.3]**.



[f7.3] Absorption of ferric ions by carboxylated and sulfated mucosubstances.

The excess reagent is washed out and the classic Prussian blue reaction is used to demonstrate iron bound to the tissue:

$$4\ Fe^{3+} + 3\ K_4Fe(CN)_6 \rightarrow Fe_4[Fe(CN)_6]_3\downarrow$$

■ *Fixative*

10% Neutral-buffered formalin, Carnoy solution, or alcoholic formalin are preferred. Avoid chromate fixatives.

■ *Equipment*

Hot plate, Coplin jars, Erlenmeyer flasks, graduated cylinders, filter paper, pipettes

■ *Technique*

Cut paraffin sections at 4 to 5 µm.

■ *Quality Control*

A section of unautolyzed small bowel, appendix, or colon may be used as a control.

■ *Reagents*

Ferric Chloride, 29% Solution

Ferric chloride, $FeCl_3 \cdot 6H_2O$	29 g
Distilled water	100 mL

Müller Colloidal Iron (Stock) Solution

Bring 250 mL of distilled water to a boil. While the water is still boiling, pour in 4.4 mL of freshly prepared 29% ferric chloride solution and stir. When the solution has turned dark red, remove from heat and allow to cool. Store in an amber bottle and label "stock colloidal iron." This reagent is dark red, clear, and stable for many months. If the water is not kept boiling during the addition of the ferric chloride, the conversion to colloidal (hydrous) ferric oxide will not be complete and results obtained with the use of the solution will be faulty.

Working Colloidal Iron Solution

Stock colloidal iron	10 mL
Distilled water	18 mL
Glacial acetic acid	12 mL
Prepare just before use	

Potassium Ferrocyanide Solution, 2% Solution

Potassium ferrocyanide	2 g
Distilled water	100 mL

Hydrochloric Acid, 2% Solution

Hydrochloric acid	2 mL
Distilled water	100 mL

Ferrocyanide-Hydrochloric Acid Solution

Just before use, mix:	
Potassium ferricyanide, 2%	25 mL
Hydrochloric acid, 2%	25 mL

Acetic Acid, 12% Solution

Glacial acetic acid	24 mL
Distilled water	176 mL

Nuclear-Fast Red Solution

See alcian blue, pH 2.5, p145

■ *Conventional Procedure*

1. Deparaffinize and hydrate sections to distilled water.
2. Rinse the slides briefly in 12% acetic acid solution. This prevents watery dilution of the colloidal iron solution.
3. Stain sections in working colloidal iron solution for 1 hour.
4. Rinse in 3 changes of 12% acetic acid solution, 3 minutes each.
5. Immerse in ferrocyanide-hydrochloric acid solution for 20 minutes at room temperature.
6. Wash in running tap water for 5 minutes.
7. Counterstain sections in nuclear-fast red solution for 5 minutes.
8. Wash slides in running water for at least 1 minute.
9. Dehydrate, clear, and mount with synthetic resin.

■ *Microwave Procedure [Churukian 1993]*

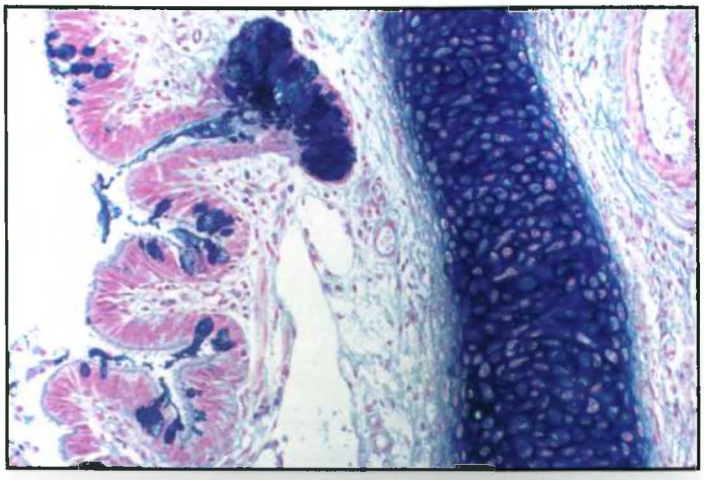
1. Deparaffinize and hydrate sections to distilled water.
2. Rinse the slides for 1 minute in 12% acetic acid solution. This prevents watery dilution of the colloidal iron.
3. Place sections in working colloidal iron solution in a glass Coplin jar, and microwave at power level 1 (60 W) for 3 minutes. Dip the slides up and down several times, and allow them to remain in the warm solution for 10 minutes.
4. Rinse in 4 changes of 12% acetic acid solution, 1 minute each.
5. Immerse in ferrocyanide-hydrochloric acid solution in a glass Coplin jar, and microwave at power level 1 (60 W) for 2 minutes. Dip the slides up and down several times, and allow them to remain in the warm solution for 2 minutes.
6. Wash in running tap water for 1 minute.
7. Rinse in 2 changes of distilled water.
8. Counterstain sections in nuclear-fast red solution for 3 minutes.
9. Wash slides in running water for at least 1 minute.
10. Dehydrate, clear, and mount with synthetic resin.

■ *Results* [i7.16], [i7.17]

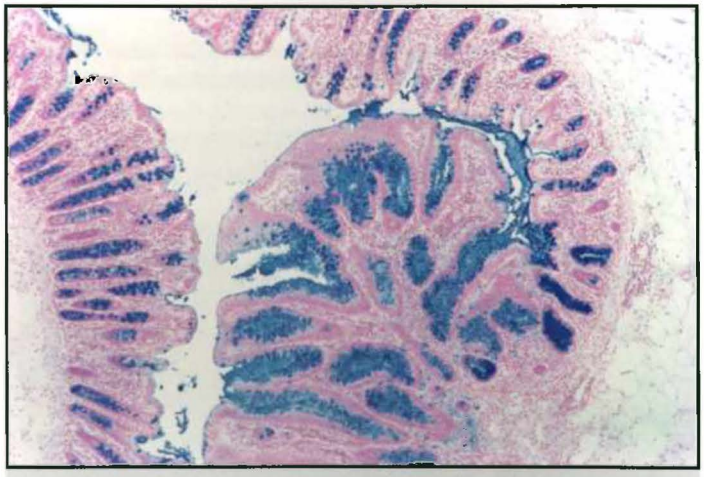
- Acid mucopolysaccharides and sialomucins — Deep blue
- Nuclei — Pink-red
- Cytoplasm — Pink

■ *Technical Notes*

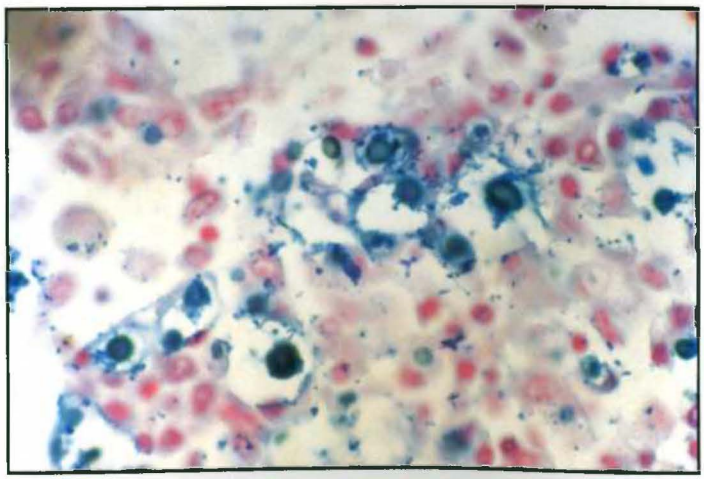
1. Strongly acid mucins that do not stain with alcian blue also do not stain with colloidal iron.
2. The PAS stain can be used as a counterstain for this procedure also. PAS-positive material will be magenta, mixtures of neutral and acidic mucosubstances will be purple, and acidic mucosubstances will be blue.
3. This method usually gives a more intense color than alcian blue, but colloidal iron is not considered specific for acid mucopolysaccharides.
4. Hyaluronidase digestion also can be used with this procedure. (See the alcian blue procedure with hyaluronidase digestion for reagent preparation and times of digestion.)
5. This method is excellent for the demonstration of *Cryptococcus neoformans* [i7.18].



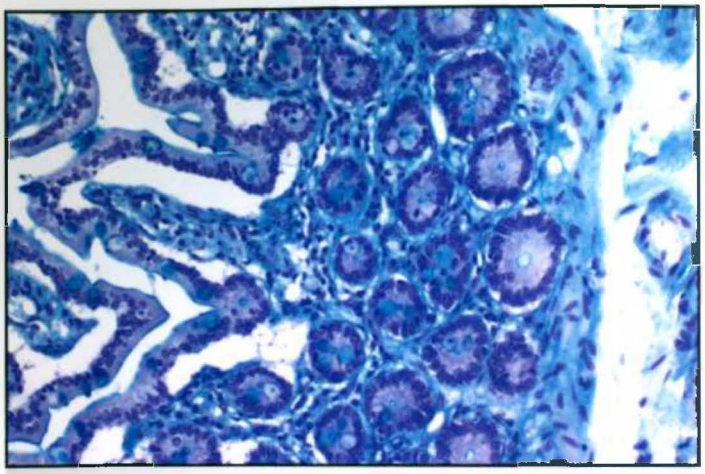
[i7.16] A section of lung containing a small bronchus stained with colloidal iron. Note the strong staining of the cartilage, goblet cells, and a mucin-secreting gland.



[i7.17] A duplicate of the sections shown in **[i7.1], [i7.11], [i7.12]** stained with colloidal iron. This method stains acid mucopolysaccharides and acidic glycoproteins, but it is not considered as specific as the alcian blue techniques.



[i7.18] *Cryptococcus neoformans* stained with the microwave colloidal iron procedure. [Image courtesy of Churukian 1993]



[i7.19] This section of small intestine was stained with colloidal iron and counterstained with hematoxylin. The marked nonspecific background staining is not acceptable. Fresh colloidal iron solution should be prepared according to the directions, and then the stain should be repeated. The slide was also dried too quickly after mounting the section from the waterbath.

6. Some background staining may be seen in some sections because of the presence of *connective tissue mucin*; however, if strong background staining **[i7.19]** is noted, the stain should be repeated using all fresh solutions, beginning with those used in the preparation of the colloidal iron solution.

7. If the slides are not washed well with water after the nuclear-fast red, clouding will result when the slides are placed in the alcohols.

Amyloid

Virchow coined the name *amyloid* (starchlike) in 1854 because the reaction with iodine led him to believe that amyloid was carbohydrate in nature. Amyloid is predominantly a fibrillar protein that deposits in tissue under certain pathologic conditions. Amyloid also contains approximately 1% to 2% carbohydrate, mostly acid mucopolysaccharides. The exact composition of this carbohydrate moiety of amyloid has been intensively studied and debated during the past half century, and has been found to include chondroitin, heparin, and dermatan sulfates.

Amyloidosis is a disease characterized by an amorphous, eosinophilic, extracellular deposit that gradually replaces cellular elements of vital organs and causes progressive loss of function and eventual death. The composition of amyloid varies between patients and between organs in the same patient. In the past, amyloid was classified into 1 of 4 groups as follows [Bancroft 1982]:

1. Primary amyloid occurs spontaneously in the absence of any predisposing disease. The organs most often affected are muscle, heart, skin, and tongue.

2. Secondary amyloid is associated with predisposing disease, frequently inflammatory, such as rheumatoid arthritis and tuberculosis. This type of amyloid is most frequently deposited in the kidney, liver, spleen, and adrenal glands, and most of the patients with this type of amyloidosis have nephritic syndrome.

3. Myeloma-associated amyloid is associated with diseases of the immune system and resembles primary amyloid in distribution.

4. Tumor-associated amyloid is found in association with many tumors, especially those of the amine precursor uptake and decarboxylation (APUD) system.

Currently, 20 different amyloids are known [Vowles 2002], and they are identified by an abbreviation of their originating protein preceded by an A (eg, AA is serum amyloid A-derived, AL is immunoglobulin light chain-derived, AH is immunoglobulin heavy chain-derived, etc). This scheme is the universally accepted method of amyloid classification, but the entire scheme is beyond the scope of this book. The interested reader is referred to Vowles and Frances [2002]. Most patients seen in the United States with systemic amyloid have the immunoglobulin light chain amyloid (AL) [Gertz 2004].

ALKALINE CONGO RED METHOD [PUCHTLER 1962]

■ *Purpose*
The demonstration of amyloid in tissues

■ *Principle*
Green birefringence following Congo red staining is considered the most specific technique for the demonstration of amyloid. However, false-positive results may be obtained, and the method used is of the utmost importance. Congo red is a benzidine derivative that can react with cellulose; Virchow regarded amyloid as an isomer of cellulose, and amyloid resembles cellulose in its chemical reactions [Vacca 1985]. In this method, pretreatment with alkali aids in the release of native internal hydrogen bonds between adjacent protein chains; as a result, more potential sites are available for dye binding. Amyloid is a linear molecule, and this configuration allows azo and amine groups of the dye to form hydrogen bonds with similarly spaced hydroxyl radicals of the amyloid. Whether the binding of the dye occurs with the polysaccharide or the protein component of amyloid is indefinite at this time.

■ *Fixative*
Alcohol or Carnoy solution is preferred; 10% neutral-buffered formalin, Bouin solution, or Zenker solution may be used. Prolonged storage in 10% formalin will cause a gradual decrease in staining intensity.

■ *Equipment*
Mechanical stirrer, Coplin jars, filter paper, Erlenmeyer flasks, graduated cylinders.

■ *Technique*
Cut paraffin sections at 8 to 10 μm. Sections not in this range may not show the green birefringence.

■ *Quality Control*
Sections containing amyloid must be used. According to Bancroft and Cook [1994], it is better not to keep too many control sections cut, because the staining intensity has been reported to decrease with the age of the sections. Also, massive, presumably

long-standing, deposits give less intense histochemical reactions than small, newly formed deposits.

■ *Reagents*

Stock 80% Alcohol Saturated with Sodium Chloride

Sodium chloride	20 g
Distilled water	200 mL
Stir until the salt is dissolved; then, with continued stirring on a magnetic stirrer, add:	
Ethyl alcohol, 100%	800 mL
Some salt should precipitate out, indicating that the solution is saturated; this method ensures a saturated solution [Lillie 1976]	

Alkaline Salt Solution

Stock 80% alcohol saturated with sodium chloride	50 mL
Sodium hydroxide, 1% solution	0.5 mL
Filter and use within 15 minutes	

Stock Congo Red Staining Solution

Congo red	1 g
Stock 80% alcohol saturated with sodium chloride	500 mL
This is a solution saturated with both sodium chloride and Congo red; stir well on the magnetic stirrer and let stand overnight	

Working Congo Red Staining Solution

Stock Congo red solution	50 mL
Sodium hydroxide, 1% solution	0.5 mL
Filter and use within 15 minutes	

■ *Procedure*

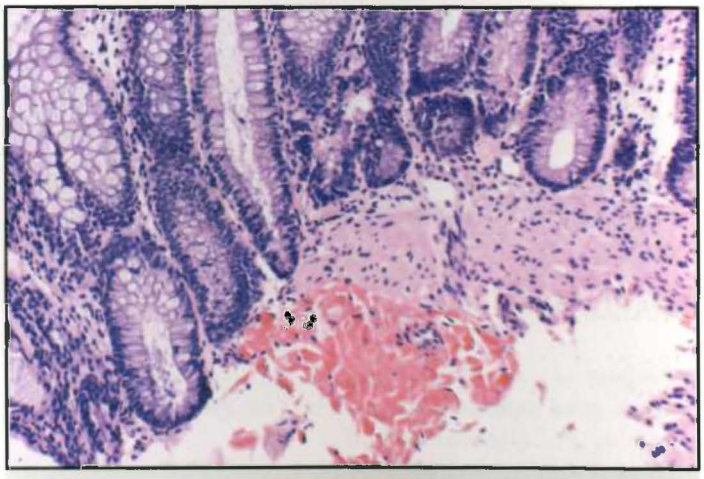
1. Deparaffinize sections, and bring to water as usual.
2. Stain in Harris hematoxylin with acetic acid (2 mL/48mL hematoxylin) for 2½ minutes.
3. Wash in running tap water for several minutes.
4. Place sections in alkaline salt solution for 20 minutes.
5. Stain in working Congo red solution for 20 minutes.
6. Dehydrate rapidly in 3 changes of absolute alcohol, 5 or 6 good dips in each.
7. Clear in 2 or 3 changes of xylene and mount in synthetic resin.

■ *Results* [i7.20]

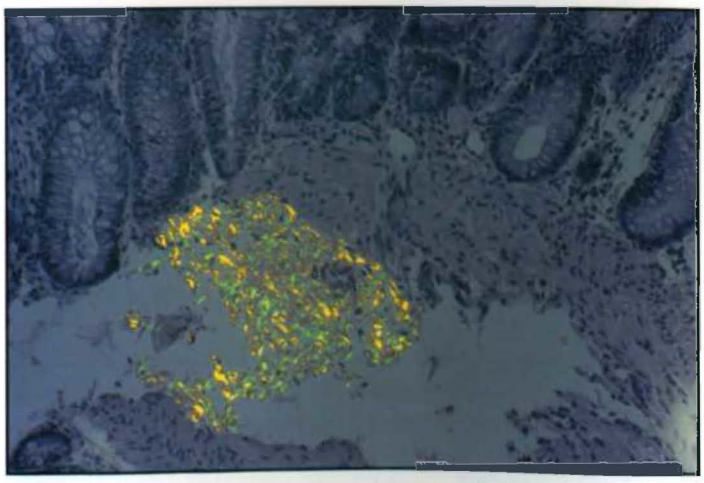
- Amyloid — Deep pink to red
- Elastic tissue — Pale pink
- Nuclei — Blue

■ *Technical Notes*

1. Following Congo red staining, bright apple-green birefringence exhibited with polarized light is considered specific for amyloid [i7.21].
2. Green birefringence is an intrinsic property of the amyloid fibril-Congo red complex and is a function of the parallel alignment of dye molecules and amyloid fibrils. The thickness of the section is critical, with 8 to 10 µm optimal; sections that



[i7.20] A section from a colon biopsy specimen stained with Congo red. Red-stained amyloid can be seen in the center bottom of the photograph.



[i7.21] The Congo red-stained section shown in **[i7.20]** examined with polarized light. Apple-green birefringence is now apparent in the stained amyloid deposit.

are too thin show faint red colors and sections that are too thick show yellow birefringence [Bancroft 1982].

3. Differentiation is avoided in this procedure by including a high concentration of sodium chloride in the dye solution, which also enhances hydrogen bonding of the Congo red to amyloid [Vowles 2002].

4. Kiernan, however, states that hydrophobic and van der Waals forces are probably the most important factors in dye binding and not hydrogen bonding.

5. False-positive staining may be obtained with many of the Congo red staining methods [Carson 1980, Elghetany 1988]; false-positive birefringence is closely related to the excess dye retained in the tissue. When performed correctly, the method of Puchtler [1962] does not tend to give false-positive results.

6. The sodium chloride and the high alcohol content present in the dye solvent tend to depress dye ionization and acid-base type staining, resulting in a stained section with a clean background.

7. Elghetany and Saleem [1988] reviewed the methods for staining amyloid and concluded that although several new techniques have been developed since the Congo red method was first described, this technique is still the mainstay for the demonstration of amyloid.

8. Saturation of the solution, where indicated, is very important, and the instructions should be followed exactly.

CRYSTAL VIOLET [LIEB 1947, SHEEHAN 1980]

■ *Purpose*

This is a good rapid screening method for amyloid but is not as specific as the Congo red method.

■ *Principle*

The exact mechanism of the staining of amyloid with crystal violet has not been defined. It has been assumed, but not proved, that the "metachromatic" staining of amyloid is because of the mucopolysaccharide content; however, amyloid will induce only weak metachromasia with thionine and toluidine blue, and investigators have been unable to find a parallel reaction with methyl violet in a wide range of mucopolysaccharides. Crystal violet and methyl violet have been shown to be mixtures of basic dyes; therefore, it is more likely that the amyloid selectively reacts with 1 of the dye components; therefore, polychromatic rather then metachromatic is a more appropriate term for the staining mechanism. The addition of acid to the staining solution will prevent overstaining of cytoplasmic components. This method has low sensitivity, and lacks specificity, so it is rarely used today for the detection of amyloid.

■ *Fixative*

10% neutral-buffered formalin or alcohol

■ *Equipment*

Hot plate, mechanical stirrer, Coplin jars, graduated cylinders, pipettes, filter paper

■ *Technique*

Cut paraffin sections at 10 to 12 µm.

■ *Quality Control*

A section containing amyloid must be used. (See the "Quality Control" section in the Congo red procedure, p152)

■ *Reagents*

Stock Saturated Crystal Violet Solution

Crystal violet, to saturate approximately	14 g
Alcohol, 95%	100 mL

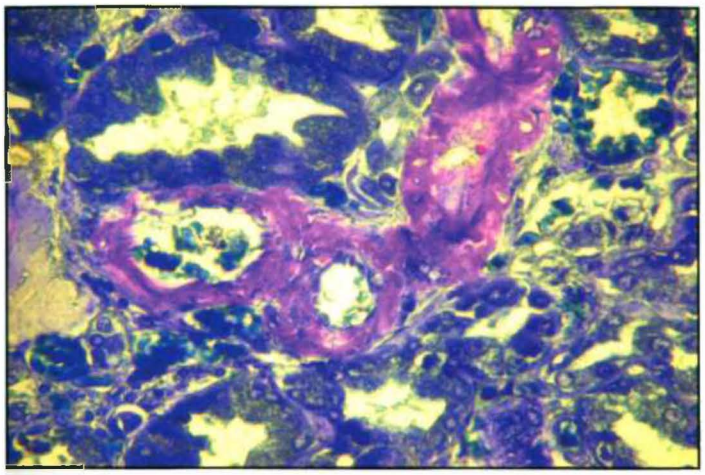
Working Crystal Violet Solution

Crystal violet, stock solution	2 mL
Distilled water	60 mL
Hydrochloric acid, concentrated	0.2 mL

Modified Apathy Mounting Medium

Acacia (gum arabic)	50 g
Cane sugar	50 g
Distilled water	100 mL
Sodium chloride	10 g
Thymol	0.1 g

Bring water to a boil and slowly add the acacia and cane sugar, stirring until both are dissolved. Restore the volume with distilled water. Add and dissolve the sodium chloride, then add the thymol. Refrigerate to remove air bubbles. The sodium chloride prevents bleeding of the crystal violet and the thymol acts as a preservative. Store in the refrigerator.



[i7.22] A section of kidney stained with crystal violet. Note the amyloid deposits in the wall of the blood vessels. The crystal violet stain has been considered a metachromatic stain in much of the literature, but is probably a polychromatic stain. The rose staining of amyloid probably results from a reaction between the amyloid and one of the various dye components of crystal violet. The method is not specific but serves as a good screening technique. [Image courtesy of Lott R, Birmingham, AL]

■ *Procedure*

1. Deparaffinize sections, and hydrate to distilled water.
2. Stain sections in working crystal violet solution for 5 minutes.
3. Rinse sections well in tap water.
4. Mount with Apathy mounting medium
5. Seal edges of coverslip with fingernail polish.

■ *Results* [i7.22]

• Amyloid	Purplish violet
• Other tissue elements	Blue

■ *Technical Notes*

1. "Bleeding," or diffusion into the surrounding mounting medium, of basic aniline dyes tends to occur with aqueous mounting media. About 20% by weight of potassium acetate or about 60% of 1 of the sugars suffices to prevent bleeding of crystal violet stains for amyloid [Lillie 1976]. Because modified apathy mounting medium contains 50% sugar and 10% salt, diffusion of the dye does not occur with this mounting medium.
2. The stained sections may be allowed to air dry completely, then dipped in xylene and mounted with synthetic resin.
3. Luna [1992] recommended a minimum of 5 hours staining time in the working crystal violet solution, with overnight staining providing the ultimate in staining of amyloid with the crystal violet method. He states that at first the entire specimen stains a dense deep purple blue, with the increase in color continuing for several hours, at which point the section is heavily stained. Some time after 3 hours, the hydrochloric acid in the staining solution begins to selectively differentiate the dye from the section, resulting in the removal of the reddish-purple color from the nonamyloid portion of the section, but leaving the amyloid reddish-purple.

THIOFLAVINE T FLUORESCENT METHOD [VASSAR 1959; JANIGAN 1965; CARSON 1984]

■ *Purpose*

This is a good method for amyloid, but it is not as specific as the Congo red method with polarization, even when the fluorescence microscope is fitted with the optimum filters.

■ *Principle*

Thioflavine T is a fluorescent dye that attaches to amyloid. The background nuclear fluorescence is quenched by staining with aluminum hematoxylin.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

Coplin jars, graduated cylinders, filter paper

■ *Technique*

Cut paraffin sections at 6 to 10 µm.

■ *Quality Control*

A section containing amyloid must be used. (See the Congo red procedure.)

■ *Reagents*

Thioflavine T, 1% Solution

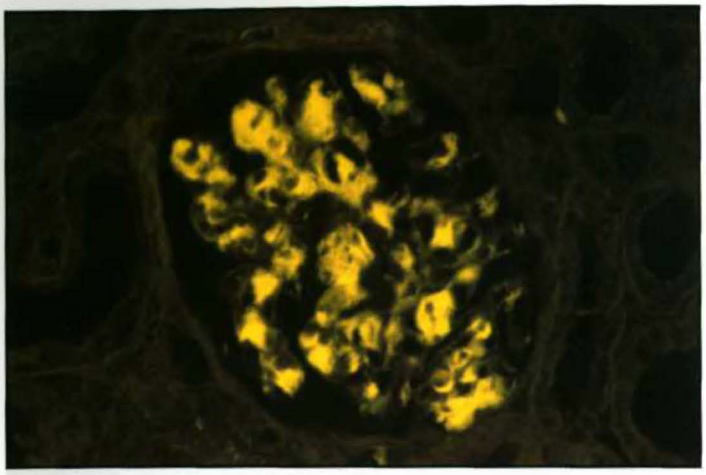
Thioflavine T	1 g
Distilled water	100 mL

Acetic Acid, 1% Solution

Acetic acid, glacial	1 mL
Distilled water	100 mL

Mayer Hematoxylin

See the hematoxylin formulation, p111



[i7.23] A section of kidney with amyloid deposits in the glomeruli stained with thioflavin T and examined with fluorescence microscopy. [Image courtesy of Wenk PA, Royal Oak, MI]

■ *Procedure*

1. Deparaffinize and hydrate sections to distilled water.
2. Stain in Mayer hematoxylin for 2 minutes to quench the nuclear fluorescence.
3. Wash in water for 3 to 5 minutes.
4. Stain in filtered thioflavine T for 3 minutes.
5. Rinse in distilled water.
6. Differentiate in 1% acetic acid for 20 minutes.
7. Wash in running water for 2 minutes.
8. Blot dry.
9. Mount with a nonfluorescent mountant.

■ *Results* **[i7.23]**

• Amyloid	Fluoresces yellow to yellow-green

■ *Technical Notes*

1. Burns [1967] found that thioflavine T at an acid pH increases the selectivity of the dye for amyloid. A pH of 1.4 is recommended.
2. The stained slides may be examined using the BG12 exciter filter and an OG4 and/or OG5 barrier filter; however, a UG1 or UG2 exciter filter and a colorless UV barrier filter will give a brighter yellow fluorescence of the amyloid against a blue background and will also show the finest deposits.
3. If preferred, the sections may be mounted from water using Apathy mounting medium.
4. Lipid granules, juxtaglomerular granules, and mast cells may give a yellow fluorescence, but should be differentiated easily from amyloid.

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LEARNING ACTIVITIES

1. **Perform the PAS, PAS with digestion, Mayer mucicarmine, alcian blue (pH 2.5 and 1.0), colloidal iron, alcian blue-PAS, Congo red, and crystal violet procedures. You may choose any tissue that will demonstrate positive staining.**

2. **Microscopically examine each stained section, and compare the results with those given in the procedure. Compare with the color plate demonstrating the results. If the results are unsatisfactory, analyze the procedural steps for possible sources of error. If a mistake is identified, repeat the stain after correcting the problem, and reexamine the slides.**

CHAPTER 8

Connective and Muscle Tissue

OBJECTIVES

On completing this chapter, the student should be able to do the following:

1. Identify the 3 connective tissue fibers, and briefly describe each
2. Identify the 3 types of muscle fibers, and briefly describe each
3. Identify 5 connective tissue cells, and briefly describe each
4. Classify the following techniques as to the fiber(s) or cell demonstrated:
    a. Masson trichrome
    b. Gomori 1-step trichrome
    c. van Gieson
    d. Verhoeff
    e. Gomori aldehyde fuchsin
    f. Orcein
    g. Russell modification of the Movat pentachrome stain
    h. Gomori silver impregnation
    i. Gordon and Sweets silver impregnation
    j. methenamine-silver
    k. Mallory phosphotungstic acid-hematoxylin
    l. oil red O
    m. Sudan black B
    n. osmium tetroxide
    o. toluidine blue
    p. methyl green-pyronin
5. Outline each of the above staining procedures, considering the following characteristics:
    a. most desirable fixative
    b. if another fixative has been used, what can be done
    c. primary reagents or dyes and their purpose
    d. results of the stain
    e. appropriate control material
    f. sources of error and appropriate correction
    g. mode of action
    h. special requirements (eg, chemically clean glassware)
    i. what type of microscope used
6. Identify reasons for using each of the techniques listed in objective 4
7. Describe the tissue preparatory techniques(s) used for the following stains:
    a. oil red O
    b. osmium tetroxide
    c. Sudan black B
8. Identify a unique feature of mast cells that aids in their demonstration
9. Identify the cytoplasmic substance stained by pyronin
10. Define impregnation

Connective Tissue

Connective tissue is 1 of the 4 basic tissues, and it functions to provide structural and metabolic support for the other tissues and organs in the body. It consists of 3 different components: fibers, cells, and amorphous ground substance. These components vary in amount within the different connective tissue types, which are

1. connective tissue proper
2. cartilage
3. bone
4. blood

Most commonly, when we use special stains to demonstrate connective tissue elements in the histopathology laboratory, we are interested in the fibers or cells of connective tissue proper. The ground substance of connective tissue proper is a mucopolysaccharide and is demonstrated with carbohydrate staining techniques. The fibers of connective tissue proper are described below:

1. **Collagen fibers** provide strength; the more collagen present, the stronger the tissue is. A dense regular arrangement of collagen fibers is found in tendons, organ capsules, and the dermis. At least 7 different types of collagen have been identified, with type 1 being the most common in humans. The differences between the various types are unnecessary for our understanding of the affinity of collagen for certain stains. Collagen is very eosinophilic and readily visible with light microscopy, is birefringent upon polarization, and reveals a characteristic pattern of cross-striations with the electron microscope. Collagen is demonstrated using the Masson and Gomori [1950b] trichrome techniques, and with the van Gieson stain. The procedures are given later in the chapter, p165.

2. **Elastic fibers** are present in most fibrous connective tissue, but are most abundant in tissue requiring flexibility, because the elastic fibers allow tissues to stretch. The size and arrangement vary among different tissues, from fenestrated sheets or lamellae in the aorta to scattered fibers in loose connective tissue. These fibers usually cannot be seen on hematoxylin-eosin (H&E)-stained sections but require special stains, such as the Verhoeff iron hematoxylin, Weigert resorcin fuchsin, orcein, or Gomori aldehyde fuchsin stains, for demonstration [Gomori 1950a].

3. **Reticular fibers** have been identified as a type of collagen. These fibers are not apparent in ordinary H&E-stained sections but may be demonstrated with an argyrophilic reaction, because they have the ability to adsorb silver from solution. The silver may then be reduced chemically to its visible metallic form. Reticular fibers form delicate networks and are much smaller than most collagen fibers.

4. The cells found in connective tissue proper, whether fixed, or free and transient, are:

   a. **Fibroblasts**, the most common cell in connective tissue, which produce the connective tissue fibers (extracellular, nonliving elements). Frequently, only flattened fibroblast nuclei can be distinguished among bundles of collagen. These cells are not commonly of interest in tissues; rather, it is the product of fibroblasts that we demonstrate with special techniques.

   b. **Mesenchymal cells,** which may be indistinguishable from fibroblasts. These are primitive, relatively undifferentiated cells that may develop into various differentiated cell types if the need arises for replacement.

   c. **Adipose**, or fat, cells, which synthesize and store lipid and are common in most loose connective tissue. In some areas of the body, this is the predominant cell type and the tissue is known as adipose tissue. The nucleus in an adipose cell becomes very flattened as the lipid accumulation of the cell grows. These are very long-lived cells, and even though the fat store may be depleted, the cell remains, awaiting a new accumulation. Fat, or simple lipid, is not preserved in paraffin sections, but can be demonstrated in frozen sections with special techniques. Lipids are discussed separately in most special staining manuals, but because adipose tissue is a type of connective tissue, I have elected to present the techniques for simple lipids as a part of the connective tissue section.

   d. **Mast cells**, which contain abundant secretory granules that, with special stains, frequently obscure the nucleus. Mast cell granules contain histamine and heparin, and exhibit metachromasia when stained with toluidine blue, a reaction that primarily is the result of the heparin content of the granules. Mast cells are most prominent along small blood vessels and closely resemble the basophilic leukocyte found in blood. Both mast cells and basophils can degranulate to increase vascular permeability.

   e. **Macrophages** are "big eaters," or scavenger cells that are found not only in connective tissue proper but in various other tissues such as liver, myeloid, and lymphatic tissues. Monocytes (blood leukocytes) are the precursor of macrophages, and are also known as histiocytes. These cells play an important role in immune mechanisms, processing antigenic material for presentation to the lymphocytes. We are not asked to demonstrate this cell in routine histopathology.

   f. **Plasma cells**, which are derived from B lymphocytes, produce immunoglobulins. Before immunoenzyme techniques were used for the demonstration of immunoglobulins, the methyl green-pyronin stain was frequently used to help identify immunoblastic sarcomas, a type of B-cell lymphoma.

   g. **Blood cells** of all types may be found in tissue, but these will not be discussed in this chapter.

Basement Membrane

The basement membrane, frequently referred to as the *basal lamina*, is found beneath epithelium, and separates the epithelium from the underlying connective tissue. The basement membrane consists of type IV collagen, laminin (a glycoprotein), and a proteoglycan rich in heparin sulfate. A similar structure surrounds muscle cells, Schwann cells, and other cells of mesenchymal origin. Basement membranes are illustrated by techniques that demonstrate the carbohydrate component. This is because of both the glycoprotein present in the membrane and the fact that the collagen present in the basement membrane contains much more sugar in some of its side chains than is normally present in ordinary collagen.

The primary function of the basement membrane is to provide physical support for epithelium; it also provides for cell attachment and for ultrafiltration. In the kidney, the basement membrane of capillary endothelium acts as a sieve, holding back molecules on the basis of size, shape, and electrostatic charge. The ultrastructural appearance of the glomerular basement membrane can be seen in chapter 14, **[i14.13, p346]**; the glomerular basement membrane stained with periodic acid-Schiff (PAS) is demonstrated in chapter 7 **[i7.2, p139]** and stained with a silver technique in this chapter.

Muscle

Muscle is also 1 of the 4 basic types of tissue and, based on the differences in structure and in function, is classified as follows:

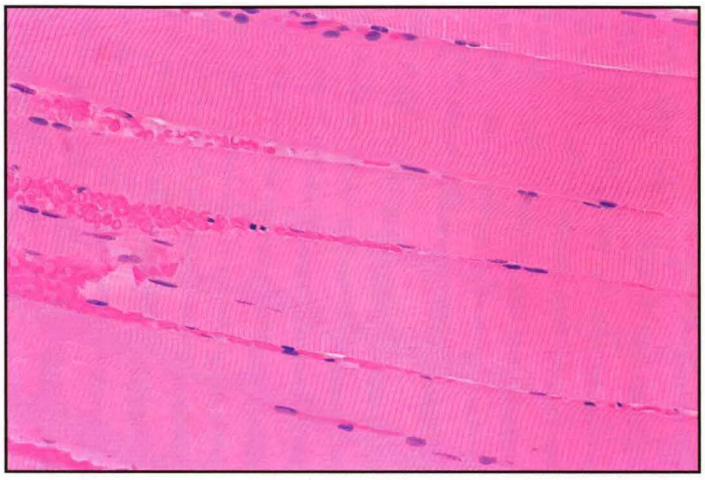
1. **Skeletal muscle**. This type of muscle also may be classified as:

   a. **striated**, because of the characteristic dark (A) and light (I) bands seen microscopically, and

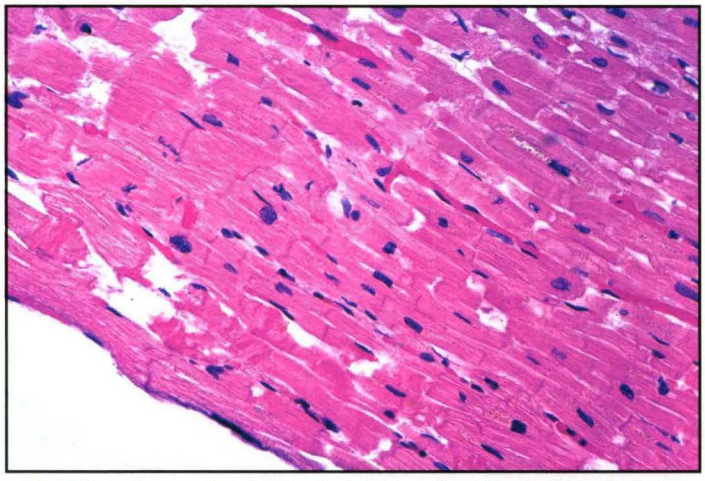
   b. **voluntary**, because contraction can be brought about at will.

   Because cardiac muscle is also striated, skeletal muscle should be referred to as skeletal muscle, not striated muscle. Individual muscle fibers, or cells, may be quite long and contain multiple peripherally located nuclei **[i8.1]**. Actin and myosin are the major contractile proteins of skeletal muscle, and the arrangement of these 2 proteins imparts the striated appearance seen in longitudinal sections. Traditionally, the phosphotungstic acid-hematoxylin (PTAH) method of Mallory was used to demonstrate these cross-striations, but today monoclonal antibodies are used with immunohistochemical methods to demonstrate pathologic skeletal muscle components.

2. **Cardiac muscle**. This is also a striated but involuntary type of muscle. It is similar to skeletal muscle, except that the cells branch and anastomose, and each cell usually has only 1 centrally located nucleus. Cardiac muscle cells anastomose with specialized intercellular junctions called intercalated discs. Cardiac muscle can be seen in **[i8.2]**.

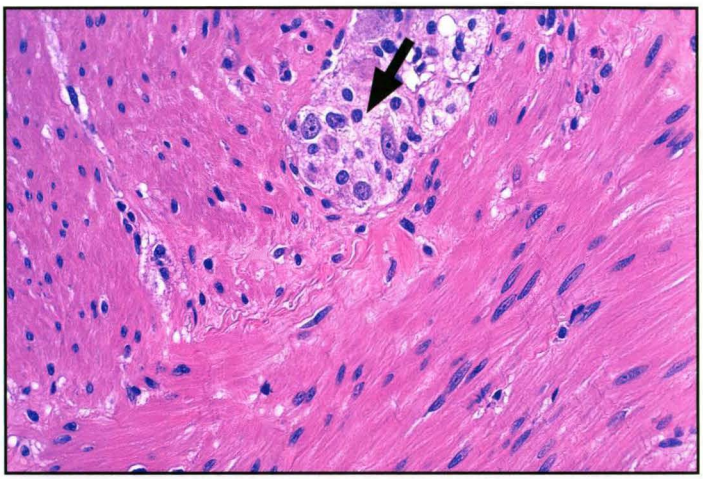


[i8.1] Skeletal muscle in longitudinal section. The muscle was isometrically fixed so that the dark (A) and light (I) bands, which give skeletal muscle its characteristic striated appearance, are demonstrated with the H&E stain. Note that each muscle cell or fiber contains multiple peripheral nuclei.

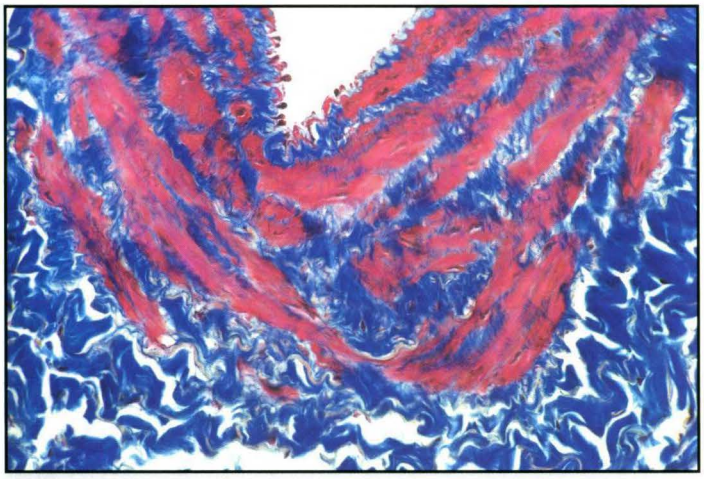


[i8.2] Cardiac muscle in longitudinal section. Note that the fibers branch, and each contains 1 centrally located nucleus. Although cardiac muscle is striated, the striations are not as obvious as in skeletal muscle **[i8.1]**. A dark line, identified as an intercalated disk, can be seen crossing the fibers occasionally.

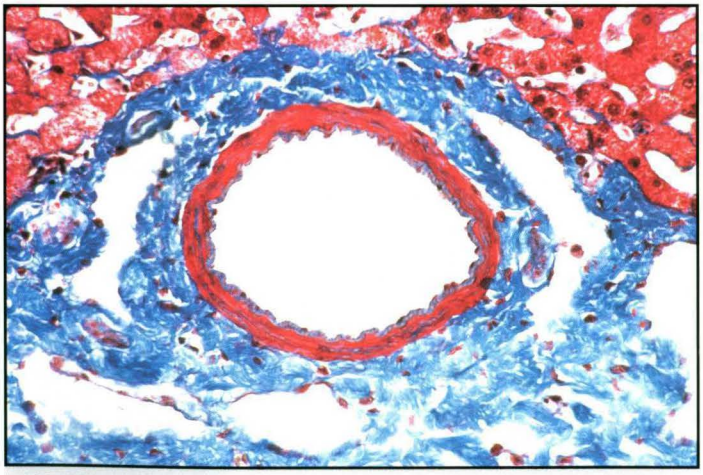
3. **Smooth muscle**. This is a nonstriated, involuntary type of muscle that is commonly arranged in layers. The muscle fibers are long and tapered and contain a single centrally located nucleus **[i8.3]**. Actin and myosin are also present in smooth muscle, but they are present in a different ratio than in striated muscle, and these proteins also are organized very differently. Smooth muscle regulates luminal size in hollow organs and tubular structures. **[i8.4]**, **[i8.5]** show smooth muscle fibers in the wall of a blood vessel stained red.



[i8.3] Smooth muscle in cross (top left) and longitudinal (bottom right) section, characteristic of the external muscle layers of the gastrointestinal tract. The single nuclei are elongated and centrally placed in the longitudinally sectioned fibers, and round and centrally placed in the cross-sectioned fibers. The arrow is located in a ganglion or a collection of nerve cells occurring outside the central nervous system.



[i8.4] Masson trichrome-stained section of a large vein. The lumen can be seen at the top. Smooth muscle cells are bright red and collagen fibers are blue.



[i8.5] Masson trichrome-stained section of liver shows the portal vein containing a few layers of smooth muscle in a portion of the portal triad. A large vein or a muscular artery in a section is best for judging the quality of staining. Red staining of epithelial cells but not of smooth muscle cells indicates a problem with the procedure or reagents.

Staining Techniques for Connective Tissue Fibers

MASSON TRICHROME STAIN [LUNA 1968, SHEEHAN 1980]

■ *Purpose*

Trichrome stains are frequently used to differentiate between collagen and smooth muscle in tumors and to identify increases in collagenous tissue in diseases such as cirrhosis of the liver.

■ *Principle*

Trichrome procedures are so named because 3 dyes, which may or may not include the nuclear stain, are used. The mechanism of the stain is not totally understood, and may be related in part to the size of different dye molecules. Sections are first stained with an acid dye such as Biebrich scarlet; all acidophilic tissue elements, such as cytoplasm, muscle, and collagen, will bind the acid dyes. The sections are then treated with phosphotungstic and/or phosphomolybdic acid. Because cytoplasm is much less permeable than collagen, phosphotungstic and phosphomolybdic acids cause Biebrich scarlet to diffuse out of the collagen but not out of the cytoplasm of cells. Phosphotungstic and phosphomolybdic acid have numerous acidic groups that most likely act as a link between the decolorized collagen and aniline blue, the collagen dye. Probably the pH of the phosphotungstic/phosphomolybdic acid solution also increases selective collagen staining and aids in the diffusion or removal of Biebrich scarlet.

■ *Fixative*

Bouin solution is preferred, but 10% neutral-buffered formalin may be used.

■ *Equipment*

56°C to 58°C oven, Coplin jars, Erlenmeyer flasks, graduated cylinders, pipettes

■ *Technique*

Cut paraffin sections at 4 to 5 μm.

■ *Quality Control*

Practically every tissue has an internal control, so no other control sections are needed; however, if a control is desired, uterus, small intestine, appendix, or fallopian tube will provide good material.

■ *Reagents*

Bouin Solution

Picric acid, saturated aqueous solution	75 mL
Formaldehyde, 37% to 40%	25 mL
Glacial acetic acid	5 mL

Weigert Iron Hematoxylin Solution

Solution a	
Hematoxylin	10 g
Alcohol, 95%	1,000 mL
Solution b	
Ferric chloride, 29% aqueous solution	20 mL
Distilled water	475 mL
Glacial acetic acid	5 mL
Working Solution	
*Mix equal parts of solutions **a** and **b***	

Biebrich Scarlet-Acid Fuchsin Solution

Biebrich scarlet, 1% aqueous solution	360 mL
Acid fuchsin, 1% aqueous solution	40 mL
Glacial acetic acid	4 mL

Phosphomolybdic/Phosphotungstic Acid Solution

Phosphomolybdic acid	25 g
Phosphotungstic acid	25 g
Distilled water	1,000 mL

Aniline Blue Solution

Aniline blue	25 g
Glacial acetic acid	20 mL
Distilled water	1,000 mL

Acetic Acid, 1% Solution

Glacial acetic acid	1 mL
Distilled water	99 mL

■ *Conventional Procedure*

1. Deparaffinize sections, and hydrate to distilled water.
2. Rinse well in distilled water.
3. Mordant formalin-fixed sections in Bouin solution for 1 hour at 56°C.
4. Remove slides from oven, allow to cool, and wash in running water until the yellow color disappears.
5. Rinse in distilled water.
6. Stain sections in Weigert iron hematoxylin for 10 minutes.
7. Wash in running water for 10 minutes.
8. Rinse in distilled water.
9. Stain sections in Biebrich scarlet-acid fuchsin solution for 2 minutes. If desired, the solution may be saved for 1 more run only.
10. Rinse in distilled water.
11. Place the slides in phosphomolybdic/phosphotungstic acid solution for 10 to 15 minutes. Discard this solution.
12. Stain sections in aniline blue solution for 5 minutes. If desired, the solution may be saved for 1 more run only.
13. Rinse the slides in distilled water.
14. Place the slides in 1% acetic acid solution for 3 to 5 minutes. Discard this solution.
15. Dehydrate with 95% and absolute alcohols, 2 changes each.
16. Clear with 2 or 3 changes of xylene, and mount with synthetic resin.

■ *Results* **[i8.4], [i8.5]**

- Nuclei — Black
- Cytoplasm, keratin, muscle fibers — Red
- Collagen and mucin — Blue

■ *Technical Notes*

1. If desired, collagen may be counterstained with light green instead of aniline blue [**i8.6**]. The following changes are made;

   - Step 11. Place the sections in a 5% aqueous solution of phosphotungstic acid.

   - Step 12. Stain 5 minutes in 2% light green:

Light Green Counterstain

Light green, SF yellowish	2 g
Distilled water	99 mL
Glacial acetic acid	1 mL

2. Light green is a better counterstain when collagen is predominant, however, when only small amounts are to be demonstrated, the aniline blue is the better counterstain.

3. Decreased red staining usually indicates that the staining solution has aged or been overused and should be discarded [**i8.7**]. If blue staining of connective tissue appears faded, the section has probably been overdifferentiated in the acetic acid solution. Pathologically altered collagen, such as that seen in burns, may lose its affinity for aniline blue and bind the acid dye instead [Vacca 1985].

4. Sections fixed in 10% neutral-buffered formalin will stain poorly and unevenly if not mordanted in either Bouin or a mercuric chloride solution; however, mercuric fixatives should not be used because of the toxicity.

5. An iron hematoxylin solution is used for nuclear staining in the trichrome procedures because iron hematoxylin is more resistant than aluminum hematoxylin to decolorization in the subsequently used acidic dye solution.

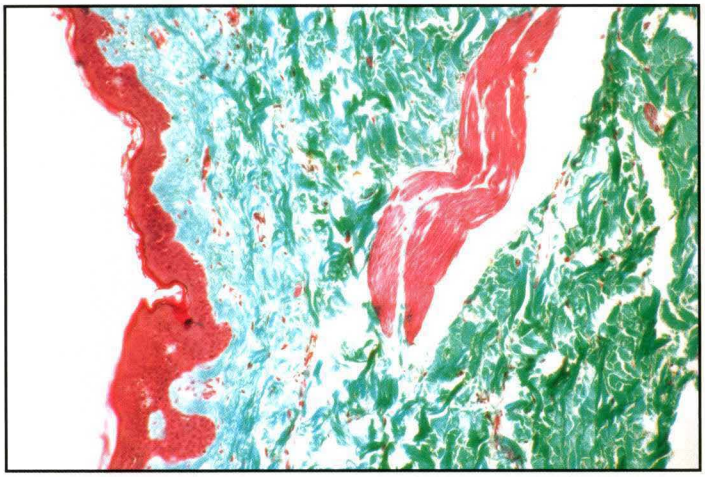
6. Although most texts state that Weigert iron hematoxylin should be prepared fresh, I find that it is good for several days.

7. Picric acid containing less than 10% water is very explosive; therefore, it is important that solutions not be spilled in the oven and then allowed to evaporate. For this reason, the staining jar containing picric acid should be place inside another container while in the oven.

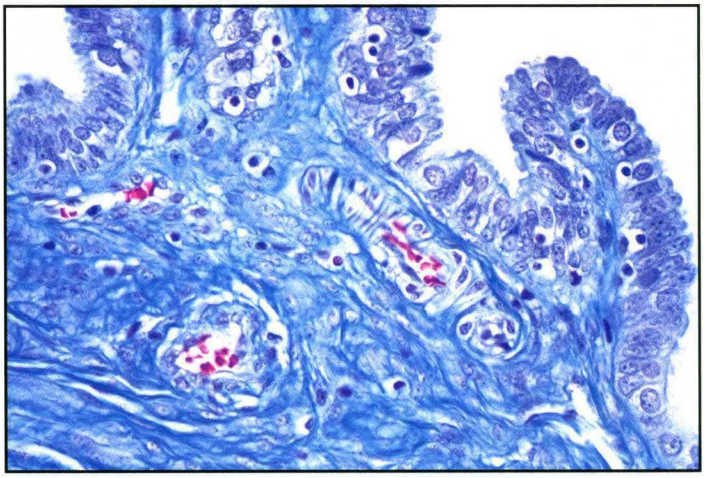
■ ***Microwave Procedure*** *[Crowder 1991]*

1. Deparaffinize the sections, and hydrate to distilled water.

2. If the sections were not originally fixed in Bouin solution, they should be mordanted by placing in a vented plastic Coplin jar of Bouin solution. Microwave at 70% power for 45 seconds. If originally fixed in Bouin solution, skip to step 4.



[i8.6] A section of skin stained with Masson trichrome. Light green has been used as the counterstain instead of aniline blue. An erector pili muscle and the epithelium are stained red, and the collagen is stained green.



[i8.7] A section of fallopian tube stained with Masson trichrome. Note that neither the smooth muscle surrounding the blood vessels nor the epithelium is stained red, but the red blood cells do show red staining. Red blood cells should never be used to judge the quality of the stain. This is a bad stain and should be repeated with new reagents.

3. Rinse sections in running water until colorless.

4. Place slides in Gill hematoxylin III in a vented plastic Coplin jar. Microwave on high for 15 seconds.

5. Rinse slides in tap water.

6. Blue the nuclei with an alkaline blueing solution.

7. Rinse well with tap water.

8. Rinse slides in distilled water.

9. Place sections in Biebrich scarlet-acid fuchsin solution in a vented plastic Coplin jar. Microwave at 70% power for 30 seconds.

10. Rinse sections in distilled water.

11. Place sections in phosphomolybdic/phosphotungstic acid solution in a vented plastic Coplin jar. Microwave at full power for 15 seconds. **Do not rinse.**

12. Place in aniline blue solution in a vented Coplin jar. Microwave at 70% power for 30 seconds.

13. Rinse slides in distilled water.

14. Place slides in 1% acetic acid for 2 to 3 minutes.

15. Dehydrate sections with 95% and absolute alcohol, 2 changes each.

16. Clear with xylene, and mount with synthetic resin.

■ *Results*
Same as [**i8.4**], [**i8.5**]

- Nuclei — Dark blue
- Cytoplasm, keratin, muscle fibers — Red
- Collagen and mucin — Blue

■ *Technical Notes*
1. All reagents should be discarded after use.

2. Because picric acid is explosive when allowed to dry, the Coplin jar should be placed in a partially closed plastic bag to trap any boil-over or spill of this reagent.

GOMORI 1-STEP TRICHROME STAIN
[GOMORI 1950b, SHEEHAN 1980]

■ *Purpose*
Identification of an increase in collagenous connective tissue fibers or differentiation between collagen and smooth muscle fibers

■ *Principle*
In the 1-step trichrome procedure, a plasma stain (chromotrope 2R) and a connective tissue fiber stain (fast green FCF, light green, or aniline blue) are combined in a solution of phosphotungstic acid to which glacial acetic acid has been added. Phosphotungstic acid favors the red staining of muscle and cytoplasm. The tungstate ion is specifically taken up by collagen, and the connective tissue fiber stain is subsequently bound to this complex.

■ *Fixative*
Any well-fixed tissue may be used. Bouin solution is used as a mordant to intensify the color reactions.

■ *Equipment*
56°C to 58°C oven, Coplin jars, Erlenmeyer flasks, graduated cylinders, pipettes

■ *Technique*
Cut paraffin sections at 4 to 5 µm.

■ *Quality Control*
Practically every tissue has an internal control, so no other control sections are needed; however, if a control is desired, uterus, small intestine, appendix, or fallopian tube will provide good material.

■ *Reagents*

Bouin Solution

Picric acid, saturated aqueous solution	75 mL
Formaldehyde, 37% to 40%	25 mL
Glacial acetic acid	5 mL

Weigert Iron Hematoxylin Solution

Solution a	
Hematoxylin	10 g
Alcohol, 95%	1,000 mL
Solution b	
Ferric chloride, 29% aqueous solution	20 mL
Distilled water	475 mL
Glacial acetic acid	5 mL
Working Solution	
*Mix equal parts of solutions **a** and **b***	

Gomori Trichrome Stain

Chromotrope 2R	0.6 g
Fast green FCF, light green, or aniline blue	0.3 g
Phosphotungstic acid	0.8 g
Glacial acetic acid	1 mL
Distilled water	100 mL
Store this solution in the refrigerator	

Acetic Acid, 0.5% Solution

Glacial acetic acid	0.5 mL
Distilled water	99.5 mL

■ *Procedure*

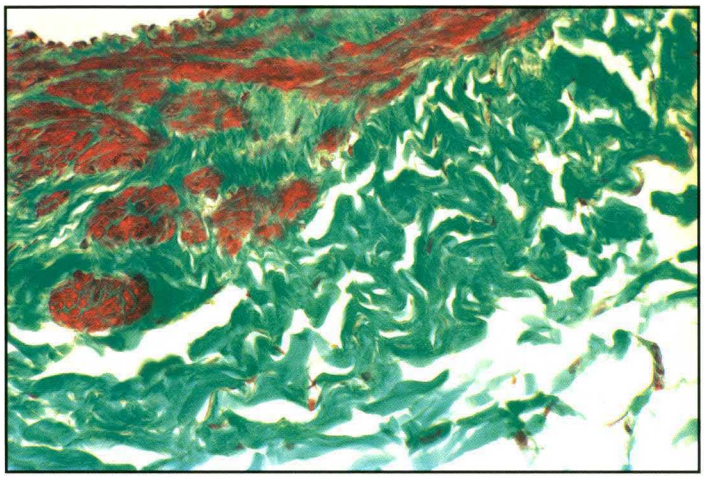
1. Deparaffinize sections, and hydrate to distilled water.
2. Rinse well in distilled water.
3. Mordant sections in Bouin solution for 1 hour at 56°C.
4. Remove slides from the oven, allow to cool, and wash in running water until the yellow color disappears.
5. Rinse in distilled water.
6. Stain sections in Weigert iron hematoxylin for 10 minutes.
7. Wash in running water for 10 minutes.
8. Stain sections for 15 to 20 minutes in Gomori trichrome stain.
9. Differentiate for 2 minutes in 0.5% acetic acid.
10. Dehydrate, clear, and mount with synthetic resin.

■ *Results* **[i8.8]**

- Nuclei — Black
- Cytoplasm, keratin, muscle fibers — Red
- Collagen and mucin — Green or blue

■ *Technical Notes*

1. Sweat [1968] states that coloration of fine connective tissue fibers is affected by the dye solution pH, with maximum binding occurring around pH 1.3. The pH of Gomori trichrome is about 2.5, which decreases affinity for anions by approximately 50%, so these investigators suggest that by replacing the acetic acid with hydrochloric acid, a pH of approximately 1.3 can be obtained. The intensity of coloration of the fine connective tissue fibers can be varied by altering the pH.
2. Churukian [1993] finds that zinc formalin allows good trichrome staining without mordanting in Bouin solution.



[i8.8] Gomori 1-step trichrome-stained section of a large vein. Light green was used instead of aniline blue in preparing the solution. Smooth muscle cells are stained bright red and collagen fibers are stained green.

VAN GIESON PICRIC ACID-ACID FUCHSIN STAIN
[MALLORY 1942, SHEEHAN 1980]

■ *Purpose*

Although the van Gieson technique may be considered a primary connective tissue stain, it is rarely used as such; however, it serves as an excellent counterstain for other methods such as the Verhoeff elastic technique, referred to in many institutions as the Verhoeff-van Gieson (VVG) stain. Other institutions refer to it as the elastic-van Gieson (EVG) stain.

■ *Principle*

In a strongly acidic solution, collagen is selectively stained by acid fuchsin, an acid aniline dye. Picric acid provides the acidic pH necessary and also acts as a stain for muscle and cytoplasm. The low pH is very important, as selective staining of collagen will not occur at higher pH levels. The addition of 0.25 mL of hydrochloric acid to 100 mL of van Gieson solution will sharpen the differentiation between collagen and muscle [Lillie 1976]. Saturated picric acid solutions are important in the preparation of the stain and again in the selective staining of collagen. If the picric acid solution is not saturated, collagen may stain pale pink to pale orange, and collagen, cytoplasm, and muscle may all stain the same color.

■ *Fixative*

Any well-fixed tissue may be used.

■ *Equipment*

Mechanical stirrer, Coplin jars, Erlenmeyer flasks, graduated cylinders, pipettes

■ *Technique*

Cut paraffin sections at 4 to 5 μm.

■ *Quality Control*

Practically every tissue has an internal control, so no other control sections are needed; however, if a control is desired, uterus, small intestine, appendix, or fallopian tube will provide good material.

■ *Reagents*

Weigert Iron Hematoxylin Solution

Solution a	
Hematoxylin	10 g
Alcohol, 95%	1,000 mL
Solution b	
Ferric chloride, 29% aqueous solution	20 mL
Distilled water	475 mL
Glacial acetic acid	5 mL
Working Solution	
*Mix equal parts of solutions **a** and **b***	

Acid Fuchsin, 1% Solution

Acid fuchsin	1 g
Distilled water	100 mL

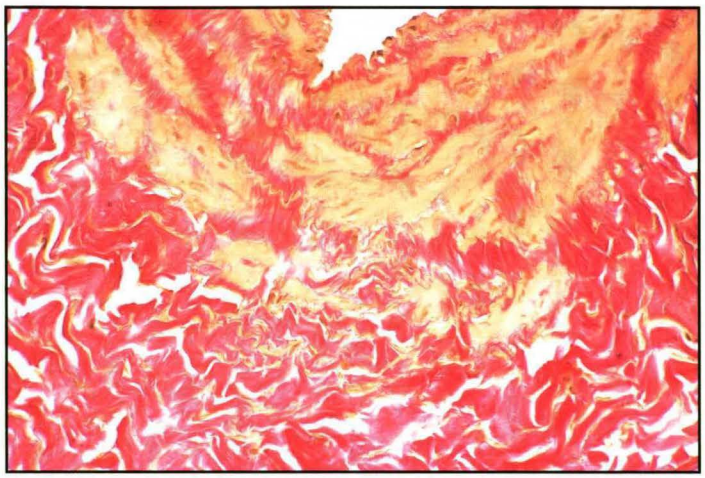
Picric acid, Saturated Solution

Picric acid	13 g
Distilled water	1,000 mL

Stir the solution on the mechanical stirrer for several hours. Some picric acid should remain undissolved in the bottom of the flask. The solubility of picric acid is 1.23 g/100 mL water at 20°C. The amount used may have to be adjusted, depending on whether water has been added to the stock powder to ensure that the water content does not drop below at least 10%.

van Gieson Solution

Acid fuchsin, 1% solution	5 mL
Picric acid, saturated solution	95 mL



[i8.9] van Gieson-stained section of a large vein. Smooth muscle cells are stained yellow and collagen fibers are stained red.

■ *Procedure*

1. Deparaffinize sections, and hydrate to distilled water.
2. Stain sections with Weigert iron hematoxylin for 10 to 20 minutes. Sections should be overstained, as they will be slightly decolorized by the picric acid.
3. Wash in running tap water for 10 minutes.
4. Stain sections in van Gieson stain for 5 minutes. Discard solution.
5. Place slides in 95% alcohol.
6. Dehydrated as usual, clear with xylene, and mount with synthetic resin.

■ *Results* [i8.9]

• Nuclei	Black
• Collagen	Brilliant red
• Muscle and cytoplasm	Yellow

■ *Technical Notes*

1. An iron hematoxylin solution is used for nuclear staining in the trichrome procedures, because iron hematoxylin is more resistant than aluminum hematoxylin to decolorization in subsequent acidic dye solutions.
2. Although many texts state that Weigert iron hematoxylin should be prepared fresh, I find that the solution is good for several days.
3. If a sharp color differentiation is not obtained between collagen and muscle, check the preparation of the saturated picric acid solution, as the acidic pH provided by this solution is very important. Also the addition of 0.25 mL of hydrochloric acid to100 mL van Gieson solution may sharpen the color differentiation.

VERHOEFF ELASTIC STAIN [MALLORY 1942, SHEEHAN 1980]

■ *Purpose*

Elastic fiber techniques are used for the demonstration of pathologic changes in elastic fibers. These include atrophy of the elastic tissue, thinning or loss that may result from arteriosclerotic changes, and reduplication, breaks, or splitting that may result from other vascular diseases. The techniques also may be used to demonstrate normal elastic tissue, as in the identification of veins and arteries, and to determine whether or not the blood vessels have been invaded by tumor.

■ *Principle*

The tissue is overstained with a soluble lake of hematoxylin-ferric chloride-iodine. Both ferric chloride and iodine serve as mordants, but they also have an oxidizing function that assists in converting hematoxylin to hematein. The mechanism of dye binding is probably by formation of hydrogen bonds, but the exact chemical groups reacting with the hematoxylin have not been identified. Because this method requires that the sections be overstained and then differentiated, it is a regressive method. Differentiation is accomplished by using excess mordant, or ferric chloride, to break the tissue-mordant-dye complex. The dye will be attracted to the larger amount of mordant in the differentiating solution and will be removed from the tissue. The elastic tissue has the strongest affinity for the iron-hematoxylin complex and will retain the dye longer than the other tissue elements. This allows other elements to be decolorized and the elastic fibers to remain stained. Sodium thiosulfate is used to remove excess iodine. Although van Gieson solution is the most commonly used counterstain, other counterstains may be used.

■ *Fixative*

Any well-fixed tissue may be used, but neutral-buffered formalin or Zenker solution is preferred.

■ *Equipment*

Mechanical stirrer, Coplin jars, Erlenmeyer flasks, graduated cylinders

■ *Technique*

Cut paraffin sections at 4 to 5 µm.

■ *Quality Control*

Most laboratories use a section of aorta embedded on edge, but I prefer a cross-section of a muscular artery.

■ *Reagents*

Lugol Iodine

Iodine	10 g
Potassium iodide	20 g
Distilled water	1,000 mL

Put the iodine and potassium iodide in a flask with 200 mL of the water. Stir on a mechanical stirrer until the iodine dissolves, and then add the remaining water.

Ferric Chloride, 10% Solution

Ferric chloride	50 g
Distilled water	500 mL
Store in the refrigerator	

Ferric Chloride, 2% Solution

Ferric chloride, 10% solution	10 mL
Distilled water	40 mL

Alcoholic Hematoxylin, 5% Solution

Hematoxylin	5 g
Alcohol, 95%	100 mL

Verhoeff Elastic Stain

Prepare fresh each time and mix in order	
Alcoholic hematoxylin, 5%	30 mL
Ferric chloride, 10% solution	12 mL
Lugol iodine	12 mL

van Gieson Solution

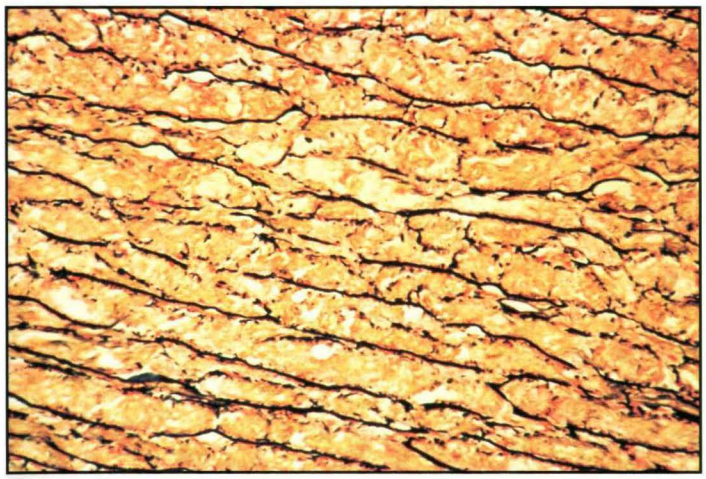
See van Gieson picric acid-acid fuchsin stain, p167

Sodium Thiosulfate, 5% Solution

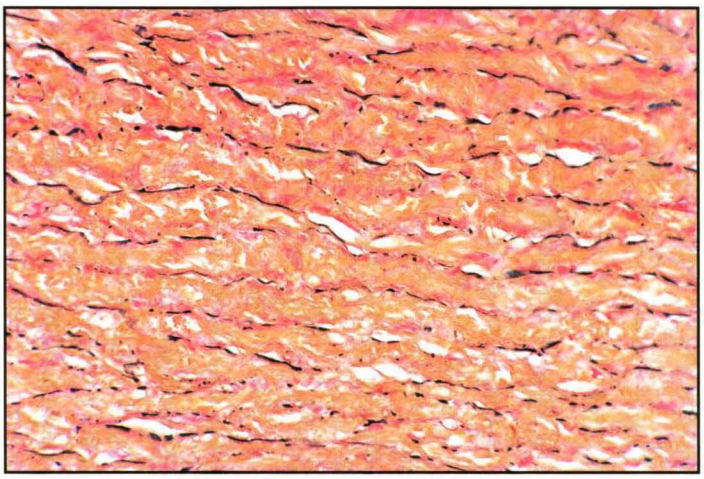
Sodium thiosulfate	50 g
Distilled water	1,000 mL

■ *Procedure*

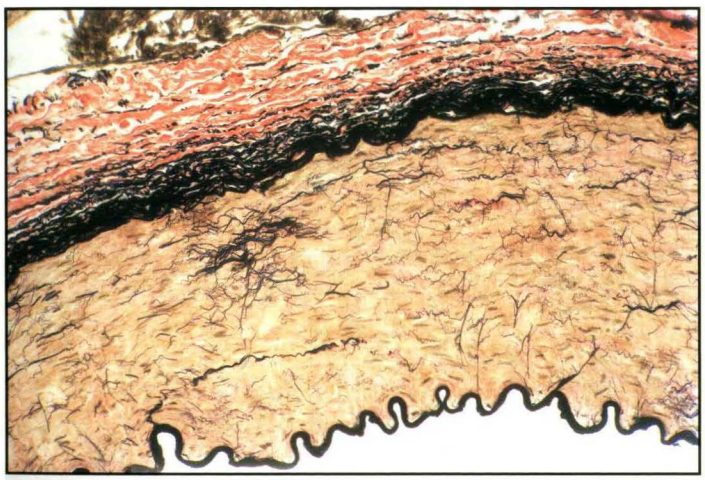
1. Deparaffinize sections, and hydrate to distilled water.
2. Place sections in Verhoeff elastic tissue stain for 1 hour.
3. Wash in 2 changes of distilled water.
4. Differentiate sections microscopically in 2% ferric chloride until the elastic fibers are distinct and the background is colorless to light gray. If the sections are differentiated too far, restain.
5. Rinse sections in distilled water.



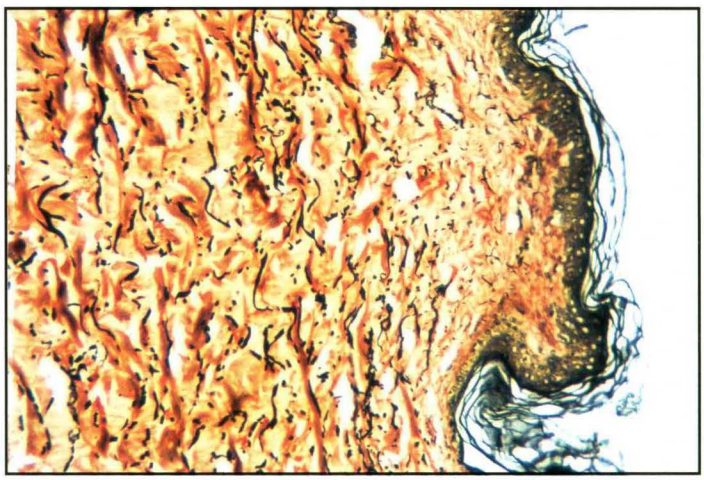
[i8.10] A Verhoeff-van Gieson-stained section from the wall of the aorta, an elastic artery. The tunica media (middle layer) is composed of concentrically arranged perforated laminae of elastic tissue stained black. The spaces between the elastic laminae are occupied by smooth muscle cells (yellow) and collagen fibers (red).



[i8.12] A duplicate of **[i8.10]** stained with the Verhoeff-van Gieson stain, but overdifferentiated. Note how much less elastic tissue is demonstrated. The lack of the slightly grayed background, along with very clear, bright yellow muscle fiber staining, is frequently indicative of overdifferentiation. It is imperative that each section be differentiated microscopically.



[i8.11] Part of a cross-section of a muscular artery stained with the Verhoeff-van Gieson stain. Both the internal and external elastic laminae are very prominent, and very fine elastic fibers (black) can be seen in the yellow muscular tunica media. The collagen fibers of the tunica adventitia are stained red.



[i8.13] A section of skin stained with the Verhoeff-van Gieson stain. The collagen should be stained bright red in a correctly done stain, but it is yellow-orange in this section, indicating that the picric acid used to prepare the van Gieson stain was not saturated. This stain should be repeated with correctly prepared van Gieson stain.

6. Place section in sodium thiosulfate for 1 minute.
7. Wash in running tap water for 5 minutes.
8. Counterstain sections in van Gieson stain for 1 minute.
9. Differentiate in 95% alcohol.
10. Dehydrate in absolute alcohol, clear in xylene, and mount with synthetic resin.

■ *Results* [i8.10], [i8.11]

• Elastic fibers	Blue-black to black
• Nuclei	Blue to black
• Collagen	Red
• Other tissue elements	Yellow

■ *Technical Notes*

1. It is easy to overdifferentiate this stain **[i8.12]**. If the background is completely colorless, so that a clear yellow counterstain is obtained, the section may be overdifferentiated. It is probably better to err on the side of underdifferentiation.
2. Overdifferentiated sections may be restained at any step, provided they have not been treated with alcohol.
3. Do not prolong staining with van Gieson solution, because picric acid also will differentiate the stain further.
4. It is not necessary to remove mercury deposits before staining, because they will be removed by the staining solution; however, because of the toxicity, mercuric fixatives should not be used.
5. The preparation of van Gieson solution is critical for proper differentiation of muscle and collagen. If the picric acid is not saturated, collagen will not stain red, and cytoplasm, muscle, and collagen may all stain the same color **[i8.13]**.

6. To prepare Verhoeff elastic staining solution, the reagents must be added in the order given, with mixing after each addition, or poor staining may result.

7. The staining jar that contained Verhoeff solution may be cleaned easily by transferring the 2% ferric chloride to the jar for a few minutes before discarding the solution.

8. For optimum results, slides must be individually differentiated, because the time of differentiation is somewhat dependent on the amount of elastic tissue present. Do not depend on the control for timing the differentiation of all sections.

9. Because proper differentiation is sometimes difficult, it is helpful to use duplicate sections differentiated to a slightly different end point.

ALDEHYDE FUCHSIN ELASTIC STAIN [GOMORI, 1950a, SHEEHAN 1980]

■ *Purpose*

Refer to the Verhoeff elastic stain, p170

■ *Principle*

Hydrochloric acid and paraldehyde are added to an alcoholic solution of basic fuchsin to form aldehyde fuchsin. Schiff bases are formed by the aldehyde and the fuchsin, but the affinity of elastic fibers for this solution is not understood. A number of other tissue elements will also stain with aldehyde fuchsin. These elements include pancreatic β cell granules and sulfated mucosubstances. Staining is intensified by prior oxidation.

■ *Fixative*

10% neutral-buffered formalin is preferred; chromate fixatives should be avoided. Formalin- and Bouin-fixed tissues will show a colorless background, and mercury-fixed tissue will show a pale lilac background [Sheehan 1980].

■ *Equipment*

Coplin jars, Erlenmeyer flasks, graduated cylinders, pipettes, and Whatman No. 2 filter paper.

■ *Technique*

Cut paraffin sections at 4 to 5 µm.

■ *Quality Control*

Use a section of aorta embedded on edge or a cross-section of a muscular artery. Skin also provides a good control.

■ *Reagents*

Aldehyde Fuchsin Solution

Pararosaniline (basic fuchsin, CI 42500)	1 g
Ethyl alcohol, 70%	200 mL
Hydrochloric acid, concentrated	2 mL
Paraldehyde (must be fresh)	2 mL
Mix well and let stand at room temperature for 2 to 3 days or until the stain is deep purple; store in the refrigerator	

Light Green Stock Solution

Light green SF yellowish	0.2 g
Distilled water	100 mL
Glacial acetic acid	0.2 mL
Mix well	

Light Green Working Solution

Light green stock solution	10 mL
Distilled water	50 mL

■ *Procedure*

1. Deparaffinize sections, and hydrate to 70% alcohol.

2. Stain sections in aldehyde fuchsin solution for 10 to 40 minutes. With good solutions, 10 minutes is usually sufficient for staining.

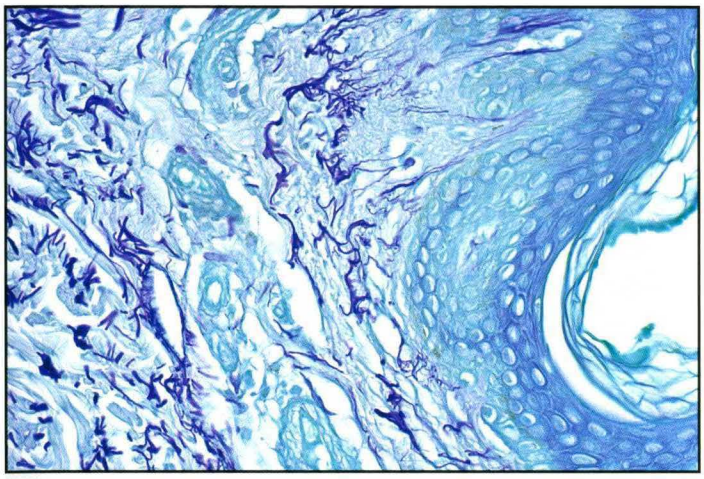
3. Rinse off the excess stain with 70% alcohol.

4. Wash the sections in water and check microscopically for staining of elastic fibers. If a deeper stain is desired, rinse sections briefly in 70% alcohol and return to the aldehyde fuchsin. If further differentiation is needed, return sections to the 70% alcohol. Differentiation is stopped by rinsing the sections with distilled water. The stain may be filtered and reused.

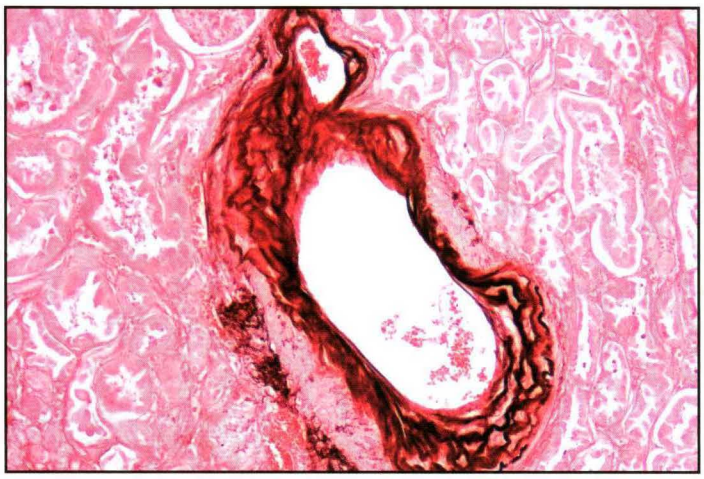
5. Rinse the sections with distilled water.

6. Counterstain sections with the light green working solution for 1 to 2 minutes. Discard the solution.

7. Dehydrate in 2 changes each of 95% and absolute alcohols, clear in xylene, and mount with synthetic resin.



[i8.14] A section of skin stained with the Gomori aldehyde fuchsin procedure. Elastic tissue is stained a dark purple. The keratinized stratified squamous epithelium, or the epidermis, and the dense irregular connective tissue of the dermis are all stained green.



[i8.15] Orcein-stained sections of a small artery demonstrating the elastic fibers.

■ *Results* [i8.14]

• Elastic fibers	Deep blue to purple
• Other tissue elements	Green

■ *Technical Notes*

1. The paraldehyde used for preparation of the aldehyde fuchsin reagent should be fresh. Do not use reagent that was opened previously.

2. Old solutions of aldehyde fuchsin may not stain well, and the staining time may need to be prolonged.

3. Do not use rosaniline (CI 42510) for the preparation of this reagent; it is not satisfactory [Mowry 1979].

4. The shelf life of aldehyde fuchsin may be prolonged by refrigerating a small amount and freezing aliquots of the remainder.

5. Acetaldehyde is cheaper and may be obtained without a DEA number. The solution can be prepared as shown below [Wenk 1995].

Alcoholic Basic Fuchsin, 0.5% Solution

Basic fuchsin (CI 42500)	2.5 g
Ethyl alcohol, 70%	500 mL

Stir until dissolved, and store at room temperature; stable for several months

Aldehyde Fuchsin Solution

Alcoholic basic fuchsin, 0.5% solution	50 mL
Acetaldehyde	2.5 mL
Hydrochloric acid, concentrated	1.0 mL

Stir until dissolved. Cover tightly, and allow to stand overnight at room temperature. Filter and store at 4°C. Allow to warm to room temperature and shake before using. The solution may be reused until staining is weak. It is stable for about 3 weeks.

NOTES ON OTHER ELASTIC STAINS

In the United States, the Verhoeff-van Gieson stain is probably the most widely used for the demonstration of elastic fibers. The second most widely used is the aldehyde fuchsin stain. Other stains for elastic tissue that are not used often in the United States, and not given in this text, are orcein and resorcin fuchsin. Orcein is one of the oldest methods for elastic fibers [Kiernan 1990]; however, it gives a less intense color than the Verhoeff. Orcein is used in an acidified alcoholic solution, and elastic fibers are stained brown with this stain **[i8.15]**. Resorcin fuchsin [Vacca 1985] uses an acidified alcoholic solution of resorcin fuchsin, and usually the counterstain is van Gieson; the results look very much like the Verhoeff-van Gieson.

In Europe, the Miller technique for elastic tissue is widely used [Luna 1992]. This is a modification of the original Weigert resorcin fuchsin method. Miller elastic stain is composed of:

1. a mixture of Victoria blue 4R, new fuchsin, and crystal violet dissolved in water, and to this is added in order

2. resorcin, dextrin, and ferric chloride; finally hydrochloric acid is added

RUSSELL MODIFICATION OF THE MOVAT PENTACHROME STAIN [LUNA 1992]

■ *Purpose*

The demonstration of mucin, fibrin, elastic fibers, muscle, and collagen

■ *Principle*

Acidic mucosubstances are stained by alcian blue. The alkaline alcohol solution that follows converts the alcian blue to monastral fast blue, which is insoluble. Complete conversion is necessary because alcian blue will be decolorized during the remainder of the procedure. Iron hematoxylin is used to stain the elastic fibers, which are then differentiated with ferric chloride (see the Verhoeff method). Sodium thiosulfate removes any residual iodine. Crocein scarlet and acid fuchsin are acid dyes that stain muscle, cell cytoplasm, collagen, and ground substance. Phosphotungstic acid differentiation removes the stain from the collagen and ground substance. The acetic acid removes the phosphotungstic acid, and collagen is then counterstained with alcoholic safran.

■ *Fixative*

10% neutral-buffered formalin is preferred.

■ *Equipment*

Coplin jars, Erlenmeyer flasks, graduated cylinders, pipettes

■ *Technique*

Cut paraffin sections at 4 to 5 μm.

■ *Quality Control*

Use a section of lung, skin, or colon.

■ *Reagents*

Alcian Blue, 1% Solution

Alcian blue-GS	1 g
Distilled water	100 mL
Glacial acetic acid	1 mL
Mix well, and store at room temperature	

Alkaline Alcohol Solution

Ammonium hydroxide	10 mL
Alcohol, 95%	90 mL
Prepare fresh	

Iodine-Iodide Solution

Iodine	2 g
Potassium iodide	4 g
Distilled water	100 mL
Add the iodine and potassium iodide to about 25 mL of water, and mix until dissolved. Add the remaining water.	

Absolute Alcoholic Hematoxylin, 10% Solution

Hematoxylin	10 g
Absolute alcohol	100 mL
Mix until dissolved; cap tightly, and store at room temperature	

Ferric Chloride, 10% Solution

Ferric chloride	10 g
Distilled water	100 mL
Mix until dissolved, and store at room temperature	

Working Hematoxylin Solution

Absolute alcoholic hematoxylin, 10%	25 mL
Absolute alcohol	25 mL
Ferric chloride, 10% aqueous	25 mL
Iodine-iodide solution	25 mL
Prepare just before use	

Ferric Chloride, 2% (for differentiation)

Ferric Chloride, 10% solution	10 mL
Distilled water	40 mL
Prepare just before use	

Sodium Thiosulfate, 5% Solution

Sodium thiosulfate	5 g
Distilled water	100 mL
Mix until dissolved, and store at room temperature	

Crocein Scarlet-Acid Fuchsin Solution

Solution a (stock)	
Crocein scarlet	1 g
Distilled water	99.5 mL
Glacial acetic acid	0.5 mL
Mix until dissolved, and store at room temperature	
Solution b (stock)	
Acid fuchsin	0.1 g
Distilled water	99.5 mL
Glacial acetic acid	0.5 mL
Mix until dissolved, and store at room temperature	
Working solution	
Solution **a**	40 mL
Solution **b**	10 mL
Prepare just before use	

Phosphotungstic Acid, 5% Solution

Phosphotungstic acid	5 g
Distilled water	100 mL
Mix until dissolved, and store at room temperature	

Alcoholic Safran Solution

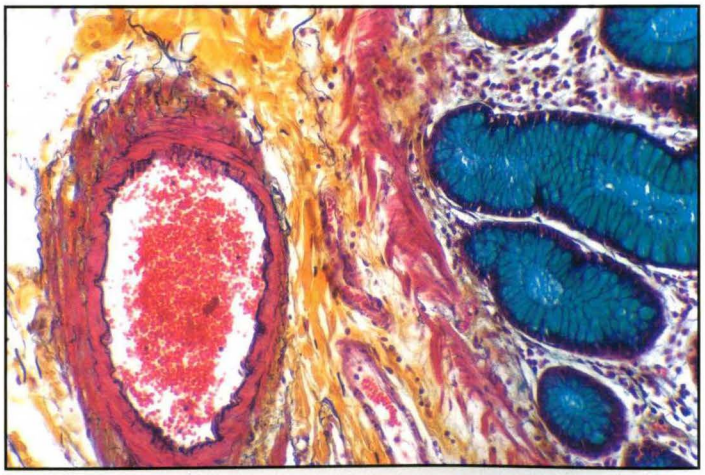
Safran du Gatinais	6 g
Absolute alcohol	100 mL
Mix well, and keep tightly closed to prevent hydration or evaporation	

■ ***Procedure***

1. Deparaffinize and hydrate to distilled water.
2. Stain in alcian blue for 20 minutes.
3. Wash in running tap water for 5 minutes.
4. Place slides in alkaline alcohol for 30 minutes.
5. Wash in running tap water for 10 minutes.
6. Rinse in distilled water.
7. Stain in working hematoxylin solution for 15 minutes.
8. Rinse in several changes of distilled water.
9. Differentiate in 2% aqueous ferric chloride until the elastic fibers contrast sharply with the background.
10. Rinse in distilled water.
11. Place slides in sodium thiosulfate for 1 minute.
12. Wash in running tap water for 5 minutes; rinse in distilled water.
13. Stain in Crocein scarlet-acid fuchsin solution for 1 minute.
14. Rinse in several changes of distilled water.
15. Rinse in 0.5% acetic acid solution for 30 seconds.
16. Place slides in 5% phosphotungstic acid solution, 2 changes of 5 minutes each.
17. Rinse in 0.5% acetic acid solution.
18. Rinse in 3 changes of absolute alcohol.
19. Stain in alcoholic safran solution for 15 minutes.
20. Rinse in 3 changes of absolute alcohol.
21. Clear in 2 or 3 changes of xylene, and mount with synthetic resin.

■ ***Results*** **[i8.16]**

• Nuclei and elastic fibers	Black
• Collagen	Yellow
• Ground substance and mucin	Blue
• Fibrinoid, fibrin	Intense red
• Muscle	Red



[i8.16] A section of colon stained with the Movat pentachrome procedure. Note the blue-stained mucin in the goblet cells, red-stained smooth muscle, yellow-stained collagen, and black elastic fibers.

■ *Technical Notes*

1. The differentiation of the elastic fibers is usually complete in 2 to 3 minutes.

2. The complete removal of alkaline alcohol with running water is very important. Failure to remove all of the alkaline alcohol will inhibit the subsequent staining steps.

3. This stain also may be used to demonstrate *Cryptococcus neoformans*, staining the organism a bright blue.

SILVER TECHNIQUES FOR RETICULAR FIBERS

Many variations of silver techniques can be used for the demonstration of reticular fibers; the principles, however, are the same for most of the techniques and closely resemble those of the PAS technique. The major steps are:

1. Oxidation of the adjacent glycol groups of the hexose sugars in reticulin to aldehydes. Reagents vary with the technique used, but the most frequently used oxidizers are phosphomolybdic acid, potassium permanganate, and periodic acid.

2. Sensitization, which usually is a metallic impregnation step. Impregnation is the deposition of metallic salts on or around, but not in, the tissue element to be demonstrated. The exact chemical reaction of sensitizers is not known, but according to Sheehan and Hrapchak [1980], the metallic salt used in this step probably forms a metal-organic compound with the reticulin. The sensitizing metal is then replaced by silver. Commonly used sensitizers are uranyl nitrate, ferric ammonium sulfate, and dilute solutions of silver nitrate.

3. Silver impregnation involves treating tissue with an ammoniacal or diamine silver complex, $[Ag(NH_3)_2]^+$. Kiernan [1990] states that 4 atoms of silver will be deposited at the site of each reactive sugar residue in the reticulin, and that the aldehyde groups present will reduce the diamine silver to metallic silver; however, this is not enough silver to provide adequate visibility. Further deposition occurs when incompletely washed sections are transferred to formaldehyde.

4. Reduction uses formaldehyde in all methods. Residual silver diamine ions are reduced to metallic silver by the formaldehyde. Kiernan [1990] states that metallic silver catalyzes the reaction, so the metal precipitates because of formaldehyde reduction mainly at the original sites of the sugar molecules of the reticulin. This further deposition of silver by the formaldehyde reduction gives a highly visible precipitate. The reduction step in silver impregnation techniques is sometimes termed *developing.*

5. *Toning* is the term used when bound metallic silver is treated with gold chloride, and the color of the impregnated component is changed from brown to black. The metallic silver is replaced by metallic gold in the following reaction:

$$3Ag^{\circ} + AuCl_3 \longrightarrow Au^{\circ} \downarrow + 3AgCl$$

A more stable compound is formed, and section contrast and clarity are improved. The yellow color is removed from the background by this step; however, toning can be overdone, and a violet to red background instead of the desired gray one will result.

6. Unreduced silver is removed by treating the sections with sodium thiosulfate (hypo). This step will prevent any nonspecifically bound silver remaining in the section from being reduced by a later exposure to light.

7. Counterstaining may or may not be used, depending on the type of tissue stained and personal preferences of the pathologists.

Only 2 representative, reliable methods for reticular fibers will be presented. Silver stains are notoriously capricious, and the method that consistently gives the best results in your laboratory should be used. If one of these methods does not work well in your hands, refer to the literature for other techniques. In my experience, the results of the 2 methods presented are consistently better than those obtained with either the Wilder or the Snook technique. It is very important that the amount of reticulin to be demonstrated in the control section be known and that it be checked carefully each time to determine how completely the reticulin is stained. A summary of the most common reticulin methods and the variations in the first 4 steps is given in **[t8.1]**.

GOMORI STAIN FOR RETICULAR FIBERS [LUNA 1986]

■ *Purpose*

The demonstration of reticular fibers in tissue sections can be important in the differential diagnosis of certain types of tumors. A change from the normal reticular fiber pattern, as is seen in some liver diseases, is also an important diagnostic finding.

■ *Principle*

The hexose sugars of reticulin are demonstrated by oxidation to aldehydes. Potassium permanganate is the oxidizing agent in this procedure, and the excess is removed by potassium metabisulfite. Ferric ammonium sulfate acts as the sensitizer and is subsequently replaced by silver from the diamine silver solution. Following impregnation, formalin is used to reduce the silver to its visible metallic form. Follow with toning with gold chloride and removal of unreacted silver with sodium thiosulfate. The final step is to counterstain, if desired.

■ *Fixative*

10% neutral-buffered formalin is preferred.

■ *Equipment*

Nonmetallic forceps, chemically clean glassware (Coplin jars, graduated cylinders, Erlenmeyer flasks, and pipettes), filter paper

[t8.1] Variations in the first 4 steps of 6 of the most common methods for reticulin

Reagent	Snook	Gordon and Sweets	Gomori	Laidlaw	Nasher and Shanklin	Wilder
Oxidizer	Potassium permanganate	Potassium permanganate	Potassium permanganate	Potassium permanganate	Potassium permanganate plus sulfuric acid	Phosphomolybdic acid
Sensitizer	Uranyl nitrate	Ferric ammonium sulfate	Ferric ammonium sulfate	None	Silver nitrate	Uranyl nitrate
Impregnating Solution	Ammoniacal silver (silver nitrate plus sodium hydroxide, precipitate almost dissolved with ammonium hydroxide)	Ammoniacal silver (silver nitrate plus sodium hydroxide, precipitate almost dissolved with ammonium hydroxide)	Ammoniacal silver (silver nitrate plus sodium hydroxide, precipitate almost dissolved with ammonium hydroxide)	Lithium silver (silver nitrate plus lithium carbonate, precipitate almost dissolved with ammonium hydroxide)	Ammoniacal silver (ammonium hydroxide plus silver nitrate until slight turbidity; pyridine added at end)	Ammoniacal silver (silver nitrate plus sodium hydroxide, precipitate almost dissolved with ammonium hydroxide)
Reducing Solution	Formaldehyde	Formaldehyde	Formaldehyde	Formaldehyde	Formaldehyde plus absolute alcohol	Formaldehyde plus uranyl nitrate

The remaining steps (gold chloride, sodium thiosulfate, and counterstain) may vary only in reagent concentration or counterstain choice. [Table courtesy of NSH/CAP HQIP Final Critique 2004A]

■ *Technique*

Cut paraffin sections at 4 to 5 μm.

■ *Quality Control*

Liver is a very good control tissue.

■ *Reagents*

Silver Nitrate, 10% Solution

Silver nitrate	10 g
Distilled water	100 mL

Potassium Hydroxide, 10% Solution

Potassium hydroxide	10 g
Distilled water	100 mL

Ammoniacal Silver Solution

Combine 20 mL of 10% silver nitrate solution and 4 to 5 mL of 10% aqueous solution of potassium hydroxide. Add concentrated ammonium hydroxide, drop by drop, while shaking the container continuously, until the precipitate is completely dissolved. Cautiously add 10% silver nitrate solution, drop by drop, until 1 drop causes the solution to become permanently cloudy. Only a faint cloudiness is desirable. Measure the resulting solution, dilute with an equal amount of distilled water, and filter into a chemically clean Coplin jar.

Potassium Permanganate, 0.5% Solution

Potassium permanganate	2.5 g
Distilled water	500 mL

Potassium Metabisulfite, 2% Solution

Potassium metabisulfite	10 g
Distilled water	500 mL

Ferric Ammonium Sulfate, 2% Solution

Ferric ammonium sulfate	10 g
Distilled water	500 mL

Formalin Solution

Formaldehyde, 37% to 40%	10 mL
Distilled water	40 mL

Gold Chloride, 0.2% Solution

Stock gold chloride solution (1%)	10 mL
Distilled water	40 mL

Sodium Thiosulfate, 2% Solution

Sodium thiosulfate	10 g
Distilled water	500 mL

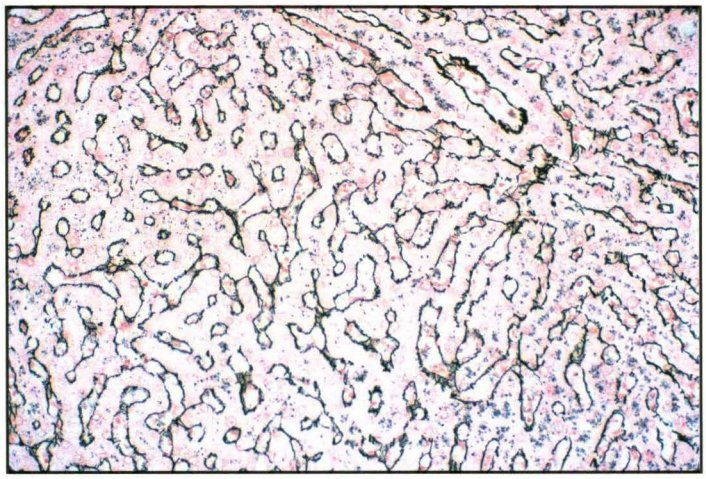
Nuclear-fast red (Kernechtrot) Solution

Nuclear-fast red (Kernechtrot)	0.5 g
Aluminum sulfate	25 g
Distilled water	500 mL

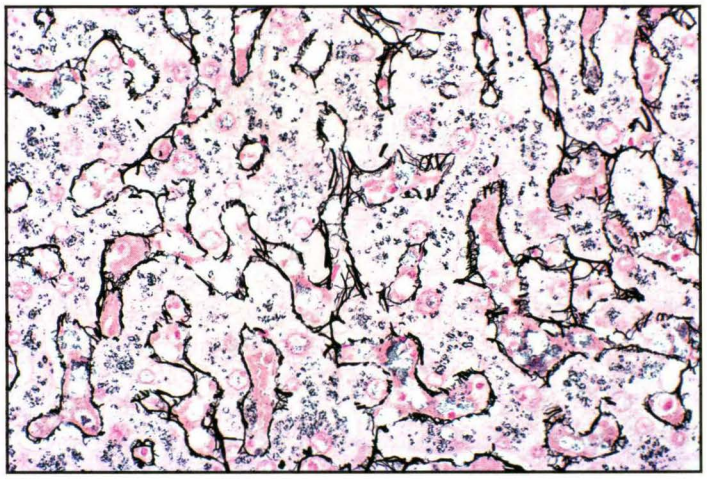
Dissolve the aluminum sulfate in the distilled water and then dissolve the nuclear-fast red in this solution using heat. Cool, filter, and add a few grains of thymol as a preservative.

■ *Procedure*

1. Deparaffinize sections, and hydrate to distilled water.
2. Oxidize sections in 0.5% potassium permanganate solution for 1 minute.
3. Rinse in tap water for 2 minutes.
4. Differentiate in 2% potassium metabisulfite for 1 minute.
5. Wash in tap water for 2 minutes.
6. Sensitize sections in 2% ferric ammonium sulfate for 1 minute.
7. Wash slides in tap water for 2 minutes followed by 2 changes of distilled water for 30 seconds each.
8. Impregnate sections with the ammoniacal silver solution for 1 minute.
9. Rinse in distilled water for 20 seconds.
10. Reduce for 3 minutes in the 20% formalin solution.
11. Wash in tap water for 3 minutes.
12. Tone in 0.2% gold chloride solution for 10 minutes.
13. Rinse in distilled water.
14. Place sections in 2% potassium metabisulfite for 1 minute.
15. Place sections in 2% sodium thiosulfate for 1 minute.
16. Wash in tap water for 2 minutes.
17. Counterstain, if desired, with nuclear-fast red for 5 minutes.



[i8.17] A section of liver stained with the Gomori reticulin stain and lightly counterstained with nuclear-fast red. As in this photograph, the pattern should be readily assessed with the 10× objective.



[i8.18] The same section as shown in **[i8.17]** viewed with the 40× objective. The reticulin fibers stand out sharply in **[i8.17]**, **[i8.18]**. Some cellular organelles have stained black with the silver, and rare suggestions of silver precipitate can be seen in the sinusoids.

18. Wash well in tap water.
19. Dehydrate in 95% and absolute alcohols.
20. Clear in xylene, and mount with synthetic resin.

■ *Results* **[i8.17]**, **[i8.18]**

- Reticulin — Black
- Collagen — Taupe
- Other tissue elements reflect the counterstain used

■ *Technical Notes*

1. It is important that a hint of turbidity remain in the silver solution. An excess of ammonia decreases the sensitivity and results in incomplete impregnation of reticular fibers.

2. The rinse between the diamine silver solution and the formaldehyde is critical for good reticulin demonstration. If the wash is prolonged, the staining of the reticulin will be reduced, and if it is insufficient, there will be excessive background staining.

3. The glassware must be chemically cleaned with commercial cleaning agents or bleach. The older method of using a mixture of sulfuric acid and potassium dichromate is not recommended because of the hazards involved in the preparation and use of this solution.

4. Many types of plastic or coated (eg, Teflon) forceps are available commercially and should be used in this method. Metallic forceps can be coated with paraffin, but this is less than satisfactory because then heated solutions or paraffin solvents must be avoided.

5. Some texts refer to the ferric ammonium sulfate as iron alum. Alum is a term used for salts that are double or bisulfates, such as potassium aluminum sulfate, ammonium aluminum sulfate, chromium potassium sulfate, and ferric ammonium sulfate.

6. If nuclear-fast red is used as a counterstain, wash the slides well with water after staining. If the slides are not washed well, or are transferred from the counterstain directly to alcohol, they will develop a cloudiness that can be removed only by backing the slides up to water.

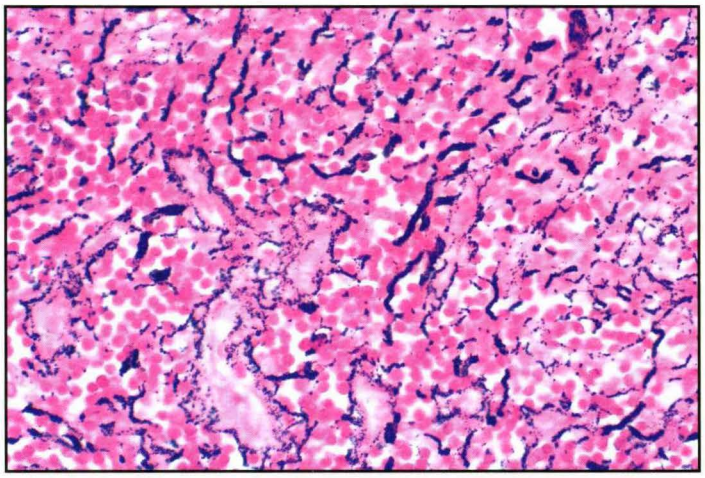
7. Many times, the pattern of reticular fiber staining is very important, as in liver biopsy specimens, and should be seen easily with the scanning lens of the microscope. The easy visualization of the reticulin pattern with the 10× objective is a guide to the quality of the stain. The counterstain, however, may obscure this easy visualization and therefore should not be used, or should be extremely light, when visualization of the pattern is important.

8. If silver staining of the nuclei is a problem, this may be helped by using acetified potassium permanganate.

Sulfuric acid, 3%	2.5 mL
Potassium permanganate, 0.5%	47.5 mL

9. When the ammonium hydroxide has lost strength because of the loss of ammonia from the solution, a fresh bottle should be opened. The decrease in strength is usually noticed because more than the usual amount of ammonium hydroxide is required, and the reticulin may stain gray-black rather than a sharp black.

10. Churukian [1993] used the ammoniacal silver solution for several days, storing it in the refrigerator and bringing it to room temperature before use. He feels that the reticulin stains better after the ammoniacal silver has aged for a few days.



[i8.19] A section of liver stained with the Snook reticulin stain [Sheehan 1980] shows granular, rather than linear, staining of the reticulin. This may be because of the use of old reagents, especially old uranium nitrate. This stain should be repeated.

11. The deposition of silver should be in a linear pattern. Stains exhibiting a granular deposition, as seen in **[i8.19]**, should be repeated.

12. Diamine silver is a very alkaline solution, with a pH in the range of 11 to 12, and sections will sometimes wash off the slides. It may be helpful to use special coated slides or charged slides for picking up the sections. If this is not sufficient, sections may be treated with celloidin before staining.

13. Ammoniacal silver solutions may form explosive compounds, so extreme care must be exercised in the preparation, use, and storage of these solutions. Storage of these solutions in the refrigerator inhibits the formation of explosive compounds; exposure to direct sunlight should be avoided.

GORDON AND SWEETS STAIN FOR RETICULAR FIBERS [GORDON 1936]

■ *Purpose*

The demonstration of reticular fibers in tissue sections can be important in the differential diagnosis of certain types of tumors. A change from the normal reticular fiber pattern, as seen in liver diseases such as cirrhosis, hepatocellular fibrosis, and/or necrotic liver disease, is also an important diagnostic finding.

■ *Principle*

The tissue is first oxidized by potassium permanganate to enhance subsequent staining of reticular fibers, and excess permanganate is removed by oxalic acid. Ferric ammonium sulfate acts as the sensitizer and is subsequently replaced by silver from the diamine silver solution. After impregnation, formalin is used to reduce the silver to its visible metallic form. Before toning with gold chloride, unreacted silver is removed with sodium thiosulfate. The final step is to counterstain, if desired.

■ *Fixative*
10% neutral-buffered formalin is preferred.

■ *Equipment*
Nonmetallic forceps, chemically clean glassware (Coplin jars, graduated cylinders, Erlenmeyer flasks, and pipettes), filter paper

■ *Technique*
Cut paraffin sections at 4 to 5 µm.

■ *Quality Control*
Liver is a very good control tissue.

■ *Reagents*

Silver Nitrate, 10% Solution

Silver nitrate	10 g
Distilled water	100 mL

Sodium Hydroxide, 3% Solution

Sodium hydroxide	3 g
Distilled water	100 mL

Ammoniacal Silver Solution

Place 5 mL of 10% silver nitrate solution in an Erlenmeyer flask, and add concentrated ammonium hydroxide, drop by drop, while shaking the container continuously, until the precipitate that forms is completely dissolved. Do not add any excess ammonium hydroxide. Add 5 mL of 3% sodium hydroxide solution, and cautiously redissolve the precipitate with concentrated ammonium hydroxide until only a faint cloudiness remains. If this step is carried too far and no cloudiness remains, add 10% silver nitrate solution, drop by drop, until 1 drop causes the solution to become permanently cloudy. Only a faint cloudiness is desirable. Dilute the resulting solution to 50 mL with distilled water, and filter into a chemically clean Coplin jar.

Potassium Permanganate, 1% Solution

Potassium permanganate	1 g
Distilled water	100 mL

Oxalic Acid, 1% Solution

Oxalic acid	1 g
Distilled water	100 mL

Ferric Ammonium Sulfate, 2.5% Solution

Ferric ammonium sulfate	2.5 g
Distilled water	100 mL

Formalin, 10% Solution

Formaldehyde, 37% to 40%	10 mL
Distilled water	90 mL

Gold Chloride, 0.2% Solution

Stock gold chloride solution (1%)	10 mL
Distilled water	40 mL

Sodium Thiosulfate, 5% Solution

Sodium thiosulfate	5 g
Distilled water	100 mL

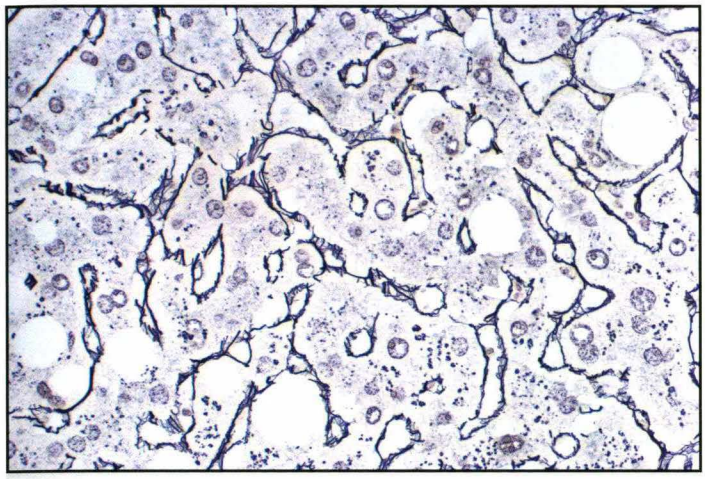
Nuclear-Fast Red (Kernechtrot) Solution

Nuclear-fast red (Kernechtrot)	0.5 g
Aluminum sulfate	25 g
Distilled water	500 mL

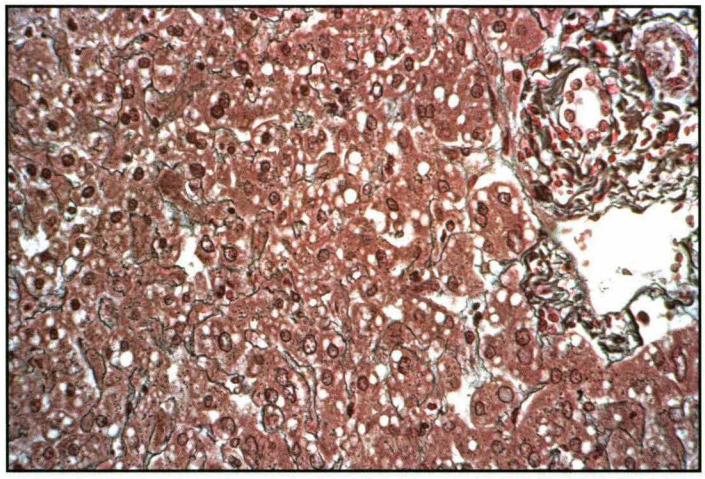
Dissolve the aluminum sulfate in the distilled water, and then dissolve the nuclear-fast red in this solution using heat. Cool, filter, and add a few grains of thymol as a preservative.

■ *Procedure*

1. Deparaffinize sections, and hydrate to distilled water.
2. Oxidize sections in 1% potassium permanganate solution for 5 minutes.
3. Rinse in tap water for 2 minutes.
4. Bleach in 1% oxalic acid for 2 minutes or until sections are colorless.
5. Wash in tap water for 2 minutes.
6. Sensitize sections in 2.5% ferric ammonium sulfate for at least 15 minutes.
7. Wash in several changes of distilled water.



[i8.20] A section of liver stained with the Gordon and Sweets technique. No counterstain has been applied, the reticular network lining the sinusoids is well demonstrated, and the reticulin pattern can be evaluated easily with lower magnification.



[i8.21] This is an example of a poor reticulin stain. The reticulin pattern is totally obscured by the strong counterstaining. The stain is unacceptable and should be repeated.

8. Impregnate sections with the silver solution for 2 minutes.
9. Rinse well with distilled water.
10. Reduce sections for 2 minutes in the 10% formalin solution.
11. Wash in tap water for 3 minutes.
12. Tone in 0.2% gold chloride solution for 10 minutes.
13. Wash in tap water for 2 minutes.
14. Place slides in 5% sodium thiosulfate for 1 minute.
15. Rinse in distilled water.
16. Counterstain, if desired, with nuclear-fast red for 5 minutes.
17. Wash well in distilled water.
18. Dehydrate in 2 changes each of 95% and absolute alcohols, clear in xylene, and mount with synthetic resin.

■ *Results* [i8.20]

- Reticulin — Black
- Other tissue elements depend on counterstain used

■ *Technical Notes*

1. See the technical notes under the Gomori technique for reticular fibers.
2. The Gordon and Sweets method gives much less background and nuclear staining than most of the more common methods.
3. Silver stains are capricious, and when poor stains are obtained [i8.19], [i8.21], the stain should be repeated. All fresh reagents should be used, and particular attention should be paid to the preparation of the diamine silver solution.
4. The 2 methods presented do not use uranyl nitrate, which has been banned in some laboratories because of its toxicity.

Staining Techniques for Muscle

MALLORY PTAH TECHNIQUE FOR CROSS-STRIATIONS AND FIBRIN [MALLORY 1942, CARSON 1984, SHEEHAN 1980]

■ *Purpose*

The demonstration of muscle cross-striations and fibrin. Cross-striations are a diagnostic feature of rhabdomyosarcomas or tumors arising from striated muscle. Nemaline rods, present in some skeletal muscle diseases, may also be demonstrated by the method. The PTAH has also been used for the demonstration of glial fibers and myelin. This method is rarely used today because it has been replaced by immunohistochemical techniques.

■ *Principle*

The amount of phosphotungstic acid in the staining solution is far greater than the amount of hematein (20:1), and it is believed that tungsten binds all available hematein to give a blue lake. This metal-hematein lake stains selected tissue components blue, while the phosphotungstic acid is thought to stain the red-brown components. This stain has been referred to as a polychrome stain because 1 solution gives 2 major colors. The components colored red-brown will lose this color with water or prolonged alcohol washes, and therefore dehydration of the section after staining must be rapid.

■ *Fixative*

Zenker solution is preferred, but 10% neutral-buffered formalin may be used.

■ *Equipment*

Mechanical stirrer, Coplin jars, Erlenmeyer flasks, graduated cylinders

■ *Technique*

Cut paraffin sections at 4 to 6 μm.

■ *Quality Control*

Use longitudinal sections of skeletal or cardiac muscle to demonstrate cross-striations, a section containing fibrin for the demonstration of fibrin.

■ *Reagents*

PTAH Solution

Hematoxylin	1 g
Phosphotungstic acid	20 g
Distilled water	1,000 mL

Dissolve the solid ingredients in separate portions of water, dissolving the hematoxylin with the aid of heat. When cool, combine. No preservative is necessary. The solution that is allowed to ripen naturally (approximately 4 to 6 months) is a better stain and lasts longer, but if time is not available for natural ripening, 0.2 g of potassium permanganate may be added to the solution. This chemically ripened stain may be used immediately, but the best results are not obtained until the solution has been ripened for at least 2 weeks or longer.

Gram Iodine

Iodine	3 g
Potassium iodide	6 g
Distilled water	900 mL

Place the iodine, potassium iodide, and about 150 mL of the water in a flask, and stir until dissolved. Add remaining water.

Sodium Thiosulfate, 5% Solution

Sodium thiosulfate	5 g
Distilled water	100 mL

Potassium Permanganate, 0.25% Solution

Potassium permanganate	0.25 g
Distilled water	100 mL

Oxalic acid, 5% Solution

Oxalic acid	5 g
Distilled water	100 mL

■ *Procedure*

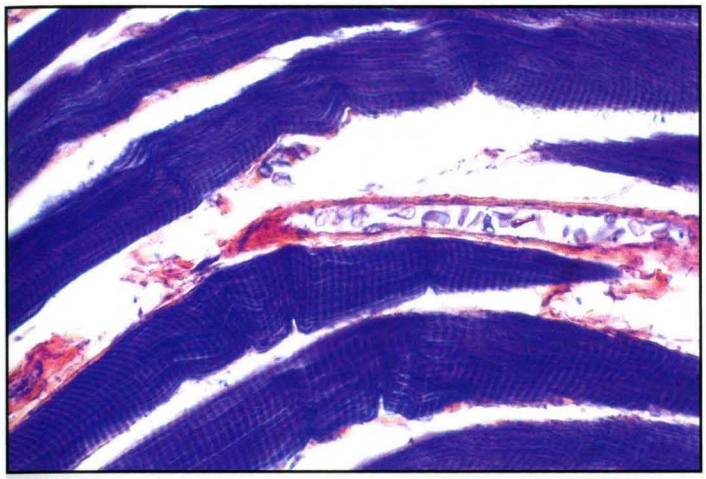
1. Deparaffinize sections, and hydrate to distilled water.
2. If sections are formalin-fixed, mordant in Zenker fixative containing 5% acetic acid overnight at room temperature.
3. Rinse in tap water.
4. Place in Gram iodine for 15 minutes.
5. Rinse in tap water.
6. Place in 5% aqueous sodium thiosulfate for 3 minutes.
7. Wash in tap water for 10 minutes.
8. Place sections in 0.25% potassium permanganate for 5 minutes.
9. Rinse in tap water.
10. Place in 5% oxalic acid for 1 minute.
11. Wash in running tap water for 10 minutes.
12. Stain in PTAH solution overnight at room temperature.
13. Dehydrate rapidly through 2 changes each of 95% and absolute alcohol, clear in xylene, and mount with synthetic resin.

■ *Results [i8.22], [i8.23]*

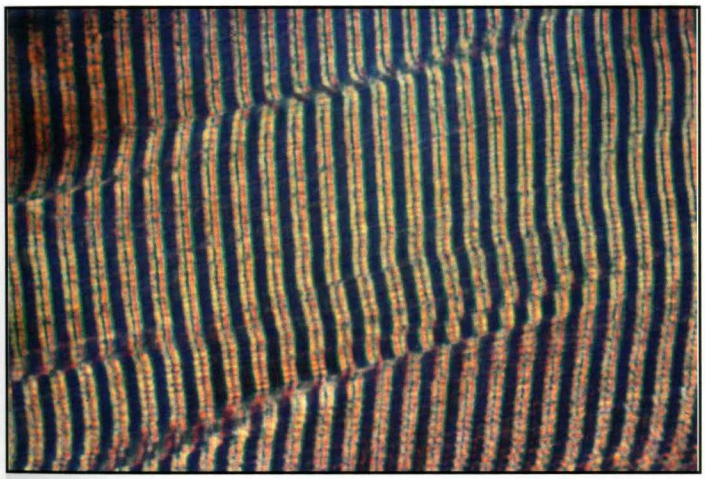
- Cross-striations, fibrin — Blue
- Nuclei — Blue
- Collagen — Red-brown

■ *Technical Notes*

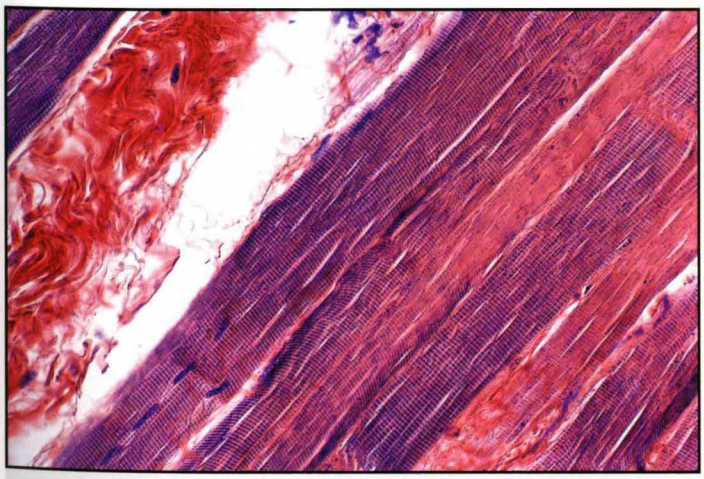
1. A chemically oxidized staining solution has a shorter shelf-life than a naturally ripened staining solution, because chemical



[i8.22] A section of Zenker-fixed skeletal muscle stained with the Mallory phosphotungstic acid-hematoxylin (PTAH) technique.



[i8.23] A very high magnification of a section of Zenker-fixed skeletal muscle stained with the Mallory PTAH technique. The A- and I-bands are well demonstrated, and the Z-lines can also be seen. The stain is rarely used today for the identification of cross-striation in tumors thought to be rhabdomyosarcomas; immunohistochemical staining is now preferred.



[i8.24] A section of formalin-fixed skeletal muscle stained with PTAH. The sections were mordanted overnight in Zenker fixative. This uneven staining appears to be characteristic of formalin-fixed tissue. Note that the collagen is stained red in **[i8.22]**, **[i8.23]**, **[i824]**. This is an important differentiation in correctly performed PTAH staining.

oxidation may cause overoxidation. When this occurs, there is a failure to show the proper density of the blue tones. Solutions should be stored in amber glass bottles to retard overoxidation by light.

2. According to Meloan and Puchtler [1988], thorough washing of sections before staining is essential, because hydration of the tissue structures will greatly facilitate uptake of dye molecules. They also state that sodium thiosulfate interferes with binding of the PTAH, so sections should be washed very well after application of sodium thiosulfate.

3. Bouin solution will also work as a mordant. It should be used for 1 hour at 60°C, and then the sections should be washed in running water until the yellow color is gone. Skip the iodine step. This gives better results than mordanting in Zenker solution or than the chromate method that follows [Wenk 2007]. Fixation in zinc formalin may also provide satisfactory PTAH staining, but I have not tried this variant.

4. Fortunately, the PTAH technique has been largely replaced by immunohistochemical methods; although fibrin will stain very well after formaldehyde fixation, we consistently get better results when staining for muscle cross-striations if the tissue is mordanted, or postfixed, in a mercuric solution. The stain is not as good when formalin-fixed sections are mordanted as when the original fixative is mercuric; very uneven staining frequently occurs in tissue originally fixed in formalin **[i8.24]**. Stevens and Wilson [1996] described the following technique, which avoids mercuric solutions and uses acidic dichromate treatment instead.

PTAH WITHOUT MERCURIC SOLUTIONS [BANCROFT 1996]

■ *Reagents*

Acidic Dichromate Solution

HCl, 10% in absolute alcohol	12 mL
Potassium dichromate, 3% aqueous solution	36 mL

Acidic Potassium Permanganate Solution

Potassium permanganate, 0.5% aqueous solution	50 mL
Sulfuric acid, 3% solution	2.5 mL

Oxalic Acid, 1% Solution

Oxalic acid	1 g
Distilled water	100 mL

■ *Procedure*

1. Deparaffinize sections, and hydrate to distilled water.
2. Place slides in acidic dichromate solution for 30 minutes.
3. Wash in tap water.
4. Place slides in acidic permanganate solution for 1 minute.
5. Wash in tap water.
6. Bleach sections in 1% oxalic acid solution.
7. Rinse in tap water.
8. Stain in PTAH solution overnight.
9. Dehydrate rapidly through 95% and absolute alcohols, clear in xylene, and mount with synthetic resin.

The results of this stain should be the same as those described for the technique using mercuric solutions for either primary fixation or post-fixation mordanting.

■ *Technical Note*

Potassium dichromate is a hazardous chemical that is toxic on inhalation and ingestion. It is corrosive to eyes, skin, and mucous membranes, and is also a carcinogen; however with care, the solutions pose little risk when used under normal conditions. It cannot be put down the drain.

Staining Technique for Basement Membranes

PERIODIC ACID-METHENAMINE SILVER MICROWAVE PROCEDURE FOR BASEMENT MEMBRANES [BRINN 1983, SHEEHAN 1980]

■ *Purpose*

This procedure best delineates basement membranes, and is most often used in the histopathology laboratory for the detection of abnormalities or diseases manifested in the glomerular basement membrane.

■ *Principle*

The carbohydrate component of basement membranes is oxidized to aldehydes by periodic acid. The aldehydes formed by oxidation bind the silver ions from the methenamine silver complex and reduce the silver to its metallic form. Methenamine gives the solution the alkaline properties necessary for the proper reaction, and the sodium borate acts as a buffer. Toning is with gold chloride, and any unreduced silver is removed by sodium thiosulfate.

■ *Fixative*

10% neutral-buffered formalin is preferred. Mercury-containing fixatives are not recommended.

■ *Equipment*

Microwave oven or 50°C to 60°C water bath, vented plastic staining jars, graduated cylinders, Erlenmeyer flasks

■ *Technique*

Paraffin sections cut at 2 µm.

■ *Quality Control*

Kidney has an internal control. No other control slide is necessary.

■ *Reagents*

Stock Methenamine Silver

Methenamine, 3% aqueous (15 g/500 mL)	400 mL
Silver nitrate, 5% aqueous (5 g/ 100 mL)	20 mL
Mix and keep solution refrigerated at 4°C	

Borax (Sodium Borate), 5% Solution

Sodium borate	5 g
Distilled water	100 mL

Working Methenamine Silver Solution

Stock methenamine silver	25 mL
Distilled water	25 mL
Borax (sodium borate), 5% solution	2 mL
Prepare just before use	

Periodic Acid, 1% Solution

Periodic acid	1 g
Distilled water	100 mL

Sodium Thiosulfate, 2% Solution

Sodium thiosulfate	10 g
Distilled water	500 mL

Gold Chloride, 0.02% Solution

Gold chloride, 1% solution	1 mL
Distilled water	49 mL

Stock Light Green, 0.2% Solution

Light green SF (yellowish)	1 g
Distilled water	500 mL
Glacial acetic acid	1 mL

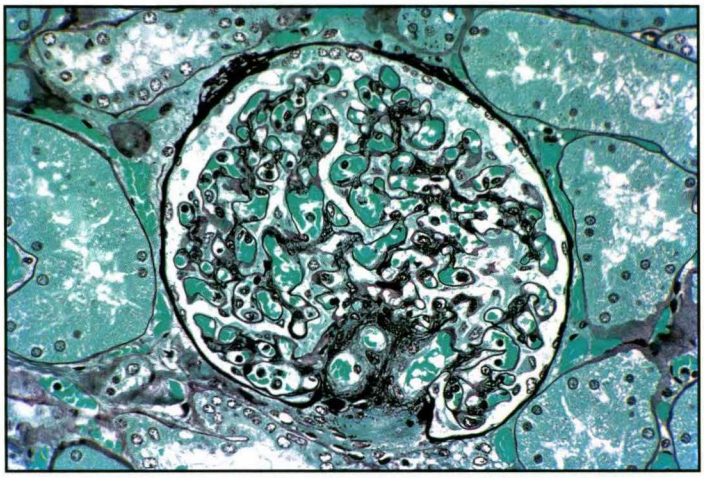
Working Light Green Solution

Light green stock solution	10 mL
Distilled water	50 mL

■ *Procedure*

This procedure is for 5 slides. If you do not have 5 slides, then include blank slides. Do not use more than 5 slides. This procedure should be followed exactly for optimum results.

1. Deparaffinize sections, and hydrate to distilled water.
2. Place sections in 1% periodic acid solution for 15 minutes at room temperature.
3. Rinse in distilled water.
4. Place slides (5) in a vented plastic Coplin jar containing 50 mL of methenamine working solution.* Apply the vented cap, and place in the microwave oven. Also place a vented plastic Coplin jar containing exactly 50 mL (measured) of distilled water in the oven. Microwave on full power for exactly 70 seconds (see technical note 2). Remove both jars from the oven, mix the staining solution with a plastic Pasteur pipette, and let stand on the counter. Check the slides frequently until the desire staining intensity is achieved. This will take approximately 15 to 20 minutes.
5. Rinse slides in the heated distilled water.
6. Tone sections in 0.02% gold chloride.*
7. Rinse slides in distilled water.
8. Treat sections with 2% sodium thiosulfate for 1 minute.
9. Wash in tap water.
10. Counterstain in the working light green solution for 1½ minutes.
11. Dehydrate with 2 changes each of 95% and absolute alcohols.
12. Clear with xylene and mount with synthetic resin.



[i8.25] A kidney glomerulus stained with a periodic acid-methenamine silver technique. A good stain should look almost as if the basement membrane had been drawn with ink. Although the tubular basement membranes are well-stained, they should not be used to judge the end point of the impregnation; use the glomerular basement membrane only.

*If for some reason, the microwave oven cannot be used, substitute the following solutions and staining times:

Methenamine Silver Solution

Stock methenamine silver solution	50 mL
Borax, 5% solution	5 mL
Preheat the solution, and stain slides at 56°C-60°C for 40-90 minutes	

Gold Chloride, 0.2% solution

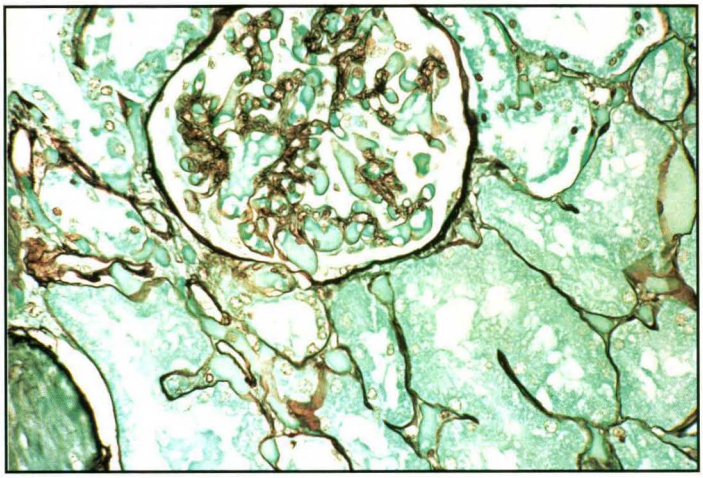
Gold chloride, 1% solution	10 mL
Distilled water	40 mL
Tone for 30 seconds to 1 minute	

■ *Results* **[i8.25]**

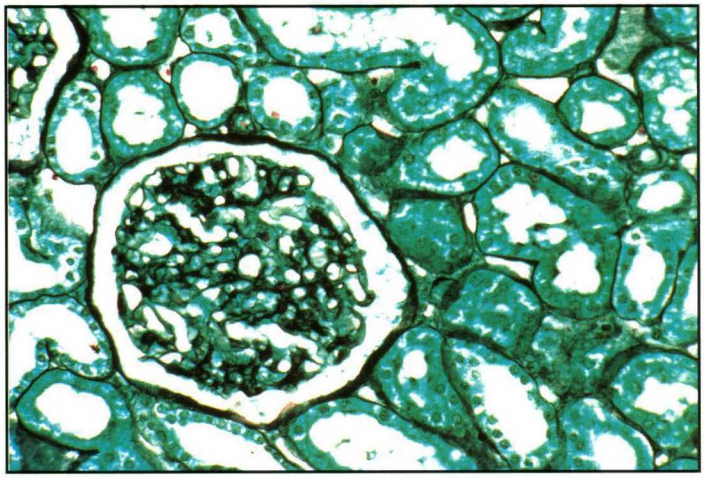
- Basement membrane — Black
- Background — Green

■ *Technical Notes*

1. Sharper staining of the basement membrane and less background staining can be obtained with the use of the microwave oven for silver techniques.
2. The temperature is critical and should be just below boiling or approximately 95°C, immediately after removal from the microwave oven. Each oven should be calibrated for the time required to reach the correct temperature.



[i8.26] This kidney glomerulus has been understained with a periodic acid-methenamine silver technique. Compare the staining here with that in **[i8.25].** Note that the basement membrane exhibits continuous staining in **[i8.25]** and interrupted staining here.



[i8.27] Too much counterstain has been applied to this section of kidney, and the periodic acid-methenamine silver staining is masked. The section is also too thick; sections should be 2 µm thick for demonstration of the glomerular basement membrane.

3. This is a very difficult stain to perform correctly. Although the tubular membranes will stain, the end-point should be determined by the glomerular basement only. When well-stained, the glomerular basement membrane should appear as a continuous black line. Stopping the silver impregnation too soon will result in uneven or interrupted staining **[i8.26]**. The application of too much counterstain will mask the silver stain and decrease contrast **[i8.27]**.

4. As with all silver stains, chemically cleaned glassware and plastic forceps should be used.

5. The PAS technique is also use to stain basement membranes and can be found in chapter 7, "Carbohydrates," p137.

Staining Techniques for Lipid

Because the lipid stains requested in the routine histopathology laboratory are for simple lipids, only a selection of techniques for simple fats will be presented.

OIL RED O METHOD FOR NEUTRAL FATS [PEARSE 1968]

■ *Purpose*

The oil red O method is used to demonstrate neutral lipids in frozen tissue sections. Fat occurring in an abnormal place such as fatty emboli that may develop after either a bone fracture an injury that crushes a fatty body area may be demonstrated. The fat stain may verify that the emboli caused death. Degenerating material containing fat, such as cell membranes or myelin, may coalesce into fat droplets that are demonstrable with fat stains, and tumors arising from fat cells (liposarcomas) can be differentiated from other types of tumors.

■ *Principle*

Staining with oil-soluble dyes is based on the greater solubility of the dye in the lipoid substances than in the usual hydroalcoholic dye solvents. This is a physical method of staining and the dye used must:

1. be more soluble in the tissue lipid than in the solvent in which it is dissolved
2. not be water soluble
3. be strongly colored
4. must act with tissue constituents only by solution

The solvent used is critical, with isopropanol removing a minimal amount of lipid and propylene glycol not extracting any lipid.

■ *Fixative*

10% neutral-buffered formalin or calcium-formol. Because of the lipid-dissolving ability, no alcoholic fixative should be used.

■ *Equipment*

Cryostat, Coplin jars, Erlenmeyer flasks, graduated cylinders, filter paper

■ *Technique*

Cut frozen sections at 10 µm. Paraffin sections cannot be used because dehydrating and clearing agents dissolve the fat. If free-floating sections are not used, then sections of fixed tissue should be picked up on coated, charged, or subbed slides.

■ *Quality Control*

Most tissue contains some fat, so normally a control is not used.

■ *Reagents*

Oil Red O Stock Solution

Oil red O	2.5 g
Isopropanol, 98%	500 mL
Mix well	

Oil Red O Working Solution

Oil red O, stock solution	24 mL
Distilled water	16 mL
Mix well, and let stand for 10 minutes. Filter. The filtrate can be used for several hours.	

■ *Procedure*

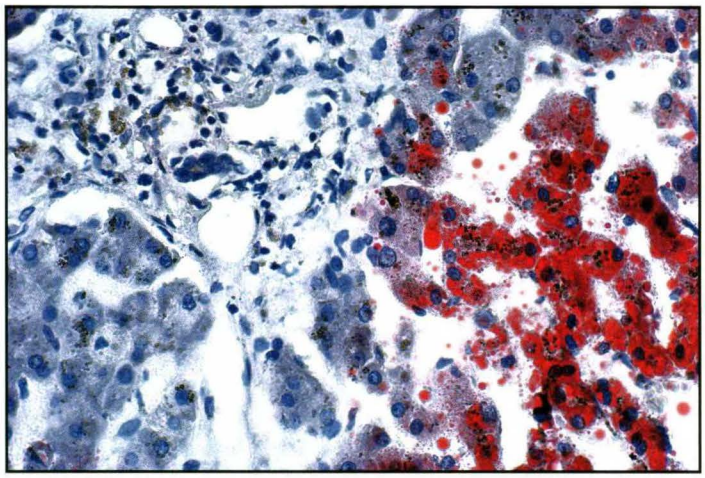
1. Cut frozen sections, fix in 40% formaldehyde for 1 minute, and wash well in tap water. Blot off excess water. If the tissue has been previously fixed, there is no need to refix.
2. Stain sections in oil red O for 10 minutes.
3. Wash sections in tap water.
4. Stain for 1 minute in Harris hematoxylin containing acetic acid (glacial acetic acid, 2 mL/hematoxylin, 48 mL).
5. Wash section in tap water.
6. Blue in ammonia water.
7. Wash in tap water.
8. Mount sections with an aqueous mounting medium.
9. Seal the edges of the coverslip with fingernail polish.

■ *Results [i8.28]*

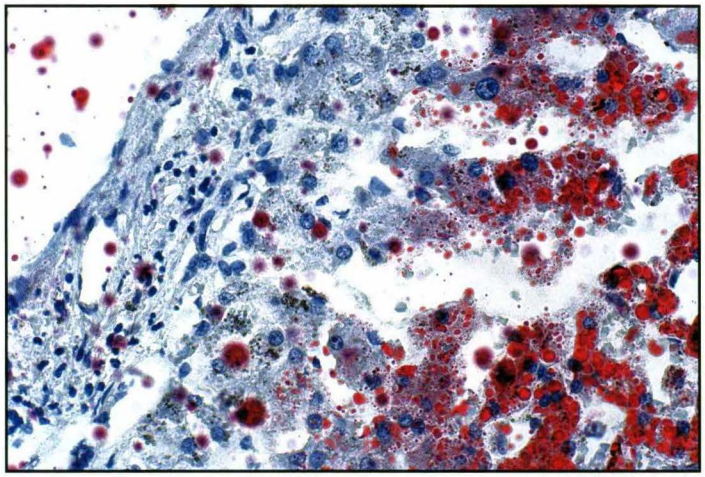
• Fat	Black
• Other tissue elements	According to method used

■ *Technical Notes*

1. Lipids are removed by any fixative or solution containing alcohol or organic solvents, so sections cannot be processed for paraffin embedding. Frozen sections are most frequently used, but the use of water-soluble wax for embedding will allow the demonstration of fat.



[i8.28] A frozen section from a fatty liver stained with oil red O. This tissue had been fixed in formalin for several weeks before freezing. Note that even with the amount of fat present, the section shows little fat displacement.



[i8.29] Air bubbles were "chased out" by mashing on the coverslip with forceps during the mounting step. Note the fat on top of this section compared with the duplicate cleanly mounted section in **[i8.28]**; fat is even present in the portal vein in this section. Fat is relatively liquid, so the mounting step must be done carefully.

2. Free-floating sections stain more readily; however, free-floating sections are difficult to obtain with the cryostat, and sections mounted on slides may be used. The staining time may need to be adjusted. Sections that have been fixed previously also may tend to loosen from the slides.
3. To improve the microtomy of frozen sections, formalin-fixed tissues may be infiltrated with 30% sucrose solution before freezing.
4. Aqueous mounting media must be used, because the organic solvent present in synthetic resinous media will dissolve the fat.
5. The fat in the section is relatively liquid and mobile, so care should be taken that no pressure is placed on the cover glass or the fat may be displaced **[i8.29]**. If air bubbles are present in the section, remove the coverslip by soaking the slide in warm water. If glycerin jelly is used for mounting, it should not be overheated, because this may melt the fat and also displace it.

6. Oil red O may be dissolved in propylene glycol instead of isopropyl alcohol. Prepare the solution and perform the stain as in steps 1 though 4 of the Sudan black B procedure that follows. Steps 5 through 9 of the above procedure above should be used to complete the stain. The oil red O in propylene glycol staining should be done at 60°C. Small fat droplets are less likely to be dissolved by propylene glycol than by alcohol.

SUDAN BLACK B IN PROPYLENE GLYCOL [CHIFFELLE 1951, CARSON 1983]

■ *Purpose*

The demonstration of neutral lipids in tissue sections as described in the oil red O procedure. Sudan black B is the most sensitive of the lipid dyes and is far more soluble in phospholipids and, to a lesser extent in cerebrosides, than oil red O. It also is used in hematopathology to aid in differentiating granulocyte precursors from leukocytes committed to lymphocytic or monocytic pathways.

■ *Principle*

Sudan black B is one of the Sudan dyes used for demonstrating lipids. In addition to the solubility of the dye in neutral fats as described with oil red O, Sudan black B is a slightly basic dye and will combine with acidic groups in compound lipids; therefore, it will also stain phospholipids.

■ *Fixative*

10% neutral-buffered formalin or sections postfixed in calcium-formalin. Because of lipid dissolving ability, no alcoholic fixative should be used.

■ *Equipment*

Cryostat, 100°C hot plate, 60°C oven, vacuum filtration apparatus with a fritted glass or Millipore filter, graduated cylinders, Erlenmeyer flasks, Coplin jars

■ *Quality Control*

Most tissue contains some fat, so normally a control is not used.

■ *Reagents*

Calcium-Formalin Solution

Formaldehyde, 37% to 40%	10 mL
Calcium chloride	1.1 g
Distilled water	90 mL

Sudan Black B Staining Solution

Sudan black B	0.7 g
Propylene glycol	100 mL

Add a small amount of the Sudan black B at a time to the propylene glycol, and stir continuously. Heat to 100°C (do not exceed 110°C) for a few minutes, stirring constantly. Filter immediately through Whatman No. 2 filter paper, cool, and refilter through a fritted glass filter or Millipore filter with a pore size suitable for filtering reagents. This reagent may be stored indefinitely in a well-capped container in a 60°C oven.

■ *Procedure*

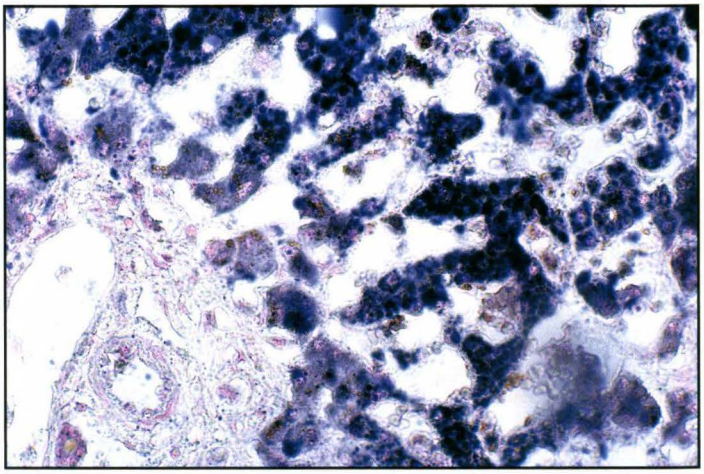
1. Place fixed and rinsed cryostat sections in 100% propylene glycol for 15 minutes. Use 2 changes. If tissue has not been previously fixed, fix the cut and mounted sections in calcium-formal for 1 minute, rinse well, and then place in propylene glycol.
2. Stain sections in Sudan black B for 10 minutes.
3. Differentiate in 85% propylene glycol.
4. Wash sections well in distilled water.
5. Counterstain sections with nuclear-fast red for 1 to 5 minutes.
6. Wash well in several changes of distilled water.
7. Mount sections with an aqueous mounting medium.

■ *Results* [i8.30]

- Fat — Blue-black
- Nuclei — Red

■ *Technical Notes*

See the notes for oil red O staining.



[i8.30] A frozen section from the same fatty liver shown in **[i8.28]** and **[i8.29]** stained with Sudan black B.

OSMIUM TETROXIDE PARAFFIN PROCEDURE FOR FAT [CARSON 1983]

■ *Purpose*

The demonstration of fat by a method that allows paraffin embedding of the tissue.

■ *Principle*

Osmium tetroxide chemically combines with fat, blackening it in the process. This is the only method for fat that is chemical, although the nature of the black reaction product is not understood. Fat that has combined with osmium tetroxide is insoluble in alcohols and xylene, and the tissue can be processed for paraffin embedding.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

Chemical hood, small capped bottles, processing cassettes

■ *Technique*

Stain the wet tissue block, and then process in the tissue processor, beginning in 70% alcohol. The gross section must be no thicker than 2 mm, or the osmium will not penetrate.

■ *Quality Control*

Most tissue contains some fat, so no control block is necessary.

■ *Reagents*

Osmium Tetroxide, 1% Solution

Osmium tetroxide	1 g
Distilled water	100 mL

Store in a dark container in the refrigerator. The vapor is harmful, so prepare and use the reagent under a fume hood.

Periodic Acid, 0.5% Solution

Periodic acid	2.5 g
Distilled water	500 mL

■ *Procedure*

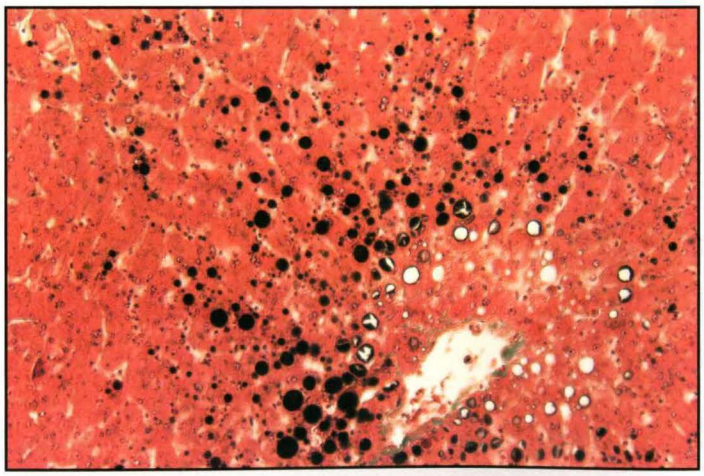
1. Trim 10% neutral buffered formalin-fixed tissue to 2 mm thick, and wash in running tap water for at least 30 minutes.
2. Rinse the tissue well in distilled water.
3. Place the tissue in a small quantity (5 mL) of osmium tetroxide solution. Cap the container, and leave for 1 to 2 hours, depending on the denseness of the tissue: heart, 2 hours; aorta, 1 hour; and lung, 1 hour. This step should be done under the hood. Periodically agitate the solution containing the tissue.
4. Rinse tissue in 2 changes of distilled water for 15 minutes each.
5. Differentiate by placing tissue in 0.5% periodic acid solution for 30 minutes. Agitate the solution periodically. The background tissue will clear and leave the fat stained black.
6. Wash the tissue in tap water for 30 minutes.
7. Process routinely, beginning with 70% alcohol; embed as usual.
8. Cut paraffin sections 4 to 5 μm, pick up on slides, and dry routinely.
9. Deparaffinize and hydrate sections as usual.
10. The sections may be stained with the routine H&E procedure or with any special stain desired. Masson trichrome provides an excellent counterstain for osmium-fixed sections.
11. After staining, the sections should be dehydrated, cleared, and mounted with a synthetic resin.

■ *Results* [i8.31]

• Fat	Black
• Other tissue elements	According to method used

■ *Technical Notes*

1. Gross amounts of fat will not be fixed by this method, but small fat droplets and individual fat cells are beautifully demonstrated.



[i8.31] This section of liver, containing a small amount of fat, was fixed with osmium tetroxide, and then processed and embedded in paraffin. The fat has been fixed and stained black by the osmium, so that it was not dissolved by the alcohol and xylene. Following processing and microtomy, this section was stained with the Masson trichrome procedure.

2. The block should be faced carefully and sections should be taken as soon as possible. Osmium has a very low penetrating power and the interior of the block may not show good fat preservation and staining. It is essential that the sections for processing be cut thin.

3. The cytoplasm will be gray as a result of the osmium, and this will affect the quality of the cytoplasmic stains.

Staining Techniques for Connective Tissue Cells

TOLUIDINE BLUE FOR MAST CELLS [LILLIE 1965, VACCA 1985]

■ *Purpose*

The demonstration of mast cells in tissue. Mast cells play a key role in inflammation, and allergic reactions, are implicated in the pathology associated with autoimmune disorders, and are found in mast cell tumors (mastocytomas) common in dogs and cats and rare in humans.

■ *Principle*

Mast cells will stain metachromatically with toluidine blue; that is, they will stain a different color from the dye solution and the rest of the tissue. The control section should show mast cells stained red-purple (metachromatic staining) and the background stained blue (orthochromatic staining). The color shift, called metachromasia, generally is attributed to the cationic or basic dye and is somewhat dependent on pH, dye concentration, and temperature. Blue or violet dyes will show a red color shift, and red dyes will show a yellow color shift with metachromatic tissue elements.

■ *Fixative*

10% neutral-buffered formalin is preferred.

■ *Equipment*

Coplin jars, Erlenmeyer flask, graduated cylinder

■ *Technique*

Cut paraffin sections at 4 to 5 µm.

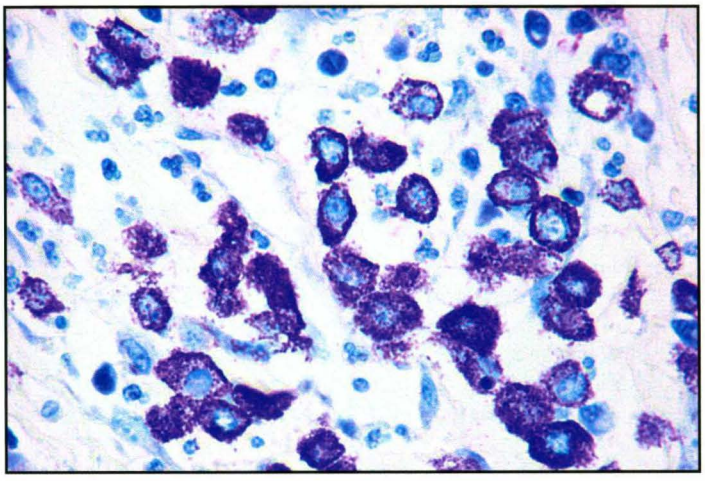
■ *Quality Control*

A section containing mast cells must be used as a control.

■ *Reagents*

Toluidine Blue Solution

Toluidine blue	0.1 g
Distilled water	100 mL



[i8.32] A section of skin from a mastocytoma in a dog. Note the many metachromatically stained mast cells in the section. [Image courtesy of Lott R, Birmingham, AL]

■ *Procedure*

1. Deparaffinize sections, and hydrate to distilled water.
2. Stain sections in toluidine blue solution for 10 minutes.
3. Rinse in distilled water.
4. Quickly dehydrate with 95% and absolute alcohol.
5. Clear in xylene, and mount with synthetic resin.

■ *Results* [i8.32]

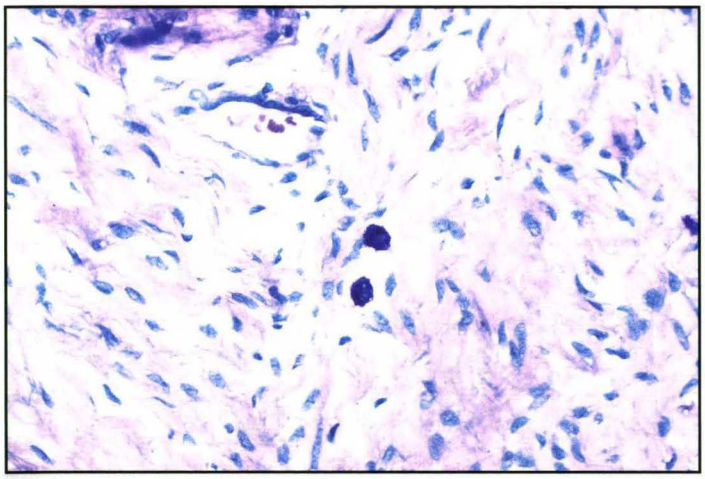
• Mast cells	Deep rose-violet
• Background	Blue

■ *Technical Notes*

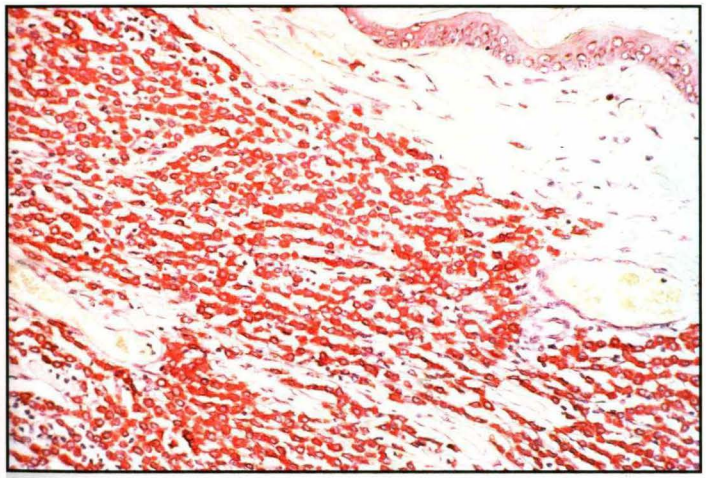
1. Alcohol cannot be used for dehydration in most metachromatic staining procedures, but metachromasia of mast cell granules is stable and will not be lost after alcoholic dehydration.
2. A rapid screening method for mast cells is to simply apply a light methylene blue stain as if you were counterstaining an acid-fast stain. The mast cells are very well demonstrated **[i8.33]**.
3. Mast cells will also be stained orange-red by the methyl green-pyronin stain as shown in **[i8.34]**.

METHYL GREEN-PYRONIN Y

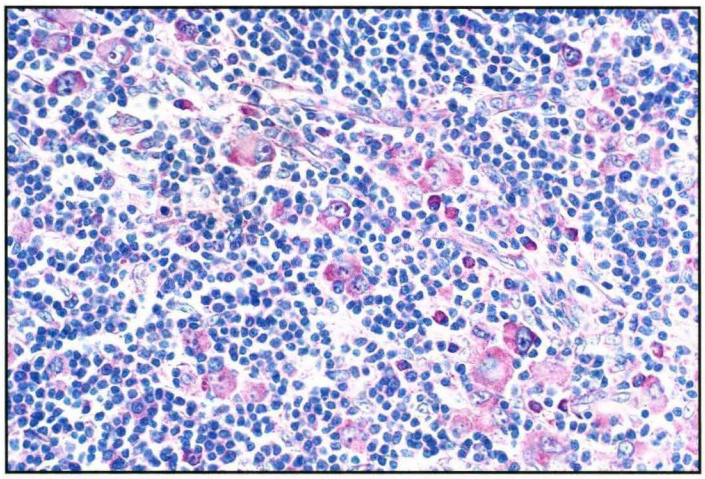
This method was described in detail in chapter 6, p124, as a method of differentiating DNA and RNA. It is mentioned again in this chapter because it stains plasma cells, which are connective tissue cells. Because of the rough endoplasmic reticulum present, with its high RNA content, the cytoplasm of mature plasma cells and immunoblasts will be stained pink to red by pyronin **[i8.35]**.



[i8.33] 2 mast cells can be seen in this section, which was stained with the methylene blue counterstain used in the Kinyoun acid-fast technique. This solution must be applied so that a very light stain is achieved, but it provides a rapid, easily performed screening method for mast cells.



[i8.34] This mast cell tumor of the skin has been stained with the methyl green-pyronin stain. Mast cell granules are very pyroninophilic, but the color is more orange than that seen with RNA staining.



[i8.35] Several mature plasma cells of this lymph node stained with the methyl green-pyronin stain show dark rose-stained cytoplasm (RNA). The larger immunoblasts have more and paler rose-stained cytoplasm. The nuclei (DNA) are stained blue-green and the nucleoli (RNA), prominent in the immunoblasts, are also stained rose.

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Wenk P [2007] Personal communication.

LEARNING ACTIVITIES

1. **Perform the Masson trichrome, Gomori trichrome, van Gieson, Verhoeff, aldehyde fuchsin, Russell modification of the Movat pentachrome, silver impregnation for reticulum, Mallory PTAH, oil red O, Sudan black B, toluidine blue, methyl green-pyronin, and methenamine-silver staining procedures. You may choose any tissue that will demonstrate positive staining. (Note: the van Gieson technique may be used as a counterstain with the Verhoeff stain and not done as a separate stain.)**

2. **Microscopically examine each stained section, and compare the results with those given in the procedure. If the results are unsatisfactory, analyze the procedural steps for possible sources of error. If a mistake is identified, repeat the stain after correcting the problem, and reexamine the slides.**

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CHAPTER 9

Nerve

OBJECTIVES

On completing this chapter, the student should be able to do the following:

1. Define:
    a. neuron
    b. glia and glial fibers
    c. Nissl substance
    d. Myelin
    e. axon (axis cylinder)
2. Classify the following techniques as to element demonstrated:
    a. cresyl echt violet
    b. Bodian
    c. Holmes silver nitrate
    d. Bielschowsky
    e. Sevier-Munger
    f. Thioflavin S
    g. Phosphotungstic acid-hematoxylin (PTAH)
    h. Holzer
    i. Cajal
    j. Weil
    k. Luxol fast blue
3. Outline each of the above techniques, considering the following:
    a. most desirable fixative
    b. if another fixative has been used, what can be done
    c. primary reagents and dyes and their purposes
    d. results of staining
    e. appropriate control material
    f. sources of error and appropriate correction
    g. mode of action
    h. special requirements (eg, chemically clean glassware)
    i. microscope used

The Nervous System

The nervous system may be divided anatomically into 2 parts: the central nervous system (CNS), which comprises the brain and spinal cord, and the peripheral nervous system (PNS), which consists of all other nervous tissue. Functionally, the nervous system is divided into the somatic nervous system (voluntary, or under conscious control) and the autonomic nervous system (involuntary). Histologically, nervous tissue consists of cells and cell processes, and the stains for demonstrating the various components of nerve tissue usually fall into 3 groups. These groups of stains are for:

1. neuronal cell bodies and processes
2. glial cells and processes
3. the myelin sheath

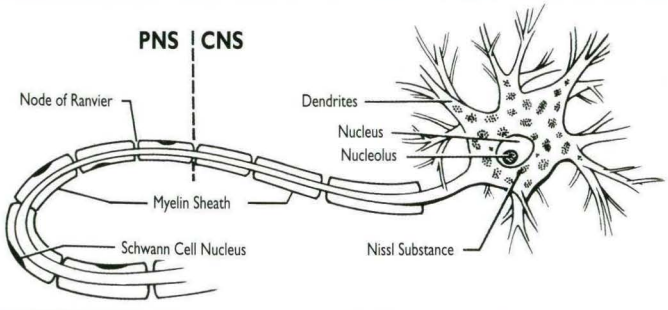
The morphology of each component will be described, and then representative methods for demonstration will be presented.

Neurons

It has been estimated that the brain contains at least 14 billion neurons, or nerve cells. A neuron consists of a cell body (perikaryon) that contains the nucleus and 1 or more cell processes (axon and dendrites). Neuron cell bodies vary in shape and generally are larger than other cells, varying from 4 to 135 μm in diameter. Usually, each cell has only 1 nucleus that contains predominantly euchromatin and a very prominent nucleolus. A neuron with the various structural components is shown in **[f9.1]**.

NISSL SUBSTANCE

Nissl substance, also called tigroid substance and chromidal substance, refers to basophilic material in the cytoplasm of the neuron. Ultrastructurally, this material can be identified as large aggregates of rough endoplasmic reticulum, with the RNA content providing the basis for demonstration by special light microscopic techniques. Because of the RNA content, Nissl substance is sharply stained with basic aniline dyes such as thionin and cresyl echt violet. Nissl substance varies in form, size, and distribution in different types of neurons. Injury of a neuron may cause the Nissl substance to disappear, first from around the nucleus and then entirely; this loss is referred to as *chromatolysis*, and demonstrating this loss is useful in assessing neuronal damage.



[f9.1] The neuron with its various structural components.

NERVE CELL PROCESSES

Two types of processes, axons and dendrites, arise from the neuron cell body. Dendrites are usually short, highly branched processes that function as the major sites of information input for the neuron. Dendrites do not have a myelin sheath. Axons, frequently referred to as nerve fibers, are the neuronal processes that carry nerve impulses over long distances. Each neuron has a single axon that originates from a cone-shaped elevation (the axon hillock) of the cell body and terminates on the dendrites or cell body of other neurons (a synapse), or in a specialized ending associated with an effector organ such as muscle. In older literature, the axon is frequently referred to as the axis cylinder.

The cytoplasm of the cell body, the axon, and the dendrites contains neurofibrils that can be seen ultrastructurally to consist of aggregates of microtubules and neurofilaments. Silver methods are used to demonstrate both nerve fibers and neurofibrils.

Neuroglia

Neuroglia (nerve glue) provide the supporting network for the CNS. Neuroglia may be thought of as neural connective tissue, because connective tissue proper is not found in the CNS except in the meninges covering the brain and in the blood vessels. Except where they are in synaptic contact, neurons are surrounded and insulated by glia. The glia produce the myelin sheath covering many axons and also function to regulate the neuronal microenvironment. There are 4 types of glial cells: oligodendroglia, astroglia, microglia, and ependymal cells.

OLIGODENDROGLIA

Oligodendroglia are small cells that function in the CNS to produce, and probably maintain, the myelin sheath surrounding many axons. They are the most numerous of the glial cells and are found in both the gray (composed primarily of nerve cell bodies) and the white (composed primarily of nerve fibers, many myelinated) matter. Special stains for the demonstration of this type of glial cell are rarely requested in routine histopathology laboratories.

ASTROCYTES

Astrocytes are stellate cells of 2 types: protoplasmic, which occur in the gray matter, and fibrous, which occur in the white matter. Following injury or trauma of the CNS, astrocytes function in scar formation by proliferation of cell processes and the formation of an area of gliosis. Astrocytes provide support for nerve fiber tracts; this type of glial cell also participates in the exchange of fluids, gases, and metabolites among nervous tissue, blood, and cerebrospinal fluid. Stains for astrocytes and for the astrocytic processes have been used frequently in histopathology and neuropathology laboratories; however, most of these techniques rarely are used today, because they have been replaced by immunohistochemical procedures.

MICROGLIA

Microglia are fixed phagocytic cells found throughout the brain and spinal cord; stains for the demonstration of microglia rarely are needed except for research purposes.

EPENDYMAL CELLS

Ependymal cells are true epithelial cells that line the ventricles and spinal canal. They form a selective barrier between the cerebrospinal fluid and nervous tissue.

Myelin

Myelin is a complex, white, fatty, nonliving material containing protein, cholesterol, phospholipids, and cerebrosides. It is largely lost during routine paraffin processing with only neurokeratin, a resistant proteolipid, left in the embedded tissue. The myelin sheath is formed by oligodendroglia in the CNS and by Schwann cells in the peripheral nervous system. In response to injury or diseases that cause a breakdown of myelin, a simple lipid that becomes increasingly sudanophilic is formed. Luxol fast blue and iron hematoxylin methods are commonly used for the demonstration of the myelin sheath.

Special Staining Techniques

NISSL SUBSTANCE: CRESYL ECHT VIOLET METHOD I [LUNA 1960]

■ *Purpose*

Identification of neurons in tissue sections, or demonstration of the loss of Nissl substance (chromatolysis). This loss occurs when the axons are transected, injured, or destroyed. This is a reversible change in response to axonal injury and is apparently related to the need for the cell to increase protein synthesis as the cell attempts to regenerate a new axon. When the need for increased protein synthesis is ended, the Nissl substance will return to normal. However, if the axon is injured very close to the cell body, the neuron may just disappear.

■ *Principle*

Neurons contain Nissl substance, which is primarily composed of rough endoplasmic reticulum, with the amount, form, and distribution varying in different types of neurons. Because of the RNA content, Nissl substance is very basophilic and will be very sharply stained with basic aniline dyes. By varying the pH and the degree of differentiation, both Nissl substance and nuclei, or only Nissl substance, may be demonstrated.

■ *Fixative*

10% neutral-buffered formalin is preferred

■ *Equipment*

Coplin jars, Whatman #1 filter paper, Erlenmeyer flasks, graduated cylinders, balance

■ *Technique*

Cut paraffin sections at 6-8 μm.

■ *Quality Control*

A section of spinal cord is a good control.

■ *Reagents*

Cresyl Echt Violet Solution

Cresyl echt violet	0.5 g
Distilled water	100 mL
Ripen for 24-48 hours and filter before use	

Balsam-Xylene Mixture

Canada balsam (Aldrich Chemical Co)	25 mL
Xylene	25 mL

■ *Procedure*

1. Deparaffinize sections, and hydrate to distilled water.
2. Stain for 3-5 minutes in cresyl echt violet solution.
3. Rinse in 2 changes of distilled water.
4. Place sections in 95% alcohol for 30 seconds.
5. Transfer sections to absolute alcohol for 30 seconds.
6. Place in xylene for 1 minute.
7. Place in balsam-xylene mixture for 2 minutes.
8. Differentiate in absolute alcohol, 2 changes for 10-30 seconds each. Check the sections microscopically.
9. Take through several changes of xylene.
10. Steps 7 through 9 probably will have to be repeated several times. When differentiation is complete, the background should be colorless, with nuclei and Nissl substance well demonstrated.
11. Mount sections with synthetic resin.

■ *Results* [i9.1]

• Nissl substance	Blue to purple
• Nuclei	Blue to purple
• Background	Colorless

■ *Technical Notes*

1. The differentiation should be repeated until the background is colorless. This usually will require that the differentiation steps be repeated several times.

2. The alcohol that follows the balsam-xylene will become cloudy and should be changed frequently.

NISSL SUBSTANCE: CRESYL ECHT VIOLET METHOD II [VACCA 1985]

■ *Purpose*

Identification of neurons in tissue sections or demonstration of loss of Nissl substance (chromatolysis)

■ *Principle*

See the description under the previous procedure. This method uses cresyl echt violet at an acid pH. Staining is restricted to nuclei and to DNA- and RNA-containing structures; the contrast of the Nissl substance and nuclei with the unstained background is enhanced.

■ *Fixative*

10% neutral-buffered formalin is preferred

■ *Equipment*

Coplin jars, Whatman #1 filter paper, Erlenmeyer flasks, graduated cylinders, balance

■ *Technique*

Cut paraffin sections at 6-8 µm.

■ *Quality Control*

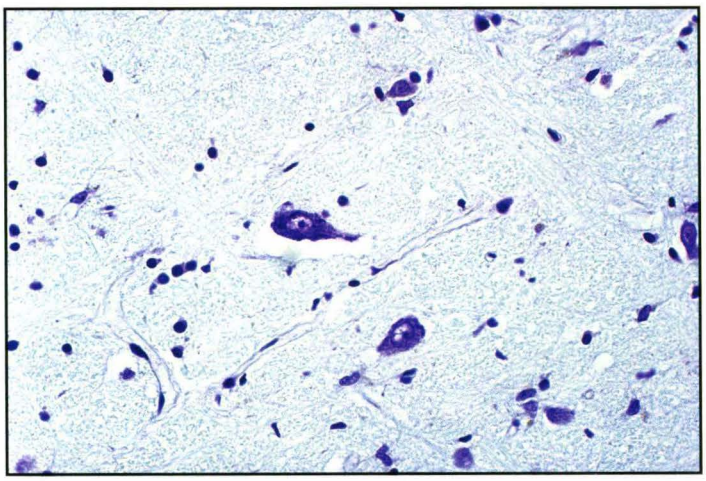
A section of spinal cord is a good control.

■ *Reagents*

Stock Cresyl Echt Violet Solution

Cresyl echt violet (CI 51010)	0.5 g
Distilled water	80 mL
Absolute alcohol	20 mL

Warm the distilled water, add the cresyl echt violet, mix, and then add the absolute alcohol



[i9.1] Several neurons stained with cresyl echt violet (method I) can be seen. The Nissl substance is well demonstrated, and the nuclei of glial cells are also stained. Note the practically colorless background.

Working Cresyl Echt Violet Solution, pH 2.5

Cresyl echt violet stock solution	45 mL
Acetic acid, glacial	15 drops

■ *Procedure*

1. Deparaffinize and hydrate the sections to distilled water.

2. Stain sections in working cresyl echt violet for 8 minutes.

3. Dehydrate sections with 95% and absolute alcohol, 2 changes each.

4. Clear in 2 changes of xylene and mount with synthetic resin.

■ *Results* [i9.2]

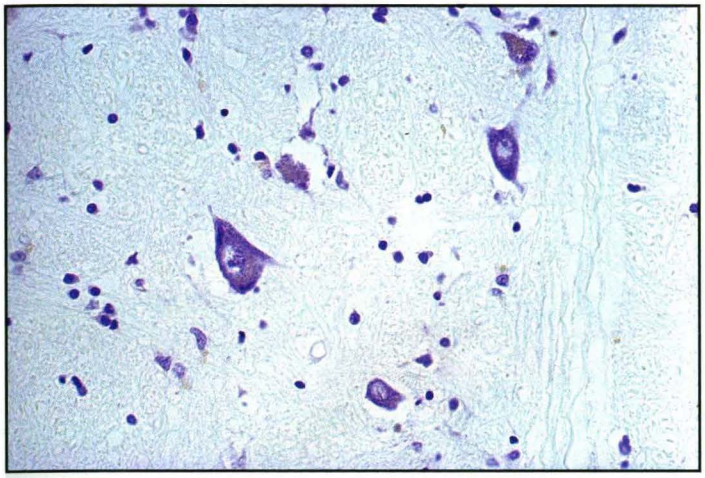
• Nissl substance and nuclei	Blue-purple
• Background	Colorless

■ *Technical Notes*

1. The sections will appear unstained macroscopically and possibly lead to a false conclusion that the stain is not working.

2. Cresyl fast violet, cresyl echt violet, and cresyl violet acetate appear to be used interchangeably; the techniques presented were used with Chroma-Gesellschaft cresyl echt violet (Roboz) and the results are shown in [i9.1], [i9.2]. Other forms of the dye were not tried.

3. The cresyl echt violet solution used in the Luxol fast blue-cresyl echt violet (LFB-CEV) method described in this chapter may also be used to stain Nissl substance. Deparaffinize the sections, hydrate to distilled water, and follow steps 10 through 12 of the LFB-CEV procedure.



[i9.2] Nissl substance is stained using the method of Vacca (method II) in this section. Note that the Nissl substance is less intensely stained than in **[i9.1]** and the background is colorless.

NERVE FIBERS, NERVE ENDINGS, NEUROFIBRILS: BODIAN METHOD [MALLORY 1961, SHEEHAN 1980]

■ *Purpose*

This technique is useful for staining nerve fibers in tissue sections. When an axon is severely or irreversibly injured, all of the axon distal to the injury disappears along with its myelin sheath. This is known as Wallerian degeneration. This injury is readily demonstrated with silver stains.

■ *Principle*

Protargol (Winthrop Laboratories, New York, NY), a brand name for silver proteinate, is used to impregnate tissue sections. Copper is added to the impregnating solution to "destain" connective tissue, allowing a greater degree of differentiation between neural and connective tissue elements. It is thought that copper is more reactive than silver and replaces the silver that has impregnated the connective tissue fibers. Hydroquinone is used to reduce silver salts that have been deposited on certain tissue structures to visible metallic silver. Sections are toned with gold chloride, as in the diamine silver methods for reticulum. Oxalic acid may be used to reduce the gold, intensifying the stain by increasing the deposit of metallic gold on the section. Sodium thiosulfate removes any unreduced silver from the section. Luna [1964] modified the original method and achieved more consistent results by combining formalin, instead of sodium sulfite, with the hydroquinone used for the reduction step. He also increased the impregnation time from 24-48 hours.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

Chemically clean Coplin jars, 37°C incubator, small beakers, graduated cylinders, Erlenmeyer flasks

■ *Quality Control*

A section of peripheral nerve or cerebral cortex provides the best control. Spinal cord is not good, because most nerve fibers will appear in cross-section. The glassware should be chemically clean and nonmetallic forceps should be used.

■ *Reagents*

Protargol, 1% Solution

Protargol	1 g
Distilled water	100 mL

Sprinkle the Protargol on the surface of the water, and allow it to remain undisturbed at 37°C until it dissolves

Reducing Solution

Hydroquinone	1 g
Formaldehyde, 37% to 40%	5 mL
Distilled water	100 mL

Gold Chloride, 1% Solution

Use as purchased

Oxalic Acid, 2% Solution

Oxalic acid	2 g
Distilled water	100 mL

Sodium Thiosulfate (Hypo), 5% Solution

Sodium thiosulfate	5 g
Distilled water	100 mL

Aqua Regia

Hydrochloric acid, concentrated	15 mL
Nitric acid, concentrated	5 mL

Be very careful when handling this reagent. Wear goggles, gloves, and apron; prepare and use the reagent in a fume hood

Aniline Blue Solution

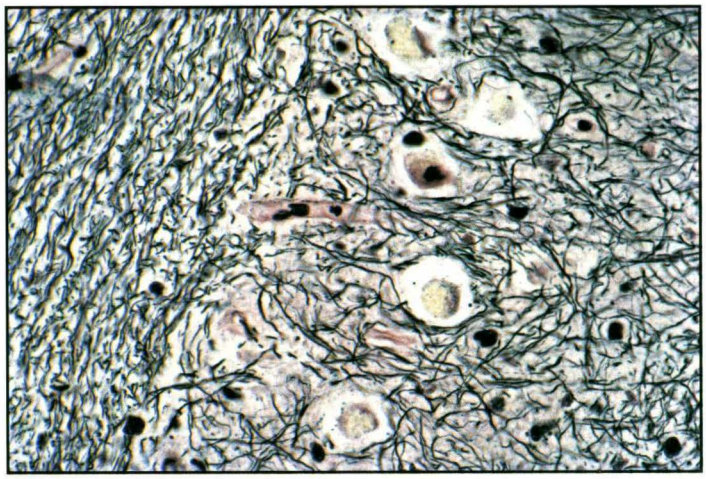
Aniline blue	0.1 g
Oxalic acid	2 g
Phosphomolybdic acid	2 g
Distilled water	300 mL

■ *Procedure*

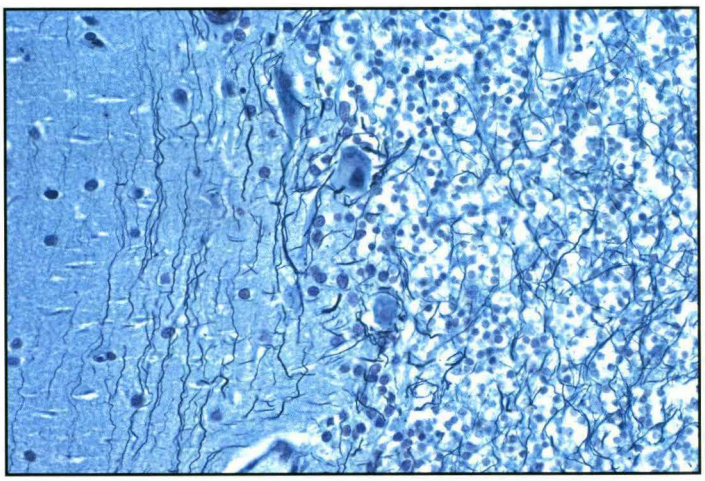
1. Deparaffinize and hydrate sections to distilled water.
2. For every 100 mL of Protargol solution, add 4-6 g of clean copper shot (cleaned with aqua regia and rinsed very well with distilled water). Place slides in this solution and let stand at 37°C for 48 hours.
3. Rinse sections in 3 changes of distilled water.
4. Place slides in the reducing solution for 10 minutes.
5. Rinse in 3 changes of distilled water.
6. Tone sections in gold chloride solution for 10 minutes. This solution may be reused.
7. Rinse in 3 changes of distilled water.
8. Develop in oxalic acid solution, checking with the microscope, until the background is gray and the nerve fibers appear clearly stained (approximately 3-5 minutes). Oxalic acid treatment should not be prolonged because overtreatment will ruin the silver proteinate reaction.
9. Rinse in 3 changes of distilled water.
10. Treat sections with sodium thiosulfate for 5 minutes.
11. Rinse in distilled water.
12. Counterstain if desired, with aniline blue solution (2 or 3 quick dips to give a light blue background). See technical note 4.
13. Dehydrate in 95% and absolute alcohols, 2 changes each.
14. Clear in 2 changes of xylene.
15. Mount with synthetic resin.

■ *Results* **[i9.3], [i9.4]**

- Nerve fibers — Black
- Background — Light gray or blue
- Nuclei — Black



[i9.3] Adjacent gray (right) and white (left) matter stained with the Bodian technique. Nerve fibers are stained black; unstained neuron cell bodies can be seen surrounded by an artifactual space.



[i9.4] A light aniline blue counterstain has been applied to this Bodian-stained section of cerebellum.

■ *Technical Notes*

1. After use, the aqua regia should be gradually poured into a very large volume of water and then discarded in the sink. Do not pour directly into the sink, and do not add the water to the acid. This dilution should be done in a fume hood.
2. It is important that the Protargol be left undisturbed until it is completely dissolved.
3. Chemically clean glassware and nonmetallic forceps should be used, or stain precipitate and a dirty background may be obtained. Glassware may be cleaned with household bleach or a commercial cleaning product.
4. Care must be taken not to overcounterstain with aniline blue, or contrast will be lost **[i9.5]**.
5. Some individuals may have trouble microscopically differentiating blue from black, and a nuclear-fast red counterstain may be used [**Hrapchak 1980**].

NERVE FIBERS AND NEUROFIBRILS: HOLMES SILVER NITRATE METHOD [HOLMES 1943, SHEEHAN 1980]

■ *Purpose*

Demonstration of nerve fibers and neurofibrils in tissue sections

■ *Principle*

Holmes [1943] attributed inconsistent results obtained with the Bodian technique to the fact that the Protargol solution never reaches the alkalinity necessary for optimal impregnation, and he modified the technique by developing a buffered impregnating solution. The pyridine in the solution is an alkali, and Holmes thought that this modified the electrostatic condition of the tissue. This is an argyrophil silver method, requiring that chemical reduction be used. The purpose of gold chloride, oxalic acid, and sodium thiosulfate are identified in the description of the Bodian procedure.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

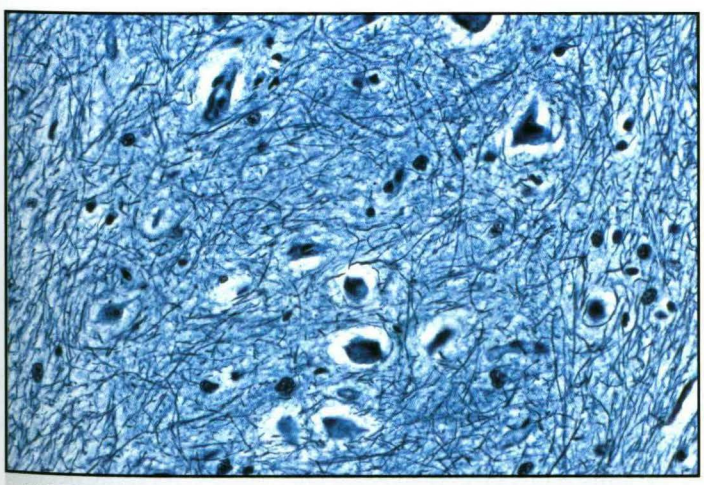
Chemically cleaned Coplin jars, 56°C to 58°C oven, 37°C incubator, graduated cylinders, Erlenmeyer flasks

■ *Technique*

Cut paraffin sections at 10-15 µm.

■ *Quality Control*

Use a section of cerebral cortex. Spinal cord is not a good control for this stain because all axons appear in cross-section. Use chemically cleaned glassware for steps 2 through 7. Nonmetallic forceps also should be used.



[i9.5] This section of cortex has been overstained with aniline blue, so contrast between the background and the black-stained nerve fibers is lost. This stain is not acceptable.

■ *Reagents*

Aqueous Silver Nitrate, 20% Solution

Silver nitrate	20 g
Distilled water	100 mL

Aqueous Silver Nitrate, 1% Solution

Silver nitrate, 20% solution	2.5 mL
Distilled water	47.5 mL

Boric Acid Solution

Boric acid	1.24 g
Distilled water	100 mL

Borax Solution

Sodium borate	1 g
Distilled water	100 mL

Pyridine, 10% Solution

Pyridine	5 mL
Distilled water	45 mL

Impregnating Solution

Boric acid solution (fresh)	27.5 mL
Borax solution (fresh)	22.5 mL
Distilled water	247 mL
Silver nitrate, 1 % aqueous solution	0.5 mL
Pyridine, 10% aqueous solution	2.5 mL

Mix boric acid solution and borax solution in a 500-mL flask. Add the water, then the aqueous silver nitrate, and then the aqueous solution of pyridine. Mix thoroughly. Make enough solution for 20 mL per slide.

Reducing Solution

Hydroquinone	1 g
Sodium sulfite (crystals)	10 g
Distilled water	100 mL
Make fresh for use	

Gold Chloride, 0.2% Solution

Gold chloride, 1% solution	20 mL
Distilled water	80 mL

Oxalic Acid, 2% Solution

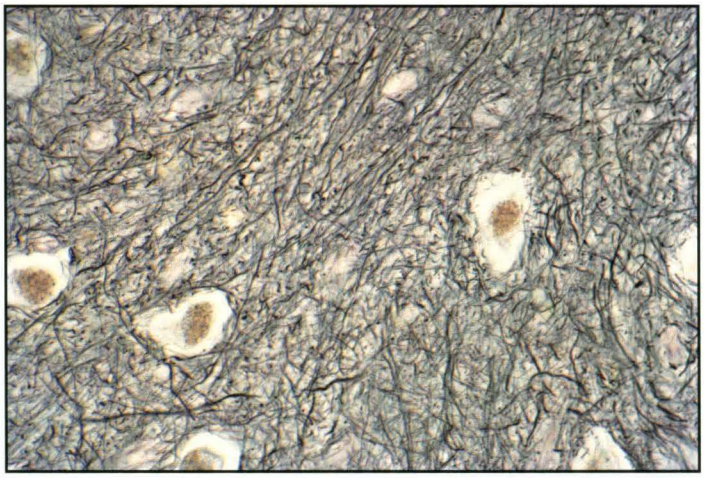
Oxalic acid	2 g
Distilled water	100 mL

Sodium Thiosulfate, 5% Solution

Sodium thiosulfate	5 g
Distilled water	100 mL

■ *Procedure*

1. Deparaffinize sections, and hydrate to distilled water.
2. Place sections in 20% silver nitrate in the dark at room temperature for 1 hour.
3. Prepare the impregnating solution.
4. Take slides from 20% silver nitrate, and wash for 10 minutes in 3 changes of distilled water.
5. Place slides in impregnating solution, allowing at least 20 mL of solution per slide. Cover jar, and incubate overnight at 37°C.
6. Remove slides, shake off superfluous fluid, and place in the reducer for at least 2 minutes.
7. Wash in running water for 3 minutes.
8. Rinse in distilled water.
9. Tone in 0.2% gold chloride for 3 minutes. This solution may be reused until a brown precipitate forms or the solution becomes cloudy.
10. Rinse in distilled water.



[i9.6] Nerve fibers are stained black with the Holmes silver nitrate stain in this section of CNS tissue. Unstained neuron cell bodies again can be seen surrounded by an artifactual space.

11. Place slides in 2% oxalic acid for 3-10 minutes. When the axons are thoroughly blue-black, stop the process.
12. Rinse in distilled water.
13. Place the slides in 5% aqueous sodium thiosulfate.
14. Wash in tap water for 10 minutes. As with the Bodian stain, a counterstain may be applied at this point.
15. Dehydrate in 2 changes each of 95% alcohol and absolute alcohol.
16. Clear in xylene, and mount with synthetic resin.

■ *Results* [i9.6]

• Axons and nerve fibers	Black
• Neurofibrils	Black

■ *Technical Note*

Pyridine is toxic by ingestion, inhalation, and skin absorption. It has an Occupational Safety and Health Administration (OSHA) time-weighted average (TWA) of 5 ppm; it should be used under a chemical fume hood, and suitable gloves and goggles should be used.

NERVE FIBERS, NEUROFIBRILLARY TANGLES, AND SENILE PLAQUES: BIELSCHOWSKY-PAS STAIN [WHITE 1989, MILLSAPS 1989]

■ *Purpose*

Demonstration of nerve fibers and the presence of neurofibrillary tangles and senile plaques in Alzheimer disease. As aging occurs, most individuals develop alteration of neurofibrils in at least some neurons; the neurofibrils may become clumped and twisted. In Alzheimer disease, tremendous numbers of the neurofibrillary tangles develop.

■ *Principle*

The tissue is impregnated with the ammoniacal silver solution, and silver is deposited on neurofibrils and axons. The silver is then reduced to metallic silver by the formaldehyde in the developer. Gold chloride is used to tone the tissue, and this step eliminates the yellow background. Sodium thiosulfate removes any unreduced silver. The Schiff reaction is used to stain both basement membranes and amyloid in the plaques.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

Chemically cleaned Coplin jars, Erlenmeyer flasks, and pipettes

■ *Technique*

Cut paraffin sections at 8-10 µm.

■ *Quality Control*

Tissue from the CNS must be used. If possible, the tissue should contain senile plaques and neurofibrillary tangles.

■ *Reagents*

Aqueous Silver Nitrate, 20% Solution

Silver nitrate	20 g
Distilled water	100 mL

Ammoniacal Silver Solution

Place 50 mL of 20% aqueous silver nitrate in an Erlenmeyer flask. With constant swirling, add concentrated ammonium hydroxide, drop by drop, until a precipitate is formed and then clears. Do not add excess ammonium hydroxide at this point. When the solution has cleared, add 2 mL of ammonium hydroxide and filter the solution.

Developer

Formaldehyde, 37% to 40%	20 mL
Distilled water	100 mL
Nitric acid, concentrated	1 drop
Citric acid	0.5 g
This solution is stable and can be stored at room temperature	

Gold Chloride, 0.5% Solution

Gold chloride, 1% solution	25 mL
Distilled water	25 mL

Sodium Thiosulfate, 5% Solution

Sodium thiosulfate	5 g
Distilled water	100 mL

Periodic Acid, 1% Solution

Periodic acid	1 g
Distilled water	100 mL

Schiff Reagent

See the PAS Procedure in chapter 7, p137.

■ *Procedure*

1. Prepare the ammoniacal silver solution before beginning the procedure.
2. Deparaffinize slides as usual to distilled water.
3. Place slides in 20% silver nitrate solution in the dark at room temperature for 20 minutes.
4. Remove the slides from the silver nitrate and wash once with distilled water.
5. Place slides in ammoniacal silver solution at room temperature for 20 minutes.
6. Wash slides in ammonia water (4 drops concentrated ammonium hydroxide to 100 mL distilled water).
7. While the slides are in the ammonia water, add 2 drops of developer to the ammoniacal silver solution used in step 5 and mix well.
8. Place slides in the mixed developer-ammoniacal silver solution. The tissue should turn brown (average time 3 minutes).
9. Wash well in ammonia water, then in distilled water.
10. Tone in gold chloride until the first gray appears, approximately 30 seconds.

11. Wash in ammonia water, then rinse in distilled water for 1 minute.

12. Place sections in 5% sodium thiosulfate (hypo) for 30 seconds.

13. Wash slides in running tap water for 5 minutes.

14. Rinse sections well in distilled water.

15. Place sections in 1% periodic acid solution for 5 minutes.

16. Rinse slides in 2 changes of distilled water.

17. Place sections in Schiff reagent for 5 minutes.

18. Wash slides in tap water for 5 minutes

19. Dehydrate with 2 changes of 95% alcohol and 2 or 3 changes of absolute alcohol.

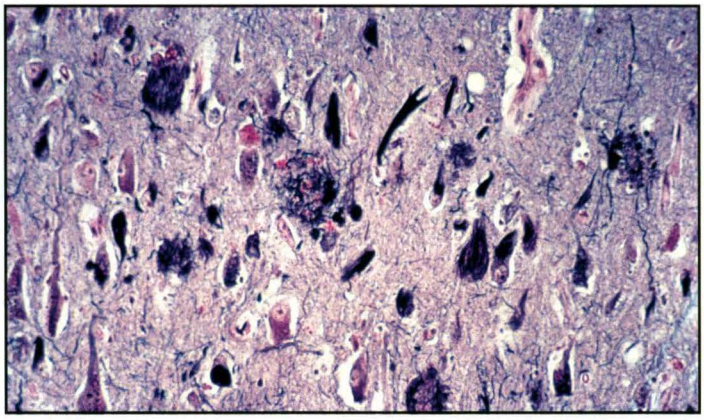
20. Clear with 2 or 3 changes of xylene and mount with a synthetic resin.

■ *Results* [i9.7], [i9.8]

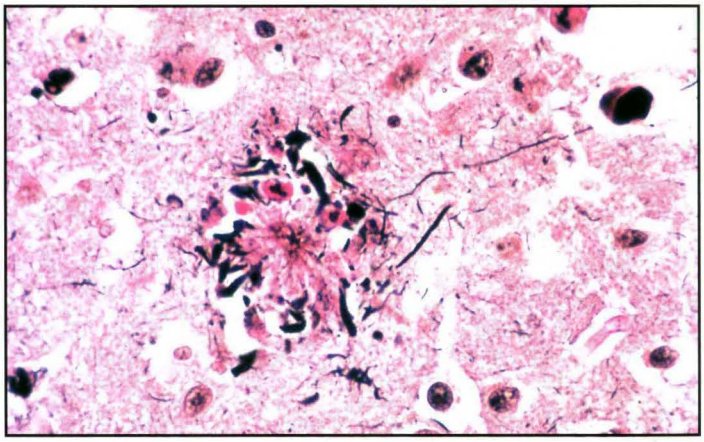
• Neurofibrillary tangles	Dark black
• Peripheral neurites of neuritic plaques	Dark black
• Axons	Black
• Amyloid (plaque cores and vascular)	Magenta
• Lipofuscin	Magenta

■ *Technical Note*

A modified Bielschowsky stain that uses much less silver was described in the *Journal of Histotechnology*. I have no experience with this technique, but the interested reader is referred to Garvey [1999].



[i9.7] A section of cortex from a patient with Alzheimer disease stained with the modified Bielschowsky-periodic acid-Schiff (PAS) technique. Numerous senile plaques and a few neurofibrillary tangles can be seen. A "classic" senile plaque is an abnormal spherical structure composed of an amyloid core (highlighted by the PAS) surrounded by dystrophic neurites (highlighted by the silver). Neurofibrillary tangles are accumulations of abnormal, fibrillary material that fill the perikaryon (cytoplasm surrounding the nucleus) of the neurons. [Image courtesy of Bigio EH, Millsaps R, University of Texas Southwestern Medical School]



[i9.8] High-power view of a section from the cortex of a patient with Alzheimer disease demonstrating a "classic" senile plaque stained with the modified Bielschowsky-PAS technique. [Image courtesy of Bigio EH, Millsaps R, University of Texas Southwestern Medical School]

NERVE FIBERS, NEUROFIBRILLARY TANGLES, AND SENILE PLAQUES: MICROWAVE MODIFICATION OF BIELSCHOWSKY METHOD [CHURUKIAN 1993]

■ *Purpose*

Demonstration of nerve fibers and the presence of neurofibrillary tangles and senile plaques in Alzheimer disease. As aging occurs, most individuals develop alteration of neurofibrils in at least some neurons; the neurofibrils may become clumped and twisted. In Alzheimer disease, tremendous numbers of the neurofibrillary tangles develop.

■ *Principle*

The tissue is impregnated with the ammoniacal silver solution. The silver deposited on neurofibrils and axons is then reduced to metallic silver by the formaldehyde in the developer. Because the sections are not toned with gold chloride in this procedure, the yellow background remains. Sodium thiosulfate removes any unreduced silver.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

Microwave oven, Coplin jars, Erlenmeyer flasks, and pipettes

■ *Technique*

Cut paraffin sections at 8 μm.

■ *Quality Control*

Tissue specimens from the CNS must be used. If possible, the tissue specimen should contain senile plaques and neurofibrillary tangles.

■ *Reagents*

Silver Nitrate, 1% Solution

Silver nitrate	0.4 g
Distilled water	40 mL
Prepare fresh	

Silver Nitrate, 5% Solution

Silver nitrate	0.5 g
Distilled water	10 mL
Store in the refrigerator	

Nitric Acid, 10% Solution

Nitric acid, concentrated	1 mL
Distilled water	9 mL
Prepare fresh, and be sure to add the acid to the water	

Developer Solution

Formaldehyde, 37% to 40%	0.4 mL
Distilled water	4 mL
Citric acid	0.2 g
10% nitric acid	0.1 mL
Prepare fresh	

Ammonium Hydroxide, 1% Solution

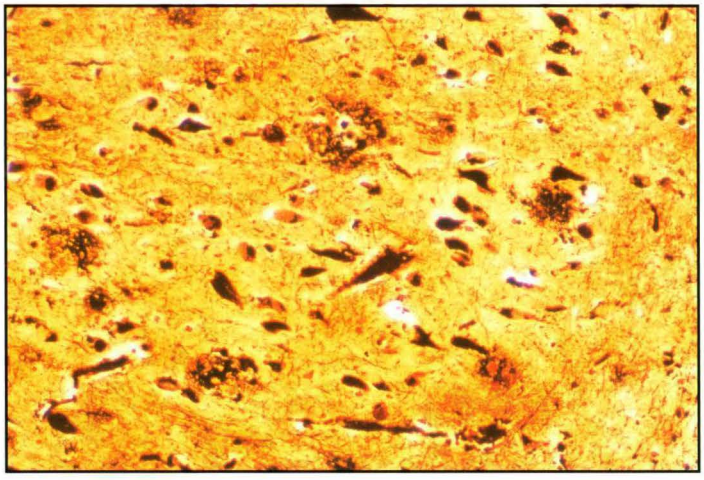
Ammonium hydroxide, concentrated	1 mL
Distilled water	99 mL

Sodium Thiosulfate, 2% Solution

Sodium thiosulfate	2 g
Distilled water	100 mL

■ *Procedure*

1. Deparaffinize the slides, and hydrate to distilled water.
2. Place slides in 40 mL of 1% silver nitrate solution in a plastic Coplin jar, and microwave at power level 3 (180 W) for 1 minute. Dip the slides up and down several times, and allow them to remain in the warm solution (50°C) for 15 minutes.
3. Place the slides in distilled water.
4. Pour the warm 1% silver nitrate used in step 2 into a 125-mL flask. Add 28% ammonium hydroxide (concentrated) drop by drop with constant shaking, until the initial precipitate disappears and the solution turns clear. Then add 5% silver nitrate drop by drop with constant shaking, until the solution becomes slightly cloudy.
5. Pour the ammoniacal silver solution prepared in step 4 into a plastic Coplin jar. Place slides in this solution and microwave at power level 3 (180 W) for 1 minute. Dip the slides up and down several times, and allow them to remain in the warm solution (60°C) for 15 minutes.
6. Place slides in 1% ammonium hydroxide solution for no longer than 20 seconds.
7. Add 3 drops of developer to the ammoniacal silver solution used in step 5. Quickly mix with a glass rod, and ***immediately*** place the slides in the solution for about 3 minutes or until the tissue sections turn brown. The solution will turn a grayish color, and a mirror of silver will form on the sides of the Coplin jar and sometimes on the slides, but not on the tissue sections.
8. Place slides in 1% ammonium hydroxide solution for no longer than 15 seconds.
9. Rinse in 3 changes of distilled water.
10. Wipe off the mirror of silver from both sides of the slides, taking care not to damage the tissue sections.
11. Place slides in 2% sodium thiosulfate solution for 30 seconds.
12. Rinse slides in 4 changes of distilled water.
13. Dehydrate in graded alcohols.
14. Clear in 3 or 4 changes of xylene, and mount with synthetic resin.



[i9.9] A section of cortex from a patient with Alzheimer disease stained with the Bielschowsky microwave procedure. Several senile plaques can be seen in the section. [Image courtesy Churukian CJ, University of Rochester Medical Center]

■ *Results* [i9.9]

• Axons	Brown to black
• Cytoplasmic neurofibrils	Brown to black
• Neurofibrillary tangles and plaques of Alzheimer disease	Dark brown or black
• Neuromelanin	Black
• Lipofuscin	Brown or black

■ *Technical Notes*

1. It is essential to use chemically cleaned glassware rinsed in double distilled water.

2. This modification of the Bielschowsky method requires much less silver nitrate and, according to Churukian [1993], stains the neurofibrillary tangles and plaques of Alzheimer disease better than the original method.

NERVE FIBERS, NEUROFIBRILLARY TANGLES, AND SENILE PLAQUES: THE SEVIER-MUNGER MODIFICATION OF BIELSCHOWSKY METHOD [SEVIER 1965, SHEEHAN 1980]

■ *Purpose*

Demonstration of nerve fibers and the presence of neurofibrillary tangles and senile plaques in Alzheimer disease. As aging occurs, most individuals develop alteration of neurofibrils in at least some neurons; the neurofibrils may become clumped and twisted. In Alzheimer disease tremendous numbers of the neurofibrillary tangles develop.

■ *Principle*

The tissue is impregnated with the ammoniacal silver solution. The silver deposited on neurofibrils and axons is then reduced to metallic silver by formaldehyde. Because the sections are not toned with gold chloride in this procedure, the yellow background remains. Sodium thiosulfate removes any unreduced silver.

■ *Equipment*

Coplin jars, Whatman #1 filter paper, Erlenmeyer flasks, and pipettes

■ *Technique*

Cut paraffin sections at 6-8 µm.

■ *Quality Control*

Tissue specimen from the CNS must be used.

■ *Reagents*

Silver Nitrate, 20% Solution

Silver nitrate	10 g
Distilled water	50 mL

Silver Nitrate, 10% Solution

Silver nitrate	10 g
Distilled water	100 mL

Formalin Solution

Formaldehyde, 37% to 40%	2 mL
Distilled water	98 mL

Sodium Carbonate Solution

Sodium carbonate	8 g
Distilled water	30 mL

Ammoniacal Silver Solution

To 50 mL of 10% silver nitrate, add concentrated ammonium hydroxide drop by drop, until the dark brown precipitate that forms has almost disappeared. Shake vigorously between drops, and avoid complete decolorization. The end point is a slightly cloudy solution. At this point, add 0.5 mL of sodium carbonate solution, and shake well. Add 25 drops of ammonium hydroxide and shake well. The solution should now be crystal clear. Filter into a 125-mL Erlenmeyer flask, and cover.

Sodium Thiosulfate, 5% Solution

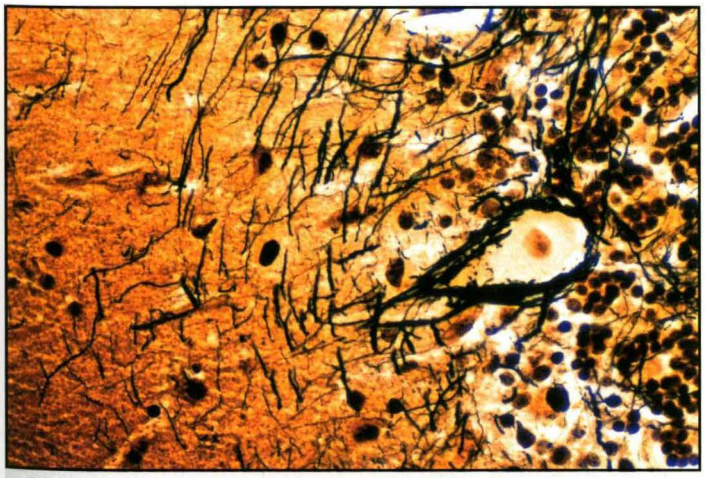
Sodium thiosulfate	5 g
Distilled water	100 mL

■ *Procedure*

1. Deparaffinize the slides, and hydrate to distilled water.
2. Preheat the 20% silver nitrate to 60°C for 15 minutes. Add the slides to the warm silver solution, and let them remain in the oven for 15 minutes.
3. Rinse 1 slide at a time in distilled water, and place in a clean, dry staining jar.
4. While shaking gently, add 10 drops of the formalin solution to the working ammoniacal silver solution. Quickly pour this solution over the slides and let develop for 5-30 minutes until golden brown. Check microscopically for completeness of the reaction. ***Do not wash while checking***. Keep in motion during development to avoid precipitation.
5. Rinse slides well in 3 changes of fresh tap water.
6. Place in sodium thiosulfate solution for 2 minutes.
7. Wash well in tap water.
8. Dehydrate, clear, and mount with synthetic resin.

■ *Results* **[i9.10]**

- Nerve endings and neurofibrils — Black
- Neurofibrillary tangles and peripheral neurites of neuritic plaques — Black



[i9.10] A section of cerebellum stained with the Sevier-Munger technique. The dendritic processes of the basket cells can be seen surrounding a Purkinje cell. Other nerve fibers also can be seen in the section.

■ *Technical Notes*

1. This is a very reliable and reproducible technique.
2. The concentration of ammonium hydroxide and formalin and their relative proportions are critical to controlled development of the stain.
3. It is very important that a few grains of silver be left in the flask after the first addition of ammonium hydroxide to the ammoniacal silver solution. Excess ammonia must not be added.
4. This is an argyrophil stain that is also useful for demonstrating the granules of some carcinoid tumor cells.

NEUROFIBRILLARY TANGLES AND SENILE PLAQUES: THIOFLAVIN S (MODIFIED) [GUNTERN 1989, GUNTERN 1992, VALLET 1992]

■ *Purpose*

Demonstration of the presence of neurofibrillary degeneration (neurofibrillary tangles, senile plaques, neuropil threads) and vascular and parenchymal amyloid deposition in Alzheimer disease.

■ *Principle*

Thioflavin dyes are fluorescent dyes that are useful in the visualization of amyloid deposits in tissues. This modification includes pretreatment of tissue sections with potassium permanganate and bleaching with potassium metabisulfite and oxalic acid, followed by treatment with sodium hydroxide and hydrogen peroxide. The $KMnO_4$ and NaOH totally remove lipid autofluorescence, resulting in improved definition of pathological lesions. Neurofibrillary tangles, senile plaque neurites, and neuropil threads are better visualized than with the routine thioflavin S, and it is not affected by prolonged fixation. This modified technique has proven more sensitive than silver methods (eg, Bielschowsky) for detecting Alzheimer neurofibrillary tangles and senile plaques; it is also faster and cheaper to perform, and allows the simultaneous demonstration of cerebrovascular amyloid on the same slide.

■ *Fixative*

10% to 20% neutral-buffered formalin

■ *Equipment*

Coplin jars, Leica staining buckets and rack, Erlenmeyer flasks, pipettes, Fisher Superfrost Plus slides, tissue flotation bath, and 58°C to 60°C oven

■ *Technique*

Cut paraffin section at 6 µm; air-dry overnight, then dry in a 58°C to 60°C oven for 10 minutes; cool.

■ *Quality Control*

Central nervous system tissue containing senile plaques and neurofibrillary tangles (eg, Alzheimer diseased brain).

■ *Reagents*

Potassium Permanganate, 0.25% Solution

Potassium permanganate	1 g
Distilled water	400 mL

Potassium Metabisulfite-Oxalic Acid, 1% Solution

Potassium metabisulfite	4 g
Oxalic acid	4 g
Distilled water	400 mL

Sodium Hydroxide-Hydrogen Peroxide Solution

Sodium hydroxide	8 g
Distilled water	400 mL
Just before application, add	
Hydrogen peroxide, 30%	12 mL

Acetic Acid, 0.25% Solution

Acetic acid, glacial	1 mL
Distilled water	400 mL

Thioflavin S Solution in 50% Alcohol

Thioflavin S	0.48 g
50% alcohol	400 mL

■ *Procedure*

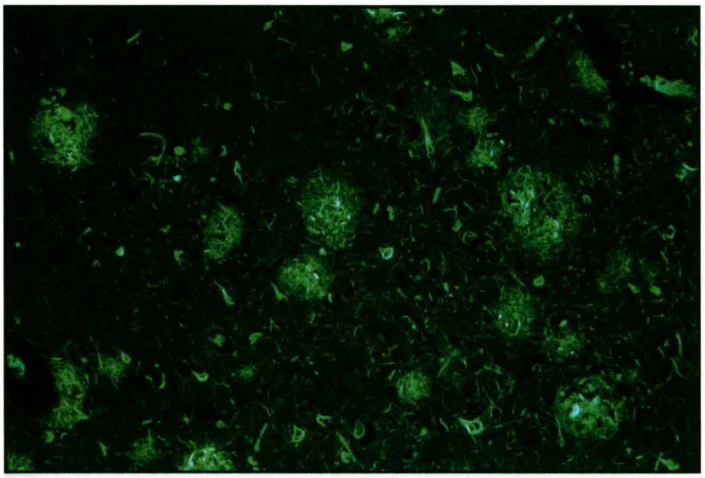
1. Deparaffinize and hydrate slides to distilled water.
2. Rinse and hold in distilled water for a minimum of 5 minutes.
3. Cover tissue slides with 0.25% potassium permanganate for 20 minutes.
4. Wash slides in running tap water for 5 minutes.
5. Treat slides with 1% potassium metabisulfite-oxalic acid solution for 2 minutes. Agitate slides during this step.
6. Wash slides in running tap water for 5 minutes.
7. Place slides in sodium hydroxide-peroxide for 20 minutes. (add the 30% hydrogen peroxide to the solution just prior to this step).
8. Wash in running tap water for 3 changes, and then do a final rinse with Millipore filtered water.
9. Place slides in 0.25% acetic acid for 1 minute.
10. Wash slides in running tap water for 5 minutes.
11. Place slides into 50% alcohol, 2 changes for 2 minutes each.
12. Place slides in 0.0125% thioflavin-S for 7 minutes (Leica staining bucket, placed on platform shaker).
13. Rinse slides with 2 changes of 50% alcohol for 2 minutes each *with agitation*.
14. Rinse slides in 2 changes of 95% alcohol for 2 minutes each.
15. Completely dehydrate with 2 changes of absolute alcohol, and clear in 3 changes of xylene. Mount with nonfluorescent mounting medium.
16. View slides on a fluorescent microscope with a fluorescence filter set that incorporates a blue-violet excitation filter (eg, excitation range 400-440 nm and a long pass barrier filter [eg, 470 nm]).

■ *Results* **[i9.11], [i9.12]**

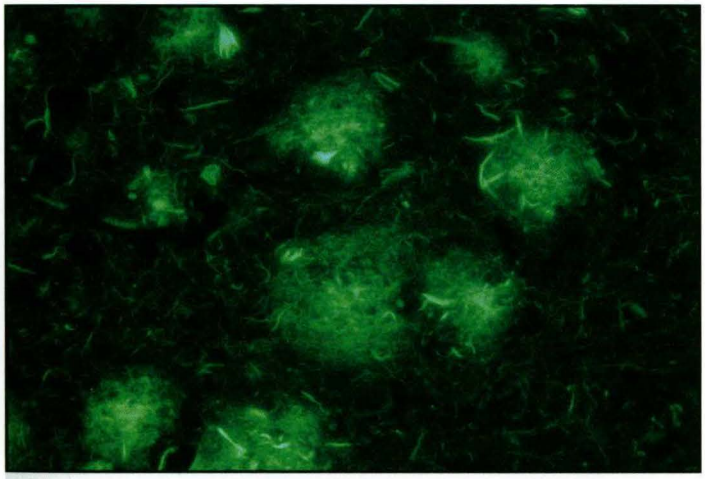
- Alzheimer neurofibrillary tangles, senile plaque neurites, neuropil threads, senile plaque amyloid, and cerebrovascular amyloid — Bright green
- Diffuse plaques and extracellular tangles — Paler yellow green
- PSP tangles and Pick bodies — Not well demonstrated

■ *Technical Notes*

1. Float tissue sections on a preheated water bath filled with distilled water. *Do not* add an adhesive compound to the water bath. The water bath should be chemically cleaned if contamination is suspected.
2. Mount sections carefully the first time, because tissue bonding begins quickly on the Superfrost Plus slides.



[i9.11] An Alzheimer brain stained with thioflavin S, demonstrating senile plaques, neurofibrillary tangles, and amyloid.



[i9.12] Senile plaques, neurofibrillary tangles, and amyloid are seen at a higher magnification of the same section as that seen in **[i9.11]** stained with thioflavin S.

3. Dry the slides completely at room temperature by draining them vertically before heating in an oven.
4. Mount sections with Cytoseal 60
5. Staining is stable for at least several months at room temperature.

GLIAL FIBERS: MALLORY PHOSPHOTUNGSTIC ACID HEMATOXYLIN (PTAH) STAIN [LUNA 1960, SHEEHAN 1980]

■ *Purpose*
Demonstration of glial fibers

■ *Principle*
The amount of phosphotungstic acid in the staining solution is far greater than the amount of hematein (20:1), and it is believed that the tungsten binds all available hematein to give a blue lake. This lake provides the blue color to selected tissue components (glial fibers, nuclei, and to a certain extent, myelin). The red-brown- or salmon-colored components (neurons) are believed to be stained by the phosphotungstic acid. Components will lose their red-brown color after water or prolonged alcohol washing, so the dehydration steps that follow staining should be rapid.

■ *Fixative*
10% neutral-buffered formalin

■ *Equipment*
Coplin jars, graduated cylinders, Erlenmeyer flasks

■ *Technique*
Cut paraffin sections at 6-8 μm.

■ *Quality Control*
Use a section of cerebral cortex (not spinal cord) for the demonstration of glial fibers.

■ *Reagents*

PTAH Solution

Hematoxylin	1 g
Phosphotungstic acid	20 g
Distilled water	1,000 mL

Dissolve the solid ingredients in separate portions of water, dissolving the hematoxylin with the aid of heat. When cool, combine the solutions. No preservative is necessary. The solution that is allowed to ripen naturally is a better stain and lasts longer, but if time is not available for natural ripening, 0.2 g of potassium permanganate may be added. The chemically ripened stain may be used immediately, but better results will be obtained if the solution is allowed to age for at least 2 weeks.

Lugol Iodine

Iodine	10 g
Potassium iodide	20 g
Distilled water	1,000 mL

Place the potassium iodide in about 150 mL of the water in a flask, and stir until dissolved. Dissolve the iodine in this concentrated solution of potassium iodide. When the iodine is dissolved, add the remaining water, and mix well.

Potassium Permanganate, 1% Solution

Potassium permanganate	1 g
Distilled water	100 mL

Oxalic Acid, 5% Solution

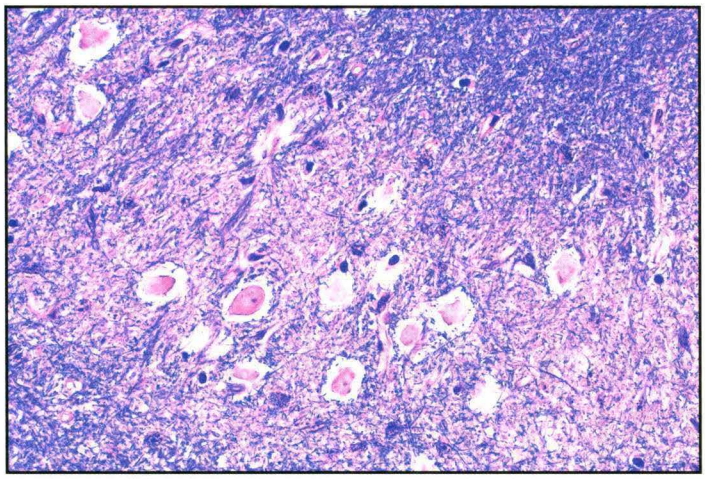
Oxalic acid	5 g
Distilled water	100 mL

■ *Procedure*

1. Deparaffinize the sections, and hydrate to distilled water.
2. Mordant the sections overnight at room temperature in Zenker solution containing acetic acid.
3. Wash the sections in running water for 15 minutes.
4. Place in Lugol iodine for 15 minutes. Do not take the slides through sodium thiosulfate (hypo), because this may impair the subsequent staining reaction.
5. Decolorize the sections in 95% alcohol for at least 1 hour.
6. Rinse rapidly in 3 changes of distilled water.
7. Place sections in 1% potassium permanganate for 5 minutes.
8. Wash in running tap water for 10 minutes.
9. Decolorize the sections in 5% oxalic acid for 5 minutes.
10. Wash in running tap water for 10 minutes.
11. Stain in PTAH solution overnight at room temperature.
12. Dehydrate rapidly through 2 changes each of 95% and absolute alcohol, clear in xylene, and mount with synthetic resin.

■ *Results* [i9.13]

• Glial fibers	Blue
• Nuclei	Blue
• Neurons	Salmon
• Myelin	Blue



[i9.13] The phosphotungstic acid-hematoxylin (PTAH) technique stains glial fibers blue to purple and neuron cell bodies salmon. Myelin is also stained blue to purple. The lack of intensity of blue staining of glial fibers and the fact that myelin also stains both make this a difficult stain to interpret. The Holzer technique is preferred.

■ *Technical Note*

Although this stain can be used for glial fibers, the Holzer stain is a better method; both stains have been replaced to a great degree by immunohistochemical methods.

GLIAL FIBERS: HOLZER METHOD [MCMANUS 1960; SHEEHAN 1980]

■ *Purpose*

Demonstration of glial fibers and areas of gliosis

■ *Principle*

Glial fibers are stained with crystal violet and are resistant to decolorization with the alkaline aniline-chloroform mixture.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

Coplin jars, staining rack, blotting pad, graduated cylinders, Erlenmeyer flasks

■ *Technique*

Cut paraffin sections at 6-8 μm.

■ *Quality Control*

Use a section of cerebral cortex (not spinal cord) for the demonstration of glial fibers.

■ *Reagents*

Aqueous Phosphomolybdic Acid, 0.5% Solution

Phosphomolybdic acid	0.25 g
Distilled water	50 mL
Prepare fresh	

Phosphomolybdic Acid-Alcohol Solution

Phosphomolybdic acid, 0.5% aqueous solution	10 mL
Alcohol, 95%	20 mL

Absolute Alcohol-Chloroform Mixture

Absolute alcohol	5 mL
Chloroform	20 mL
Prepare fresh	

Potassium Bromide, 10% Solution

Potassium bromide	10 g
Distilled water	100 mL

Crystal Violet Stain

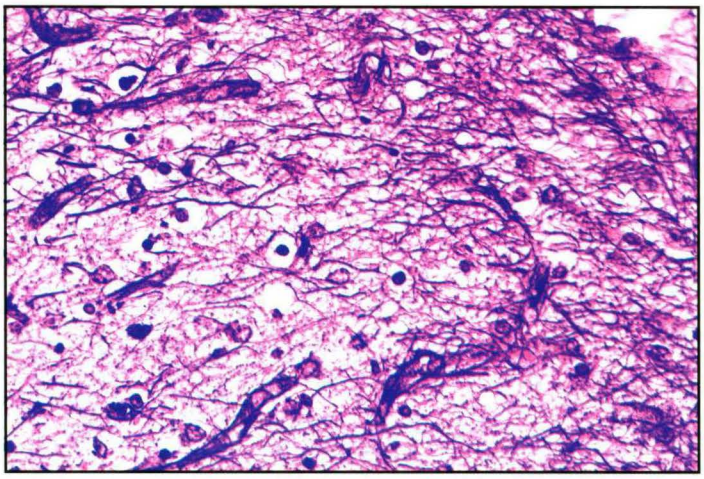
Crystal violet	1.25 g
Absolute alcohol	5 mL
Chloroform	20 mL
Prepare fresh	

Differentiating Solution

Aniline oil	30 mL
Chloroform	45 mL
Ammonium hydroxide, concentrated	5 drops

■ *Procedure*

1. Deparaffinize the sections, and hydrate to distilled water.
2. Place the sections in fresh phosphomolybdic acid-alcohol for 3 minutes.



[i9.14] Glial fibers are well demonstrated with the Holzer technique.

3. Drain off the excess fluid, place slides on a staining rack, and cover the sections with absolute alcohol-chloroform mixture. The tissue should become translucent.
4. While sections are still wet, cover them with the crystal violet stain and allow to remain for 30 seconds.
5. Replace the stain with 10% potassium bromide, washing for 1 minute with this solution.
6. Blot the sections dry, and then allow them to air-dry thoroughly.
7. Differentiate slides individually in the differentiating solution for 30 seconds.
8. Wash in several changes of xylene. Steps 7 and 8 may have to be repeated several times until the background is very pale blue or colorless.
9. Mount in synthetic resin.

■ *Results* **[i9.14]**

- Glial fibers — Blue
- Background — Very pale blue to colorless

■ *Technical Notes*

1. Crystal violet precipitate may be removed with straight aniline oil.
2. Aniline oil has a permissible exposure limit of 5 ppm. It is a sensitizer, is toxic by skin absorption, and rated by the National Institute for Occupational Safety and Health (NIOSH) to be neoplastic. Chloroform has an OSHA ceiling limit of 50 ppm, is toxic by ingestion and inhalation, is a mild skin irritant, and is rated by NIOSH as a carcinogen. Extreme caution must be used in handling these materials, and the appropriate protective measures, including using in a chemical fume hood, are advisable.

ASTROCYTES: CAJAL STAIN [MCMANUS 1960]

■ *Purpose*

Demonstration of astrocytes. This method has been replaced to a great extent by immunohistochemical procedures.

■ *Principle*

Astrocytes are selectively stained with the Cajal gold sublimate method on frozen sections.

■ *Fixative*

Formalin ammonium bromide for no less than 2 days and no more than 25 days. If the tissue has been fixed originally in 10% neutral-buffered formalin, wash and place in formalin ammonium bromide for 48 hours before proceeding with the technique.

■ *Equipment*

Cryostat, staining dishes, blotting paper, graduated cylinders, Erlenmeyer flasks

■ *Technique*

Cut frozen sections at 20-30 µm. Do not pick up on slides; the sections should be free-floating for this technique. Tissue will section better if washed in tap water for 30 minutes before freezing.

■ *Quality Control*

Use a section of cerebral cortex (not spinal cord) for the demonstration of astrocytes.

■ *Reagents*

Formalin Ammonium Bromide

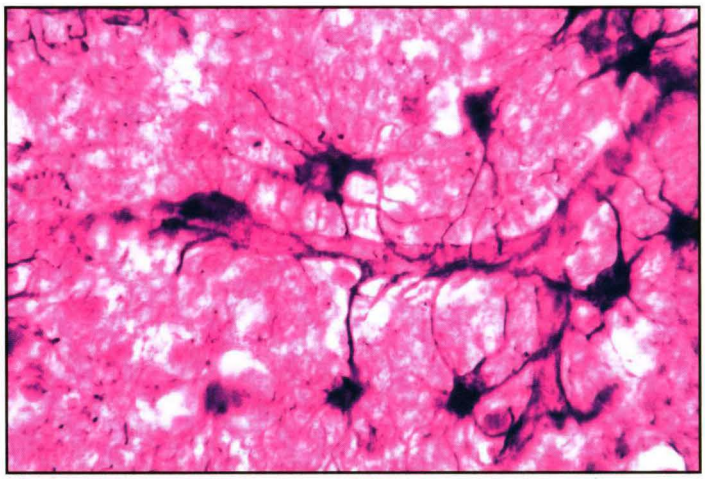
Ammonium bromide	4 g
Formaldehyde, 37% to 40%	30 mL
Distilled water	170 mL

Gold Sublimate

Gold chloride, 1% solution	5 mL
Mercuric chloride, 1% solution	25 mL
Distilled water	5 mL

Sodium Thiosulfate, 5% Solution

Sodium thiosulfate	5 g
Distilled water	100 mL



[i9.15] Astrocytes and their processes are seen in this section stained with the Cajal technique. Perivascular feet (terminal expansions of astrocytic processes) are seen on the basement membrane of a capillary.

■ *Procedure*

1. Wash the free-floating frozen sections in several changes of distilled water.
2. Transfer the sections to the gold sublimate solution, and leave in the dark for 4 hours. The sections should be purple.
3. Wash well in several changes of distilled water.
4. Treat with 5% sodium thiosulfate for 2 minutes.
5. Wash sections well in several changes of distilled water.
6. Carefully mount the sections on slides, blot with bibulous paper, and dehydrate in 95% and absolute alcohols.
7. Clear in xylene, and mount in synthetic resin.

■ *Results* [i9.15]

- Astrocytes with perivascular feet — Black

■ *Technical Notes*

1. The chemicals used should be of the utmost purity, and brown gold chloride is preferred over yellow gold chloride.
2. Protoplasmic astrocytes lose stainability after prolonged fixation.
3. The temperature of the staining solution should not exceed 30°C.
4. The mixture of the 2 chlorides, mercury and gold, is essential; either chloride used alone is not effective for demonstration of the astrocytes.

5. The sections must be flat and not overlap in the gold sublimate solution.

6. Mercuric chloride is extremely toxic and a severe environmental hazard; contact with skin can cause irritation and dermatitis. Skin absorption is possible with systemic poisoning resulting. Extreme care must be used when handling this chemical; alternate methods that do not use mercuric salts should be substituted where possible.

MYELIN SHEATH: WEIL METHOD [WEIL 1928]

■ *Purpose*

Demonstration of myelin in tissue. When an axon degenerates, the myelin sheath breaks down into simpler lipids; these simple lipids will be removed eventually. If Wallerian degeneration occurs in a "tract," or collections of large numbers of axons related to the same function, then demyelination of the tract can be demonstrated. Examples of this type of demyelination occur in syphilis and amyotrophic lateral sclerosis.

■ *Principle*

The mordant-hematoxylin solution attaches to the phospholipid component of the myelin sheath, which has an affinity for the cationic dye lake. This is a regressive staining technique with differentiation usually accomplished in 2 steps. The first differentiation is accomplished macroscopically with ferric ammonium sulfate (excess mordant differentiation), which removes most of the excess dye. The second differentiation is done microscopically with borax ferricyanide (oxidizer differentiation), which removes any remaining nonspecifically bound hematoxylin lake and forms a colorless oxidation product. Only the myelin sheath and red blood cells are left stained.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

54°C to 56°C oven, Coplin jars, graduated cylinders, Erlenmeyer flasks

■ *Technique*

Cut paraffin sections at 10-15 μm.

■ *Quality Control*

A section of spinal cord or medulla

■ *Reagents*

Ferric Ammonium Sulfate, 4% Solution

Ferric ammonium sulfate	4 g
Distilled water	100 mL

Alcoholic Hematoxylin, 10% Solution

Hematoxylin powder	10 g
Absolute alcohol	100 mL

This solution should be allowed to stand for at least 2 or 3 days, but prolonged ripening is unnecessary because the iron used in the staining solution is a strong oxidizer.

Staining Solution

Hematoxylin, 10% alcohol solution	2.5 mL
Distilled water	22.5 mL
Mix in an Erlenmeyer flask and add:	
Ferric ammonium sulfate, 4% solution	25 mL
Prepare fresh	

Differentiating Solution

Sodium borate	1 g*
Potassium ferricyanide	1.25 g
Distilled water	100 mL

■ *Procedure*

1. Deparaffinize sections, and hydrate to distilled water.

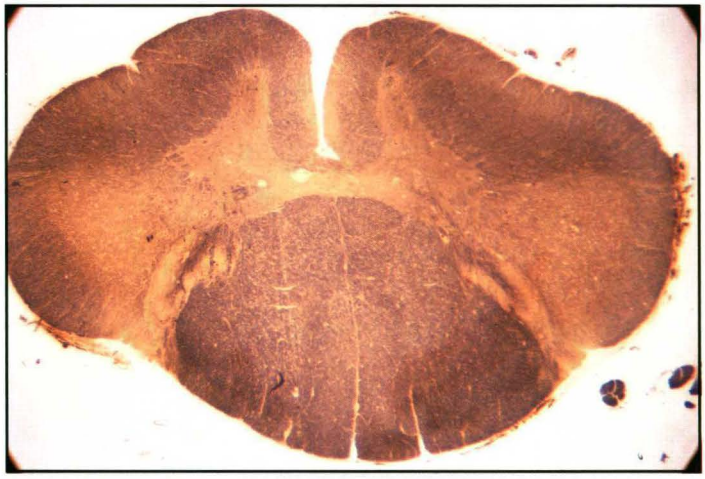
2. Transfer sections to the staining solution, and stain for 30 minutes at 54°C to 56°C.

3. Wash in 2 changes of tap water.

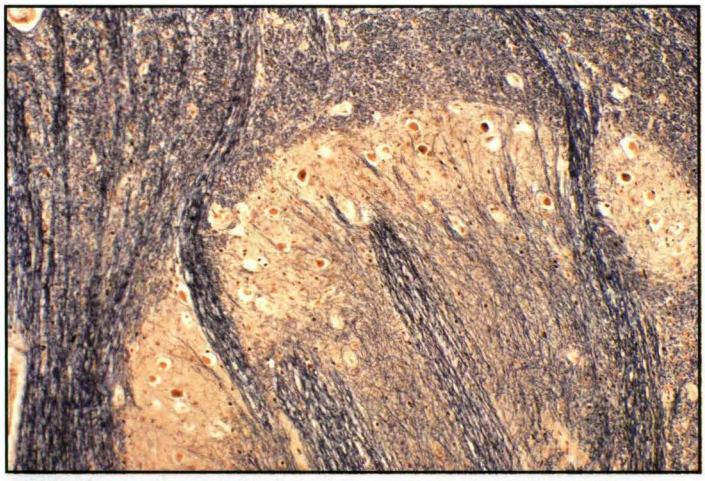
4. Differentiate in 4% ferric ammonium sulfate until the gray matter can just be distinguished from the white matter and the stain is removed from the slides.

5. Wash in 3 changes of tap water.

6. Complete differentiation of the sections in sodium borate-potassium ferricyanide solution. This differentiation should be controlled microscopically until the gray and white matter are sharply defined.



[i9.16] This cross-section of spinal cord has been stained with the Weil myelin stain. Good myelin stains will show macroscopic (naked eye) differentiation of the gray and white matter, as demonstrated in this section. One can tell macroscopically that the corticospinal tract has been demyelinated. This section is slightly underdifferentiated. (Reprinted with permission from Carson [1987])



[i9.17] Gray (lower right) and white (upper left) matter are sharply defined in this section of medulla stained with the Weil myelin stain. The myelin sheaths are stained blue-black and the background of the olivary nucleus (gray matter) is light brown.

7. Wash sections in 2 changes of tap water.

8. Treat sections with diluted ammonia water (about 6 drops to 100 mL of water).

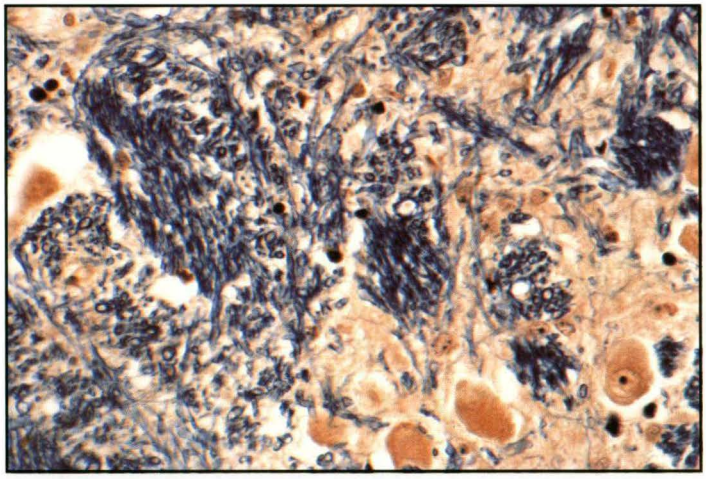
9. Wash in distilled water.

10. Dehydrate in 2 changes each of 95% and absolute alcohols.

11. Clear in xylene, and mount with synthetic resin.

■ *Results* **[i9.16], [i9.17], [i9.18]**

• Myelin sheath	Blue to blue-black
• Background	Light tan



[i9.18] A higher magnification of the section shown in **[i9.17]**; myelin is stained with the Weil method. Note the contrast between the myelin and the background. Also note that the neurons in the lower right corner are well decolorized.

■ *Technical Notes*

1. Gray matter and demyelinated white matter should be light brown and contrast sharply with the blue to blue-black myelinated white matter.

2. The quality of the myelin stain can be determined macroscopically (with the naked eye), with both gray and white matter easily distinguished. On a good myelin stain, the areas of demyelination frequently are more easily identified macroscopically than microscopically.

3. Weil [1928] allowed the hematoxylin solution to ripen for 6 months before use, but this is unnecessary because the solution is oxidized adequately by the ferric ammonium sulfate. Reed [1985] published a method for using fresh 1% hematoxylin prepared in absolute alcohol, but I prefer the method outlined in this chapter.

4. If the procedure is not used very often, then dissolve 2.5 g of hematoxylin in 25 mL of absolute alcohol. Any unused solution can be measured and diluted with 9 volumes of 95% alcohol to give a 1% solution for use in Weigert hematoxylin (used in Mayer mucicarmine and Masson trichrome stains).

MYELIN SHEATH: LUXOL FAST BLUE METHOD [KLÜVER 1953, SHEEHAN 1980]

■ *Purpose*

Demonstration of myelin in tissue sections. When an axon degenerates, the myelin covering breaks down into simpler lipids that will be removed eventually.

■ *Principle*

Luxol fast blue, like alcian blue, is of the sulfonated copper phthalocyanine type, but it is alcohol-soluble, whereas alcian blue is water-soluble. Staining is caused by lipoproteins, and the mechanism is that of an acid-base reaction with salt formation; the base of the lipoprotein replaces the base of the dye.

■ *Fixative*
10% neutral-buffered formalin

■ *Equipment*
Coplin jars, 56°C to 58°C oven, graduated cylinders, Erlenmeyer flasks

■ *Technique*
Cut paraffin sections at 10-15 µm.

■ *Quality Control*
A section of spinal cord or medulla provides a good control.

■ *Reagents*

Luxol Fast Blue, 0.1% Solution

Luxol fast blue MBSN	0.1 g
Alcohol, 95% alcohol	100 mL
Dissolve dye in alcohol, then add:	
Acetic acid, 10%	0.5 mL
The solution is stable	

Lithium Carbonate, 0.05% Solution

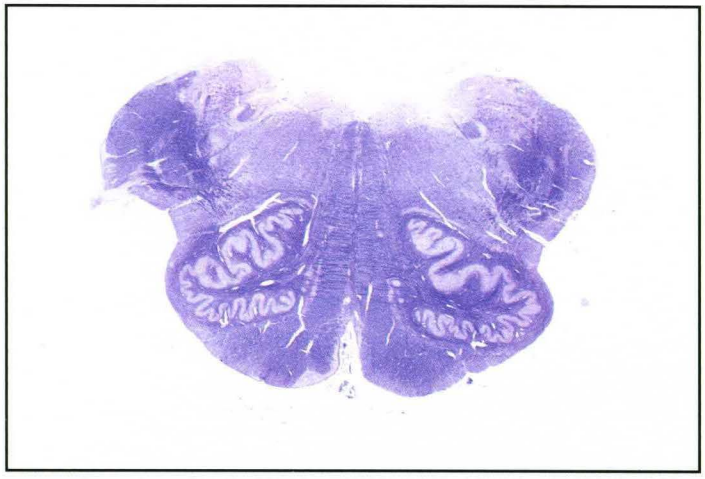
Lithium carbonate	0.25 g
Distilled water	500 mL

Alcohol, 70% Solution

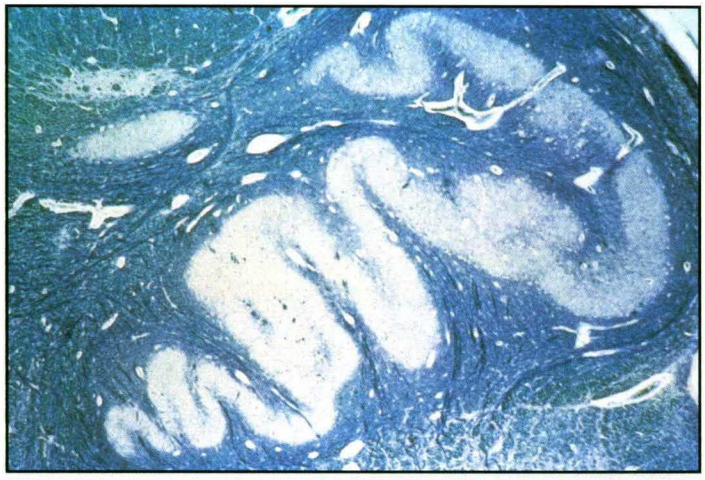
Absolute alcohol	70 mL
Distilled water	30 mL

■ *Procedure*

1. Deparaffinize sections, and hydrate to 95% alcohol.
2. Place slides in Luxol fast blue solution, and leave overnight at 56°C to 58°C. The container should be tightly capped as this alcoholic solution will evaporate readily.
3. Rinse sections in 95% alcohol to remove excess stain.
4. Rinse in distilled water.
5. Begin the differentiation by immersing the slides in lithium carbonate solution for 10-20 seconds.
6. Continue the differentiation in 70% alcohol solution until gray and white matter can be distinguished. Do not overdifferentiate.



[i9.19] A cross-section of medulla stained with the Luxol fast blue technique shows sharp differentiation between gray and white matter. A good stain will always show good macroscopic (naked eye) differentiation between gray and white matter.

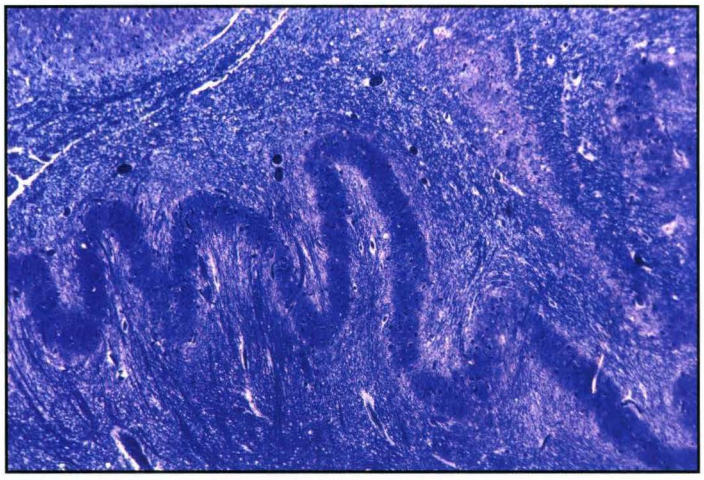


[i9.20] A section of the olivary nucleus (gray matter) can be seen in sharp contrast to the white matter (myelin) in this section stained with Luxol fast blue.

7. Wash the sections in distilled water.
8. Finish the 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. Dehydrate in several changes of 95% and absolute alcohols.
11. Clear in xylene, and mount with synthetic resin.

■ *Results* **[i9.19], [i9.20]**

• Myelin	Blue to blue-green
• Background	Colorless



[i9.21] The Luxol fast blue stain has not been differentiated enough. Although there appears to be a contrast between gray and white matter, the gray matter is stained darker than the white matter. The gray matter contains little myelin, and should be colorless, as in **[i9.20]**, when the differentiation step is correctly performed.

■ *Technical Notes*

1. Gray matter and demyelinated white matter should be almost colorless and contrast sharply with the blue-stained myelinated white matter.

2. The quality of a myelin stain can be determined macroscopically with the gray and white matter easily distinguished; on a good myelin stain, the areas of demyelination frequently are more easily identified macroscopically than microscopically.

3. There is frequently a point in this procedure where the gray matter is darker than the white matter **[i9.21]**. The differentiation should be continued until the gray matter is almost colorless and the white matter is blue. If in doubt as to the location of gray and white matter in the particular section to be stained, refer to an atlas of histology.

MYELIN SHEATH AND NISSL SUBSTANCE COMBINED: LUXOL FAST BLUE-CRESYL ECHT VIOLET STAIN [KLÜVER 1953]

■ *Purpose*

Demonstration of both myelin and Nissl substance in tissue sections. Nissl substance is lost after cell injury, and if the axon degenerates, the myelin covering also breaks down. Nuclei of neurons and glial cells are also demonstrated by this method.

■ *Principle*

Described under the Luxol fast blue and cresyl echt violet techniques

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

Coplin jars, 56°C to 58°C oven, graduated cylinders, Erlenmeyer flasks, Whatman #1 filter paper

■ *Technique*

Cut paraffin sections at 10-15 µm.

■ *Quality Control*

A section of spinal cord or medulla provides a good control.

■ *Reagents*

Acetic Acid, 10% Solution

Acetic acid, glacial	10 mL
Distilled water	90 mL

Luxol fast blue, 0.1% Solution

Luxol fast blue MBSN	0.1 g
Alcohol, 95%	100 mL
Dissolve the dye in alcohol, then add:	
Acetic acid, 10% solution	0.5 mL
The solution is stable	

Cresyl Echt Violet, 0.1% Solution

Cresyl echt violet	0.1 g
Distilled water	100 mL
Just before use, add 15 drops of 10% acetic acid solution, filter, and preheat. This solution is not very stable, so do not prepare a large amount.	

Lithium Carbonate, 0.05% Solution

Lithium carbonate	0.25 g
Distilled water	500 mL

Alcohol, 70% Solution

Absolute alcohol	70 mL
Distilled water	30 mL

■ *Procedure*

1. Deparaffinize sections, and hydrate to 95% alcohol.

2. Place slides in Luxol fast blue solution, and leave overnight at 56°C to 58°C. The container should be tightly capped as this alcoholic solution will evaporate readily.

3. Rinse sections in 95% alcohol to remove excess stain.

4. Rinse in distilled water.

5. Begin the differentiation by immersing the slides in lithium carbonate solution for 10-20 seconds.

6. Continue the differentiation in 70% alcohol solution until gray and white matter can be distinguished. Do not overdifferentiate.

7. Wash the sections in distilled water.

8. Finish the 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. Place slides in cresyl echt violet solution for 6 minutes. Add the acetic acid, filter, and preheat cresyl echt violet solution to 57°C just before use. Keep hot during staining.

11. Differentiate in several changes of 95% alcohol.

12. Dehydrate in absolute alcohol, clear in xylene, and mount with synthetic resin.

■ *Results* [i9.22]

• Myelin	Blue
• Nissl substance	Violet
• Nuclei	Violet

■ *Technical Notes*

1. Failure to add acetic acid to the cresyl echt violet solution will result in diffuse violet background staining [i9.23].

2. Failure to heat the cresyl echt violet prior to adding the slides will result in decreased staining of the Nissl substance.

3. The cresyl echt violet counterstain intensifies the staining of the myelin sheath [Klüver 1953].

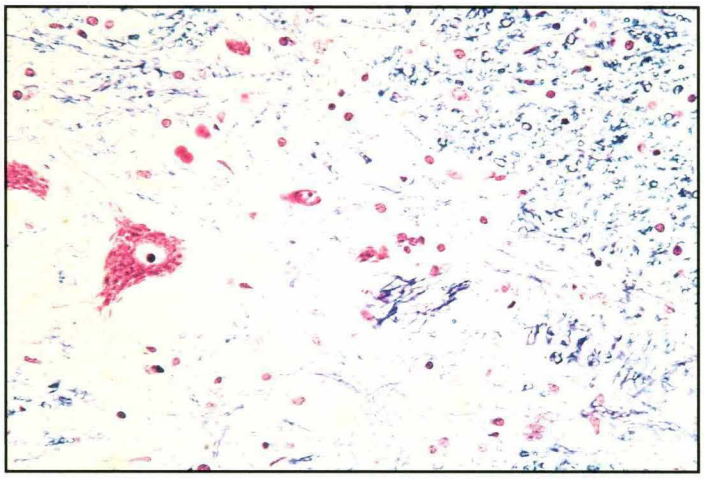
MYELIN SHEATHS AND NERVE FIBERS COMBINED: LUXOL FAST BLUE-HOLMES SILVER NITRATE METHOD [MARGOLIS 1956, CARSON 1984]

■ *Purpose*

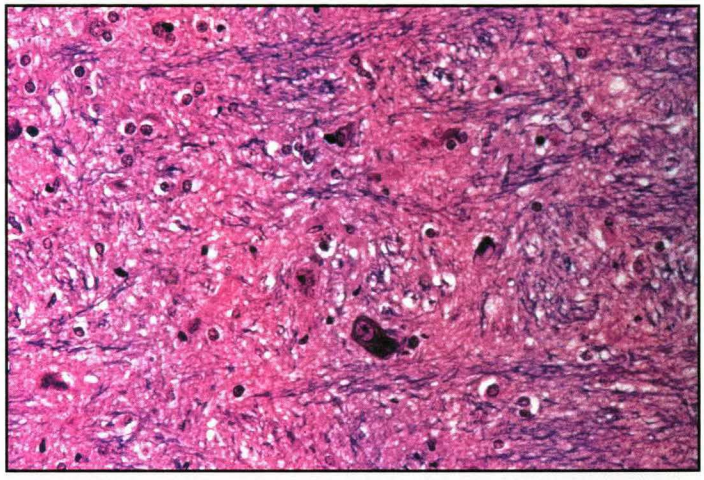
Demonstration of both myelin and nerve fibers in the same tissue section. If the axon degenerates, the myelin covering breaks down into simpler lipids that are eventually removed.

■ *Principle*

See the descriptions of the Holmes and Luxol fast blue techniques.



[i9.22] Cresyl echt violet provides a good counterstain for Luxol fast blue. Nissl substance and cell nuclei are stained violet, myelin is blue. If the Luxol fast blue has been properly differentiated, the neuron cell body should be colorless before the application of the counterstain, so that the Nissl substance should be a rose-violet. This cresyl echt violet solution can also be used as a primary stain for Nissl substance without the need for the Luxol fast blue. (Reprinted with permission from Carson [1987])



[i9.23] If the cresyl echt violet solution is not properly acidified, the background will be stained diffusely and differentiation of the Nissl substance and cell nuclei will be impossible to detect.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

Chemically cleaned Coplin jars, 56°C to 58°C oven, graduated cylinders, 37°C incubator, Erlenmeyer flasks, pipettes

■ *Technique*

Cut paraffin sections at 10-15 µm.

■ *Quality Control*

Use a section of cerebral cortex. Spinal cord is not a good control for this stain because most axons will be in cross-section. A longitudinal section of peripheral nerve also provides a good control. Use chemically cleaned glassware for steps 2 through 7.

■ *Reagents*

Aqueous Silver Nitrate, 20% Solution

Silver nitrate	20 g
Distilled water	100 mL

Aqueous Silver Nitrate, 1% Solution

Silver nitrate, 20% solution	2.5 mL
Distilled water	47.5 mL

Boric Acid Solution

Boric acid	1.24 g
Distilled water	100 mL

Borax Solution

Sodium borate	1 g
Distilled water	100 mL

Pyridine, 10% Solution

Pyridine	5 mL
Distilled water	45 mL

Impregnating Solution

Boric acid solution (fresh)	27.5 mL
Borax solution (fresh)	22.5 mL
Distilled water	247 mL
Silver nitrate, 1 % aqueous solution	0.5 mL
Pyridine, 10% aqueous solution	2.5 mL

Mix boric acid solution and borax solution in a 500-mL flask. Add the water, and with a pipette, add the aqueous silver nitrate, and then with another pipette, add the aqueous solution of pyridine. Mix thoroughly. Make enough solution for 20 mL per slide, and make just before use.

Reducing Solution

Hydroquinone	0.5 g
Sodium sulfite (crystals)	5 g
Distilled water	50 mL

Prepare just before use

Gold Chloride, 0.2% Solution

Gold chloride, 1% solution	10 mL
Distilled water	40 mL

Oxalic Acid, 2% Solution

Oxalic acid	2 g
Distilled water	100 mL

Sodium Thiosulfate, 5% Solution

Sodium thiosulfate	5 g
Distilled water	100 mL

Luxol fast blue, 0.1% Solution

Luxol fast blue MBSN	0.1 g
Alcohol, 95%	100 mL
Dissolve the dye in alcohol, then add:	
Acetic acid, 10% solution	0.5 mL
The solution is stable	

Lithium Carbonate, 0.05% Solution

Lithium carbonate	0.25 g
Distilled water	500 mL

■ *Procedure*

1. Deparaffinize sections, and hydrate to distilled water.
2. Place sections in 20% silver nitrate in the dark at room temperature for 1 hour.
3. Prepare the impregnating solution, allowing at least 20 mL of the solution per slide.
4. Take the slides from the 20% silver nitrate, and wash for 10 minutes in 3 changes of distilled water.
5. Place slides in impregnating solution. Cover the jar, and incubate at 37°C overnight.
6. Remove slides, shake off the superfluous fluid, and place in the reducer for not less than 2 minutes.

7. Wash section in running water for 3 minutes, and then rinse in distilled water.

8. Tone sections in 0.2% aqueous gold chloride for 3 minutes. This solution may be reused until a brown precipitate forms or the solution becomes cloudy.

9. Rinse in distilled water.

10. Place sections in 2% aqueous oxalic acid for 3-10 minutes. When the axons are thoroughly blue-black, stop the process.

11. Rinse sections in distilled water.

12. Place slides in 5% aqueous sodium thiosulfate for 5 minutes.

13. Wash in tap water for 10 minutes.

14. Place the slides briefly in 95% alcohol.

15. Stain in Luxol fast blue solution overnight at 60°C. The container should be tightly capped as this alcoholic solution will evaporate readily.

16. Rinse in 95% alcohol.

17. Place slides in distilled water.

18. Place in 0.05% lithium carbonate for 15 seconds.

19. Differentiate in 70% alcohol for 20-30 seconds.

20. Rinse in distilled water (repeat steps 18-20 if Luxol fast blue needs more differentiation).

21. Dehydrate in 2 changes each of 95% alcohol and absolute alcohol.

22. Clear in xylene, and mount with synthetic resin.

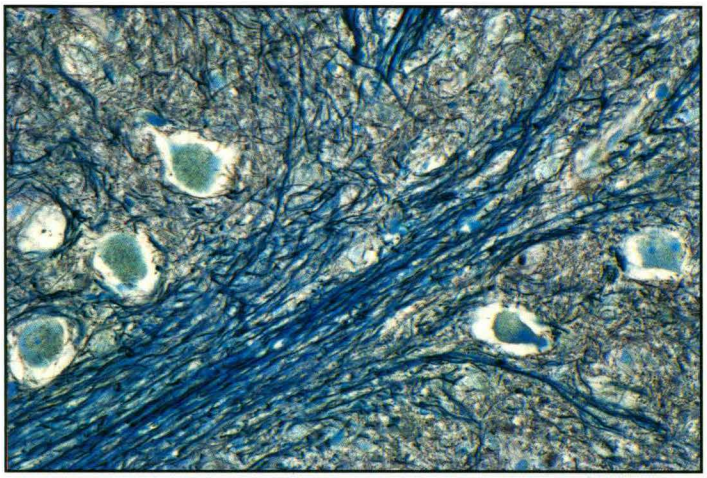
■ *Results* [i9.24], [i9.25]

- Myelin sheaths — Blue to green
- Axons and nerve fibers — Black

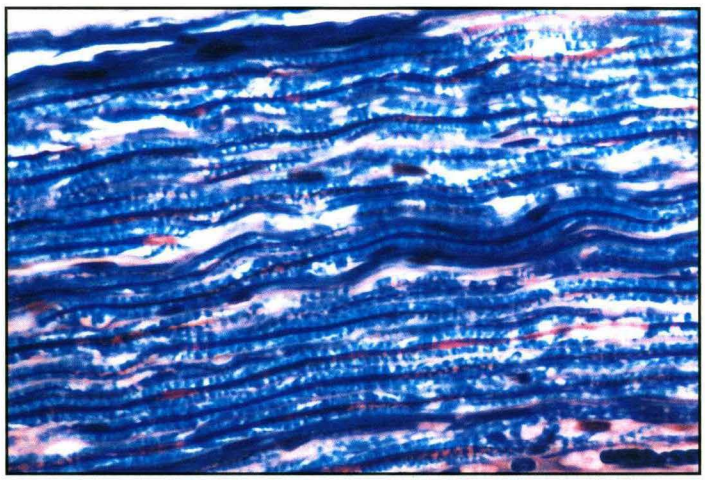
■ *Technical Note*

1. A section showing complete degeneration of both the axon and myelin sheath can be seen in [i9.26].

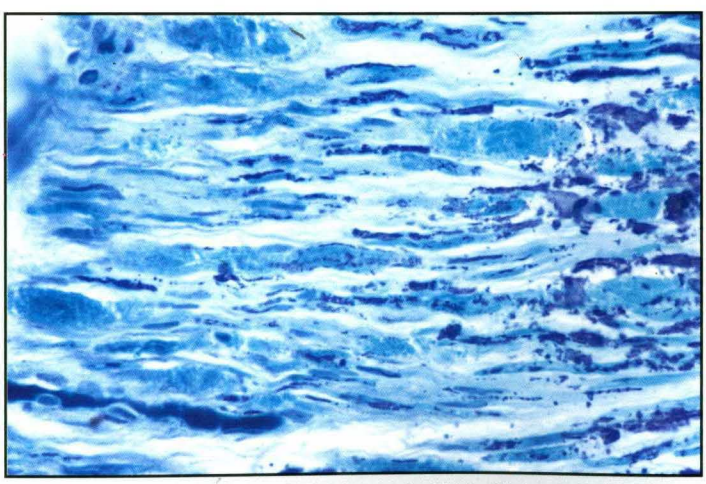
2. Pyridine is toxic by ingestion, inhalation, and skin absorption. It has an OSHA TWA of 5 ppm; it should be used under a chemical fume hood, and suitable gloves and goggles should be used.



[i9.24] Holmes silver nitrate technique combined with Luxol fast blue demonstrates both nerve fibers (black) and the myelin sheath (blue).



[i9.25] Both axons (black) and their myelin sheath (blue) are well demonstrated in this section of peripheral nerve. Holmes silver nitrate stain has been followed by the Luxol fast blue technique.



[i9.26] A section of peripheral nerve stained with the Holmes silver nitrate-Luxol fast blue technique shows complete degeneration of both the axon and the myelin sheath.

LUXOL FAST BLUE-PAS-HEMATOXYLIN [MARGOLIS 1956, CARSON 1984]

■ *Purpose*

Demonstration of the myelin sheath, basement membranes, senile plaques, fungi, and corpora amylacea. This is a particularly useful combination stain that allows a correlative study of the cellular elements, fiber pathways, and vascular components of the nervous system. Each stain is sharpened and complemented by the other.

■ *Principle*

See the principle of the Luxol fast blue stain earlier in this chapter and the principle of the PAS technique in chapter 7, p137.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

Coplin jars, 56°C to 58°C oven, graduated cylinders, Erlenmeyer flasks

■ *Technique*

Cut paraffin sections at 10-15 µm.

■ *Quality Control*

A section of medulla or cerebral cortex provides a good control.

■ *Reagents*

Luxol fast blue, 0.1% Solution

Luxol fast blue MBSN	0.1 g
Alcohol, 95%	100 mL
Dissolve the dye in alcohol, then add:	
Acetic acid, 10% solution	0.5 mL
The solution is stable	

Lithium Carbonate, 0.05% Solution

Lithium carbonate	0.25 g
Distilled water	500 mL

Schiff Solution

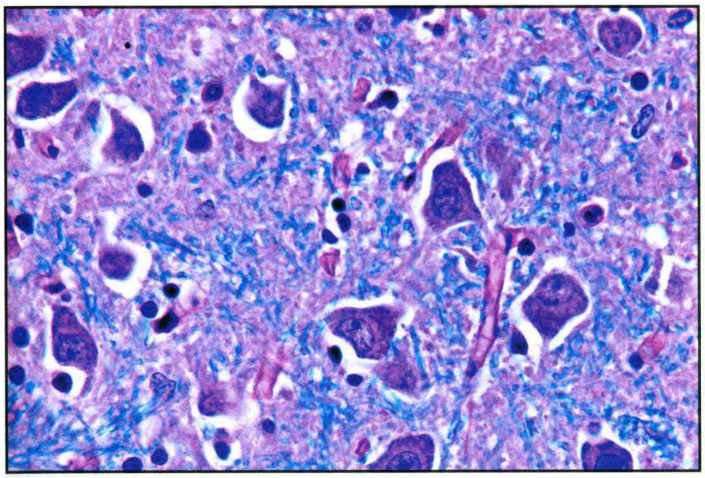
See description of the PAS procedure in chapter 7.

Periodic Acid, 0.5% Solution

Periodic acid	0.5 g
Distilled water	100 mL

■ *Procedure*

1. Deparaffinize and hydrate sections to 95% alcohol
2. Place sections in Luxol fast blue solution overnight in oven at 56°C-58°C. The container should be tightly capped because this alcoholic solution will evaporate readily.
3. Rinse sections in 95% alcohol to remove excess stain.
4. Rinse in distilled water.
5. Begin the differentiation by immersing the slides in lithium carbonate solution for 10-20 seconds.
6. Continue the differentiation in 70% alcohol solution until gray and white matter can be distinguished. Do not overdifferentiate.
7. Wash the sections in distilled water.
8. Finish the 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. (Steps 7 and 8 may be repeated if further differentiation is necessary.)
10. Place in 0.5% periodic acid solution for 5 minutes.
11. Rinse in 2 changes of distilled water.
12. Place in Schiff solution for 15 minutes.
13. Wash in tap water for 5 minutes.
14. Stain in Harris hematoxylin for 30 seconds.
15. Wash in tap water for 5 minutes. (If background is not clear, dip once in acid alcohol; wash. If nuclei are not dark blue to purple, dip briefly in dilute ammonium hydroxide; wash.)
16. Dehydrate in 95% alcohol and 2 changes of absolute alcohol.
17. Clear in 3 changes of xylene and mount with a synthetic resin.



[i9.27] A section from the central nervous system stained with the Luxol fast blue-PAS-hematoxylin technique. Small blood vessels can be seen stained with PAS, and myelin is stained with the Luxol fast blue.

■ *Results* **[i9.27]**

• Capillary basement membranes	Rose
• Fungi	Rose
• Corpora amylacea	Rose
• Senile plaques	Rose
• Myelin sheath	Blue to blue-green
• Nuclei	Purple

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LEARNING ACTIVITIES

1. **Perform the cresyl echt violet, Bodian, Holmes silver nitrate, Bielschowsky, Sevier-Munger, PTAH, Weil, and Luxol fast blue procedures. You may choose any tissue specimen that will demonstrate positive staining. You may use the cresyl echt violet as a counterstain for the Luxol fast blue and as a separate stain.**

2. **Microscopically examine each stained section and compare the results with those given in the procedure. If the stains are unsatisfactory, analyze the procedural steps for possible sources of error. If a mistake is identified, repeat the staining procedure after correcting the problem and then reexamine the slides.**

CHAPTER 10

Microorganisms

OBJECTIVES

On completing this chapter, the student should be able to do the following:

1. Define and give examples of:
   a. bacteria
   b. cocci
   c. bacilli
   d. spirochetes
   e. mycobacteria
   f. fungi
   g. yeasts
   h. protozoans
   i. hyphae
   j. mycelia
2. Classify the following techniques as to type of organism demonstrated:
   a. acid-fast techniques (Kinyoun, Ziehl-Neelsen, Fite)
   b. auramine-rhodamine
   c. Gram stain modifications
   d. Giemsa
   e. alcian yellow-toluidine blue
   f. periodic acid-Schiff (PAS)
   g. chromic acid-Schiff (CAS)
   h. Gridley fungus stain
   i. Grocott modification of Gomori methenamine-silver
   j. Mayer mucicarmine
   k. Warthin-Starry
   l. Dieterle
   m. Steiner and Steiner
3. Outline each of the above techniques, considering the following criteria:
   a. most desirable fixative
   b. if another fixative has been used, what can be done
   c. primary reagents or dyes and their purpose
   d. results of staining
   e. appropriate control material
   f. sources of error and appropriate correction
   g. mode of action
   h. special requirements (eg, chemically clean glassware)
   i. microscope used

Microorganisms are forms of life so small that they can be seen only through a microscope. Medically important microorganisms include bacteria, fungi, viruses, and protozoans.

Bacteria

Bacteria are tiny, single-celled organisms that are widely distributed in nature. The genetic material of bacteria is not enclosed in a special nuclear membrane, but each bacterial cell is a complete organism that can metabolize, grow, and reproduce. The cell walls that enclose bacteria are primarily composed of a substance called peptidoglycan, a mucopolysaccharide. Bacteria vary in size from approximately 0.2 to 10 μm in their greatest dimension. They also vary in shape, and this variation provides a basis for classification.

One way of classifying bacteria is by shape. The spherical or ovoid bacteria are classified as cocci and are subclassified according to the way they are arranged. Some cocci occur in pairs (diplococci), some occur in grapelike clusters (staphylococci), and others occur in chains (streptococci). *Staphylococcus aureus* is the agent of toxic shock syndrome and the cause of many life-threatening hospital and locker-room infections (eg, methicillin-resistant *Staphylococcus aureus* [MRSA]). Other pathogenic cocci are *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, and *Neisseria meningitidis*. The rod-shaped bacterial organisms are classified as bacilli. Some of the pathogenic bacilli are *Clostridia tetani*, *Clostridia botulinum*, and *Bacillus anthracis*. Coccobacilli are rod-shaped, but so short and wide that they resemble cocci. Examples of coccobacilli are *Haemophilus influenzae* and *Chlamydia trachomatis*.

Bacteria that are spiral or corkscrew-shaped are classified as spirochetes. *Treponema pallidum*, the causative organism of syphilis, is a very important organism in this group. *Borrelia burgdorferi*, the organism that causes Lyme disease, is also a spirochete. Silver stains are the primary technique used for the demonstration of spirochetes.

Rickettsiae, chlamydiae, and mycoplasmas are bacteria that do not possess the typical bacterial attributes. Rickettsiae and chlamydiae can reproduce only within a living host cell (ie, obligate intracellular parasites), and mycoplasmas do not form cell walls. These organisms were originally classified as viruses because of their size and the difficulties encountered in isolating the organisms.

Chlamydia trachomatis organisms cause lymphogranuloma venereum, trachoma, cervicitis, myocarditis, and other diseases in humans, while rickettsiae are responsible for such diseases as Rocky Mountain spotted fever and typhus. Examples of problems caused by mycoplasma organisms are pneumonia, still birth, and spontaneous abortion.

Another way of classifying bacteria is by means of the Gram stain, a stain developed by Christian Gram in 1884. The stain colors some bacteria deep blue (gram-positive) and leaves others either unstained or colored by the counterstain (gram-negative). It is now known that the organisms that stain deep blue have a cell wall containing teichoic acid, and those that are unstained or stain red contain lipopolysaccharides. This stain is one of the most basic stains used in microbiology. *Salmonella* organisms are gram-negative bacilli and *Clostridium botulinum* are endospore-forming gram-positive rods. Other gram-positive bacilli are *Clostridium tetani* and *Corynebacterium diptheriae*; other gram-negative bacilli are *Shigella dysenteriae, Escherichia coli*, and *Pseudomonas aeruginosa*. *Neisseria gonorrhoeae* and *Neisseria meningitidis* are gram-negative cocci, and *Streptococcus pneumoniae* and *Staphylococcus aureus* are gram-positive cocci.

Many bacteria are pathogenic (disease-producing), and proper identification aids in providing appropriate therapy for the patient. Culture, staining, and biochemical characteristics are all used in the microbiology laboratory to identify organisms, while in the histopathology laboratory, identification depends on the shape and staining characteristics of organisms found in tissue and on the way the tissue has responded to the presence of the organisms. Immunohistochemical methods are also important in the identification of some organisms.

While there are stains that simply screen for the presence of bacteria (eg, Giemsa and methylene blue), we are more concerned with the 2 common differential techniques that allow bacteria to be divided into groups.

1. **Acid-fast stains** allow bacteria to be divided into acid-fast and non-acid-fast groups

2. **Gram stains** allow bacteria to be classified as gram-positive and gram-negative organisms

Acid-fast techniques are of value in the detection of mycobacteria, rod-shaped organisms that sometimes exhibit filamentous (fungus-like) growth. The most significant disease-producing mycobacteria are *Mycobacterium tuberculosis* and *Mycobacterium leprae*. *Mycobacterium avium intercellulare* has become a common infective agent in HIV-positive individuals. Acid-fast organisms contain large amounts of lipid in the cell wall; once the cell is stained, it resists decolorization with dilute mineral acids, and resistance to acid decolorization is responsible for the application of the term *acid-fast bacteria* to these organisms.

Fungi

Fungi are unicellular or multicellular primitive plants that have a distinct membrane-bound nucleus containing genetic material [Myrvik 1988]. Although large multicellular fungi (eg, mushrooms) may resemble plants, they are without chlorophyll and cannot carry out photosynthesis. The cell wall of true fungi is composed of chitin. The study of fungi is termed mycology, and mycosis is the term used to designate a disease produced by fungi. There are 4 classes of fungi that are medically important. The fungi and related organisms are summarized in **[t10.1]**.

The filamentous fungi are also called molds. The basic structure of the filamentous fungi is the "hypha." As more and more hyphae

[t10.1] Pathologic fungi and related organisms*

Fungus	Characteristics	Stains
Actinomyces israelii	Thin, 1-mm wide, branching, gram-positive filaments occurring in tangled masses, or "sulfur granules," varying from 30 to 3,000 μm in diameter. These organisms are filamentous bacteria and not fungi.	Gram and Grocott are best for demonstration. CAS, PAS and Gridley should not be used.
Aspergillus fumigatus	3- to 6-μm-wide septate hyphae with parallel walls.	Grocott, CAS, and PAS best; Gridley and Gram can be used.
Blastomyces dermatitidis	Spherical, multinucleate, yeast-like cells, 8 to 15 μm in diameter, with thick double refractile walls, and single, broad-based buds.	Grocott, CAS, and PAS best; Gridley is also satisfactory. Alcian blue and Mayer mucicarmine may also stain the cell walls of this organism [Chandler 1995b].
Candida albicans	2- to 4-μm, thin-walled, oval, yeast-like cells that are frequently seen in mycelial forms. Pseudohyphae and septate hyphae may be seen.	Grocott, CAS, and PAS are best for demonstration. Gridley may also be used.
Coccidioides immitis	Large, spherical, 20- to 200-μm, thick-walled spherules filled with numerous one-celled, small, 2- to 5-μm endospores. Septate hyphae and chains of arthroconidia may be seen in necrotic nodules. Endemic in the southwestern United States.	Grocott, CAS, and PAS best; Gridley is also satisfactory.
Cryptococcus neoformans	2- to 20-μm, spherical to oval, yeast-like organism with single or, rarely, multiple narrow-based buds. The organism is normally surrounded by a wide mucinous capsule, although some cryptococci are capsule deficient and mucin stains will be negative. Pseudohyphae are common. Occurs worldwide and is associated with pigeon excrement.	Grocott good for demonstration, but does not demonstrate the capsule. PAS, CAS, alcian blue, Mayer mucicarmine, and toluidine blue all demonstrate the mucinous capsule and aid in the differentiation of this fungus.
Histoplasma capsulatum	Small, 2- to 5-μm, spherical to oval, yeast-like organisms found in giant cells or macrophages. Reproduces by budding. Endemic in some areas of the United States.	Grocott best for demonstration. PAS and Gridley are frequently unsatisfactory. Chromic acid should be used for oxidation in the CAS and Grocott methods.
Nocardia asteroides	Delicate, branched, individual filaments, 1 μm wide. This organism is also a bacterium not a fungus.	Gram and Grocott will demonstrate. The organisms are also usually weakly acid-fast.
Pneumocystis jirovecii (formerly *P carinii*)	5- to 8-μm, with characteristic cup shape of cyst. This organism was until recently classified as a protozoan, as it has trophozoite and cyst stages. Recently it was shown to be most closely related to the fungi, particularly the yeasts.	Grocott best for demonstration. Giemsa may also be used.
Sporothrix schenckii	Cigar-shaped, oval, or spherical, 2- to 10-μm cells with single buds.	Grocott best for demonstration. PAS, CAS, and Gridley are also good.

*Adapted from [Baron 1994, Chandler 1995b, Sheehan 1980]

are produced with fungal growth, the collection of hyphae form a "mycelium." Some hyphae are divided transversely by partitions called "septa." *Aspergillus fumigatus* organisms typically show septate hyphae in tissue sections.

Single round or oval cells that reproduce by "budding" are classified as yeasts. In budding, a protuberance is formed on the outer surface of the parent cell and the nucleus of the parent cell divides. The nucleus migrates to the bud, cell-wall material is laid down between the parent cell and the bud, and the bud breaks away from the parent cell. *Cryptococcus neoformans* exemplifies this classification of fungi.

The yeastlike type of fungus also reproduces by budding, but the buds tend to elongate into filamentous structures that do not detach from the parent cell. These structures are called "pseudohyphae." *Candida albicans*, an opportunistic pathogen, belongs to this group of fungi.

Dimorphic fungi, when grown at 37°C, have a yeastlike morphology, but when grown on artificial media at 25°C, have a filamentous morphology. Many of the systemic pathogenic fungi, such as *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Histoplasma capsulatum*, belong to this group.

Viruses

Viruses are composed of either DNA or RNA, and protein. Basically, they are just protein-coated genes that need living cells to provide energy and the machinery for duplication. While most viruses form cellular inclusions that can be seen only with the electron microscope, some viruses form cellular inclusions that can be seen with the light microscope (eg, Negri bodies of rabies and the cytomegalovirus).

Protozoans

Protozoans are single-celled microorganisms that are functionally complex structures. The means of locomotion provides the means of classification of the protozoans. Amebae move by using extensions of their cytoplasm (pseudopods), and other protozoans move by means of flagella or cilia. Some medically important protozoans include *Entamoeba histolytica*, *Giardia lamblia*, and *Toxoplasma gondii*. Until recently *Pneumocystis jirovecii* (formally *P carinii*) was classified as a protozoan, and it has trophozoite and cyst stages; however, nucleic acid sequence analysis of the small subunit ribosomal RNA gene has shown this organism to be most closely related to fungi in general and yeasts in particular [Stewart 1995].

Special Staining Techniques

KINYOUN ACID-FAST STAIN [LUNA 1968, CARSON 1984]

■ *Purpose*

Detection of acid-fast mycobacteria in tissue sections

■ *Principle*

The lipoid capsule of the acid-fast organism takes up carbol-fuchsin and resists decolorization with dilute mineral acid. Carbol-fuchsin is more soluble in the lipids of the cell wall than in acid-alcohol, but is readily removed from bacteria that lack the waxy capsule. Staining is enhanced by the phenol and alcohol, and both of these chemicals also aid in dissolving the basic fuchsin. Alcoholic, rather than aqueous, solutions of acid are used because more uniform decolorization is obtained with alcoholic solutions [Sheehan 1980]. The carbol-fuchsin methods provide a specific way of identifying mycobacteria. These organisms are not readily demonstrated by other methods such as the Gram stain. The lipoid capsule of mycobacteria is of such high molecular weight that it is waxy at room temperature, and successful penetration by the aqueous-based staining solutions used in the Gram staining procedures is prevented [Koski 1984].

■ *Fixative*

Although 10% neutral-buffered formalin is preferred, others, with the exception of Carnoy solution, may be used.

■ *Equipment*

Coplin jars, Whatman #1 filter paper, Erlenmeyer flasks, graduated cylinders, pipettes

■ *Technique*

Cut paraffin sections at 4-5 µm.

■ *Quality Control*

Tissue containing acid-fast organisms must be used for a control. Millipore-filtered (pore size 0.45 µm or smaller) water should be used in the flotation bath, and a negative control from the same day's workload must be run (cut on the same microtome and using the same water bath as is used for the diagnostic case). Do not use tap or regular distilled water before the carbol-fuchsin; use only Millipore-filtered water [Carson 1964].

■ *Reagents*

Kinyoun Carbol-Fuchsin Solution

Basic fuchsin	28 g
Phenol crystals, melted	56 mL
Alcohol, 95%	140 mL
Millipore-filtered distilled water	700 mL
Filter the solution each time before use	

Acid Alcohol, 1% Solution

Hydrochloric acid, concentrated	5 mL
Alcohol, 70%	495 mL

Stock Methylene Blue Solution

Methylene blue	1.4 g
Alcohol, 95%	100 mL

Working Methylene Blue Solution

Stock methylene blue solution	2.5 mL
Tap water	47.5 mL

■ *Procedure*

1. Deparaffinize sections through 2 changes of xylene, hydrate through absolute and 95% alcohols, and rinse in Millipore-filtered water. Remove mercury precipitate with iodine and hypo solutions if necessary.
2. Stain in Kinyoun carbol-fuchsin solution (freshly filtered) for 1 hour at room temperature or for 30 minutes at 56°C. The reagent may be poured back into the stock bottle for reuse.
3. Wash well in running tap water.

4. Differentiate in 2 changes of 1% acid alcohol until tissue is pale pink.

5. Wash the sections in running tap water. Carry slides through the remainder of the procedure 1 at a time.

6. Counterstain in working methylene blue solution for a few dips. Do not overstain; the sections should be sky blue.

7. Rinse the sections in tap water.

8. Dehydrate with 2 changes each of 95% and absolute alcohols, clear with 2 or 3 changes of xylene, and mount with synthetic resin.

■ *Results* **[i10.1]**

• Acid-fast bacteria	Bright red
• Background	Light blue

■ *Technical Notes*

1. Acid-fast organisms have been reported to exist in tap water [Carson 1964, Wang 1969], so no tap water should be used before applying the carbol-fuchsin reagent. The negative control cut from the same day's workload and using the same flotation bath helps detect any possible solution contamination. A section from a block of uterus provides a good negative control.

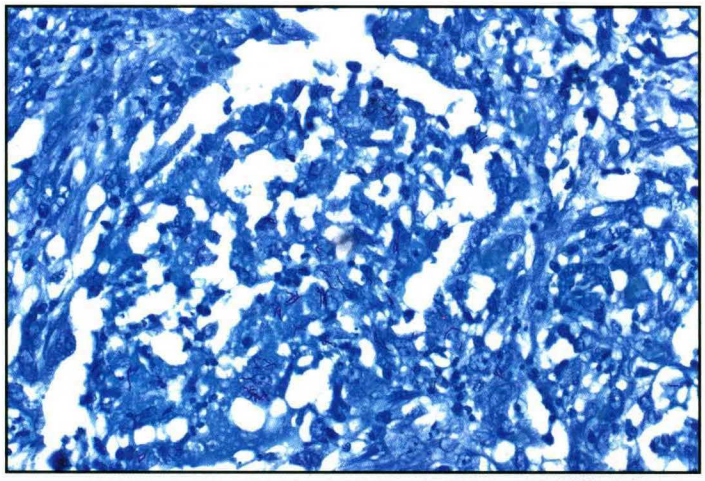
2. The counterstain is critical in this procedure, because overcounterstaining with methylene blue will mask any organisms present **[i10.2]**. If the section is overstained, take it back to the acid-alcohol to remove the methylene blue, wash with water, and then repeat the counterstaining step.

3. If the acid is not washed out of the tissue before the counterstaining step the tissue will not stain.

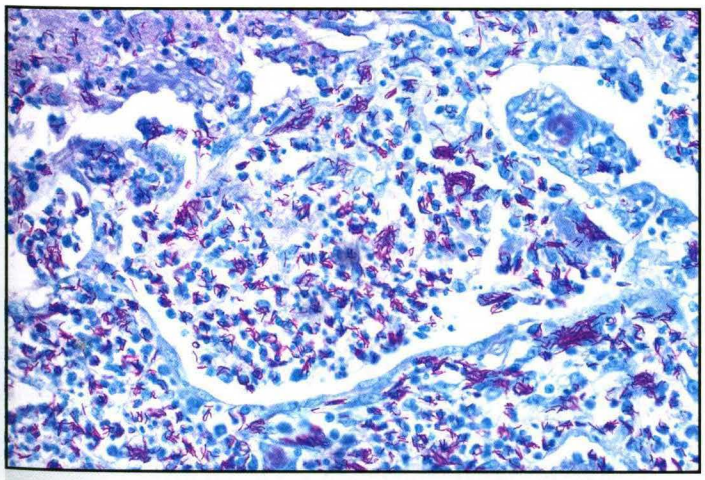
4. If the section is allowed to dry after the carbol-fuchsin stain is applied, a compound that is resistant to decolorization will be formed. Repeated attempts to remove this compound will result in complete decolorization of the acid-fast organisms.

5. This method is not satisfactory for the demonstration of *Mycobacterium leprae* **[i10.3]**. The Fite method should be used for the demonstration of this organism.

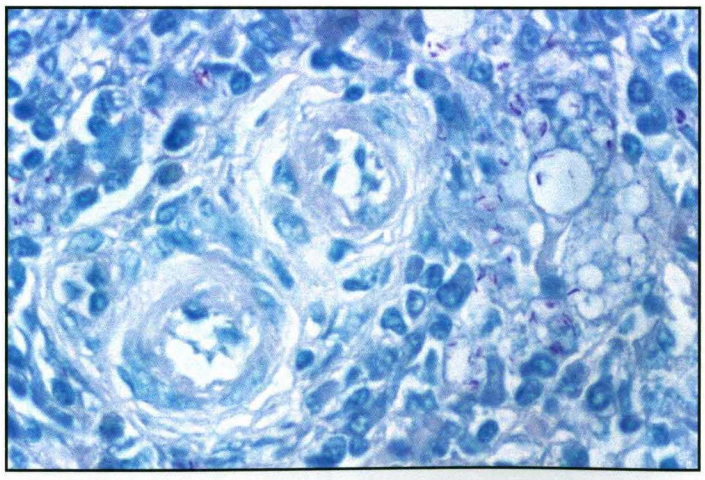
6. Phenol was known as *carbolic acid* in older literature, thus the name *carbol-fuchsin* for the solution of phenol and basic fuchsin. Penetration of this reagent is enhanced by heat and by wetting agents.



[i10.2] A section from the same block as that shown in **[i10.1]** Both were stained with Kinyoun carbol-fuchsin at the same time; however, this section has been overstained with methylene blue. Because of the intensity of the counterstain, acid-fast organisms are masked.



[i10.1] Acid-fast bacteria can be seen in this section of lung stained with the Kinyoun carbol-fuchsin method and examined with the high-dry objective. Note that the methylene blue is light so that the organisms are not masked or stained by the counterstain.



[i10.3] A section containing leprosy organisms has been stained with the Kinyoun carbol-fuchsin technique. Even in this high-power photomicrograph, the organisms are very difficult to see (compare with **[i10.5]**). The lipoid capsule of the leprosy organism is very sensitive to the alcohols and xylene used in routine acid-fast techniques, so special protective measures must be taken during steps that normally require alcohol and xylene.

7. Fixation in Carnoy solution will make acid-fast organisms non-acid-fast [Sheehan 1980].

8. Controls should not be overwhelmingly positive **[i10.4]** because of the possibility of overdecolorization without being aware, and also the danger of cross-contamination during microtomy and/or staining.

9. Phenol is highly toxic by ingestion, inhalation, and skin absorption, with a time-weighted average (TWA) of 5 ppm. Wear suitable gloves (butyl rubber recommended), and work under a chemical fume hood when using.

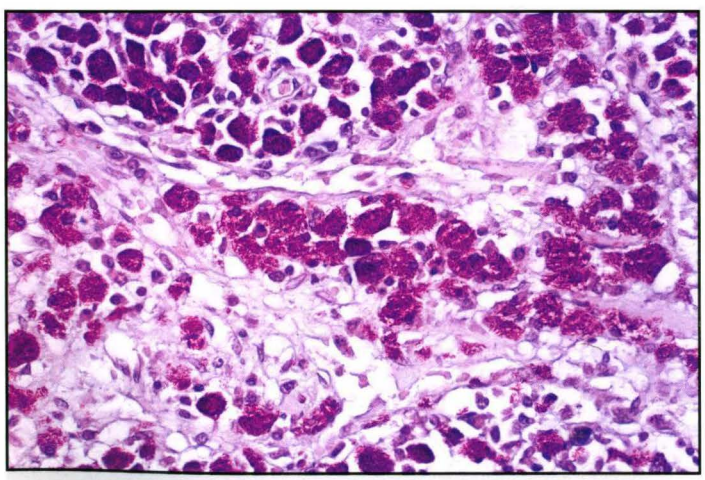
ZIEHL-NEELSEN METHOD FOR ACID-FAST BACTERIA (AFIP MODIFICATION) [PROPHET 1992]

■ *Purpose*

Detection of acid-fast mycobacteria in tissue sections

■ *Principle*

The lipoid capsule of the acid-fast organism takes up carbol-fuchsin and resists decolorization with dilute mineral acid. Carbol-fuchsin is more soluble in the lipids of the cell wall than in acid-alcohol, but is readily removed from bacteria that lack the waxy capsule. Staining is enhanced by the phenol and alcohol, and both of these chemicals also aid in dissolving the basic fuchsin. Alcoholic, rather than aqueous, solutions of acid are used because more uniform decolorization is obtained with alcoholic solutions [Sheehan 1980]. The carbol-fuchsin methods provide a specific way of identifying mycobacteria. These organisms are not readily demonstrated by other methods such as the Gram stain. The lipoid capsule of mycobacteria is of such high molecular weight that it is waxy at room temperature, and successful penetration by the aqueous-based staining solutions used in the Gram staining procedures is prevented [Koski 1984].



[i10.4] This control section contains too many acid-fast bacteria. There is the possibility of overdecolorization without recognizing it, and of cross-contamination through knife or solution metastasis.

■ *Fixative*

Any well-fixed tissue, with the exception of that fixed in Carnoy solution, may be used.

■ *Equipment*

Coplin jar, Whatman #1 filter paper, Erlenmeyer flasks, graduated cylinders, pipettes

■ *Technique*

Cut paraffin sections at 4-5 µm.

■ *Quality Control*

Tissue containing acid-fast organisms must be used for a control. Millipore-filtered (pore size 0.45 µm or smaller) water should be used in the flotation bath, and a negative control from the same day's workload must be run (cut on the same microtome and using the same water bath as is used for the diagnostic case). Do not use tap or regular distilled water before the carbol-fuchsin; use only Millipore-filtered water [Carson 1964].

■ *Reagents*

Ziehl-Neelsen Carbol-Fuchsin Solution

Phenol crystals, melted	2.5 mL
Absolute ethyl alcohol	5 mL
Basic fuchsin	0.5 g
Millipore-filtered distilled water	700 mL
Filter before use each time	

Acid Alcohol, 1% Solution

Hydrochloric acid, concentrated	5 mL
Alcohol, 70%	495 mL

Stock Methylene Blue Solution

Methylene blue	1.4 g
Alcohol, 95%	100 mL

Working Methylene Blue Solution

Stock methylene blue solution	2.5 mL
Tap water	47.5 mL

■ *Procedure*

1. Deparaffinize and hydrate the sections to Millipore-filtered water.
2. Stain the sections with freshly filtered carbol-fuchsin solution for 30 minutes.
3. Wash sections well in running water.
4. Decolorize with 1% acid alcohol solution until sections are pale pink.
5. Wash thoroughly with tap water, then with distilled water.
6. Counterstain by dipping 1 slide at a time in the methylene blue working solution. Sections should be pale blue.
7. Wash with tap water, then with distilled water.
8. Dehydrate quickly in 95% and absolute alcohols, 2 changes each.
9. Clear with 2 changes of xylene, 2 minutes each.
10. Mount with synthetic resin.

■ *Results (Same as* [i10.1]*)*

- Acid-fast bacteria — Bright red
- Background — Light blue

■ *Technical Notes*

1. This carbol-fuchsin method is preferred by many laboratorians for staining acid-fast organisms.
2. See other notes in the section on the Kinyoun procedure.

MICROWAVE ZIEHL-NEELSEN METHOD FOR ACID-FAST BACTERIA [CHURUKIAN 1993]

■ *Purpose*

Detection of acid-fast bacteria in tissue

■ *Principle*

See the Kinyoun method.

■ *Fixative*

Although 10% neutral-buffered formalin is preferred, others, with the exception of Carnoy solution, may be used.

■ *Equipment*

Coplin jars, Whatman #1 filter paper, Erlenmeyer flasks, graduated cylinders, pipettes

■ *Technique*

Cut paraffin sections at 4-5 µm.

■ *Quality Control*

Tissue containing acid-fast organisms must be used for a control. Millipore-filtered (pore size 0.45 µm or smaller) water should be used in the flotation bath, and a negative control from the same day's workload must be run (cut on the same microtome and using the same water bath as used for the diagnostic case). Do not use tap or regular distilled water before the carbol-fuchsin; use only Millipore-filtered water [Carson 1964].

■ *Reagents*

Carbol-Fuchsin Solution

Pararosaniline, CI 42500 or basic fuchsin CI 42510	0.8 g
Phenol crystals, melted	7.5 mL
Isopropyl alcohol	14 mL
Millipore-filtered distilled water	140 mL

Dissolve the pararosaniline or basic fuchsin in the isopropyl alcohol and the phenol in the distilled water. Mix the 2 solutions. Filter the solution through Whatman #1 filter paper each time before use.

Acid Alcohol, 0.5% Solution

Hydrochloric acid, concentrated	2.5 mL
Alcohol, 70%	497.5 mL

Methylene Blue Solution

Methylene blue	0.25 g
Distilled water	100 mL
Acetic acid, glacial	1 mL

■ *Procedure*

1. Deparaffinize and hydrate the slides to Millipore-filtered water.
2. Place the slides in carbol fuchsin in a glass Coplin jar, and microwave at power level 1 (60W) for 1½ minutes. Dip the slides up and down several times, and allow them to remain in the warm solution for 15 minutes.
3. Wash well in running water to remove excess stain.

4. Decolorize with acid alcohol until sections are pale pink.
5. Wash in running water for 1 minute, and rinse in 2 changes of distilled water.
6. Counterstain with methylene blue solution for 15 seconds.
7. Rinse with 95% and absolute alcohol, 2 changes each.
8. Clear in 3 or 4 changes of xylene, and mount with synthetic resin.

■ *Results* **[i10.5]**
Acid-fast bacilli including

- *Mycobacterium avium intracellulare* Red
- Erythrocytes Pink
- Mast cells Blue
- Other tissue elements Pale blue

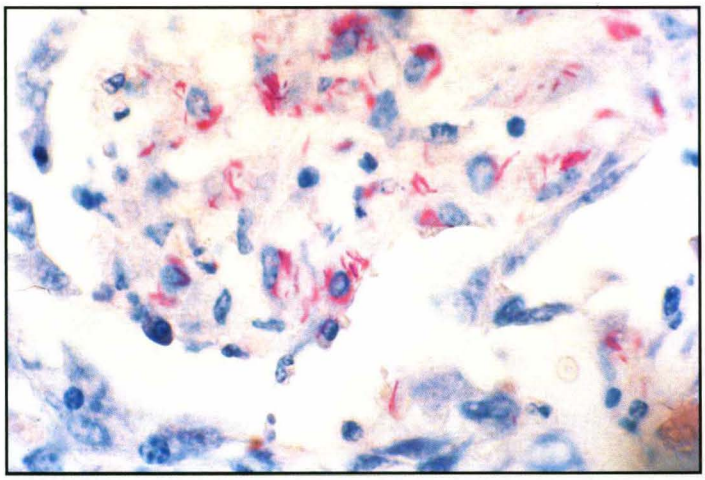
■ *Technical Notes*

1. Churukian [1993] states that carbol-fuchsin prepared with pararosaniline (CI 42500) rosaniline (CI 42510), magenta II, new fuchsin (CI 42529), or mixtures of these dyes will stain acid-fast organisms well with this procedure.
2. After trying varying amounts of microwave radiation, Churukian [1993] observed that the optimum staining results were obtained when the solution was heated as described.
3. To ensure that the temperature is equal throughout the solution, the slides must be dipped up and down after the solution is removed from the microwave oven.
4. If desired, the stain may be performed at room temperature by staining the sections for 30 minutes in the carbol-fuchsin solution [Churukian 1993].

FITE ACID-FAST STAIN FOR LEPROSY ORGANISMS [LUNA 1968]

■ *Purpose*
Detection of *Mycobacterium leprae* (causative organism of leprosy) in tissue sections

■ *Principle*
The lipoid capsule of the organism takes up carbol-fuchsin and resists decolorization with dilute mineral acid. See further discussion in the section on the Kinyoun method.



[i10.5] A section of lung containing acid-fast bacteria stained with the microwave Ziehl-Neelsen method. [Image courtesy of Churukian CJ, University of Rochester Medical Center]

■ *Fixative*
Although 10% neutral buffered formalin is preferred, others, with the exception of Carnoy solution, may be used.

■ *Equipment*
Coplin jars, Whatman #1 filter paper, Erlenmeyer flasks, graduated cylinders, pipettes

■ *Technique*
Cut paraffin sections at 4-5 µm.

■ *Quality Control*
Tissue containing leprosy organisms must be used for a control. Millipore-filtered water (see description of Kinyoun acid-fast stain) should be used in the flotation bath and a negative control from the same day's workload should be run (cut on the same microtome and using the same flotation bath, as used for the diagnostic case). Do not use tap or regular distilled water before applying the carbol-fuchsin; use only Millipore-filtered water.

■ *Reagents*

Xylene-Peanut Oil

Peanut oil	1 part
Xylene	2 parts

Acid Alcohol, 1% Solution

Hydrochloric acid, concentrated	10 mL
Alcohol, 70%	990 mL

Ziehl-Neelsen Carbol-Fuchsin Solution

Phenol crystals, melted	5 mL
Alcohol, absolute	10 mL
Basic fuchsin	1 g
Distilled water	85 mL

Stir on a mechanical stirrer. Filter before use each time. The solution keeps well at room temperature.

Methylene Blue Solution

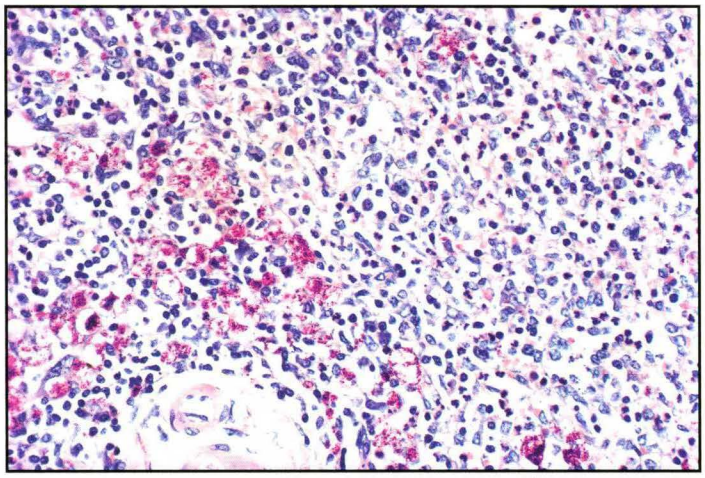
Methylene blue	0.5 g
Glacial acetic acid	0. 5 mL
Tap water	100 mL

■ *Procedure*

1. Deparaffinize sections with two 12-minute changes of xylene-peanut oil mixture.
2. Drain sections, wipe off excess oil, and blot to opacity. The residual oil helps to prevent shrinkage and injury of the sections.
3. Stain sections in freshly filtered Ziehl-Neelsen carbol-fuchsin solution for 20-30 minutes at room temperature. This solution may be saved for reuse.
4. Wash sections in running tap water.
5. Differentiate slides individually with 1% acid alcohol until the sections are faint pink.
6. Wash in tap water.
7. Counterstain sections lightly with working methylene blue solution. Do not overstain; the sections should look sky-blue.
8. Rinse off excess methylene blue in tap water.
9. Blot sections and let stand for a few minutes to air-dry completely.
10. Mount air-dried sections with synthetic resin. Do not use alcohol and xylene.

■ *Results* **[i10.6]**

- *M leprae* and other acid-fast bacteria — Bright red
- Background — Light blue



[i10.6] This section of spleen, from the same block as that shown in **[i10.3]** has been stained by the Fite carbol-fuchsin method. Even at this lower magnification, it is obvious that many more organisms are demonstrated with this method.

■ *Technical Notes*

1. See the notes on the Kinyoun acid-fast technique.
2. This method is not as good as the Kinyoun or Ziehl-Neelsen procedures for mycobacteria other than *M leprae*.
3. For the demonstration of Nocardia species, use the following modification of the Fite method [Sheehan 1980]:
   a. Stain in carbol-fuchsin for 10 minutes (time is critical).
   b. Decolorize in 1% aqueous sulfuric acid for 5-10 minutes, agitating the slides frequently to remove background color.
   c. Wash well in tap water.
   d. Follow the remainder of the Fite procedure, beginning with step 7.
4. *M leprae* and *Nocardia* spp are weakly acid-fast and not alcohol-fast [Chandler 1995a], so alcohol must be avoided.
5. Acid-fastness of the leprosy organism is enhanced when the waxy capsule is protected by the mixture of peanut oil and xylene and by the avoidance of dehydrating solutions.
6. Why a short exposure to xylene for removal of paraffin has such an adverse effect on the leprosy organism while the prolonged expose to xylene during processing does not have the same effect is an interesting consideration [Stevens 1994]. According to Stevens, once the leprosy organism is adequately stained, it will resist decolorization as tenaciously as the tubercle bacillus does; instead, the problem may be one of resistance to uptake of the stain rather than retention.

MICROWAVE AURAMINE-RHODAMINE FLUORESCENCE TECHNIQUE [TRUANT 1962, CHURUKIAN 1991]

■ *Purpose*

Detection of *Mycobacterium tuberculosis* or other acid-fast organisms

■ *Principle*

The exact mechanism of this stain is unknown. Both of the dyes used are basic dyes that fluoresce at short wavelengths. Both dyes used in combination yield better staining than either dye used alone.

■ *Fixative*

10% neutral-buffered formalin is preferred.

■ *Equipment*

Coplin jars, Whatman #4 filter paper, graduated cylinders, Erlenmeyer flasks, pipettes

■ *Technique*

Cut paraffin sections at 4-5 µm.

■ *Quality Control*

Tissue containing acid-fast mycobacteria must be used for control. Millipore-filtered water should be used in the flotation bath, and a negative control from the same day's workload should be run (cut on the same microtome and using the same flotation bath, as used for the diagnostic case). Do not use tap or regular distilled water before applying the auramine-rhodamine; use only Millipore-filtered or sterile distilled water.

■ *Reagents*

Auramine O-Rhodamine B Solution

Auramine O (CI 41000)	0.45 g
Rhodamine B (CI 45170)	0.03 g
Glycerol	90 mL
Phenol, melted crystals	12 mL
Sterile distilled or Millipore-filtered water	60 mL

Rinse all glassware used in the preparation of this solution in sterile distilled or Millipore-filtered water. Combine the liquids in a 250-mL flask, and add the dyes to the solution. Place on a hot plate stirrer, apply gentle heat, and allow to mix for about 30 minutes. Filter through Whatman #4 filter paper while warm and before use. Store the solution at room temperature.

Acid Alcohol, 0.5% Solution

Hydrochloric acid, concentrated	5 mL
Alcohol, 70%	995 mL

Use sterile distilled or Millipore-filtered water for preparation of the 70% alcohol

Eriochrome Black T, 0.3% Solution

Eriochrome black T	0.3 g
Sterile distilled or Millipore-filtered water	100 mL

■ *Procedure*

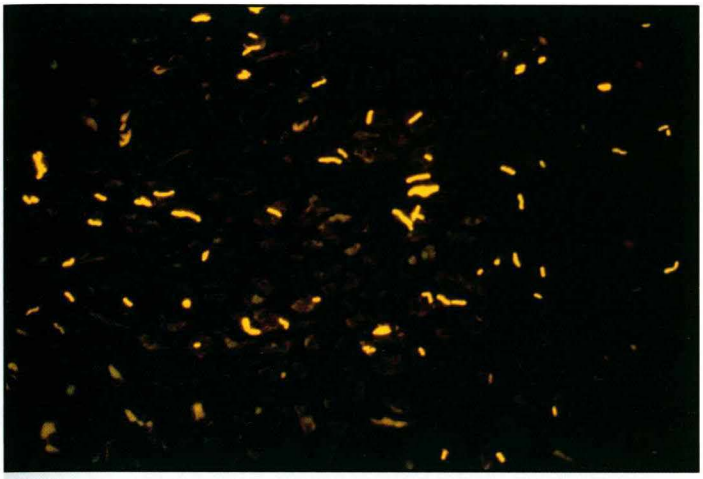
1. Deparaffinize and hydrate sections to sterile distilled or Millipore-filtered water.
2. Place the slides in 45 mL of the auramine O-rhodamine B solution in a glass Coplin jar, and microwave at power level 1 (60 W) for 4 minutes. Dip the slides up and down several times, and allow them to remain in the hot solution (80°C) for 3 minutes. Discard used solution.
3. Rinse in 3 changes of sterile distilled or Millipore-filtered water.
4. Differentiate sections in 2 changes of acid alcohol, 1½ minutes in each change.
5. Rinse in 4 changes of distilled water.
6. Stain in 0.3% eriochrome black T for 15 seconds.
7. Rinse in 3 changes of distilled water.
8. Stand slides on end, and thoroughly air dry.
9. Dip in xylene, and mount with synthetic resin.
10. Examine sections with a high-dry objective, a UG 1 or UG 2 exciter filter, and a colorless UV barrier filter.

■ *Results* [i10.7]

- Acid-fast organisms — Reddish-yellow fluorescence
- Background — Black

■ *Technical Notes*

1. This is an extremely sensitive and highly specific method for mycobacteria; however, there is an increased possibility of obtaining false positives, particularly with relatively inexperienced microscopists.



[i10.7] A section of lung containing mycobacteria stained with the Churukian modification of the Truant auramine-rhodamine fluorescence technique. This is a modification using the microwave oven. [Image courtesy of Churukian CJ, University of Rochester Medical Center]

2. Slides stained with the auramine O-rhodamine B method can be restained with carbol-fuchsin for confirmation if the results are questionable; however, carbol-fuchsin stained slides cannot be restained with auramine O-rhodamine B.

3. This method is more likely to stain dead and dying organisms than the carbol-fuchsin methods [Churukian 1991].

4. Churukian [1991] has greatly reduced the concentration of auramine O and rhodamine B in the staining solution. He found that it is especially important to use only a small amount of rhodamine B because, although it is a fluorochrome, it can act to quench fluorescence even in low concentrations. Reducing the concentration of the rhodamine B greatly intensifies the fluorescence of mycobacteria; however, even the small amount changes the fluorescence from the yellow given by the auramine O alone to orange-yellow and also gives a more intense fluorescence than auramine O alone.

5. Churukian [1991] uses glass Coplin jars for microwaving because they are easier to clean and they do not usually break when heated slowly as described.

6. Fluorescence microscopy is not satisfactory following fixation in solutions containing heavy metals because the primary fluorescence of the specimen may be quenched [Thompson 1966], so this procedure should be used with caution if zinc formalin solution is used for fixation [Guibord 1995].

BROWN-HOPPS MODIFICATION OF THE GRAM STAIN [BROWN 1978, CARSON 1984]

■ *Purpose*

Demonstration of gram-negative and gram-positive bacteria in tissue

■ *Principle*

Crystal violet is applied first and then followed by an iodine mordant forming a dye lake. At this point, both gram-negative and gram-positive organisms are stained. Although both types of bacteria have cell walls composed of peptidoglycan and lipoprotein, the cell walls of gram-positive bacteria are thicker (15-25 nm) than those of gram-negative organisms (8-12 nm). Gram-negative bacteria contain irregular layers of lipoprotein and fewer peptidoglycan layers, whereas gram-positive organisms contain up to 25 layers of peptidoglycan in the outer lipoprotein membranes. These differences in the cell wall account for differences in the way that bacteria will decolorize in the next procedural step. The large crystal violet-iodine molecular complex cannot easily be washed out of the intact peptidoglycan layers of gram-positive cells; however, it is easily removed from gram-negative bacteria, because alcohol or acetone disrupts the outer lipoprotein layer, and the remaining thin peptidoglycan cell wall cannot retain the complex. Gram-positive cell walls will retain the crystal violet-iodine complex, unless the cell walls have been damaged or disrupted for some other reason (old or dead organisms). If the cell wall of a normally gram-positive organism is damaged, the organism will then stain gram-negative. The decolorization step is a relative one, and sections can be overdecolorized, removing stain from both gram-negative and gram-positive organisms. After decolorization, a counterstain is applied to color the gram-negative organisms.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

Coplin jars, blotting paper, Erlenmeyer flasks, graduated cylinders

■ *Technique*

Cut paraffin sections at 4-5 µm.

■ *Quality Control*

Sections containing both gram-positive and gram-negative organisms should be used.

■ *Reagents*

Crystal Violet, 1% Solution

Crystal violet	5g
Distilled water	500 mL

Gram Iodine

Iodine	3 g
Potassium iodide	6 g
Distilled water	900 mL

Place the iodine and potassium iodide in approximately 150 mL of the water. Stir until completely dissolved, then add the remaining water.

Basic Fuchsin Solution

Basic fuchsin	0.1 g
Distilled water	100 mL

Gallego Solution

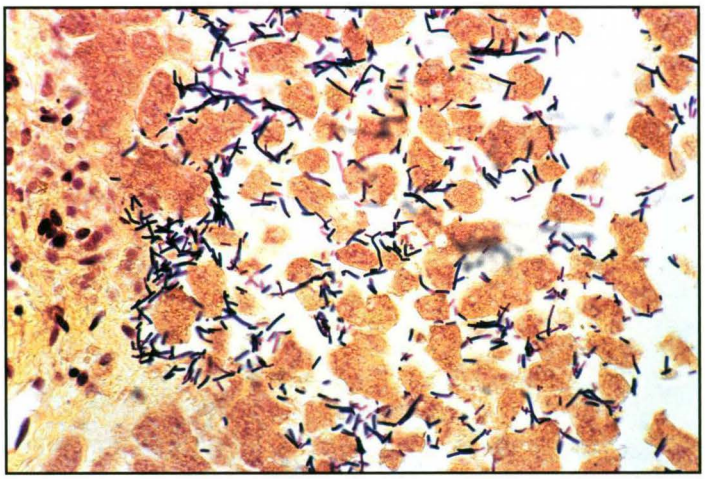
Distilled water	50 mL
Formalin, 37% to 40%	1 mL
Glacial acetic acid	0.5 mL

Picric Acid-Acetone Solution

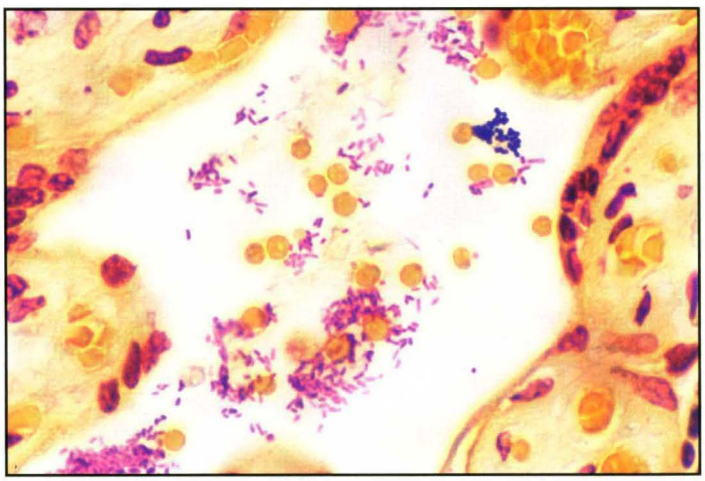
Picric acid	0.5 g
Acetone	1,000 mL

■ *Procedure*

1. Deparaffinize and hydrate sections to distilled water.
2. Stain sections with crystal violet for 2 minutes.
3. Rinse slides in distilled water.
4. Stain slides with gram iodine for 5 minutes.
5. Rinse slides in distilled water to remove excess iodine.
6. Blot 1 slide at a time with slightly damp filter paper, and decolorize quickly in acetone.
7. Rinse slides quickly but thoroughly in distilled water.
8. Stain sections with working basic fuchsin for 5 minutes.
9. Rinse slides in distilled water.
10. Differentiate sections with Gallego solution for 5 minutes.
11. Rinse slides in distilled water and blot sections, but do not blot to dryness.
12. Quickly dip slides in acetone 3 times.
13. Quickly dip slides in picric acid-acetone 3 times.
14. Quickly dip slides in acetone 3 times.
15. Pass slides through acetone-xylene mixture (1:2) for 5 quick dips, and then clear with 2 changes of xylene.
16. Mount with synthetic resin.



[i10.8] A control section stained with the Brown and Hopps modification of the Gram stain. Gram-positive organisms (blue-black bacilli) are seen in this section of necrotic liver.



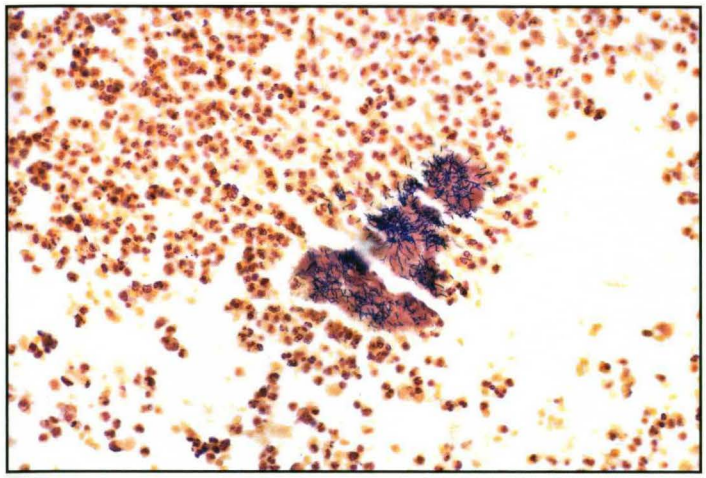
[i10.9] Red-stained, gram-negative bacilli and blue-stained, gram-positive cocci (upper right) are seen in this section of placenta stained with the Brown and Hopps modification of the Gram stain. This control was prepared by injecting fresh placental tissue with an inoculum of *Staphylococcus aureus* and *Escherichia coli* and allowing it to incubate overnight before fixation and processing.

■ *Results* [i10.8], [i10.9]

• Gram-positive bacteria	Blue
• Gram-negative bacteria	Red
• Background tissue generally	Yellow
• Nuclei	Light red

■ *Technical Notes*

1. This modification of the Brown and Brenn Gram stain is the preferred stain for gram-negative organisms and rickettsiae, but the original Brown and Brenn procedure is preferred for demonstrating gram-positive bacteria [Chandler 1995a]. Chandler also states that the depth of staining of weakly gram-negative organisms can be intensified by increasing the concentration of basic fuchsin from 0.1% to 1.0%.



[i10.10] Blue-stained gram-positive *Actinomyces* organisms are seen in this section stained with the Brown and Hopps modification of the Gram stain.

2. Luna [1992] found this a useful method for screening for the infectious agents causing actinomycosis **[i10.10]**, nocardiosis, coccidiomycosis, blastomycosis, cryptococcosis, aspergillosis, rhinosporidiosis, and amebiasis.

3. The picric acid-acetone decolorizes sections better if the picric acid used to prepare the reagent is nearly anhydrous. Slightly more than the required weight of picric acid can be dehydrated by placing it in a watch glass or glass Coplin jar lid in a desiccator overnight. Handle carefully and return any excess to the stock bottle for rehydration. Remember that picric acid containing less than 10% water is explosive.

4. Sections should not be allowed to dry at any stage of the procedure, because drying leads to the formation of insoluble compounds that are difficult or impossible to decolorize with picric acid-acetone.

5. Brown and Hopps applied the reagents in steps 2, 4, 8, and 10 to slides that are lying flat; the other steps are done in Coplin jars.

6. If picric acid cannot be stocked in your laboratory as a dry reagent, the following steps may be substituted [Ott 1987]:

   Step a. Immerse slides in 95% ethyl alcohol for 2-3 minutes.

   Step b. Immerse slides in saturated aqueous picric acid (may be obtained commercially) for 13-15 minutes.

   Step c. Dehydrate and clear as follows: 95% ethyl alcohol, 4 quick dips; 100% ethyl alcohol, 2 changes, 8-10 dips each; and xylene, 2 changes, 10 dips each.

   Step d. Mount with synthetic resin.

7. Gram-positive organisms most likely will not stain correctly if the patient is taking antibiotics, because antibiotics compromise the cell walls.

GIEMSA METHODS

Giemsa stains can be used for the demonstration of bacteria, rickettsias, and *T gondii*. The May-Grunwald Giemsa stain procedure can be found in chapter 6, "Nuclear and Cytoplasmic Staining," p127.

MODIFIED DIFF-QUIK GIEMSA STAIN FOR *HELICOBACTER PYLORI* [POTVIN 1994]

■ *Purpose*
Identification of *H pylori* in tissue sections

■ *Principle*
The Romanowsky stains, "neutral" dyes combining the basic dye methylene blue and the acid dye eosin, give a wide color range when staining tissues and blood smears. This is because of impurities present in the actual dye solution. On standing in solution, particularly at an alkaline pH, methylene blue gives rise to new substances that are metachromatic; however, today most commercial solutions are prepared with weighed amounts of the azures, and most omit methyl violet.

■ *Fixative*
10% neutral-buffered formalin

■ *Equipment*
Coplin jars, Erlenmeyer flasks, graduated cylinders, pipettes

■ *Technique*
Cut paraffin section at 4-5 µm.

■ *Quality Control*
Sections containing *H pylori*.

■ *Reagents*

Diff-Quik Solution I (Baxter Healthcare Corporation)

1 g/L xanthene dye, 100% pure dye content, buffer and sodium azide (0.01%) as preservative. Keep in a tightly capped Coplin jar at room temperature, and discard if any microbial growth is observed. Filter occasionally.

Diff-Quik Solution II (Baxter Healthcare Corporation)

1.25 g/L thiazine dye mixture, 100% pure dye content (0.625 g/l azure A, and 0.625 g/L methylene blue) and buffer. Keep in a tightly capped Coplin jar at room temperature, and discard by the expiration date on the box label. Filter occasionally.

Acetic Acid Water

Glacial acetic acid	1 mL
Distilled water	400 mL

■ *Procedure*

1. Deparaffinize sections in xylene and hydrate with 2 changes of absolute alcohol, 2 changes of 95% alcohol, and 1 change of 70% alcohol, to distilled water. Carry slides 1 at a time through the remainder of the procedure.
2. Dip the slide in Diff-Quik Solution I, 25 dips.
3. Dip slide in Diff-Quik Solution II, 25 dips
4. Rinse quickly in distilled water.
5. Differentiate in 2 changes of acetic water, 5 dips in each.
6. Rinse quickly in distilled water. Check microscopically. *H pylori* and nuclei should be dark blue, cytoplasm should be pink. If greater enhancement of the stain is desired, steps 2-6 can be repeated.
7. Dehydrate in 1 change of 95% alcohol, 15 quick dips.
8. Continue dehydration with 1 change of absolute alcohol, 15 quick dips.
9. Clear in xylene, and mount with synthetic resin.

■ *Results* **[i10.11]**

• *H pylori*	Dark blue
• Other bacteria	Blue
• Nuclei	Dark blue
• Cytoplasm	Pink

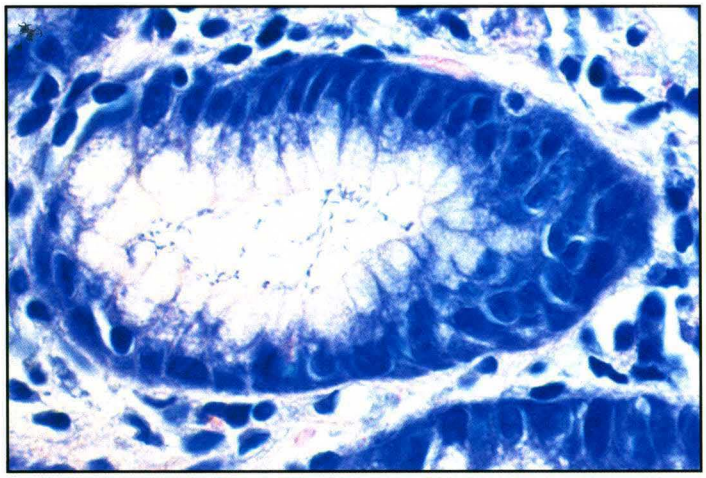
■ *Technical Notes*

1. Diff-Quik Solution I is a buffered solution of eosin Y (an anionic dye, which stains the cytoplasmic elements pink).
2. Diff-Quik Solution II is a cationic dye mixture of azure A and methylene blue, which stains the nuclei and bacteria blue.
3. Do not prolong the time in the last distilled water rinse or in the dehydrating alcohols, or it will lead to excess decolorization.
4. *H pylori* are associated with gastritis and peptic ulcer disease. More importantly, recent data suggest that infection with this organism is a significant risk factor for the development of gastric carcinoma and lymphoma.

ALCIAN YELLOW-TOLUIDINE BLUE METHOD FOR *H PYLORI* [LEUNG 1996]

■ *Purpose*

Detection of *H pylori* in tissue sections



[i10.11] Many bacilli consistent with *Helicobacter pylori* are seen in the Diff-Quik-stained section of a gastric biopsy. [Image courtesy of Potvin CA, Windsor]

■ *Principle*

Alcian yellow is a monoazo dye that reacts similar to alcian blue, staining mucin yellow. Toluidine blue is a basic dye and metachromatic stain that stains the *H pylori* organisms and nuclei blue.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

Coplin jars, Erlenmeyer flasks, graduated cylinders, pipettes

■ *Technique*

Cut paraffin section at 4-5 µm.

■ *Quality Control*

Sections containing *H pylori*

■ *Reagents*

Periodic acid, 1% Solution

Periodic acid	1 g
Distilled water	100 mL

Alcian Yellow Solution

Alcian yellow	1 g
Ethanol, 50%	100 mL
Acetic acid, glacial	3 mL
Filter before use	

Sodium Metabisulfite, 5% Solution

Sodium metabisulfite	5 g
Hydrochloric acid, 1N	1 mL
Distilled water	100 mL

Toluidine Blue Stain

Toluidine blue, 1% aqueous	0.5 mL
Sodium hydroxide, 3% aqueous	2 drops
Distilled water	50 mL
Prepare fresh	

■ *Procedure*

1. Deparaffinize and hydrate sections to distilled water.
2. Oxidize sections in 1% periodic acid for 10 minutes.
3. Wash well with water.
4. Place sections in sodium metabisulfite solution for 5 minutes.
5. Wash in running water for 2 minutes.
6. Stain with alcian yellow for 5 minutes.
7. Wash well with water.
8. Stain with toluidine blue solution for 3 minutes.
9. Wash well with water.
10. Blot sections dry.
11. Dehydrate, clear, and mount with synthetic resin.

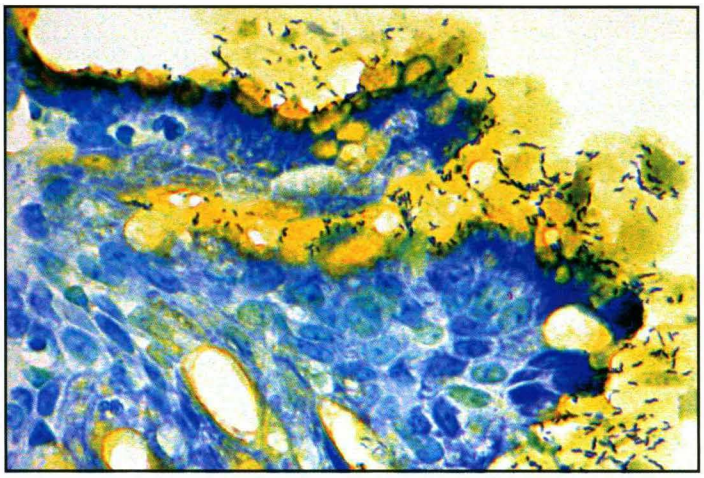
■ *Results* [i10.12]

• *H pylori* organisms	Blue
• Mucin	Yellow
• Background	Pale blue

HOTCHKISS-MCMANUS PAS REACTION FOR FUNGI [KLIGMAN 1951]

■ *Purpose*

Demonstration of fungi in tissue



[i10.12] Numerous dark blue organisms are noted in this alcian yellow-toluidine blue technique for *Helicobacter pylori*. Mucin is stained yellow, and cell nuclei are stained blue. [Image courtesy of Lott R, Birmingham, AL]

■ *Principle*

The principle of this stain is similar to that described in chapter 7, "Carbohydrates and Amyloid," p137. Polysaccharides present in the fungal cell walls are oxidized by the periodic acid to aldehydes. The aldehydes react with Schiff reagent to yield rose-colored fungi.

■ *Fixative*

10% neutral-buffered formalin, Bouin solution, or Zenker solution

■ *Equipment*

Hot plate, Coplin jars, Whatman #1 filter paper, Erlenmeyer flasks, graduated cylinders

■ *Technique*

Cut paraffin sections at 4-5 µm.

■ *Quality Control*

A section containing fungi must be used for a control.

■ *Reagents*

Periodic Acid, 1% Solution

Periodic acid	5 g
Distilled water	500 mL

Schiff Reagent

See the PAS procedure in chapter 7, p138.

1N Hydrochloric Acid

Hydrochloric acid, concentrated	83.5 mL
Distilled water	916.5 mL

Sodium Metabisulfite, 10% Solution

Sodium metabisulfite	10 g
Distilled water	100 g

Sulfurous Acid Rinse Solution

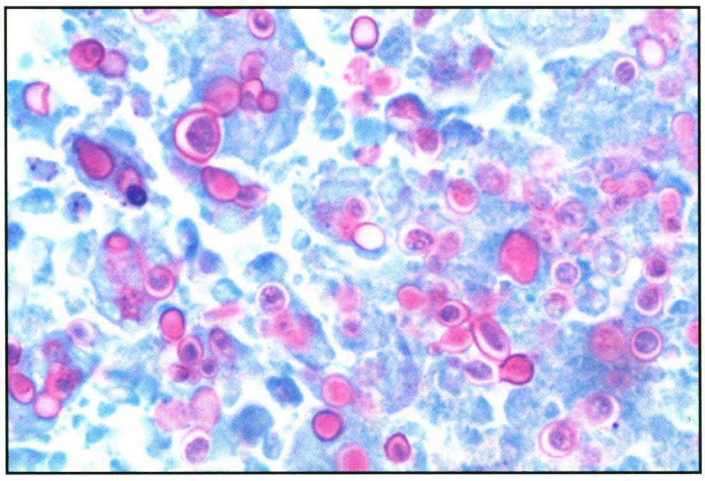
Distilled water	300 mL
1 N hydrochloric acid	15 mL
Sodium metabisulfite, 10% solution	18 mL
Prepare fresh just before use	

Fast Green, 1:5000 Solution

Fast green FCF	0.2 g
Distilled water	1,000 mL
Glacial acetic acid	2.5 mL

■ *Procedure*

1. Deparaffinize and hydrate slides to distilled water.
2. Place sections in 1.0% periodic acid solution for 5 minutes.
3. Wash slides in 3 changes of distilled water.
4. Place sections in Schiff reagent for 15 minutes.
5. Rinse sections in three 2-minute changes of sulfurous acid rinse solution.
6. Wash in running tap water for 10 minutes to develop full color.
7. Counterstain in fast green solution for 1 minute.
8. Rinse in distilled water.
9. Dehydrate with 95% and absolute alcohols, clear with xylene, and mount with synthetic resin.



[i10.13] Fungi in a section of lung stained with the PAS technique. Fungi are better demonstrated if a light green or fast green counterstain is used and the hematoxylin is omitted.

■ *Results* **[i10.13]**

• Fungi	Rose
• Background	Green

■ *Technical Notes*

1. Notes on the PAS procedure can be found in chapter 7, p137.
2. When staining for fungi, the green counterstain provides a better contrast, without masking organisms, than does hematoxylin. Light green (used in the Grocott methenamine-silver nitrate fungus stain) may also be used as a counterstain.
3. In all of the methods for fungi, it might be helpful to use diastase digestion on sections containing glycogen (eg, liver; see chapter 7, p139).
4. The oxidizing agent and the Schiff reagent must not be overused or poor stains will result **[i10.14]**. It is best if fresh oxidizing agent is used each time.

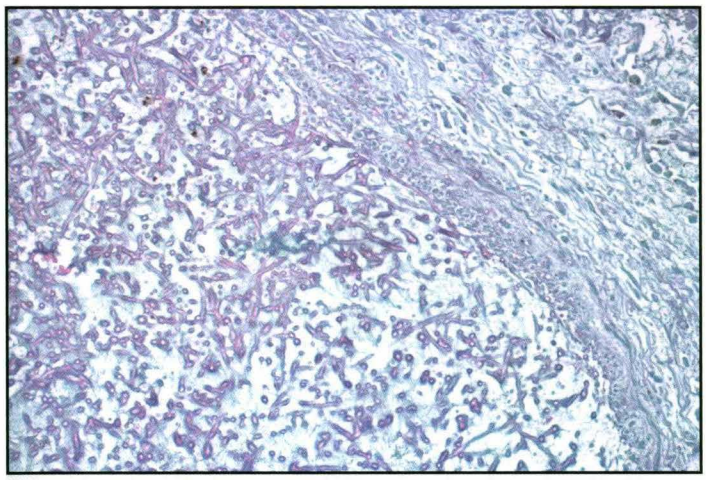
CHROMIC ACID-SCHIFF STAIN FOR FUNGI (CAS) [DELLA SPERANZA 2005]

■ *Purpose*

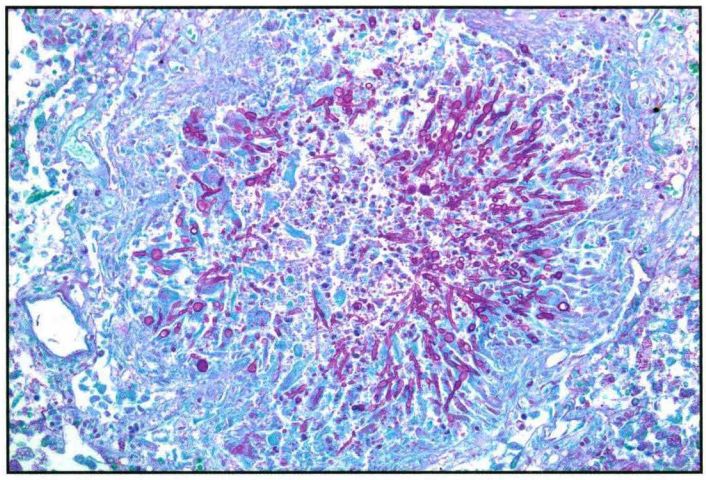
Identification of fungi in tissue

■ *Principle*

Chromic acid oxidizes the carbohydrates present in the fungal cell walls to aldehydes. Chromic acid is a strong oxidizer, and when given time it will eliminate reactive aldehydes in all but the structures with the greatest concentration of carbohydrates; this includes mucin, glycogen, and fungal cell walls. A cleaner background is provided with chromic acid oxidization than with periodic acid oxidation **[i10.15]**.



[i10.14] The fungi are barely visible in this PAS-stained section. Most likely, either the periodic acid or the Schiff reagent, or both, have been overused. This slide is unacceptable; fresh reagents should be prepared and the stain repeated.



[i10.15] The fungi in this section have been stained with the CAS technique (chromic acid-Schiff). The stronger oxidizing action of chromic acid will yield less nonspecific background staining. A light green counterstain was applied. [Image courtesy of Lott R, Birmingham, AL]

■ *Fixative*
10% neutral-buffered formalin

■ *Technique*
Cut paraffin sections at 4-5 µm.

■ *Quality Control*
Sections containing fungi must be used as a control.

■ *Reagents*

Chromic acid, 5% Solution

Chromium trioxide	5 g
Distilled water	100 mL

1N Hydrochloric acid

Hydrochloric acid, concentrated	83.5 mL
Distilled water	916.5 mL
Add the acid to the water, and mix well	

Schiff Reagent

Basic fuchsin	4.0 g
Distilled water	800 mL
Sodium metabisulfite	4 g
1N hydrochloric acid	80 mL
Heat water to boiling, then remove from heat, and add the basic fuchsin. Reheat to the boiling point. Cool the solution to 50°C, and then filter. Add 80 mL of 1N hydrochloric acid, cool completely, and add 4.0 g sodium metabisulfite. Let the solution stand in the dark overnight; it should turn light amber. Add 2.0 g of activated charcoal, and shake for 1 minute. Filter the solution, and store in the refrigerator.	

Sulfurous Acid Rinse Solution

Distilled water	300 mL
1 N hydrochloric acid	15 mL
Sodium metabisulfite, 10% solution	18 mL
Prepare fresh just before use	

Fast Green, 1:5000 Solution

Fast green FCF	0.2 g
Distilled water	1,000 mL
Glacial acetic acid	2.5 mL

■ *Procedure*

1. Deparaffinize and hydrate slides to distilled water.
2. Place sections in the 5% chromic acid solution for 8-10 minutes at 60°C or 1 hour at room temperature.
3. Wash slides in 3 changes of deionized water.

4. Place the sections in Schiff reagent for 15 minutes at room temperature.

5. Rinse sections in three 2-minute changes of sulfurous acid rinse solution.

6. Wash in running tap water for 15 minutes to develop full color

7. Counterstain in Harris hematoxylin for ½ minute or in 1:5,000 fast green for 1 minute.

8. Wash sections well. If hematoxylin is used, sections should be blued briefly in ammonia water and then rewashed.

9. Dehydrate with 95% and absolute alcohols, clear with xylene, and mount with synthetic resin.

■ *Results* **[i10.15]**

• Fungi	Deep rose to purple
• Nuclei if hematoxylin is used	Blue
• Background if fast green is used	Green

■ *Technical Notes*

1. Be sure that the Schiff reagent comes to room temperature before use.

2. Insufficient oxidation with chromic acid may lead to increased background staining; prolonged oxidation may cause reduced staining of fungal organisms, so care must be taken to carefully control the oxidation step.

3. Be sure that the chromic acid has not darkened because of reduction with alcohol remaining from the rehydration step; wash thoroughly after the alcohol.

4. The sulfurous acid rinse removes any unbound leucofuchsin after exposure to the Schiff reagent.

5. Chromic acid is highly toxic, and a confirmed carcinogen, so all skin contact should be avoided along with inhalation of any solid particles. It is an oxidizer and may explode on contact with reducing agents. Chromium compound cannot be put down drains, but must be collected and disposed of by a licensed hauler.

GRIDLEY FUNGUS STAIN [LUNA 1968]

■ *Purpose*
Demonstration of of fungi in tissue.

■ *Principle*
This is a modification of the Bauer technique, which uses chromic acid to oxidize adjacent glycol groups to aldehydes. The aldehydes are then reacted with Schiff reagent. Because chromic acid is a stronger oxidizing agent than periodic acid, it further attacks and destroys aldehydes, so fewer reactive groups are left to react with the Schiff reagent. A less intense reaction is obtained than with the PAS technique, but background staining is also decreased. The aldehyde fuchsin acts as an aldehyde and occupies uninvolved linkages of the Schiff reagent, thus reinforcing the depth of the stain.

■ *Fixative*
10% neutral-buffered formalin

■ *Equipment*
Coplin jars, Erlenmeyer flasks, graduated cylinders, pipettes

■ *Technique*
Cut paraffin section at 4-5 µm.

■ *Quality Control*
Sections containing fungi must be used.

■ *Reagents*

Chromic Acid, 4% Solution

Chromium trioxide	4 g
Distilled water	100 mL

Schiff Reagent

See CAS procedure previously described

Aldehyde Fuchsin Solution

Pararosaniline	1 g
Alcohol, 70% ethyl	200 mL
Hydrochloric acid, concentrated	2 mL
Paraldehyde	2 mL

Let stand at room temperature for 2-3 days or until the solution is deep purple. Filter and store in the refrigerator.

Metanil Yellow, 0.25% Solution

Metanil yellow	0.25 g
Distilled water	100 mL
Glacial acetic acid	0.25 g

■ *Procedure*

1. Deparaffinize the sections, and hydrate to distilled water.

2. Oxidize sections in 4% chromic acid for 1 hour.

3. Wash slides in running water for 5 minutes.

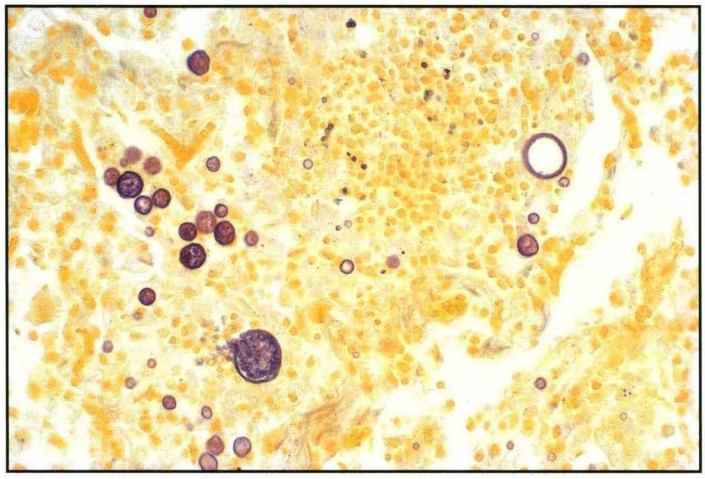
4. Stain sections in Schiff reagent for 15 minutes.
5. Wash slides in running water for 15 minutes.
6. Rinse in several changes of 70% alcohol.
7. Stain sections in aldehyde fuchsin for 30 minutes.
8. Rinse off excess stain with 95% alcohol.
9. Rinse slides in distilled water.
10. Counterstain sections with metanil yellow solution for 30 seconds to 1 minute. Do not overstain.
11. Rinse slides in distilled water.
12. Dehydrate in 2 changes each of 95% and absolute alcohol, clear in xylene, and mount with synthetic resin.

■ *Results* **[i10.16], [i10.17]**

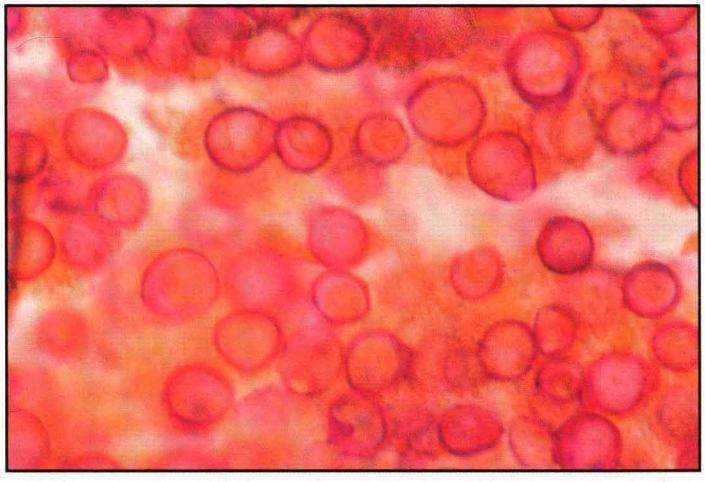
• Mycelia	Deep purple
• Conidia	Deep rose to purple
• Background	Yellow
• Elastic fibers and mucin	Deep purple

■ *Technical Notes*

1. Very old, nonviable fungi are not as well stained with this technique as with the Grocott methenamine-silver method.
2. If desired, sulfurous acid rinses may be used after the Schiff reagent.
3. Pararosaniline (CI 42500), not basic fuchsin (rosaniline, CI 42510), should be used to prepare the aldehyde fuchsin.
4. Chromic acid is a stronger oxidizing agent than periodic acid. It oxidizes all 1,2 glycols to aldehydes and then oxidizes some of the aldehydes further to acids, especially where trace amounts of polysaccharides are present. While chromic acid continues further oxidation of all polysaccharides, complete conversion to acids in the areas of heaviest concentration takes longer, so that these remain reactive with Schiff reagent (or silver). Tissue components with heavy polysaccharide concentrations (mucin, glycogen, fungi) will continue to show a positive Schiff reaction long after basement membranes and connective tissue become nonreactive. Periodic acid does not oxidize aldehydes to acids so that many more tissue components remain reactive with Schiff reagent and with silver methods that detect aldehyde groups.
5. Paraldehyde is very hazardous in case of ingestion or inhalation. Exposure limits are not available, but gloves, laboratory coat, and splash goggles should be worn when handling this chemical. Chromic acid is also highly toxic, and a confirmed carcinogen, so all skin contact should be avoided along with inhalation of any solid particles. It is an oxidizer and may explode on contact with reducing agents. Chromium compounds cannot be put down drains, but must be collected and disposed of by a licensed hauler.



[i10.16] *Coccidioides immitis* demonstrated with the Gridley technique. Note the spherule containing endospores in the top of the photograph. An empty spherule can be seen on the right.



[i10.17] *Blastomyces dermatitidis* stained with the Gridley technique, and viewed with the oil-immersion objective.

GROCOTT METHENAMINE-SILVER NITRATE FUNGUS STAIN [GROCOTT 1955, LUNA 1968]

■ *Purpose*

Demonstration of fungal organisms in tissue sections

■ *Principle*

Polysaccharides in the fungal cell wall are oxidized to aldehydes by chromic acid. Chromic acid is a strong oxidant, further oxidizing many of the newly released aldehyde groups to break down products that will not react; this helps suppress the weaker background reactions of collagen fibers and basement membranes. Only substances that possess large quantities of polysaccharides, such as fungal cell walls, glycogen, and mucins, will remain reactive with the methenamine-silver, reducing it to visible metallic silver. Methenamine gives the solution the alkaline properties necessary for proper reaction, and sodium borate acts as a buffer. Gold chloride is a toning solution and the sodium thiosulfate removes any unreduced silver.

■ *Fixative*

10% neutral-buffered formalin is preferred.

■ *Equipment*
56°C to 58°C water bath, chemically clean Coplin jars, graduated cylinders, Erlenmeyer flasks, pipettes

■ *Technique*
Cut paraffin sections at 4-5 µm or frozen sections at 6 µm.

■ *Quality Control*
A section containing fungi must be used; if staining for *Pneumocystis jirovecii*, use a *Pnuemocystis* control, because the timing in the methenamine-silver solution is different. Chemically clean glassware and nonmetallic forceps must be used.

■ *Reagents*

Chromic Acid, 5% Solution

Chromium trioxide	5 g
Distilled water	100 mL

Silver Nitrate, 5% Solution

Silver nitrate	5 g
Distilled water	100 mL

Methenamine, 3% Solution

Hexamethylenetetramine (methenamine)	27 g
Distilled water	900 mL

Borax, 5% Solution

Sodium borate	5 g
Distilled water	100 mL

Stock Methenamine-Silver Solution

Methenamine, 3% solution	900 mL
Silver nitrate, 5% solution	45 mL

A white precipitate will form but will immediately dissolve when the solution is shaken. The clear solution will remain usable for months if stored in a chemically clean amber bottle in the refrigerator.

Working Methenamine-Silver Nitrate Solution

Borax, 5% solution	2 mL
Distilled water	25 mL
Mix and add:	
Methenamine-silver nitrate stock solution	25 mL

Sodium Bisulfite, 1% Solution

Sodium bisulfate	10 g
Distilled water	1,000 mL

Gold Chloride, 0.1% Solution

Gold chloride, 1% solution	5 mL
Distilled water	45 mL

Sodium Thiosulfate, 2% Solution

Sodium thiosulfate	20 g
Distilled water	1,000 mL

Stock Light Green Solution

Light green SF (yellowish)	1 g
Distilled water	500 mL
Glacial acetic acid	1 mL

Working Light Green Solution

Light green stock solution	10 mL
Distilled water	50 mL

■ *Procedure*

1. Deparaffinize sections, and hydrate to distilled water.
2. Oxidize sections in chromic acid solution for 1 hour at room temperature or for 5-10 minutes in a solution preheated to 60°C. Begin preheating the silver solution about 20 minutes before needed. The chromic acid solution may be reused until it turns dark.
3. Wash slides in running tap water for a few seconds.
4. Rinse in 1% sodium bisulfite for 1 minute to remove any residual chromic acid.
5. Wash in tap water for 5-10 minutes.
6. Wash with 3 or 4 changes of distilled water.
7. Using nonmetallic forceps, place slides in preheated working methenamine silver solution in the water bath at 56°C to 58°C for 15 minutes or until sections turn yellowish brown (paper-bag brown). Remove the control, rinse in distilled water, and check microscopically for adequate silver impregnation. Fungi should be dark

brown at this stage. If impregnation is not sufficient, return the slide to the methenamine silver and check every 3-5 minutes.

8. Rinse slides in 6 changes of distilled water.

9. Tone in 0.1% gold chloride solution for 2-5 minutes. This solution may be used until brown precipitate appears and the solution is cloudy.

10. Rinse sections in distilled water.

11. Remove unreduced silver by placing the slides in 2% sodium thiosulfate solution for 2-5 minutes.

12. Wash thoroughly in tap water.

13. Counterstain with working light green solution for 1½ minutes.

14. Dehydrate with 2 changes each of 95% and absolute alcohols.

15. Clear with 2-3 changes of xylene, and mount with a synthetic resin.

■ *Results* **[i10.18]**

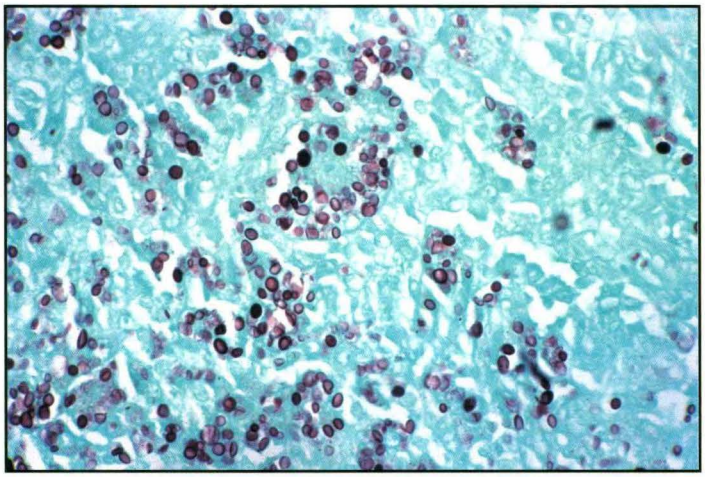
• Fungi	Cell walls should be crisp black, and the internal structures should be visible
• Mucin	Taupe to dark gray
• Background	Green

■ *Technical Notes*

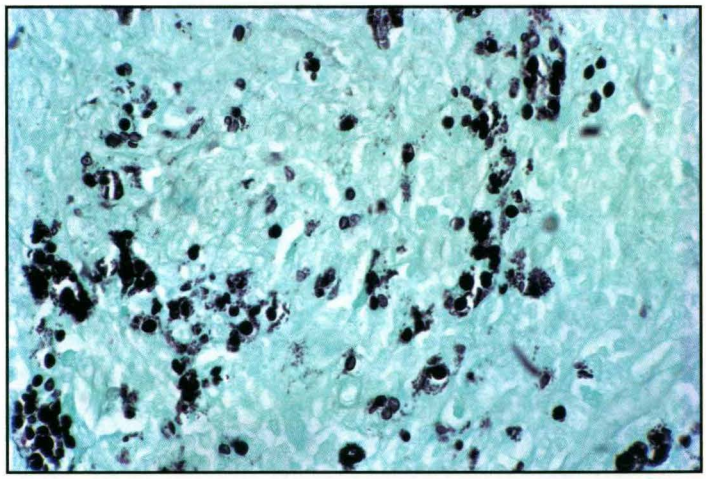
1. Failure to adequately remove the alcohol used during deparaffinization and hydration will result in reduction of the chromic acid solution; this will cause the color of the solution to change from orange to brown. The solution should be discarded when a color change is noted.

2. Usually *Pneumocystis jirovecii* takes slightly longer to become well stained than do the pathogenic fungi; therefore, for optimum staining, it is important to know which organism is suspected and to use the appropriate control. If unknown, use a *Pneumocystis* control. The fungi will be slightly overstained, but diagnostic. If the reaction is timed with a fungus control, no *Pneumocystis* organisms may be demonstrated. However, it is important that organisms not be overstained to the point of obscuring internal structures **[i10.19]**, **[i10.20]**.

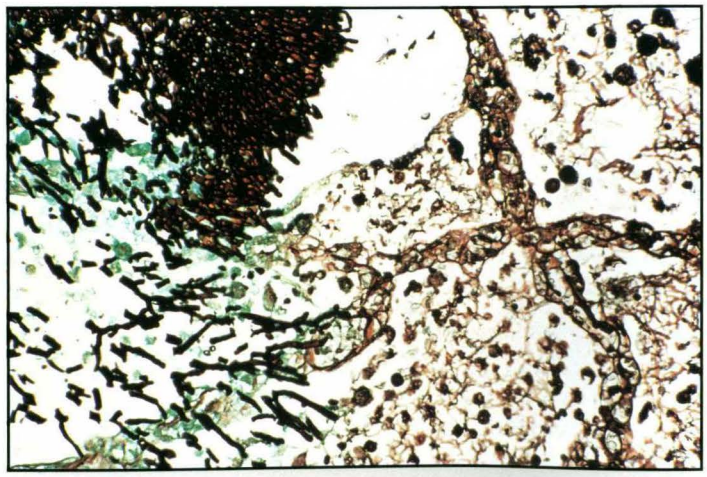
3. A water bath rather than an oven should be used for methenamine-silver staining. The solution reaches the ambient temperature in 20 minutes in a water bath, whereas it takes almost 1½ hours to reach the ambient temperature in a 60°C oven [Koski 1981].



[i10.18] *Histoplasma capsulatum* demonstrated with the Grocott methenamine-silver technique. This section was stained with the conventional method. *H capsulatum* forms oval budding cells that measure 2-4 µm in diameter. Note that the cell walls are stained black and the internal structure is visible in the organisms.



[i10.19] This section is a duplicate of the properly stained section shown in **[i10.18]**, but it has been overstained with the conventional Grocott methenamine-silver technique. Note that the organisms are well demonstrated in **[i10.18]** but that the morphology of the fungi is almost totally obscured and identification of the organisms is very difficult in **[i10.19]**.



[i10.20] This section has been both very overstained and poorly toned. There is marked nonspecific staining seen, and the internal structure of the organisms is impossible to see.

4. The silver should not be overheated, because it will lead to a breakdown of the solution and nonspecific staining.

5. In addition to fungi and *P jirovecii*, the Grocott methenamine-silver procedure will demonstrate *Actinomyces* and related species, *Nocardia asteroides*, and certain encapsulated bacteria [Swisher 1982, Chandler 1995b].

6. Because of the toxicity of chromic acid, there is a trend toward oxidation with periodic acid; however, we have found that false negatives, especially with *Histoplasma capsulatum*, may be obtained with this reagent and periodic acid oxidation should be used with caution [Carson 1999]. Because chromic acid is a stronger oxidizer than periodic acid, it has the added advantage of decreased staining of the connective tissue, thus producing a cleaner background. Only substances that possess large quantities of carbohydrates, such as the fungal cell walls, mucin, and glycogen, will remain active after chromic acid oxidation. Conversely, inadequate oxidation will cause reticulin fibers and basement membranes to stain strongly, and can mask the fungal organism when they are few in number.

7. Sections that have been stained using most other staining methods can be stained with the Grocott methenamine-silver stain; any existing stain will be removed by the chromic acid [Luna 1992].

8. Koski [1981] prepared the methenamine-silver by mixing just before use equal parts of solution I (2.1 g silver nitrate per liter) and solution II (27 g methenamine and 3.7g borax per liter). These stock solutions are more stable, contain less silver, and cause less nonspecific impregnation than the original preparations.

9. Glutaraldehyde fixative should be avoided, because the free aldehyde groups can reduce the silver and give nonspecific staining.

10. Koski [1981] stated that the methenamine-silver complex decomposes at higher temperatures as follows:

$$C_6H_{12}N_4 + H_2O \rightarrow H\text{-}CHO + NH_3$$

The formaldehyde formed by this reaction reduces silver nonselectively on tissue sections, on the sides of the container, and in the solution itself. Ideal impregnation occurs between 40°C and 50°C, but because most laboratories maintain their ovens at higher temperatures, the incubation has been done more frequently at 55°C or higher. Microwave impregnation also is done at higher temperatures, but because the sections are in the solution for less time at the higher temperatures, there is less opportunity for nonspecific reduction of silver.

MICROWAVE METHENAMINE-SILVER NITRATE PROCEDURE FOR FUNGI [BRINN 1983]

■ *Purpose*

The rapid method is most useful on cytospin preparations or on frozen sections for the diagnosis of *P jirovecii*, but is also good as a more routine procedure.

■ *Principle*

Refer to the routine Grocott methenamine-silver nitrate procedure.

■ *Fixation*

Cytospin preparations are fixed in 95% alcohol, frozen sections are fixed in 37% to 40% formaldehyde, and paraffin sections are fixed according to the routine Grocott methenamine-silver procedure.

■ *Equipment*

Microwave oven, vented plastic Coplin jars, chemically clean glass Coplin jars, graduated cylinders, Erlenmeyer flasks, pipettes

■ *Technique*

Frozen sections are cut at 6 µm, and paraffin sections are cut at 4-5 µm. Sections tend to wash off with this procedure, so special charged (+) slides should be used.

■ *Quality Control*

A section containing *Pneumocystis* organisms should be used when staining for *P jirovecii*; a section containing fungi should be used when staining for fungi. Chemically clean glassware and nonmetallic forceps must be used.

■ *Reagents*

Chromic Acid, 10% Solution

Chromium trioxide	10 g
Distilled water	100 mL

Silver Nitrate, 5% Solution

Silver nitrate	5 g
Distilled water	100 mL

Methenamine, 3% Solution

Hexamethylenetetramine (methenamine)	27 g
Distilled water	900 mL

Borax, 5% Solution

Sodium borate	5 g
Distilled water	100 mL

Stock Methenamine-Silver Solution

Methenamine, 3% solution:	900 mL
Silver nitrate, 5% solution:	45 mL

A white precipitate will form but will immediately dissolve when shaken. The clear solution will remain usable for months if stored in a chemically clean amber bottle in the refrigerator.

Working Methenamine-Silver Nitrate Solution

Borax, 5% solution	2 mL
Distilled water	25 mL
Mix and add	
Methenamine-silver nitrate stock solution	25 mL

Sodium Bisulfite, 1% Solution

Sodium bisulfate	1 g
Distilled water	100 mL

Gold Chloride, 0.2% Solution

Gold chloride, 1% solution	10 mL
Distilled water	40 mL

Sodium thiosulfate, 5% Solution

Sodium thiosulfate	5 g
Distilled water	100 mL

Stock Light Green Solution

Light green SF (yellowish)	0.2 g
Distilled water	100 mL
Glacial acetic acid	0.2 mL

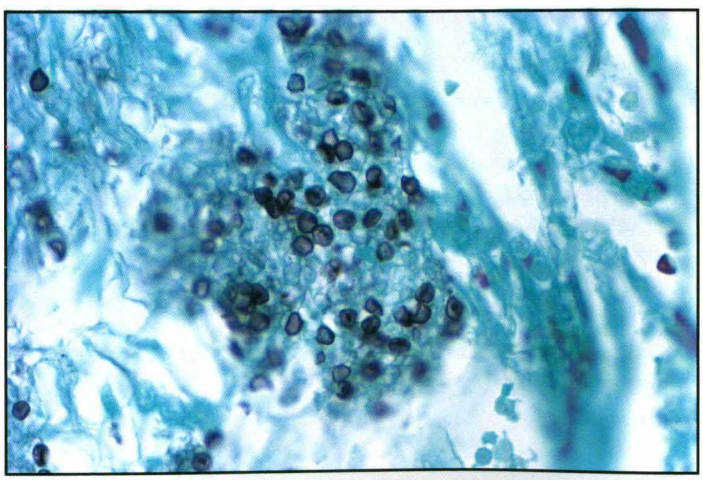
Working Light Green Solution

Light green stock solution	10 mL
Distilled water	50 mL

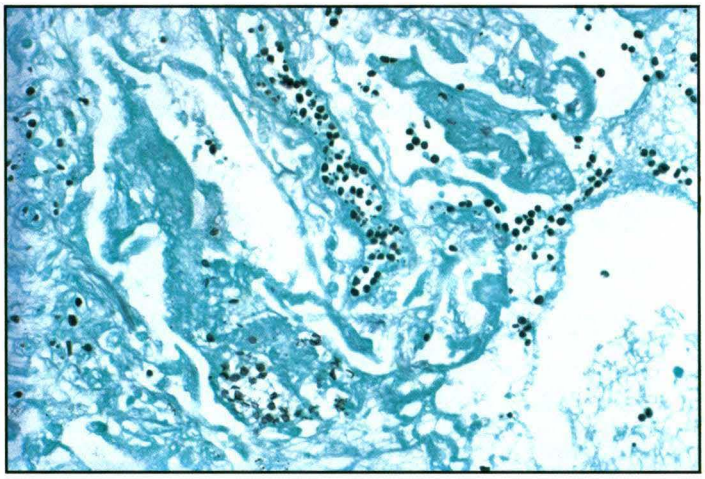
■ *Procedure*
This procedure must be followed exactly.

1. Deparaffinize sections if necessary, and hydrate to distilled water. Rinse well in distilled water.
2. Put 50 mL of 10% chromic acid into a vented plastic Coplin jar, screw the cap on loosely, and heat in the microwave at full power for 37 seconds (to approximately 70°C). Beware of fumes, because they are very caustic. Only a vented laboratory microwave should be used for this step. Remove the Coplin jar from the microwave. Pour the contents into an empty jar and then back into the original jar to mix. Immerse slides in the heated chromic acid, and let stand for 1 minute. This solution may be reused.
3. Rinse sections in distilled water to remove excess chromic acid.
4. Clear sections in 1% sodium bisulfite for 1 minute.
5. Rinse sections in 6 changes of distilled water.
6. Heat distilled water to 55°C for 27 seconds on full power in a vented Coplin jar. Place slides into heated distilled water.
7. Put working methenamine-silver solution into a vented plastic Coplin jar, screw the cap on loosely, and heat in the microwave oven at full power for 37 seconds (approximately 70°C). Remove and pour the solu tion into another Coplin jar, and then back into the original heated Coplin jar. Immerse slides and let stain for 2-6 minutes to let the silver develop. Check microscopically. Reheat the solution if needed.
8. Rinse the slides in 6 changes of distilled water.
9. Tone in 0.2% gold chloride for 45 seconds to 1 minute. This solution may be reused until a brown precipitate forms and the solution turns cloudy.
10. Rinse slides in distilled water.
11. Place in 5% sodium thiosulfate for 2-5 minutes.
12. Wash slides thoroughly in tap water.
13. Counterstain in light green for 1½ minutes.
14. Dehydrate and clear as with the routine Grocott methenamine-silver procedure.

■ *Results* [i10.21]
- Fungi and *P jirovecii* cell walls — Black
- Background — Green



[i10.21] *Pneumocystis jirovecii* stained with the microwave Grocott methenamine-silver method and viewed with the oil-immersion objective. Note that the cell walls are stained black and the internal structure of the organisms remains visible.



[i10.22] The majority of stained structures in this photomicrograph are red blood cells, with a cluster of *Pneumocystis jirovecii* organisms seen only at the arrow. Preparation such as this must be examined very carefully under high magnification.

■ *Technical Notes*

1. If the slides are left in the methenamine-silver solution too long during either the routine or the microwave process, reticular fibers, red blood cells, and other tissue structures may also be stained. The nonspecific staining may mimic the appearance of fungi or obscure small numbers of fungi present in the section **[i10.22]**.

1. It is important that fungi not be overstained and that the internal structure is always visible.

2. See the routine Grocott methenamine-silver procedure for other notes.

3. Chromic acid is very toxic. Instead of microwaving the solution, oxidation can be done in a preheated solution at 58°C for 10 minutes.

MAYER MUCICARMINE AND ALCIAN BLUE TECHNIQUES FOR *CRYPTOCOCCUS NEOFORMANS*

The Mayer mucicarmine and alcian blue, pH 2.5, techniques are given in chapter 7, "Carbohydrates and Amyloid," p142. The mucoid capsule of *C neoformans* can be demonstrated by these procedures, thus differentiating this organism from other yeastlike fungi of similar size and shape (see **[i7.9, p144]**, **[i7.17, p151]**). These techniques may also stain the cell walls of *Blastomyces dermatitidis* and *Rhinosporidium seeberi* [Chandler 1995b].

WARTHIN-STARRY TECHNIQUE FOR SPIROCHETES [LUNA 1968]

■ *Purpose*

Demonstration of spirochetes in tissue sections

■ *Principle*

This is an argyrophil method; that is, the spirochetes have the ability to bind silver ions from a solution, but they do not have the ability to reduce the silver to a visible metallic form. A chemical reducer, hydroquinone, is used for that purpose.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

54°C to 56°C and 43°C water baths, plastic 50-mL centrifuge tubes, graduated cylinders, 500-mL beaker, chemically clean Coplin jars, nonmetallic forceps

■ *Technique*

Cut paraffin sections at 4-5 µm.

■ *Quality Control*

The tissue must contain spirochetes. New plastic centrifuge tubes and chemically clean glassware must be used.

■ *Reagents*

Citric Acid, 1% Solution

Citric acid	1 g
Triple-distilled water	100 mL

Acidulated Water

Triple-distilled water	500 mL
1% citric acid: Add enough to bring the water to pH 4.0	

Silver Nitrate (for Developer), 2% Solution

Silver nitrate crystals	1 g
Acidulated water	50 mL

Silver Nitrate (for Impregnation) 1% Solution

Silver nitrate, 2% solution (see above)	25 mL
Acidulated water	25 mL
Do not preheat this solution	

Gelatin, 5% Solution

Gelatin, high-grade sheet (Fisher)	2.5 g
Acidulated water	50 mL

Hydroquinone, 0.15% Solution

Hydroquinone crystals, photographic grade	0.075 g
Acidulated water	50 mL

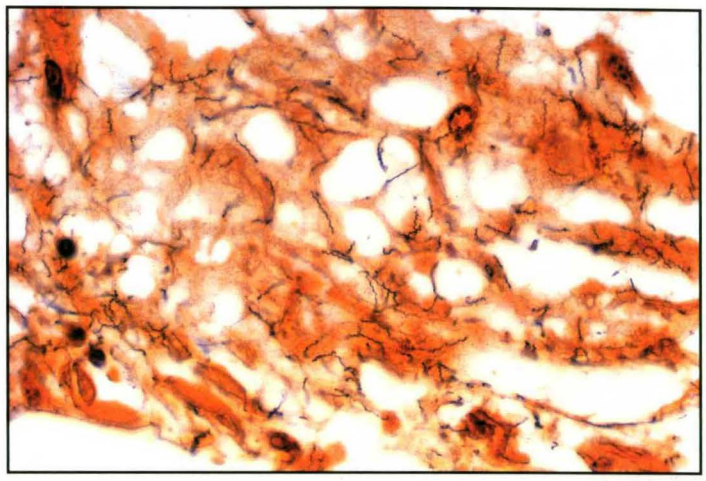
Developer Solution

Silver nitrate, 2% solution	12 mL
Gelatin, 5% solution	30 mL
Hydroquinone, 0.15% solution	16 mL

Use a prewarmed graduated cylinder, and combine ingredients in the order given, making certain the solutions are mixed well after each addition. Prepare immediately before use.

■ *Procedure*

1. Place the 2% silver nitrate, 5% gelatin, and hydroquinone solutions in separate 50-mL plastic centrifuge tubes. Heat in a water bath at 54°C for at least 1 hour.
2. Place a 100-mL graduated cylinder and a chemically clean Coplin jar in the oven for a least 1 hour (for developer).
3. Deparaffinize and hydrate sections to acidulated water.
4. Place slides in the 1% silver nitrate impregnating solution in a water bath at 43°C for 30 minutes. Do not preheat the solution.
5. Just before the slides are due out of the impregnating solution, prepare the developer (place in the warm Coplin jar) and place in the 54°C water bath.
6. Put slides in the developer for 3-4 minutes. Check after 2 minutes and continue checking frequently until they are ready.
7. Wash slides quickly and thoroughly in distilled water.
8. Dehydrate sections in 95% and absolute alcohols, and clear in xylene (2 changes of each).
9. Mount sections with synthetic resin.



[i10.23] Spirochetes are stained black with the Warthin-Starry silver technique.

■ *Results* **[i10.23]**

• Spirochetes	Black
• Other bacteria	Black
• Background	Pale yellow to light brown

■ *Technical Notes*

1. If the sections have been overdeveloped, they may be treated with iodine and sodium thiosulfate for color removal and then restained.
2. All bacteria are nonselectively blackened by silver impregnation methods such as the Warthin-Starry, Dieterle, and Steiner and Steiner. These methods best demonstrate small, weakly gram-negative bacteria, such as the *Legionella* species. According to Chandler [1995a], when compared with Gram stains, silver impregnation procedures provide much greater sensitivity when screening for small numbers of both gram-positive and gram-negative bacteria.
3. Any reducing substances present in the tissue (eg, formalin pigment) will also give a positive reaction.

MICROWAVE MODIFICATION OF THE WARTHIN-STARRY METHOD FOR BACTERIA [CHURUKIAN 1988, CHURUKIAN 1993]

■ *Purpose*

Demonstration of spirochetes and other bacteria in tissue sections

■ *Principle*

See the conventional Warthin-Starry method.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*
Microwave oven, graduated cylinders, chemically clean flasks and Coplin jars, nonmetallic forceps

■ *Technique*
Cut paraffin sections at 4-5 µm.

■ *Quality Control*
The tissue must contain bacteria. Chemically clean glassware must be used.

■ *Reagents*

Glycine-Acetic Acid Stock Solution

Glycine	2.4 g
Glacial acetic acid	0.3 mL
Distilled water	100 mL
The pH of this solution is approximately 3.9. Store in the refrigerator.	

Glycine-Acetic Acid Working Solution

Glycine-acetic acid stock solution:	5 mL
Distilled water	500 mL
The pH of this solution is approximately 4.2.	

Silver Nitrate, 0.5% Solution

Silver nitrate	0.2 g
Glycine-acetic acid working solution	40 mL

The following solutions are prepared immediately before use:

Silver Nitrate, 2% Solution

Silver nitrate	0.2 g
Glycine-acetic acid working solution	10 mL
Place in a 56°C-60°C oven	

Gelatin, 4% Solution

Gelatin	1 g
Glycine-acetic acid working solution	25 mL
Dissolve by placing on a magnetic stirrer and applying gentle heat. Place in a 56°C-60°C oven.	

Hydroquinone, 0.1% Solution

Hydroquinone	0.015 g
Glycine-acetic acid working solution:	15 mL
Place in a 56°C to 60°C oven	

Silver Nitrate-Gelatin-Hydroquinone Developer

Immediately before use, combine the following in order, mixing after each addition:

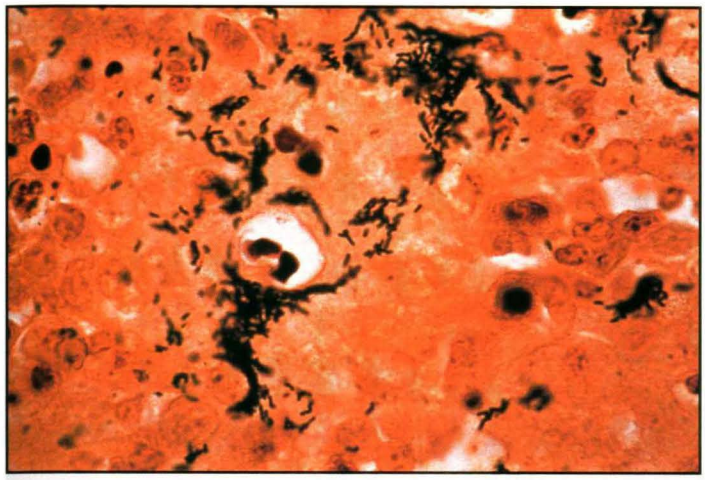
Silver nitrate, 2% solution:	10 mL
Gelatin, 4% solution	25 mL
Hydroquinone, 0.1% solution:	15 mL

■ *Procedure*

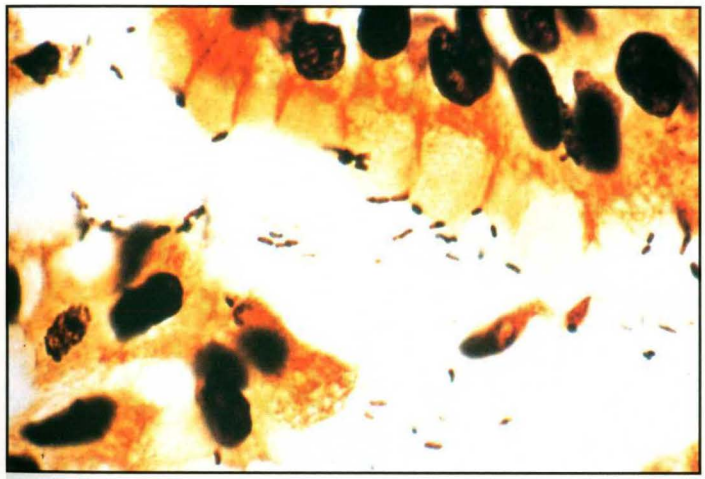
1. Deparaffinize and hydrate slides to the working glycine-acetic acid solution.
2. Place slides in 40 mL of 0.5% silver nitrate in a plastic Coplin jar, with the cap loosely applied. Place in a microwave oven, and microwave at power level 1 (60W) for 4 minutes. Allow the slides to remain in the hot solution (80°C) for 2 or more minutes.
3. Place slides in freshly prepared silver nitrate-gelatin-hydroquinone developer in a plastic Coplin jar, with the cap loosely applied. Place in a microwave oven, and microwave at power level 1 for 1 minute. Allow the slides to remain in the solution until the sections appear grayish brown. This usually takes 30-60 seconds but may take longer.
4. Wash quickly and thoroughly in hot running water.
5. Rinse in 2 changes of distilled water.
6. Dehydrate in graded alcohols.
7. Clear in 3 or 4 changes of xylene.
8. Mount with synthetic resin.

■ *Results* **[i10.24], [i10.25]**

- *Alipia felis* (cat-scratch bacillus) Black
- *Legionella pneumophila* Black
- *N asteroides* Black
- *H pylori* Black



[i10.24] *Alipia felis* (cat-scratch bacteria) are seen in this section of lymph node stained with the Churukian and Schenk microwave modification of the Warthin-Starry silver stain. [Image courtesy of Churukian CJ, University of Rochester Medical Center]



[i10.25] *Helicobacter pylori* organisms are seen in this gastric biopsy specimen stained with the Churukian and Schenk microwave modification of the Warthin-Starry silver stain, and viewed with the oil-immersion objective. [Image courtesy of Churukian CJ, University of Rochester Medical Center]

• Nuclei	Brown
• Erythrocytes	Brown

■ *Technical Notes*

1. The type of gelatin used in the method is very important. Churukian [1988] found that BBL (BBL bacterial gelatin, Baxter Healthcare Corp) or Fisher type A gave less background staining.

2. The results are more consistent and reliable when staining and developing are done at the lowest power levels. Churukian [1988] attributes this to a more uniform distribution of heat in the solutions at the lower power levels.

3. Some xylene substitutes will cause rapid fading of the stain.

4. Spirochetes are not well demonstrated by this method. To demonstrate spirochetes [Churukian 1988], immediately after step 1:

   a. Place sections in 40 mL of 1% uranyl nitrate prepared with the acetic acid-glycine working solution. Microwave at power level 7 for 1 minute. Allow the slides to remain in the solution for 1 more minute.

   b. Rinse with 5 changes of working acetic acid-glycine solution.

   c. Proceed with step 3 of the procedure above.

DIETERLE METHOD FOR SPIROCHETES AND *LEGIONELLA* ORGANISMS [VAN ORDEN 1997]

■ *Purpose*

Demonstration of spirochetes or the causative organism of legionellosis

■ *Principle*

Spirochetes are argyrophilic; that is, they will adsorb silver from a silver solution but the adsorbed silver must be chemically reduced to the visible metallic form. Hydroquinone is the reducing agent or "developer."

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

55°C to 58°C oven, chemical hood, chemically clean Coplin jars, Erlenmeyer flasks, Whatman #1 filter paper

■ *Technique*

Cut paraffin sections at 4-5 μm.

■ *Quality Control*

Tissue containing spirochetes or *Legionella* organisms must be used. Use chemically clean glassware, and take care that no metal comes in contact with the staining solutions.

■ *Reagents*

Alcoholic Uranyl Nitrate, 5% Solution

Uranyl nitrate	10 g
Ethyl alcohol, 70%	200 mL

Alcoholic Gum Mastic, 10% Solution

Gum mastic	50 g
Absolute alcohol	500 mL

Allow 2-3 days for the gum mastic to dissolve, then filter and store in a well-stoppered bottle in the refrigerator.

Silver Nitrate, 1% Solution

Silver nitrate	5 g
Distilled water	500 mL

Developer

Mix in order under the hood:

Hydroquinone	1.5 g
Sodium sulfite	0.25 g
Distilled water	60 mL
Acetone	10 mL
Formaldehyde, 37% to 40%	10 mL
Pyridine	10 mL
Alcoholic gum mastic, 10% solution	10 mL

Solution becomes milky tan as gum mastic solution is added, and medium brown on standing in a well-lighted area. Developer should be made when the procedure is begun, as aging (about 6 hours) of the developer is required for proper development. The developer may be used for 2-3 days, or until the color becomes dark brown.

Formic Acid, 10% Solution

Formic acid, concentrated	5 mL
Distilled water	45 mL

■ *Procedure*

1. Preheat the 5% alcoholic uranyl nitrate solution and the 1% silver nitrate solution in a 56°C to 58°C oven for at least 30 minutes. (Do not exceed a temperature of 60°C, as silver will precipitate.) If steps 9 through 17 will not be done until the following day, do not preheat silver.
2. Deparaffinize and hydrate sections to distilled water. Use 3 control slides.
3. Place sections in preheated 5% alcoholic uranyl nitrate in a 55°C to 58°C oven for 30 minutes to 1 hour.
4. Dip sections once in distilled water.
5. Dip sections once in 95% alcohol.
6. Place slides in 10% alcoholic gum mastic for 3 minutes.
7. Dip sections once quickly in 95% alcohol.
8. Place sections in distilled water for 1 minute, then allow slides to drain for 15-20 minutes until almost dry. Slides may be left overnight at this point.
9. Place sections in preheated 1% silver nitrate solution in a 55°C to 58°C oven, in the dark, for 5 hours. Sections from very old blocks may require longer incubation.
10. Quickly dip slides twice in distilled water.
11. Place sections in developer, and dip until the sections are tan to gold. Check a control at 4, 8, and 12 minutes. Finish all sections when the control is ready.
12. Quickly dip twice in distilled water.
13. Place in 10% formic acid for 45 seconds.
14. Dip twice in distilled water.
15. Dip twice in 95% alcohol.
16. Dip twice in acetone.
17. Clear in 2 changes of xylene, and mount with synthetic resin.

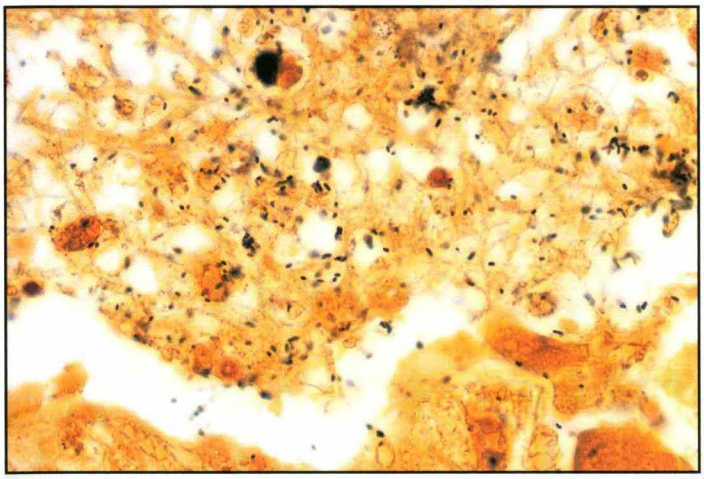
■ *Results* **[i10.26]**

• Spirochetes, bacteria	Brown to black
• Background	Pale yellow or tan

Other structures that may stain brown to black are melanin granules, chromatin, formalin pigment, and some foreign materials present in macrophages.

■ *Technical Notes*

Pyridine is toxic by ingestion, inhalation, and skin absorption. It has an Occupational Safety and Health Administration time-weighted average of 5 ppm; it should be used under a chemical fume hood, and suitable gloves and goggles should be used. Uranyl nitrate is also highly toxic, with an added radiation hazard from inhalation. Extreme care should be used when handling the powder; solutions are safer to handle.



[i10.26] *Legionella pneumophila*, the causative organism of legionellosis, demonstrated with the Dieterle technique and viewed with the oil-immersion objective.

MICROWAVE STEINER AND STEINER PROCEDURE FOR SPIROCHETES, *HELICOBACTER*, AND *LEGIONELLA* ORGANISMS [SWISHER 1982]

■ *Purpose*

Demonstration of spirochetes, *H pylori*, or the causative organism of legionellosis

■ *Principle*

The organisms demonstrated by this method are argyrophilic; that is, they will absorb silver from a silver solution, but the absorbed silver must be chemically reduced to the visible metallic form. Hydroquinone is the reducing agent, or "developer," in this procedure.

■ *Fixative*

10% neutral-buffered formalin gives the best results. Mercurial and chromate fixatives should be avoided.

■ *Equipment*

Microwave oven, 45°C to 50°C water bath, vented plastic Coplin jars, glass Coplin jars, Erlenmeyer flasks, Whatman #1 filter paper

■ *Technique*

Cut paraffin sections at 4-5 µm.

■ *Quality Control*

Tissue containing spirochetes, *H pylori*, or *L pneumophila* must be used depending on the organisms demonstrated. Use chemically clean glassware, and take care that no metal comes in contact with the staining solutions.

■ *Reagents*

Uranyl Nitrate, 1% Solution

Uranyl nitrate	1 g
Distilled water	100 mL
This solution may be reused, but discard it after 2 months	

Silver Nitrate, 1% Solution

Silver nitrate	0.5 g
Distilled water	50 mL
Make fresh each time and filter before use	

Silver Nitrate, 0.04% Solution

Silver nitrate	0.04 g
Distilled water	100 mL
Refrigerate and use for only 1 month	

Gum Mastic, 2.5% Solution

Gum mastic	2.5 g
Absolute alcohol	100 mL
Allow gum mastic to dissolve in the alcohol for 24 hours, then filter the solution until it is clear yellow. Refrigerate at 4°C. This solution may be reused, but do not pour the used solution back into the stock bottle. Discard after 2 months.	

Hydroquinone, 2% Solution

Hydroquinone	1 g
Distilled water	50 mL
Make fresh each time. Fresh hydroquinone always should be used, and the anhydrous hydroquinone crystals should be discarded and replaced after 1-2 years.	

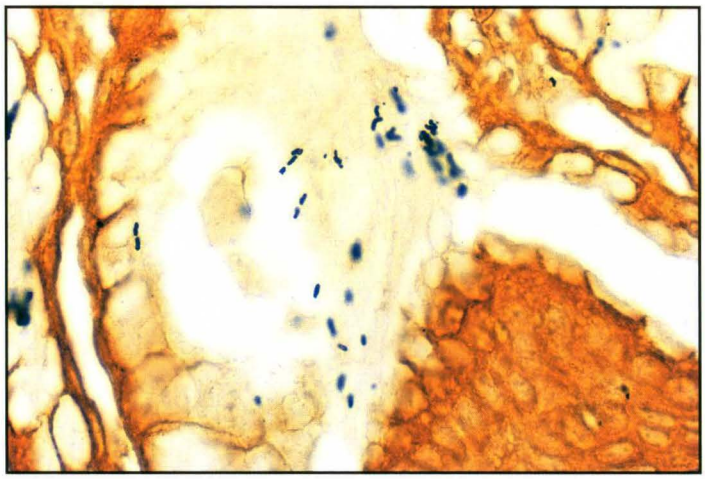
Reducing Solution

Gum mastic, 2.5% solution	10 mL
Hydroquinone, 2% solution	25 mL
Absolute alcohol	5 mL
Make just before each use, filter, and add 2.5 mL of 0.04% silver nitrate. Do not filter after adding the silver. The solution will have a milky appearance when the gum mastic is added.	

■ *Procedure*

Before staining, place a plastic Coplin jar in a 45°C to 50°C water bath to heat. Prepare the reducing solution and place in the preheated Coplin jar. (Use only vented plastic Coplin jars in the microwave oven, be sure that they are loosely capped, and place them inside a loosely closed plastic bag.)

1. Deparaffinize and rehydrate sections to distilled water.
2. Sensitize sections by placing them in room temperature 1% aqueous uranyl nitrate and then heating them in the microwave oven to just below the boiling point (approximately 42 seconds). Do not boil. Immediately remove sections from the oven, and transfer them to distilled water.
3. Rinse slides in distilled water until the possibility of cross contamination is eliminated.
4. Place sections in room temperature 1% silver nitrate and then heat in the microwave oven to just below the boiling point (about 42 seconds). Do not boil. Remove slides from the oven, and allow the slides to stand in hot silver nitrate for 10 minutes.
5. Rinse slides in 3 changes of distilled water.
6. Rinse in 2 changes of 95% alcohol.
7. Rinse in 2 changes of 100% alcohol.
8. Place slides in gum mastic for 5 minutes.
9. Air-dry sections for 1 minute.
10. Rinse in 2 changes of distilled water. The slides may stand in distilled water if necessary.
11. Reduce in reducing solution in a 45°C water bath for 10-25 minutes or until sections have developed satisfactorily, with black spirochetes and a light yellow background (15-20 minutes for *H pylori*, 20-25 minutes for spirochetes and *L pneumophila*).
12. Rinse sections in distilled water to stop reduction.
13. Dehydrate through graded alcohols, and clear in xylene.
14. Mount with synthetic resin.



[i10.27] *Helicobacter pylori* in a gastric biopsy specimen demonstrated with the microwave Steiner silver technique and viewed with the oil-immersion objective.

■ *Results* [i10.27]

• Spirochetes	Dark brown to black
• *H pylori*	Dark brown to black
• *L pneumophila*	Dark brown to black
• Other nonfilamentous bacteria	Dark brown to black
• Background	Light yellow

■ *Technical Notes*

1. A modification (conventional and microwave) of the Steiner procedure was published by Garvey [1995]. The authors claim enhanced visibility of the spirochetes with their modification. I have been unable to try the method, so it is not included in this text, however, the reader should be aware of the availability of the method.
2. Garvey [1995] states that removal of calcium and formalin pigment is essential when only a few microorganisms are present.
3. Some of the xylene substitutes will cause rapid fading of the silver stains of microorganisms.

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LEARNING ACTIVITIES

1. **Using control slides, perform the following staining techniques: acid-fast (Kinyoun, Ziehl-Neelsen, Fite), any Gram stain modification, Giemsa, alcian yellow-toluidine blue, PAS, CAS, Gridley fungus stain, Grocott modification of Gomori methenamine-silver, and the Warthin-Starry, Dieterle, or Steiner and Steiner method. Both conventional and microwave procedures should be tried.**

2. **Microscopically examine each stained section, and compare the results with those given in the procedure. If the results are unsatisfactory, analyze the procedural steps for possible sources of error. If a mistake is identified, repeat the staining procedure after correcting the problem, and reexamine the slides.**

CHAPTER 11

Pigments, Minerals, and Cytoplasmic Granules

OBJECTIVES

On completing this chapter, the student should be able to do the following:

1. Define and give examples of:
    a. endogenous pigment
    b. exogenous pigment
    c. hematogenous pigment
    d. anthracotic pigment
    e. endogenous, nonhematogenous pigment
    f. mineral
2. Compare and contrast the argentaffin and argyrophil reactions
3. Classify the following techniques as to substance demonstrated:
    a. Prussian blue
    b. Turnbull blue
    c. Schmorl ferric-ferricyanide reduction test
    d. Fontana-Masson
    e. Grimelius
    f. Churukian-Schenk
    g. Gomori methenamine-silver
    h. Hall (Fouchet)
    i. von Kossa
    j. alizarin red S
    k. rhodanine
4. Outline each of the techniques listed in objective 3, considering the following characteristics:
    a. most desirable fixative
    b. if another fixative has been used, what can be done
    c. primary reagents and/or dyes and their purpose
    d. results of staining
    e. appropriate control material
    f. sources of error and appropriate correction
    g. mode of action
    h. special requirements (eg, chemically clean glassware)
    i. microscope used
5. Describe the Prussian blue reaction, and state how it differs from the Turnbull blue reaction
6. Identify 3 body sites where melanin is found
7. List at least 4 other granules or minerals not demonstrated by the techniques listed in objective 3, and give at least 1 method for demonstrating each.
8. Outline the method for bleaching melanin pigment.
9. State how anthracotic pigment can be differentiated from other dark brown to black pigments (eg, formalin, melanin)

Pigments

The term *pigment* refers to any of the various coloring agents deposited, frequently as cytoplasmic inclusions or granules, in cells and tissues. Pigments are colored, so it is not always necessary to stain them with biologic dyes; however, special stains may be necessary for the differentiation of similarly colored pigments. Pigments may be classified into 3 broad groups: artifact, exogenous, and endogenous. Artifact pigments are deposited in tissue as a result of chemical action (eg, mercury, formalin, and chrome pigments that may result from fixation). Exogenous pigments are formed externally and then taken into the body through various routes, while endogenous pigments are formed within the body.

ARTIFACT PIGMENTS

Artifact pigments are produced in tissue during processing and most commonly result from fixation. These pigments usually lie on top of the tissue and not within the cell; however, formalin pigment has been reported within cell cytoplasm [Thompson 1978]. Mercury pigment is deposited in tissues fixed in any mercury-containing fixative, and it may be removed easily by treating sections with a solution of iodine followed by a solution of sodium thiosulfate. Chrome pigment may form if tissue fixed in solutions containing potassium dichromate is not washed in running water before dehydration. Formalin pigment results when tissue is fixed in acidic formaldehyde solutions. Formalin pigment is one of the acid hematins; the others are malarial pigment and a hydrochloric acid hematin [**Lillie 1976**]. The acid hematins are birefringent, microcrystalline, dark brown pigments that give no iron reaction, are dissolved by alcoholic picric acid or alkalis, and are fairly resistant to strong acids. Vacca [**1985**] classifies both the formalin pigment and the malarial pigment as endogenous, hematogenous pigments.

EXOGENOUS PIGMENTS

Carbon (anthracotic pigment) is a black pigment commonly seen in sections from the lung and associated lymph nodes. Carbon resists both bleaching and extraction procedures. It is insoluble in concentrated sulfuric acid, a characteristic that aids in differentiating carbon from other black pigments that are dissolved by concentrated sulfuric acid (formalin pigment, malarial pigment, and melanin pigment).

Asbestos fibers are birefringent fibers of magnesium silicate that may be found in the lungs of persons exposed to large quantities of asbestos. The fibers cause a tissue reaction that may eventually result in the development of mesothelioma. After inhalation, the fibers become coated with an iron-containing protein and are known as asbestos bodies. Birefringence is lost when the fibers become coated, but the asbestos bodies may now be demonstrated by the Prussian blue reaction.

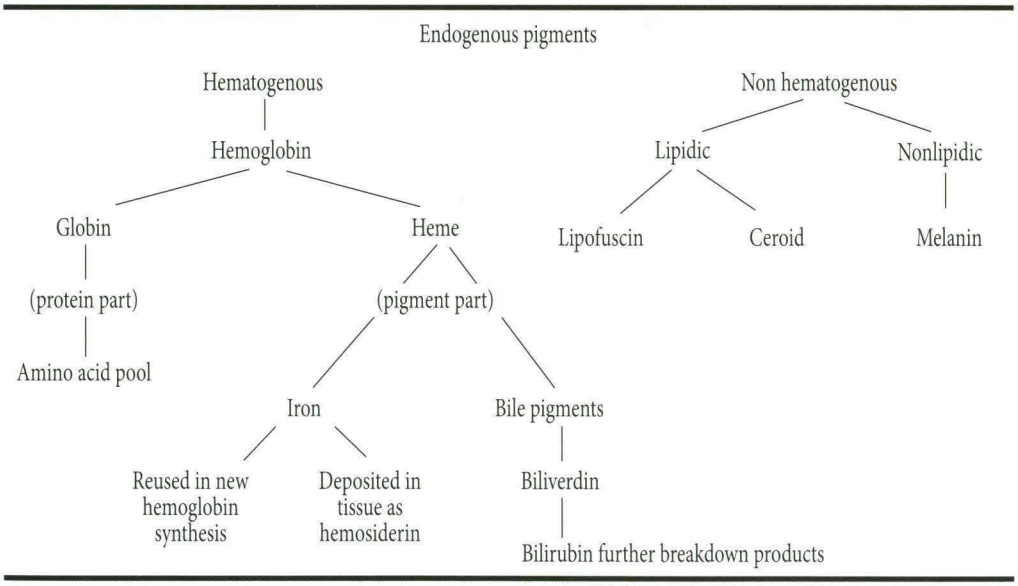
Tattoo pigments are usually found in skin that has been tattooed, but occasionally may be found in associated lymph nodes. These pigments include various organic and inorganic pigments that rarely need specific identification.

Metals are also deposited in tissues, either as normal stores (eg, iron) or in pathologic conditions (eg, copper in Wilson disease or silver in argyria). The metals are discussed in the sections on minerals.

ENDOGENOUS HEMATOGENOUS PIGMENTS

Endogenous pigments are classified as hematogenous (derived from blood) and nonhematogenous. The principal hematogenous pigments are hemoglobin, hemosiderin, and bile pigments **[f11.1].**

Hemoglobin is a conjugated protein that is found normally in red blood cells and is responsible for transporting oxygen from the lungs to other parts of the body. Hemoglobin may be found pathologically in areas of recent hemorrhage or in renal tubules after excessive hemolysis. Hemoglobin stains vividly with acid (anionic) dyes such as eosin. The Okajima technique also may be used to demonstrate hemoglobin.



[f11.1] Summary of the formation of endogenous pigments (reprinted with permission [Hrapchak 1977]).

Erythrocytes have a normal life span of about 120 days; after circulating for that period, they are destroyed by splitting open (hemolysis) or by phagocytic cells (macrophages) in the spleen. Hemoglobin breaks down into 2 parts: globin (protein that is returned to the amino acid pool) and heme (iron-containing). The heme portion splits again into iron and a greenish bile pigment (biliverdin).

Iron is conserved by the body for use in the production of new hemoglobin. If the iron is not needed immediately, it is stored primarily in the bone marrow and spleen as hemosiderin, a yellow to brown pigment. Much of the iron is needed for production of new red blood cells; therefore, large deposits of hemosiderin are found only in pathologic conditions. If the production and destruction of red cells are not balanced (eg, increased destruction in hemolytic anemia), there may be increased deposition of hemosiderin in tissues. Hemochromatosis, a disease caused by excessive absorption of dietary iron, is also characterized by excessive hemosiderin deposits. Hemosiderin is differentiated from other yellowish-brown pigments with the Prussian blue reaction.

The bile pigment biliverdin also results from destruction of red blood cells and further breakdown of the heme portion of hemoglobin. Biliverdin is transported to the liver, where it undergoes reduction to bilirubin. Bilirubin is not normally deposited in tissue but is removed from circulation by the liver and then secreted as a component of bile. An obstruction of the normal bile flow may cause abnormal accumulations of bile pigments in the blood and may impart a yellowish coloration to the skin, a condition known as jaundice. In obstructive jaundice, bile pigment may be seen in the liver in bile canaliculi and also deposited in the cytoplasm of both Kupffer cells and hepatocytes. Bile is demonstrated with techniques relying on the oxidation of bilirubin (yellow-brown) to biliverdin (green).

Hematoidin is a pigment similar to bilirubin and it is also oxidized to biliverdin by bile-demonstrating techniques. Hematoidin is formed in tissues as a result of hemorrhage and reduced oxygen tension. Bancroft and Stevens state that hematoidin may represent a heme breakdown product that has been trapped at the site of formation, has undergone minor chemical changes, and has not been transported to the liver for processing.

ENDOGENOUS NONHEMATOGENOUS PIGMENT

The second group of endogenous pigments comprises those that are not derived from blood. These nonhematogenous pigments may be divided into lipidic and nonlipidic pigments. The primary lipidic pigments are lipofuscin and ceroid, and the primary nonlipidic pigment is melanin **[f11.1]**.

■ *Nonlipidic Pigments*

Melanin, a mixture of substances closely bound to proteins, is the most important of the endogenous nonhematogenous pigments. Melanin is derived from tyrosine or tyrosine-containing compounds and is characteristically a brown-black pigment present normally in the hair, skin, retina, iris, and certain parts of the central nervous system. The amount and distribution of melanin varies among individuals and is responsible for differences in skin, hair, and eye color. The general steps in the synthesis of melanin are as follows:

$$\text{tyrosine (amino acid)} \xrightarrow[\text{tyrosinase (enzyme)}]{\text{oxidation by}} \text{dopa (3,4-dihydroxyphenylalanine)}$$

$$\text{dopa} \xrightarrow[\text{through several steps}]{\text{further oxidation}} \text{melanin}$$

This reaction has allowed the histochemical demonstration of melanocytes, the cells responsible for the formation of melanin pigment. When frozen sections of tissue are exposed to a buffered solution of dopa, melanin or a melaninlike pigment will be deposited in any melanocytes present. The formation of melanin is attributed to dopa oxidase, an enzyme present in melanocytes.

When melanin pigment is present in large amounts, cell detail may be obscured. In th is case, it can be useful to bleach the melanin with an oxidizing agent such as 10% hydrogen peroxide (1-2 days) or 0.25% potassium permanganate (1-4 hours). Potassium permanganate must be followed by oxalic acid (1%-5%) to clear the sections of color. Vacca [1985] states that the blacker the melanin, the slower the decolorization or bleaching. Besides the ability to be bleached, melanin has other characteristics that aid in identification. Melanin is dissolved by strong alkali but is insoluble in weak acids and bases or in organic solvents. Melanin is strongly basophilic, reduces silver nitrate to metallic silver (argentaffin), and gives a positive Schmorl reaction. It will form complexes with the ferrous ion, which then may be demonstrated with the Turnbull blue reaction.

■ *Lipidic Pigments*

Lipofuscin, commonly referred to as "wear-and-tear" pigment, collects in the more permanent cells (eg, heart, liver, and neurons) of older persons. This is a yellow-brown pigment that stains with oil red O, Sudan black B, and the PAS reaction.

Ceroid is a brownish-yellow pigment first described in hepatocytes and macrophages of rats with experimentally induced cirrhosis of the liver. Ceroid is rarely seen in humans; it stains with the same stains as lipofuscin, but these pigments may be differentiated by virtue of positive acid-fast staining of the ceroid.

Both lipofuscin and ceroid stain with the oil-soluble dyes, oil red O and Sudan black B, after routine processing and paraffin embedding.

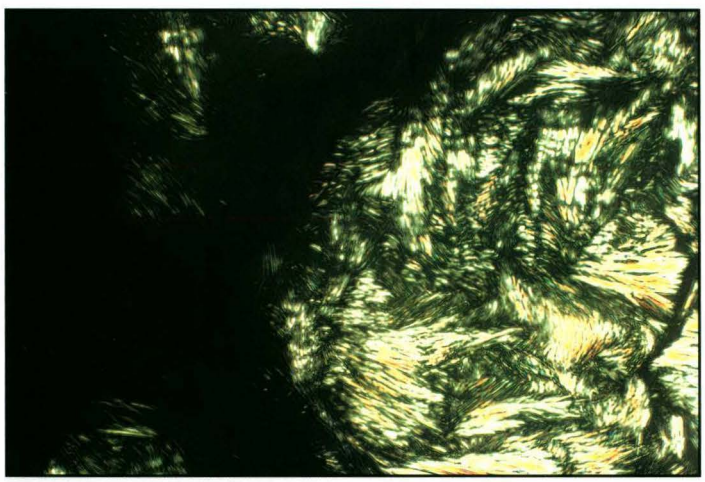
Endogenous Deposits

Urates are deposited in tissue (gouty tophi) or around joints in persons suffering from gout, a disorder in uric acid metabolism. In chronic gout, large masses of uric acid and urate crystals may accumulate and form "tophi" in soft tissue. The sodium urate crystals are very soluble in water and are dissolved if tissue is fixed in the routine aqueous fixatives; therefore, alcoholic fixation must

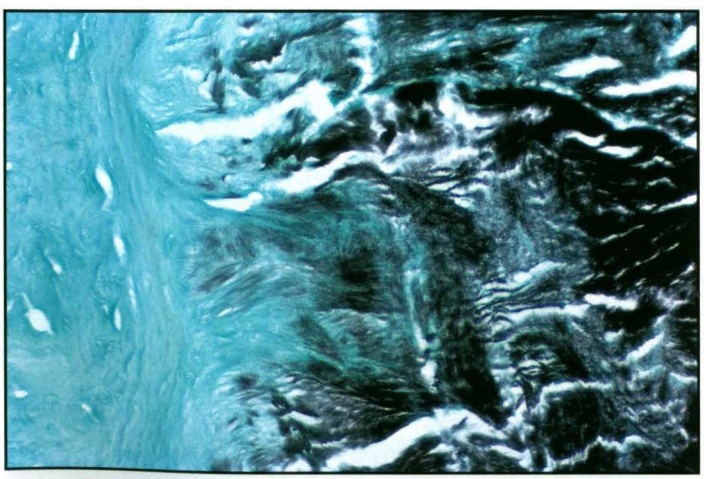
be used when the deposits are to be demonstrated. Urate crystals are birefringent and can be identified easily by polarizing microscopy **[i11.1]**; they also may be demonstrated by an argentaffin reaction such as a methenamine-silver technique **[i11.2]**.

Minerals

In the biologic sense, minerals are metallic and nonmetallic ions necessary for growth and other bodily functions. Some of the ions that are demonstrated with special stains are calcium (Ca^{2+}), ferrous/ferric (Fe^{2+}/Fe^{3+}), cupric (Cu^{2+}), phosphate (PO_4^{3+}), and carbonate (CO_3^{2+}). Some metallic elements (eg, silver, lead, copper, and gold) are deposited pathologically in tissue, and also can be demonstrated with special techniques. Methods for the demonstration of iron, calcium, and copper are described in this chapter. It is rare that lead, silver, or gold need to be demonstrated in tissue in routine histopathology, but Sheehan and Hrapchak [1980], and Bancroft and Stevens [1982] have described useful techniques for this purpose.



[i11.1] Urate crystals in an H&E-stained section as seen with polarized light. Absolute alcohol fixation was used to preserve the urate crystals in this section.



[i11.2] Urate crystals (seen with polarized light in an H&E-stained section in [i11.1]) stained black with a modified Gomori methenamine-silver technique.

Microincineration is a method used to study inorganic substances found in tissue. Paraffin sections 4-µm thick are mounted (not floated, because water should be avoided) on special glass or quartz slides that will not melt at high temperatures. The sections are incinerated to burn off all organic matter by gradually increasing the temperature to 650°C. After incineration is completed, the slides are allowed to cool slowly inside the oven, coverslips are applied with glycerol mounting medium, and the slides are microscopically examined. Further stains and chemical tests can be done on the ash to aid in specific identification of the inorganic components.

Fixation and processing should neither add nor remove inorganic matter from tissue to be examined with microincineration techniques. Metallic fixatives cannot be used because metals would be deposited in the tissue. The preferred fixative is formalin alcohol (9 parts alcohol and 1 part concentrated formaldehyde solution), although 10% neutral-buffered formalin may be used.

Cytoplasmic Granules

Adrenal chromaffin cells, pancreatic endocrine cells, gastrointestinal enterochromaffin cells, "C" cells of the thyroid, and some pituitary cells are neuroendocrine cells that, by histochemical investigation, have been shown to possess some common metabolic processes related to hormone synthesis. All of these cells have a high uptake of amine precursors and the ability to decarboxylate, hence the name *amine precursor uptake and decarboxylation* (APUD) cells. These cells are included in this chapter because they are demonstrated through the *argyrophil* and/or *argentaffin* reactions. Argyrophil cells have the ability to be impregnated with silver, but they do not have the ability to reduce the silver to a visible metallic form; an external reducing agent (light or chemical) must be applied. Argentaffin cells have both the ability to be impregnated with silver and to reduce the silver. Argentaffin cells also will give a positive reaction with argyrophil techniques, but argyrophil cells will not give a positive reaction with argentaffin techniques.

The choice of fixative is very important for preserving cytoplasmic granules. For example, chromaffin granules present in cells of the adrenal medulla are best preserved by primary chromate fixatives (Orth), argentaffin granules in cells found in the gastrointestinal tract are destroyed by alcoholic fixation, and Paneth cell granules are destroyed by acetic acid [Sheehan 1980].

Special Staining Techniques

PRUSSIAN BLUE STAIN FOR FERRIC IRON [LILLIE 1965]

■ *Purpose*

Detection of ferric (Fe^{3+}) iron in tissues. Ferric iron is normally found in small amounts in the bone marrow and the spleen. Because excess iron is not excreted from the body by a specific mechanism, it is lost only by bleeding or hemorrhage, menstrual flow, sloughing of cells, and transfer to a fetus. If there is a defect in

the control of iron absorption, too much iron is stored in a condition termed *idiopathic hemochromatosis*. Multiple transfusions, increased dietary consumption, hemolysis, and severe congestion can also lead to the storage of too much iron. When iron storage in the body increases beyond normal limits, the excess is stored as hemosiderin primarily in the liver and spleen, but also can be found in heart, muscle, and nerve; this storage can reach a point at which organ function is impaired and death results.

■ *Principle*

The Prussian blue staining method detects the ferric ion in loosely bound protein complexes (as in hemosiderin). Iron that is strongly bound, as in hemoglobin (about 70%), will not react. In this reaction, sections are treated with an acidic solution of potassium ferrocyanide, and any ferric iron present reacts to form an insoluble bright blue pigment called Prussian blue.

$$3K_4Fe(CN)_6 + 4Fe^{3+} \xrightarrow{HCl} \underset{\text{(Prussian blue)}}{Fe_4[Fe(CN)_6]_3\downarrow} + 12K^+$$

■ *Fixative*

Alcohol or 10% neutral-buffered formalin

■ *Equipment*

60°C oven, nonmetallic forceps, chemically clean Coplin jars, graduated cylinders

■ *Technique*

Cut paraffin sections at 4 to 5 μm.

■ *Quality Control*

A section containing ferric iron must be used. Excessive amounts of iron are not desirable in the control, because the reaction product is slightly soluble and may contaminate the incubating solution, giving a background stain on all sections. Coplin jars that have been used for iron hematoxylin solutions and not adequately cleaned may also contaminate the staining solutions.

■ *Reagents*

Potassium Ferrocyanide, 2% Solution

Potassium ferrocyanide	10 g
Distilled water	500 mL

Hydrochloric Acid Solution, 2%

Hydrochloric acid, concentrated	10 mL
Distilled water	490 mL

Nuclear-Fast Red (Kernechtrot) Solution

Nuclear-fast red	0.5 g
Aluminum sulfate	25 g
Distilled water	500 mL

Dissolve the aluminum sulfate in the distilled water and then dissolve the nuclear-fast red in this solution using heat. Cool, filter, and add a few grains of thymol as a preservative.

■ *Procedure*

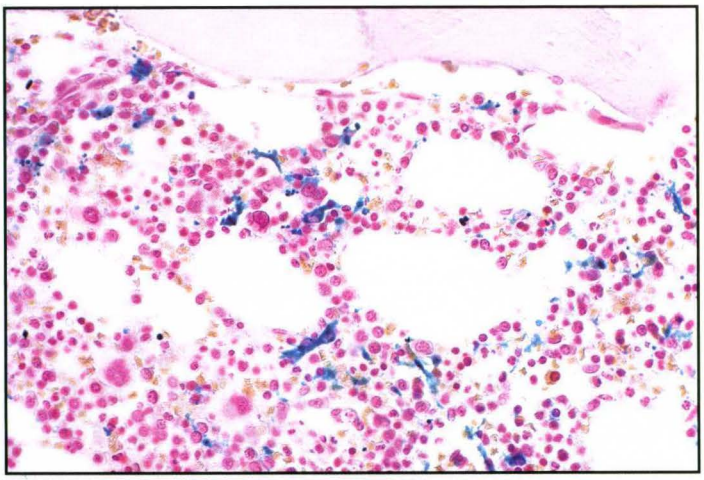
1. Deparaffinize and hydrate the section to distilled water. Handle slides in the following steps with nonmetallic forceps.
2. Place slides in a freshly prepared mixture of equal parts of 2% potassium ferrocyanide and 2% hydrochloric acid, and heat for 20 minutes at 60°C.
3. Wash sections thoroughly in several changes of distilled water.
4. Counterstain sections in nuclear-fast red for 5 minutes.
5. Rinse in running tap water for at least 1 minute.
6. Dehydrate sections in 95% alcohol and 2 changes of absolute alcohol.
7. Clear in 3 changes of xylene, and mount with synthetic resin.

■ *Results* **[i11.3]**

• Nuclei and hemofuchsin	Bright red
• Hemosiderin (iron)	Blue
• Background	Pink

■ *Technical Notes*

1. Be sure the staining jar is chemically clean. If the jar previously contained iron solutions (eg, iron hematoxylin or ferric chloride) and has not been cleaned adequately, diffuse background staining will occur, as described in the "Quality Control" section.
2. Iron may be dissolved by some of the acidic fixatives and decalcification solutions; however, iron is still demonstrable on bone biopsy specimens that are fixed overnight in Zenker solution containing 3% acetic acid. Both fixation and decalcification are accomplished, and the iron is preserved. Because Zenker solution contains mercury, it would be better to use another fixative and then decalcify briefly in 3% to 5% acetic or formic acid.



[ill.3] Iron stored in the bone marrow is revealed in this section with the use of the Prussian blue reaction. The bone marrow was both fixed and decalcified with Zenker solution containing 3%, rather than 5%, acetic acid. The hemosiderin is not dissolved with this method of fixation and decalcification; however, sections containing cortical bone cannot be totally decalcified by the 3% acetic acid, and another method of decalcification must be used for these specimens.

3. Lillie and Fullmer [1976] state that heating the ferrocyanide solution is apt to produce a finely granular, blue deposit throughout the sections. They suggest that the reaction be performed at room temperature for 1 hour.

4. It is important that iron-free reagents, especially iron-free hydrochloric acid, be used.

5. If the excess nuclear-fast red counterstain is not adequately removed with running water before beginning dehydration, cloudy slides will result. If this occurs, return the sections to running water and wash well, and then redehydrate and clear the sections.

TURNBULL BLUE STAIN FOR FERROUS IRON [LILLIE 1976]

■ *Purpose*

Detection of ferrous (Fe^{2+}) iron in tissues. Iron is not normally stored in tissue in the ferrous form, because ferrous iron is very toxic. Ferrous iron is more easily absorbed from the intestine, but it is rapidly converted to the ferric form. The Turnbull blue reaction is used in other stains, such as the Schmorl technique.

■ *Principle*

This method detects the ferrous ion in tissue with the Turnbull blue reaction. Sections are treated with an acidic solution of potassium ferricyanide, and any ferrous iron present reacts to form an insoluble bright blue pigment called Turnbull blue (ferrous ferricyanide).

$$\text{ferrous iron} + \text{potassium ferricyanide} \xrightarrow{HCl} \text{ferrous ferricyanide}\downarrow \text{ (Turnbull blue)}$$

■ *Fixative*

Alcohol or 10% neutral-buffered formalin

■ *Equipment*

Nonmetallic forceps, chemically clean Coplin jars, graduated cylinders

■ *Technique*

Cut paraffin sections at 4 to 5 μm.

■ *Quality Control*

A section containing ferrous iron must be used.

■ *Reagents*

Hydrochloric Acid, 0.06N Solution

Hydrochloric acid, concentrated	2.5mL
Distilled water	497.5 mL

Potassium Ferricyanide Staining Solution

Potassium ferricyanide	0.4 g
Hydrochloric acid, 0.06N solution	40 mL
Prepare fresh just before use	

Acetic Acid, 1% Solution

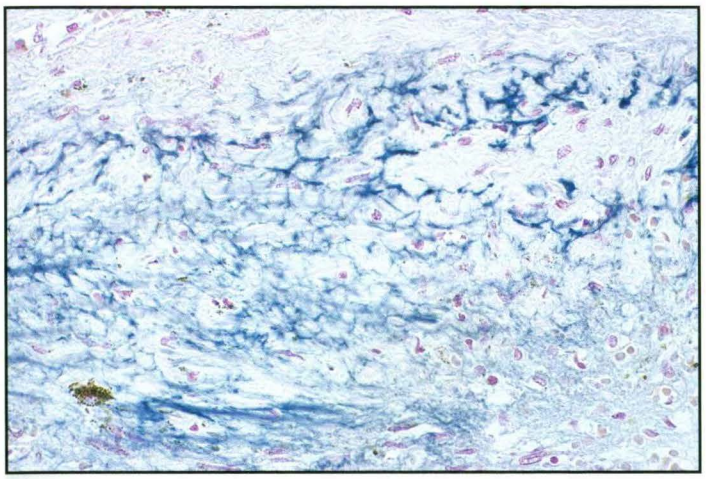
Acetic acid, glacial	1 mL
Distilled water	100 mL

Nuclear-Fast Red Solution

See the Prussian blue stain for ferric iron in this chapter, p256

■ *Procedure*

1. Deparaffinize and hydrate the sections to distilled water. Handle slides in the following steps with nonmetallic forceps.
2. Place slides in a freshly prepared ferricyanide staining solution, and leave for 1 hour at room temperature.
3. Wash sections in 1% acetic acid.
4. Counterstain sections in nuclear-fast red for 5 minutes
5. Rinse well in distilled water.
6. Dehydrate sections in 2 changes of 95% alcohol and 2 changes of absolute alcohol.
7. Clear in 3 changes of xylene, and mount with synthetic resin.



[i11.4] Ferrous iron has been stained with the Turnbull blue reaction in this section of spleen from a patient with hemochromatosis. Some ferrous iron is seen encrusting the collagen fibers. Careful examination will also reveal accumulations of yellow-brown hemosiderin that are unstained by this reaction.

■ *Results* [i11.4]

• Ferrous iron	Blue
• Background	Pink-red

SCHMORL TECHNIQUE FOR REDUCING SUBSTANCES [LUNA 1968]

■ *Purpose*

To indicate reducing substances present in tissue. Melanin, argentaffin granules, and even formalin pigment will be stained.

■ *Principle*

Reducing substances present in tissue reduce the ferric ions present in the staining solution to ferrous ions. The ferrous ions immediately combine with the ferricyanide present in the staining solution to form an insoluble precipitate of ferrous ferricyanide (Turnbull blue).

$$\text{reducing substance} + \text{ferric iron } (Fe^{3+}) \rightarrow \text{ferrous iron } (Fe^{2+})$$

$$\text{ferrous iron } (Fe^{2+}) + \text{ferricyanide} \rightarrow \underset{\text{(Turnbull blue)}}{\text{ferrous ferricyanide}} \downarrow$$

■ *Fixative*

10% neutral-buffered formalin is preferred.

■ *Equipment*

Nonmetallic forceps, pH meter, chemically clean Coplin jars, graduated cylinders, Erlenmeyer flasks

■ *Technique*

Cut paraffin sections at 4 to 5 μm.

■ *Quality Control*

A section containing melanin or argentaffin granules must be used.

■ *Reagents*

Ferric Chloride, 1% Stock Solution

Ferric chloride	1.5 g
Distilled water	150 mL

Potassium Ferricyanide Stock Solution, 0.1%

Potassium ferricyanide	0.1 g
Distilled water	100 mL

Ferric Chloride-Potassium Ferricyanide Working Solution

Ferric chloride stock solution	150 mL
Potassium ferricyanide stock solution	50 mL
Adjust the pH to 2.4 with 1N hydrochloric acid if necessary	

Mayer Mucicarmine Solution

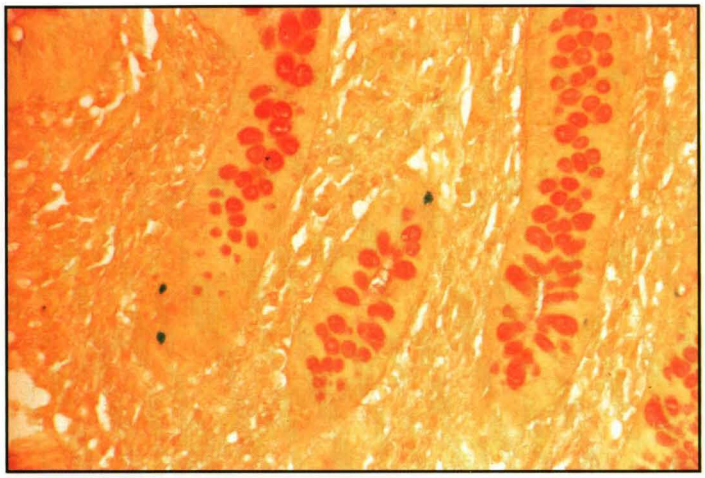
See Mayer mucicarmine stain in chapter 7, p143

Metanil Yellow, 0.25% Solution

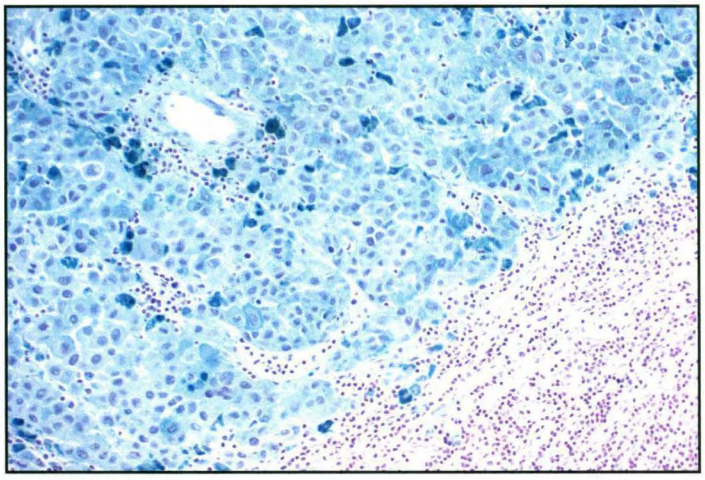
Metanil yellow	0.25 g
Distilled water	100 mL
Acetic acid, glacial	0.25 mL

■ *Procedure*

1. Deparaffinize and hydrate sections to distilled water.
2. Place slides in ferric chloride-potassium ferricyanide working solution, 3 changes for 7 minutes each.
3. Rinse sections in distilled water.
4. Stain in working Mayer mucicarmine solution for 1 hour.
5. Rinse sections rapidly in distilled water.
6. Counterstain in metanil yellow for a few seconds.
7. Rinse rapidly in distilled water.



[i11.5] Argentaffin cells are stained green and goblet cells are stained red in this section of gastrointestinal tract. This modification of the Schmorl technique uses mucicarmine and metanil yellow counterstains that change the blue of the Turnbull blue reaction to green.



[i11.6] A section from a lymph node containing metastatic melanoma stained with the Schmorl technique. This section has been counterstained with nuclear-fast red. The diffuse blue staining is the result of melanin in the tumor cells and is not background staining. With fresh solutions and well-stained sections, very little background staining should occur.

8. Dehydrate sections in 95% alcohol and 2 changes of absolute alcohol

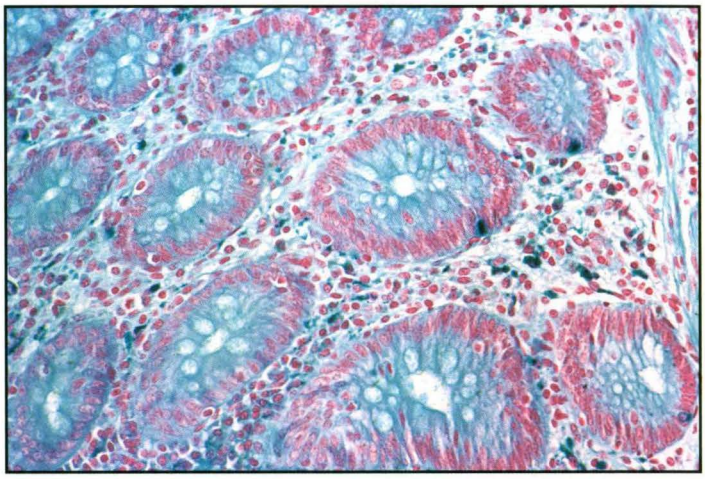
9. Clear in 3 changes of xylene, and mount with synthetic resin.

■ *Results* [i11.5]

• Reducing substances	Blue-green
• Goblet cells, mucin	Rose
• Background	Yellow-green

■ *Technical Notes*

1. This technique has been used in the past primarily for the demonstration of melanin and argentaffin granules but has been replaced today, to a great degree, by immunohistochemical techniques.



[i11.7] This section of colon has been stained with the Schmorl technique and counterstained with nuclear-fast red. The argentaffin cells in the glands show dark blue staining of the granules. The pseudomelanin present in the lamina propria also stains; this staining cannot be avoided because pseudomelanin is an argentaffin substance, but it should not be confused with argentaffin staining of the enteroendocrine cells present in the epithelium. There is slight nonspecific staining of the lamina propria, the epithelium, and the small amount of smooth muscle present in the section.

2. This particular modification works especially well on sections from the gastrointestinal tract because the mucin is also demonstrated. If preferred, steps 4 to 6 may be omitted, and the sections can be counterstained with nuclear-fast red [**i11.6**]. The instructions for preparing and staining with nuclear-fast red are given in the description of the Prussian blue technique in this chapter, p257.

3. The use of glassware contaminated with iron-containing reagents may lead to nonspecific background staining.

4. Background staining [**i11.7**] is undesirable. More specific staining with less background staining is obtained if the solutions are fresh.

FONTANA-MASSON STAIN FOR MELANIN AND ARGENTAFFIN GRANULES [MASSON 1928, LILLIE 1965, LUNA 1968]

■ *Purpose*

Demonstration of argentaffin substances such as melanin, argentaffin granules of carcinoid tumors, and some neurosecretory granules

■ *Principle*

Certain tissue components are argentaffin; that is, they possess the ability to bind silver from a silver solution and to reduce it to visible metallic silver without the need for a separate reducing agent.

■ *Fixative*

10% neutral-buffered formalin. Alcohol should be avoided, because it dissolves argentaffin granules.

■ *Equipment*

Erlenmeyer flasks, 56°C water bath, graduated cylinders, chemically clean Coplin jars, Whatman #1 filter paper

■ *Technique*
Cut paraffin sections at 4 to 5 μm.

■ *Quality Control*
Use a section of skin as a control for melanin and a section of small intestine or appendix as a control for argentaffin granules. Chemically cleaned glassware and nonmetallic instruments must be used.

■ *Reagents*

Silver Nitrate, 10% Solution

Silver nitrate	10 g
Distilled water	100 mL

Fontana Silver Solution

Place 25 mL of the 10% silver nitrate in an Erlenmeyer flask, and while swirling, add concentrated ammonium hydroxide dropwise until the solution clouds, then clears. Add 10% silver nitrate dropwise until the first permanent turbidity appears. Let the solution stand several hours if possible. For use, filter and dilute 20 mL of the diamine silver solution with 40 mL of distilled water.

Gold Chloride, 0.2% Solution

Gold chloride, 1% solution	10 mL
Distilled water	40 mL

Sodium Thiosulfate, 5% Solution

Sodium thiosulfate	25 g
Distilled water	500 mL

Nuclear-Fast Red

See the Prussian blue stain for ferric iron, p257

■ *Procedure*

1. Deparaffinize and hydrate sections to distilled water.
2. Immerse slides in silver nitrate solution in a 56°C water bath for 1 hour. Check slides and return for longer incubation if indicated. The reaction should be stopped when granules are dark brown and the background is colorless.
3. Rinse sections in distilled water.
4. Immerse in gold chloride solution for 10 minutes. This solution may be reused.
5. Rinse in distilled water.
6. Place sections in sodium thiosulfate solution for 5 minutes
7. Rinse in distilled water.
8. Counterstain in nuclear-fast red for 5 minutes.
9. Wash for at least 1 minute in running water.
10. Dehydrate and clear with 2 changes each of 95% and absolute alcohol, and clear with xylene.
11. Mount with synthetic resin.

■ *Results* **[i11.8]**, **[i11.9]**, **[i11.10]**

- Melanin — Black
- Argentaffin granules — Black
- Nuclei — Pink

■ *Technical Notes*

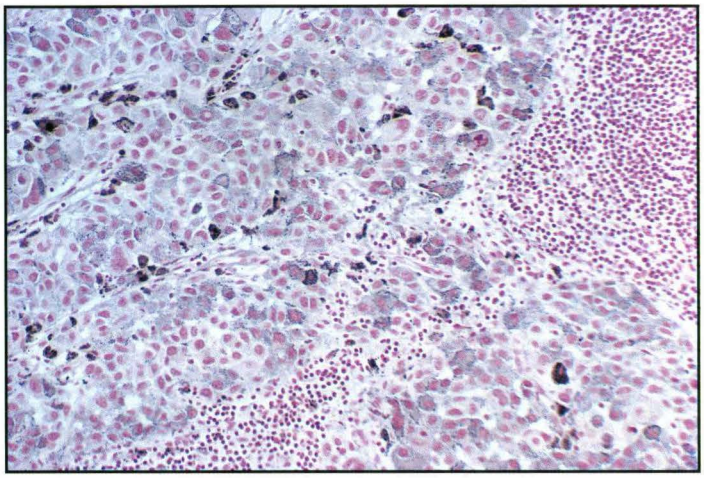
1. This technique is not specific for melanin and argentaffin granules. Other reducing substances such as formalin pigment will also give a positive reaction.
2. This technique is not used as frequently as in the past; immunohistochemistry is used for more specific identification of the granules that yield a positive argentaffin reaction.
3. Overstaining will lead to a dirty gray background and a loss of contrast **[i11.11]**.
4. After standing for several days, silver solutions may form an extremely explosive compound. To minimize explosion hazards, all ammoniacal and alkaline silver solutions should be discarded on the day of use [Vacca 1985].

MICROWAVE FONTANA-MASSON STAIN [BRINN 1983, CHURUKIAN 1979]

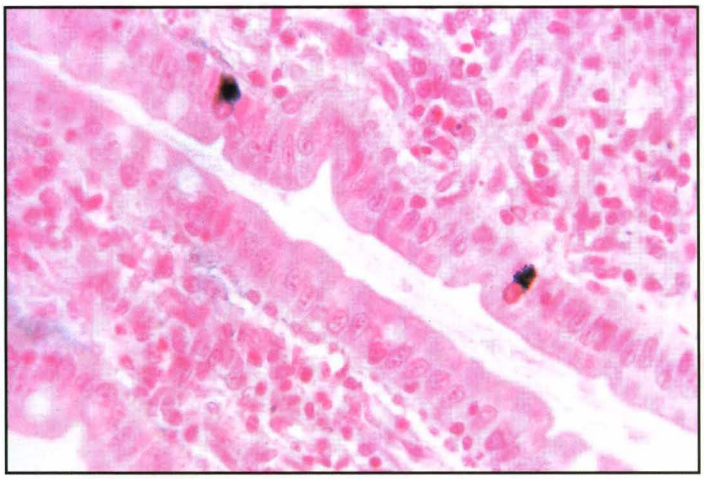
■ *Purpose*
Demonstration of argentaffin substances such as melanin, argentaffin granules of carcinoid tumors, and some neurosecretory granules.

■ *Principle*
Certain tissue components are argentaffin; that is, they possess the ability to bind silver from a silver solution and to reduce it to visible metallic silver without the need for a separate reducing agent.

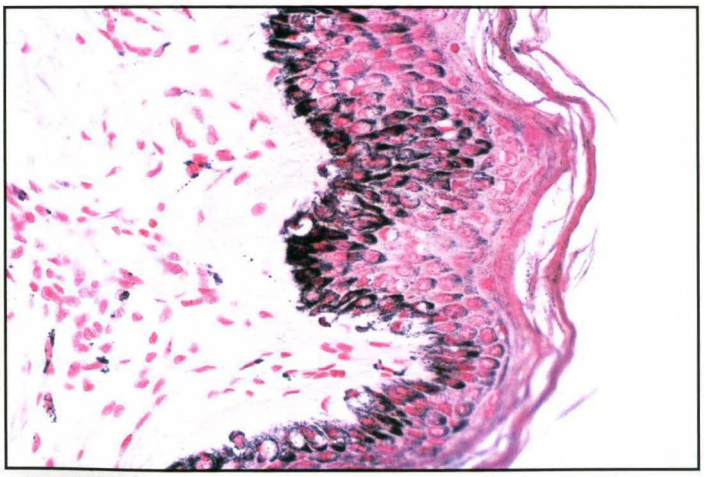
■ *Fixative*
10% neutral-buffered formalin. Alcohol should be avoided, because it dissolves argentaffin granules.



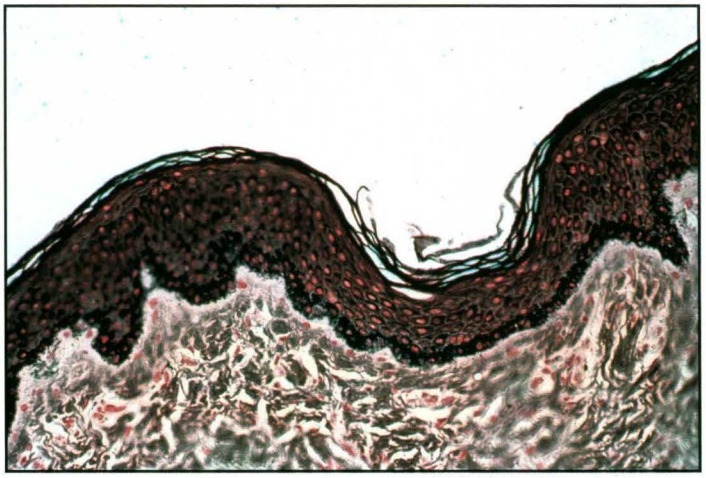
[ill.8] A section from the same lymph node shown in **[ill.6]** stained with the Fontana-Masson stain. Melanin is stained black, and again, the lighter, fine gray-black staining is caused by melanin and is not background staining.



[ill.9] Enteroendocrine (argentaffin) cells in this section of small intestine are stained black with the Fontana-Masson.



[ill.10] A section of skin from an African-American patient stained with the Fontana-Masson technique. Melanin is present in the basal layer of the epidermis and also in the cells of the other layers of the epidermis. This is normal and is not an example of nonspecific staining. A few melanin-containing cells are also present in the dermis, indicating that some melanin has been engulfed by phagocytic cells. There is no nonspecific staining in this section.



[ill.11] A section of Fontana-Masson-stained skin that shows marked nonspecific staining. This stain is not acceptable and must be repeated.

■ *Equipment*

Erlenmeyer flasks, microwave oven, graduated cylinders, chemically clean Coplin jars, Whatman #1 filter paper.

■ *Technique*

Cut paraffin sections at 4 to 5 µm.

■ *Quality Control*

Use a section of skin as a control for melanin and a section of small intestine or appendix as a control for argentaffin granules. Chemically cleaned glassware and nonmetallic instruments must be used.

■ *Reagents*

Fontana Silver Nitrate Solution

Dissolve 0.5 g of silver nitrate in 35 mL of distilled water. To this solution, add strong ammonium hydroxide, until the precipitate that initially forms just dissolves. Add, drop by drop, 5% silver nitrate to cause the clear solution to become slightly cloudy. Pour into a graduated cylinder, and add enough distilled water to make 40 mL of solution.

Gold Chloride, 0.2% Solution

Gold chloride, 1% aqueous	20 mL
Distilled water	80 mL

Sodium Thiosulfate, 2% Solution

Sodium thiosulfate	2 g
Distilled water	100 mL

Nuclear-Fast Red

See the Prussian blue stain for ferric iron, p257

■ *Procedure*

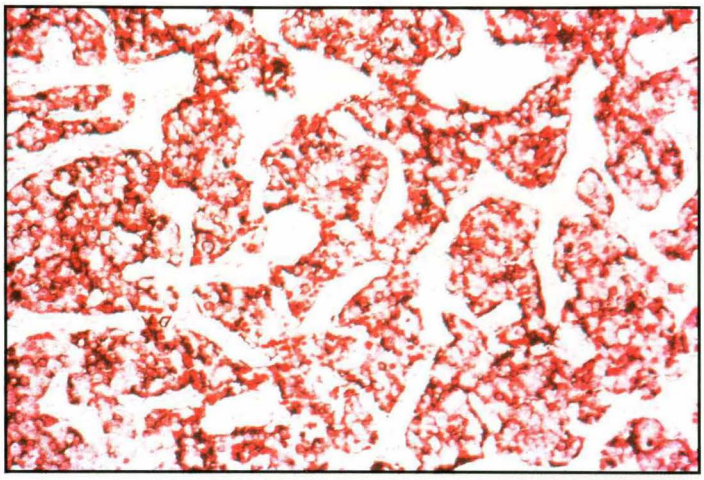
1. Deparaffinize slides, and hydrate to distilled water.
2. Place slides in the freshly prepared Fontana silver nitrate solution (40 mL) in a plastic Coplin jar, with the cap loosely applied. Microwave at power level 1 (60 W) for 5 minutes. Allow the slides to remain in this hot solution (about 88°C) for 1 to 2 minutes or until the sections turn brown.
3. Rinse the sections with 4 changes of distilled water.
4. Tone in the gold chloride solution for 1 minute.
5. Rinse with 3 changes of distilled water.
6. Place slides in sodium thiosulfate solution for 1 minute.
7. Rinse with 4 changes of distilled water.
8. Stain sections in nuclear-fast red solution for 1 minute.
9. Rinse with 3 changes of distilled water.
10. Dehydrate sections in 2 changes each of 95% and absolute alcohols.
11. Clear in 3 or 4 changes of xylene, and mount with synthetic resin.

■ *Results* [i1.12]

• Argentaffin cell granules	Black
• Chromaffin granules	Black
• Melanin	Black
• Other argentaffin substances	Black
• Nuclei	Pink

■ *Technical Notes*

1. To demonstrate that the stained material in either the conventional or microwave procedures is melanin, duplicate sections should treated with the melanin bleach procedure. If the pigment is melanin, it will disappear with this treatment.
   a. Treat deparaffinized and hydrated slides with 0.25% potassium permanganate for 30 minutes.
   b. Wash well in tap water.
   c. Place slides in 2% oxalic acid for 2 minutes
   d. Wash in running tap water for 3 minutes, and then rinse in distilled water.
   e. Stain for 3 minutes with nuclear-fast red solution.
   f. Rinse well with distilled water, dehydrate, clear, and mount.



[i1.12] A section of an argentaffin-positive carcinoid tumor of the small bowel stained with the microwave Fontana-Masson procedure. [Image courtesy of Churukian CJ, University of Rochester Medical Center]

GRIMELIUS ARGYROPHIL STAIN [LACK 1977, ROUSH 1985]

■ *Purpose*

Demonstration of argyrophil granules in neurosecretory tumors

■ *Principle*

Certain tissue components have the ability to bind silver ions from solution but no inherent ability to reduce the silver to its visible metallic form. An external or chemical reducer is used for this purpose. These components are referred to as argyrophil.

■ *Fixative*

10% neutral-buffered formalin.

■ *Equipment*

40°C to 50°C and 58°C to 60°C water baths, pH meter, chemically clean Coplin jars, Erlenmeyer flasks, and graduated cylinders

■ *Technique*

Paraffin sections cut at 4 to 5 μm

■ *Quality Control*

An argyrophil-positive carcinoid tumor is preferred, but a section of small intestine can be used. Glassware must be chemically clean, and nonmetallic forceps are required to transfer the slides.

■ *Reagents*

Silver Nitrate, 1% Solution

Silver nitrate	0.1 g
Distilled water	10 mL
Prepare fresh each time	

Acetic Acid, 0.2M Solution

Acetic acid, glacial	6 mL
Distilled water	94 mL
Store solution in refrigerator.	

Sodium Acetate, 0.2M Solution

Sodium acetate, trihydrate	2.75 g
Distilled water	100 mL
Store solution in refrigerator	

Acetic Acid-Sodium Acetate Buffer, 0.2M Solution

Acetic acid, 0.2M solution	5 drops
Sodium acetate, 0.2M solution	18 mL
Adjust to pH 5.6; make fresh each time	

Working Silver Solution

Acetic acid- acetate buffer pH 5.6	5 mL
Distilled water	48.5 mL
Silver nitrate, 1% solution	1.5 mL
Prepare fresh each time.	

Reducing Solution

Hydroquinone	0.5 g
Sodium sulfite, anhydrous	1.25 g
Distilled water	50 mL
Prepare just before use	

Nuclear-Fast Red Solution

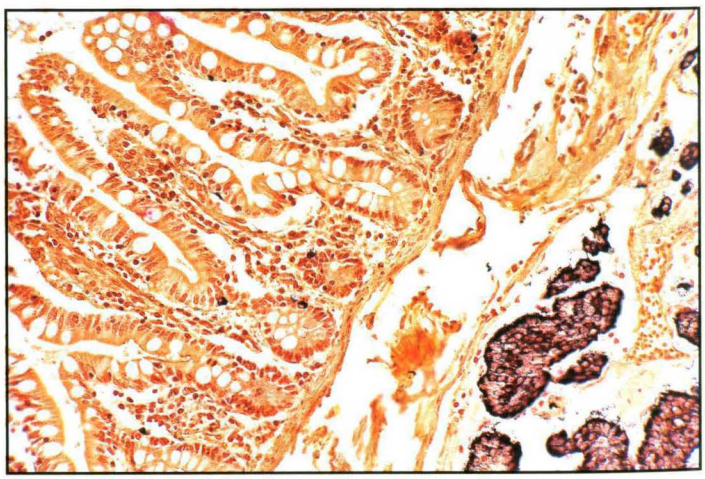
See the Prussian blue stain for ferric iron, p257

■ *Procedure*

1. Deparaffinize slides through xylene and alcohols to distilled water.
2. Place slides in room temperature working silver solution. Incubate in a 60°C water bath for 1 hour. Stir solution occasionally.
3. Drain slides briefly.
4. Place slides in freshly prepared reducing solution (preheated to 40°C to 50°C) for 1 minute.
5. Rinse slides well in distilled water.
6. Repeat step 2 for 10 minutes.
7. Drain slides briefly, and place in reducing solution again for 1 minute.
8. Rinse sections in distilled water.
9. Counterstain with nuclear-fast red for 5 minutes.
10. Rinse sections in distilled water.
11. Dehydrate, clear, and mount with synthetic resin.

■ *Results* **[i11.13]**

• Argentaffin granules	Dark brown to black
• Argyrophil granules	Dark brown to black
• Nuclei	Red
• Background	Pale yellow-brown



[i11.13] A section of small intestine containing a carcinoid tumor stained with the Grimelius stain. Note the black-stained argyrophil cells present in the intestinal epithelium and the black-stained islands of tumor in the submucosa. [Reprinted with permission from Test your knowledge. *J Histotechnol* 1987;10:231]

■ *Technical Note*

Argentaffin substances will also stain with this method because they will bind and reduce the silver. To differentiate between argentaffin and argyrophil substances, both the Grimelius and the Fontana-Masson techniques must be used and the results compared.

CHURUKIAN-SCHENK METHOD FOR ARGYROPHIL GRANULES [CHURUKIAN 1979, CHURUKIAN 1993]

■ *Purpose*

Demonstration of argyrophil granules in neurosecretory tumors

■ *Principle*

Certain tissue elements have the ability to bind silver ions from solution but no inherent ability to reduce the silver to its visible metallic form. An external or chemical reducer is used for this purpose. These substances are referred to as argyrophil.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

43°C and 58°C to 60°C water baths, pH meter, chemically clean Coplin jars, Erlenmeyer flasks, and graduated cylinders

■ *Technique*

Cut paraffin sections at 4 to 5 µm.

■ *Quality Control*

An argyrophil-positive carcinoid tumor is preferred, but a section of small intestine can be used. Glassware must be chemically clean, and nonmetallic forceps must be used to transfer the slides.

■ *Reagents*

Citric Acid, 0.3% Solution

Citric acid	0.3 g
Distilled water	100 mL

Acidified Water

Distilled water	100 mL
Add enough 0.3% citric acid to bring the pH of the water to 4.0 to 4.2	

Silver Nitrate, 0.5% Solution

Silver nitrate	0.5 g
Distilled water	100 mL

Reducing Solution

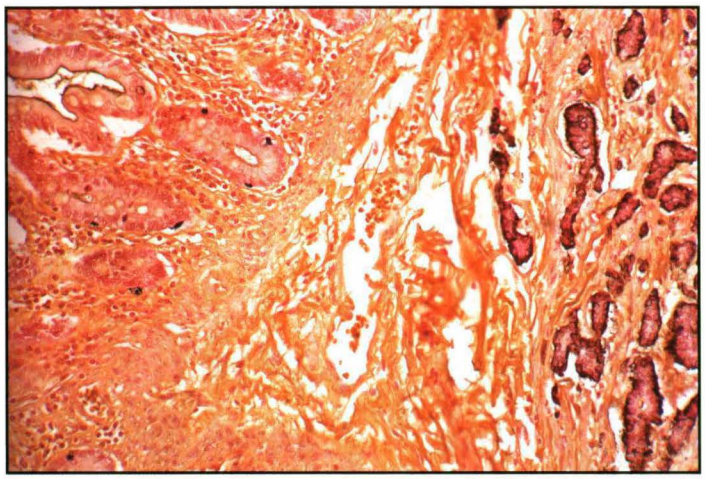
Sodium sulfite (anhydrous)	2.5 g
Hydroquinone	0.5 g
Distilled water	50mL
Prepare just before use	

Nuclear-Fast Red Solution

See the Prussian blue stain for ferric iron, p257

■ *Procedure*

1. Deparaffinize slides through xylene and alcohols to acidified water, pH 4.0 to 4.2.
2. Place slides in 0.5% silver nitrate in a 43°C water bath for 2 minutes. Transfer to a 58°C water bath for 2 hours. Cracking of the Coplin jar is prevented by first placing it in the lower-temperature bath.
3. Rinse sections in 3 changes of distilled water.
4. Transfer the slides to the reducing solution (previously heated in the 58°C water bath) for 30 to 60 minutes.
5. Rinse sections in 3 changes of distilled water.
6. Return sections to the same silver nitrate (step 2) at 58°C for 10 minutes.
7. Rinse sections in 3 changes of distilled water.
8. Place in the same reducing solution (step 4) at 58°C for 5 minutes.
9. Rinse sections in 3 changes of distilled water.
10. Counterstain in nuclear-fast red for 3 minutes.
11. Rinse in 3 changes of distilled water.
12. Dehydrate with 2 changes each of 95% and absolute alcohols.
13. Clear in xylene, and mount with synthetic resin.



[i11.14] A section from the same block as shown in **[i11.13]** stained with the Churukian-Schenk technique. Both the argentaffin cells of the epithelium and the islands of tumor are stained brown-black.

■ *Results* [i11.14]

• Argyrophil granules	Black
• Argentaffin substances	Black
• Nuclei	Red
• Background	Yellow-brown

MICROWAVE CHURUKIAN-SCHENK METHOD FOR ARGYROPHIL GRANULES [CHURUKIAN 1979, CHURUKIAN 1993]

■ *Purpose*
Demonstration of argyrophil granules in neurosecretory tumors

■ *Principle*
Certain tissue elements have the ability to bind silver ions from solution but no inherent ability to reduce the silver to its visible metallic form. An external or chemical reducer is used for this purpose. These substances are referred to as argyrophil.

■ *Fixative*
10% neutral-buffered formalin

■ *Equipment*
Microwave oven, pH meter; chemically clean plastic Coplin jars, Erlenmeyer flasks, graduated cylinders

■ *Technique*
Paraffin sections cut at 4 to 5 µm

■ *Quality Control*
An argyrophil-positive carcinoid tumor is preferred, but a section of small intestine can be used. Glassware must be chemically clean, and nonmetallic forceps must be used to transfer the slides.

■ *Reagents*

Citric Acid-Glycine Stock Solution

Citric acid	0.06 g
Glycine	1.2 g
Distilled water	100 mL

Citric Acid-Glycine Working Solution

Citric acid-glycine stock solution	5 mL
Distilled water	500 mL

Silver Nitrate, 0.5% Solution

Silver nitrate	0.2 g
Citric acid-glycine working solution	40 mL

Reducing Solution

Sodium sulfite (anhydrous)	2 g
Hydroquinone	0.4 g
Distilled water	40 mL
Prepare just before use	

Nuclear-Fast Red Solution

See the Prussian blue stain for ferric iron, p257

■ *Procedure*

1. Deparaffinize and hydrate to citric acid-glycine working solution.
2. Place sections in 40 mL of 0.5% silver nitrate solution in a plastic Coplin jar, and cap loosely. Microwave at power level 5 (300 W) for 1 minute. Dip the slides up and down several times, and allow them to remain in the hot solution (80°C) for 2 minutes.
3. Rinse in 4 changes of distilled water.
4. Place 40 mL of the reducing solution in a plastic Coplin jar, and cap loosely. Microwave at power level 6 (360 W) for 40 seconds. Immediately place the slides in this hot solution, and allow them to remain in the warm solution (65°C) for 1 minute.
5. Rinse in 4 changes of distilled water.

6. Place the slides in the same 0.5% silver nitrate as used in step 2 for 5 minutes.

7. Rinse in 4 changes of distilled water.

8. Place slides in the same reducing solution as in step 4 for 1 minute.

9. Rinse in 4 changes of distilled water.

10. Place the slides back in the 0.5% silver nitrate solution, and microwave at power level 5 for 15 seconds. Dip the slides up and down several times, and allow them to remain in the hot solution for 1 minute.

11. Rinse in 4 changes of distilled water.

12. Microwave the reducing solution used in steps 4 and 8 at power level 5 for 15 seconds. Immediately place the slides in the solution, and allow them to remain in the solution for 1 minute.

13. Rinse in 4 changes of distilled water.

14. Counterstain with nuclear-fast red solution for 1 minute.

15. Rinse in 3 changes of distilled water.

16. Dehydrate in 2 changes each of 95% and absolute alcohols.

17. Clear in 3 or 4 changes of xylene, and mount with synthetic resin.

■ *Results* [i11.15]

• Argyrophil and argentaffin cells	Black
• Nuclei	Orange to red
• Background	Light yellow-orange

■ *Technical Notes*

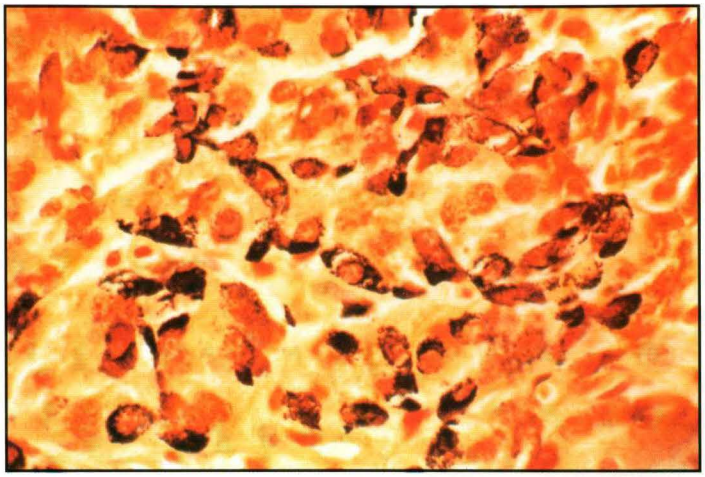
1. The shelf-life of silver nitrate and hydroquinone can be greatly increased by storing these chemicals in a refrigerator at 4°C [Churukian 1993].

2. The slides should be dipped up and down after microwaving in steps 2 and 10 to equalize the temperature of the staining solution.

GOMORI METHENAMINE-SILVER METHOD FOR URATES [PEARSE 1972, SHEEHAN 1980]

■ *Purpose*

Demonstration of urates in tissue. A disturbance of uric-acid metabolism known as gout can cause the deposition of urate crystals, especially around joints and in soft tissues. Collections of the urate crystals are known as gouty tophi.



[i11.15] Argyrophil cells in a pancreatic islet stained with the microwave Churukian-Schenk procedure. [Image courtesy of Churukian CJ, University of Rochester Medical Center]

■ *Principle*

Urates are stained black with this method, presumably by reacting with the silver, which is then reduced to its metallic form.

■ *Fixative*

Absolute alcohol is required.

■ *Equipment*

56°C to 58°C water bath, chemically clean Coplin jars, graduated cylinders, pipettes

■ *Technique*

Cut paraffin sections at 4 to 5 µm.

■ *Quality Control*

A section containing urates must be used.

■ *Reagents*

Silver Nitrate, 5% Solution

Silver nitrate	25 g
Distilled water	500 g

Methenamine Solution, 3% Solution

Hexamethylenetetramine (Eastman Kodak Co)	3 g
Distilled water	100 mL

Stock Methenamine-Silver Nitrate Solution

Methenamine, 3% solution	100 mL
Silver nitrate, 5% solution	5 mL

A white precipitate will form but will immediately dissolve upon shaking. The clear solution will remain usable for months if stored in the refrigerator.

Sodium Borate (Borax), 5% Solution

Sodium borate	5 g
Distilled water	100 mL

Working Methenamine-Silver Nitrate Solution

Methenamine-silver solution	25 mL
Distilled water	25 mL
Sodium borate, 5% solution	3 mL

Sodium Thiosulfate, 3% Solution

Sodium thiosulfate	3 g
Distilled water	100 mL

Light Green Stock Solution

Light green, SF (yellowish)	1 g
Distilled water	500 mL
Glacial acetic acid	1 mL

Working Light Green Solution

Light green stock solution	10 mL
Distilled water	50 mL

■ *Procedure*

1. Deparaffinize sections, and rinse with several changes of absolute alcohol. Do not hydrate sections.
2. Place sections in working methenamine-silver solution that has been preheated to 60°C in a water bath.
3. Incubate for 30 minutes at 60°C. The urate crystals should be black.
4. Rinse sections in distilled water.
5. Place sections in 3% sodium thiosulfate for 5 minutes.
6. Wash in running water for 2 to 3 minutes
7. Rinse in several changes of distilled water.
8. Counterstain with working light green solution for 1½ to 2 minutes
9. Dehydrate with 2 changes each of 95% and absolute alcohol.
10. Clear with 2 or 3 changes of xylene, and mount with a synthetic resin.

■ *Results* **[i11.2, p256]**

- Urates — Black
- Background — Blue-green

■ *Technical Note*

Calcium may also be demonstrated if the deposits are large enough to withstand dissolving in the silver staining solution.

BILE STAIN [HALL 1960]

■ *Purpose*

To demonstrate the presence of bilirubin in tissue and to distinguish bile pigments from other pigments found in tissue. An accumulation of bile within the liver canaliculi indicates obstructive jaundice; this may be attributed to the inability of bile to flow from the liver into the gallbladder and then into the small bowel; this intracanalicular bile is readily identifiable, but bile in hepatocytes may be confused with lipofuscin, as both are yellow-tan. In addition to bile duct obstruction caused by stones, tumors and diseases that impact bile storage and transport include primary biliary cirrhosis, biliary atresia, hepatocellular carcinoma, and tumors in the head of the pancreas.

■ *Principle*

A specific and easily identifiable green color develops when bilirubin is oxidized to biliverdin in an acid medium. This oxidation reaction is rapidly accomplished by ferric chloride in trichloroacetic acid medium.

■ *Fixative*

10% neutral-buffered formalin

■ *Equipment*

Coplin jars, Whatman #1 filter paper, graduated cylinders

■ *Technique*
Cut paraffin sections at 4 to 5 µm. Frozen sections also may be used.

■ *Quality Control*
Tissue containing bile must be used as a control.

■ *Reagents*

Ferric Chloride, 10% Solution

Ferric chloride	10 g
Distilled water	100 mL

Fouchet Reagent

Trichloracetic acid	25 g
Distilled water	100 mL
Ferric chloride, 10%	10 mL

van Gieson Solution

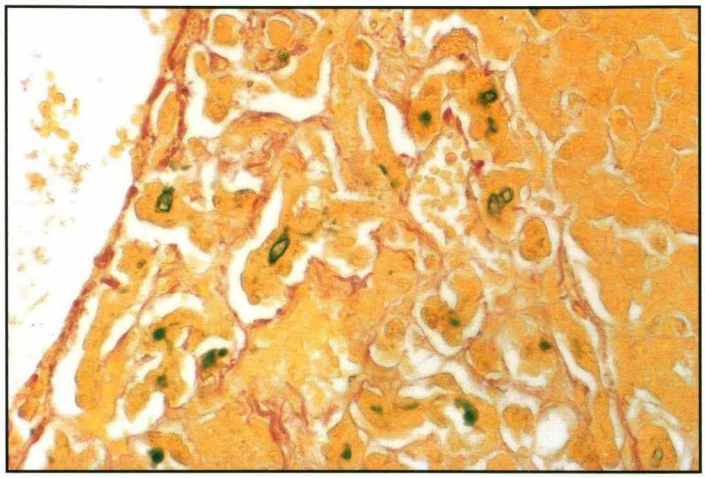
Acid fuchsin, 1% solution	20 mL
Picric acid, saturated solution	380 mL

■ *Procedure*

1. Deparaffinize and hydrate sections to distilled water.
2. Wash sections well in distilled water.
3. Stain sections for 5 minutes in freshly filtered Fouchet reagent.
4. Wash in tap water, then rinse in distilled water.
5. Stain sections in van Gieson solution for 5 minutes
6. Place slides directly into 95% alcohol and rinse well.
7. Dehydrate in 2 changes of absolute alcohol, clear in 2 changes of xylene, and mount in synthetic resin.

■ *Results* [i11.16]

- Bile or bilirubin — Emerald green to olive drab
- Background — Yellow



[i11.16] Fouchet reagent has oxidized the bilirubin to biliverdin in this section of liver that contains bile (stained emerald green).

VON KOSSA CALCIUM STAIN [LILLIE 1965, CARSON 1984]

■ *Purpose*
Identification of the presence of calcium in tissue

■ *Principle*
Although the reaction involved is a chemical one, it is an indirect way of detecting calcium. The silver reacts with the anions, primarily carbonate and phosphate, of the calcium salts. Bright light reduces the silver salt to metallic silver, and unreduced silver removed by sodium thiosulfate. An example of the reaction is as follows:

$$CaCO_3 + 2AgNO_3 \longrightarrow Ag_2CO_3 + Ca(NO_3)_2$$

$$Ag_2CO_3 + 2H^+ \xrightarrow{\text{sunlight reduction}} 2Ag^0 + CO_2 + H_2O$$

(metallic silver)

■ *Fixative*
Alcohols or 10% neutral-buffered formalin; alcohols are preferred

■ *Equipment*
Sunlight (preferred) or UV light, chemically clean Coplin jars, graduated cylinders, Erlenmeyer flasks

■ *Technique*
Cut paraffin sections at 4 to 5 µm.

■ *Quality Control*
A section containing calcium must be used.

■ *Reagents*

Silver Nitrate, 5% Solution

Silver nitrate	5 g
Distilled water	100 mL

Sodium Thiosulfate, 5% Solution

Sodium thiosulfate	5 g
Distilled water	100 mL

Nuclear-Fast Red Solution

See the Prussian blue stain for ferric iron, p257

■ *Procedure*

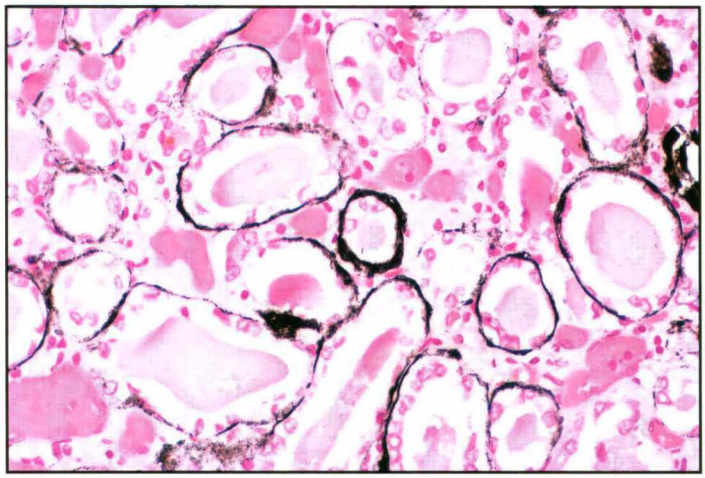
1. Deparaffinize and hydrate paraffin sections to distilled water.
2. Place sections in silver nitrate solution and expose to bright sunlight for 10 to 20 minutes. If the day is overcast, longer incubation will be necessary. Check the slides periodically and stop the reaction when the calcium salts are brown-black.
3. Rinse sections in distilled water.
4. Place slides in 5% sodium thiosulfate for 2 to 3 minutes.
5. Wash slides well in distilled water.
6. Counterstain for 5 minutes in nuclear-fast red.
7. Wash sections well in water.
8. Dehydrate and clear in 2 changes each of 95% alcohol, absolute alcohol, and xylene.
9. Mount with synthetic resin.

■ *Results* [i11.17]

- Calcium salts — Black
- Background — Red

■ *Technical Notes*

1. As noted before, this method detects anions combined with calcium, and not the calcium.
2. Alcoholic iodine solution used for the removal of mercury pigment may also remove some of the calcium salts from the tissue [Della Speranza 1987].



[i11.17] Nephrocalcinosis is demonstrated in this section of kidney stained with the von Kossa reaction. Calcium deposits around the renal tubules are stained black.

3. Most texts state that calcium stains black with the von Kossa reaction, and that has been my experience [i11.17]; however, Meloan and Puchtler [1985] state that, as emphasized by von Kossa, the black deposits in this reaction are caused by a reduction of silver by organic matter in the sections. The black reaction product is an artifact that occurs only when the sections are exposed to strong light. If the reaction is carried out in subdued light, yellow to yellowish-brown silver phosphate is selectively demonstrated. Silver carbonate is soluble in sodium thiosulfate and is not demonstrated.
4. Luna [1979] notes that problems encountered with this technique may be the result of the use of unbuffered formalin (formalin pigment will reduce silver), the use of artificial light (produces a brown reaction product), or inadequate exposure to the silver solution.

ALIZARIN RED S CALCIUM STAIN [MCGEE-RUSSELL 1958]

■ *Purpose*

Identification of the presence of calcium in tissue

■ *Principle*

Alizarin red S will react with the cations calcium, magnesium, manganese, barium, and strontium, but normally only calcium is present in tissue in sufficient quantities for demonstration. Calcium forms an alizarin red S-calcium complex in a chelation process.

■ *Fixative*

Alcoholic formalin or 10% neutral-buffered formalin

■ *Equipment*

Coplin jars, graduated cylinders, Erlenmeyer flasks, pH meter

■ *Technique*
Cut paraffin sections at 4 to 5 µm.

■ *Quality Control*
A section containing calcium must be used.

■ *Reagents*

Alizarin Red S Stain, 2% Solution

Alizarin red S	2 g
Distilled water	100 mL

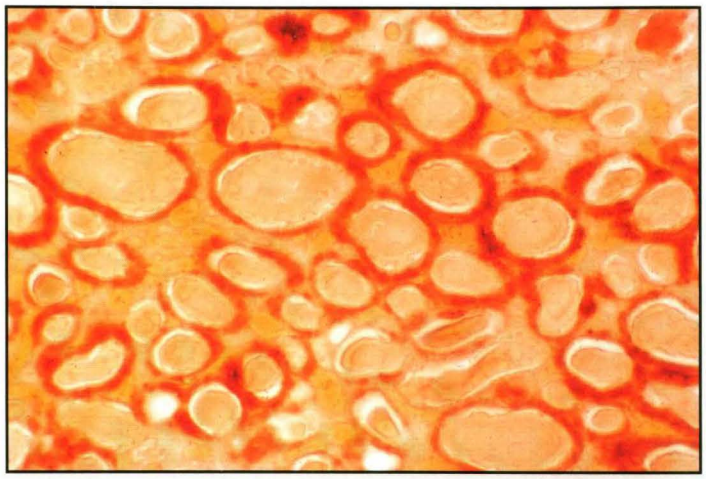
Mix the solution, and adjust the pH to 4.1 to 4.3 with 0.5% ammonium hydroxide. The pH of the solution is critical.

■ *Procedure*

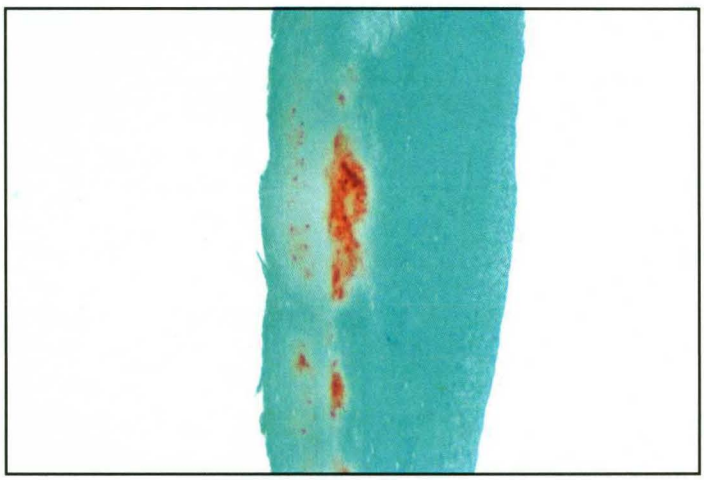
1. Deparaffinize and hydrate paraffin sections to 50% alcohol.
2. Rinse sections rapidly in distilled water.
3. Place slides in the alizarin red S staining solution. Check the reaction microscopically, and remove slides when an orange-red lake forms (30 seconds to 5 minutes).
4. Shake off the excess dye, and carefully blot the sections.
5. Dehydrate in acetone (10 to 20 seconds) and in acetone-xylene (10 to 20 seconds).
6. Clear in xylene, and mount in synthetic resin.

■ *Results* **[i11.18]**
- Calcium deposits — Orange-red

■ *Technical Notes*
1. The reaction product is birefringent.
2. The reaction is controlled microscopically to prevent diffusion artifact.
3. Vacca [1985] states that during calcification, amorphous calcium phospate is first deposited in tissue. Calcium is soluble in this form, but it is gradually transformed into the less soluble crystalline apatitelike compounds. Amorphous calcium phosphate deposits are very soluble below pH 8.5 and are easily lost during routine fixation and/or staining. Vacca describes procedures for staining at pH 9.0 (alkali-soluble calcium deposits), pH 7.0 (alkali-insoluble calcium deposits), and pH 4.0 (bone and other hard calcium deposits).



[i11.18] A section of the same kidney shown in **[i11.17]** stained with alizarin red S. Calcium deposits are stained orange-red. Note that the calcium deposits appear heavier than in the von Kossa-stained section because of slight diffusion of the calcium during the staining process.



[i11.19] A section of calcified artery has been stained with the Dahl alizarin red procedure which uses a light green counterstain. [Image courtesy of Jones ML, Winston-Salem, NC]

4. The procedure of Dahl as described by Luna [1968] may also be used, but care must be taken if a counterstain is applied **[i11.19].** Lillie and Fullmer [1976] consider the method of McGee-Russell to be better than that of Dahl.

RHODANINE METHOD FOR COPPER [LINDQUIST 1969, SHEEHAN 1980]

■ *Purpose*
Detection of copper in tissue, especially in liver, in Wilson disease

■ *Principle*
This method appears to be more sensitive than the rubeanic acid method; however, it has been suggested that rhodanine demonstrates the protein to which the copper binds rather than the copper itself. Therefore, although considered more sensitive, rhodanine may be less specific than the rubeanic acid methods and false-positive results may be obtained.

■ *Fixative*
10% neutral-buffered formalin

■ *Equipment*
Plastic Coplin jars, graduated cylinders, Erlenmeyer flasks, Whatman #1 filter paper.

■ *Technique*
Cut paraffin sections at 6 to 8 µm.

■ *Quality Control*
A section containing copper must be used.

■ *Reagents*

Saturated Rhodanine Solution (Stock)

5-(*p*-dimethylamino-benzylidine) rhodanine	0.2 g
Absolute ethanol	100 mL

Working Rhodanine Solution

Saturated rhodanine solution	3 mL
Distilled water	47 mL
Filter before use	

Diluted Mayer Hematoxylin

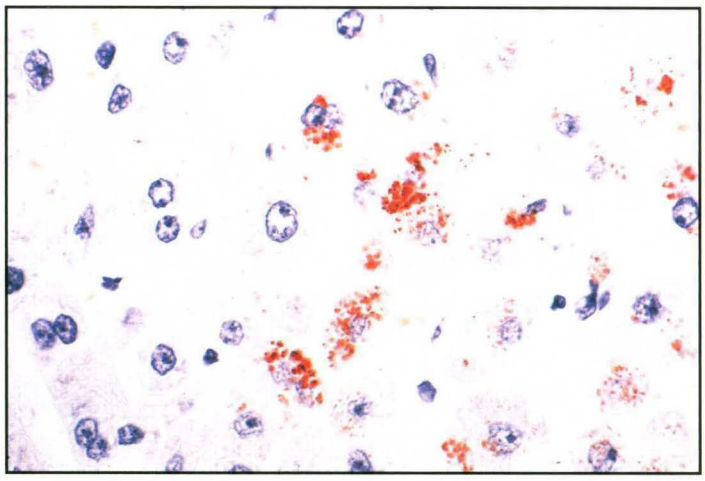
Mayer hematoxylin (see chapter 6)	25 mL
Distilled water	25 mL

Sodium Borate (Borax), 0.5%

Sodium borate	0.5 g
Distilled water	100 mL

■ *Procedure*

1. Deparaffinize sections, and hydrate to distilled water.
2. Using plastic Coplin jars, place slides in 50 mL of working rhodanine solution. Leave 18 hours at 37°C .
3. Rinse sections well in distilled water.
4. Stain 10 minutes in dilute Mayer hematoxylin.



[i11.20] This section from the liver of a patient with Wilson disease was stained for copper with the Lindquist rhodanine method. Copper is stained red. [Image courtesy of Churukian CJ, University of Rochester Medical Center]

5. Rinse with distilled water
6. Rinse slides quickly in 0.5% sodium borate solution.
7. Rinse with distilled water.
8. Dehydrate, clear, and mount sections with synthetic resin.

■ *Results* **[i11.20]**

• Copper	Bright red to red yellow
• Nuclei	Light blue

■ *Technical Notes*

1. If the copper concentration is low, fading may occur after coverslips are applied, and the stained copper may be difficult to distinguish from lipofuscin [Sheehan 1980].
2. Fetal liver, fixed no longer than 24 hours before paraffin processing, provides a good control for copper. Fetal liver always contains 3 to 4+ copper within hepatocytes [Churukian 1993].
3. Do not overstain with the hematoxylin, or the copper may be masked.

MICROWAVE RHODANINE COPPER METHOD [CHURUKIAN 1993]

■ *Purpose*
Detection of copper in tissue, especially in liver, in Wilson disease

■ *Principle*
This method appears to be more sensitive than the rubeanic acid method; however, it has been suggested that rhodanine demonstrates the protein to which the copper binds rather than the copper itself. Therefore, although considered more sensitive, rhodanine may be less specific than the rubeanic acid methods, and may lead to false-positive results.

■ *Fixative*
10% neutral-buffered formalin

■ *Equipment*
Microwave oven, plastic Coplin jars, graduated cylinders, Erlenmeyer flasks, Whatman #4 filter paper

■ *Technique*
Cut paraffin sections at 6 to 8 µm.

■ *Quality Control*
A section containing copper must be used.

■ *Reagents*

Saturated Rhodanine Solution (Stock)

5-(*p*-dimethylamino-benzylidine) rhodanine	0.2 g
Absolute ethanol	50mL
Filter the solution through Whatman #4 filter paper, and store in a refrigerator. The solution is stable for about 1 week.	

Sodium Acetate-Formalin Solution

Sodium acetate trihydrate	2 g
Distilled water	100 mL
Formalin, 37% to 40%	0.3 mL

Working Rhodanine Solution

Rhodanine stock solution	5 mL
Sodium acetate-formalin solution	45 mL
Filter before use	

Diluted Mayer Hematoxylin

Mayer hematoxylin (see chapter 6)	25 mL
Distilled water	25 mL

Sodium Borate (Borax), 0.5%

Sodium borate	0.5 g
Distilled water	100 mL

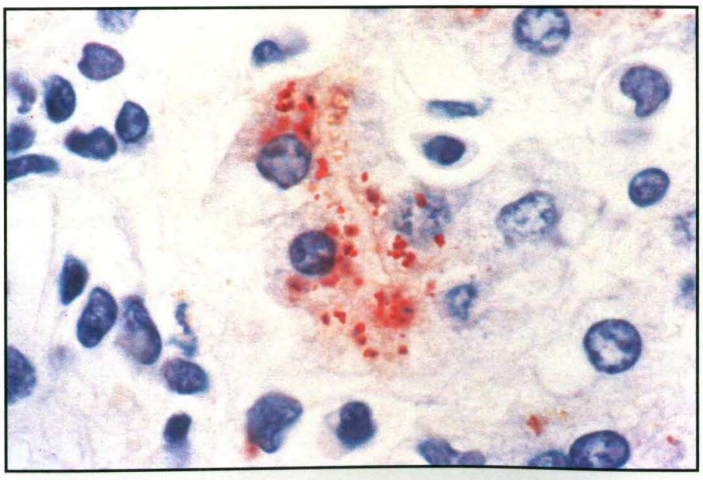
■ *Procedure*

1. Deparaffinize slides, and hydrate to distilled water.
2. Place slides in 50 mL of working rhodanine solution in a plastic Coplin jar with the cap loosely applied, and microwave at power level 1 (60 W) for 5 minutes. Dip the slides up and down several times, and allow them to remain in the hot solution for 3 minutes.
3. Return the Coplin jar to the microwave oven, and microwave at power level 1 for 90 seconds. Allow the slides to remain in the hot solution for 15 minutes.
4. Wash with 6 changes of distilled water.
5. Place in diluted Mayer hematoxylin for 10 minutes.
6. Rinse with 2 changes of distilled water.
7. Blue nuclei with 0.5% sodium borate for 5 seconds.
8. Rinse with 4 changes of distilled water.
9. Mount with an aqueous mounting medium.

■ *Results* **[i11.21]**

• Copper	Bright red or rust-red intracytoplasmic granules
• Nuclei	Blue

■ *Technical Note*
According to Churukian [1993], the results obtained with the microwave stain may not always be as good as those obtained with the overnight method conducted in a 30°C oven.



[i11.21] Copper in a liver section from a patient with Wilson disease stained with the microwave rhodanine method. [Image courtesy of Churukian CJ, University of Rochester Medical Center]

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LEARNING ACTIVITIES

1. **Perform the Prussian blue, Schmorl ferric-ferricyanide reduction test, Fontana-Masson, Grimelius or Churukian-Schenk, Gomori methenamine-silver, Hall (Fouchet), von Kossa, alizarin red S, and rhodanine staining procedures using both conventional and microwave techniques. You may choose any tissue that will demonstrate positive staining.**

2. **Microscopically examine each stained section and compare the results with those described in the text. If the results are unsatisfactory, analyze the procedural steps for possible source of error. If a mistake is identified, repeat the stain after correcting the problem, and reexamine the slides.**

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CHAPTER 12

Immunohistochemistry

OBJECTIVES

On completing this chapter, the student should be able to do the following:

1. Define:
    a. antigen
    b. antibody
    c. substrate
    d. chromogen
    e. fluorochrome
    f. epitope
    g. secondary antibody
    h. multilink secondary antiserum
2. Differentiate between polyclonal antisera and monoclonal antibodies
3. Explain each of the following immunohistochemical techniques:
    a. direct
    b. indirect
    c. peroxidase-antiperoxidase
    d. avidin-biotin complex
    e. polymeric detection system
4. List 2 fluorochromes
5. Identify 2 common chromogens used with immunoperoxidase techniques
6. Identify 2 chromogens used with immunoalkaline phosphatase techniques
7. Identify at least 3 enzyme systems used in enzyme immunohistochemistry
8. Describe the preferred method of specimen preparation for immunofluorescence
9. Identify problems that may be encountered with formaldehyde fixation of specimens for immunohistochemical staining
10. Identify fixatives other than formalin, and describe any special issues with respect to immunohistochemistry
11. Describe 2 blocking reactions, and identify the purpose of each
12. Identify the 5 heavy chains and 2 light chains that make up the different classes of immunoglobulins
13. Describe the use of negative tissue and reagent controls
14. List at least 6 problems that may occur with immunohistochemical staining, and state a corrective action for each
15. State why preparing positive controls in the laboratory is preferred to purchasing them
16. List 2 methods of epitope retrieval
17. List at least 3 solutions that have been used for heat-induced epitope retrieval
18. List at least 3 ways of heating in heat-induced epitope retrieval methods (HIER)
19. List at least 3 solutions that have been used for enzyme-induced epitope retrieval (EIER)
20. Identify alcohol-soluble and non-alcohol-soluble chromogens
21. List 4 chemicals used to intensify the 3,3'-diaminobenzidine (DAB) reaction

Introduction

In the clinical laboratory, immunohistochemistry is a heavily used technique for assisting the pathologist in making a diagnosis. Immunohistochemistry is used to determine the origin, prognosis, and treatment of a tumor. It is important to understand the basics of immunology, and the reagents and methods used, in order to achieve reproducible, high-quality patient care. Rigorous quality control (QC) is vital to ensure that results are accurate and consistent with expected staining patterns. The major role an immunohistochemist plays is troubleshooting for variables introduced by fixation, processing, and tissue types. The immunohistochemist should be familiar with both the controls used and the staining patterns of an antibody to troubleshoot unexpected results.

Techniques such as immunofluorescence and enzyme immunoassays have been available as diagnostic tools for about 50 years, but the development of immunodiagnostic methods, including immunohistochemistry, was much slower because of the unpredictable quality of polyclonal antibodies.

Since the immunohistochemistry revolution began in the 1980s, a number of dramatic advances have been made in immunology and immunodiagnostic methods; these advances have had a substantial impact on all phases of laboratory medicine. In most laboratories, the use of immunohistochemical stains has become as routine as the use of any other special stain, and in many instances these stains have completely replaced the older histochemical or empirical methods.

The next level of development in immunohistochemical staining is the introduction of standards by the College of American Pathologists related to predictive markers used for therapeutic patient care, which qualify the patient for specific drug treatment. The laboratory's role is pivotal in ensuring that all specimens are processed and treated according to the recommended guidelines.

General Immunology

The immune system exerts its control through humoral (antibody) and cellular components. The overview of the immune system presented here will emphasize only the humoral or antibody system and how it relates to the discussion of immunohistochemical techniques.

ANTIBODY

An antibody is a host protein (immunoglobulin) produced in response to the presence of foreign molecules, organisms, or other agents in the body. Antibodies, commonly known as immunoglobulins, are proteins that are produced by B lymphocytes in response to antigenic stimulation. An immunoglobulin is a Y-shaped protein molecule that is composed of both heavy and light chains. Classes of antibodies differ in structure and function, with each immunoglobulin antigenically distinct. An immunoglobulin (Ig) is composed of 2 identical heavy chains (γ, α, μ, δ, or ε) which determine the Ig subclass (IgG, IgA, IgE, IgD, and IgM). The light chain is either κ or λ. These proteins are expressed on the cell surface or membrane, and secreted into blood and other fluids by plasma cells. The upper arms of the Y are the regions of the antibody that bind to the short arms of their specific antigen. This region of the antigen is known as an epitope, and is the site at which the antibody attaches to tissue. An antibody may target more that 1 antigen, but it is specific for only 1 epitope. Proteins are very good antigens because of their large size. The fact that antibodies (eg, immunoglobulins) are proteins makes them very potent antigens.

ANTIGEN

An antigen is a molecule made up of proteins, carbohydrates, or other polymers, and is capable of producing an immune response in animals or cell cultures for the production of antibodies. Antigens in tissues bind antibodies at different cell sites: the membrane, cytoplasm, nucleus, organelles, or a combination of more than 1. Many compounds are antigenic. Because of their large size, proteins are ideal for immunization and for binding, but polysaccharides, nucleic acids, and other polymers also can be antigenic. The most common antigens that induce antibody production by the body are bacteria and viruses.

POLYCLONAL ANTISERA

If blood was drawn from your arm, and IgM antibody was isolated from it and injected into a rabbit, your IgM antibody would act as an antigen and stimulate the rabbit to make anti-human IgM antibody. Serum from the immunized rabbit could then be used as a polyclonal anti-human IgM antibody reagent. When lymphocytes are exposed to antigen, some of the lymphocytes proliferate, and each lymphocyte forms a cell line. All cells in a single clone, or cell line, produce identical antibodies, but various clones produce antibodies of different classes, with specificities to different molecular sites (epitopes) on the antigen. The result of antigenic stimulation is the production of a mixture of antibodies from many clones of lymphocytes. This pool of antibodies is known as polyclonal antiserum.

Polyclonal antiserum is highly sensitive because it binds to more than 1 epitope, but this also means that it is not as selective, resulting in some nonspecific staining (background). The titer used to stain tissue must be carefully selected to ensure no false-positive staining. Examples of hosts that are used to produce polyclonal antiserum include rabbit, goat, horse, sheep and human. Because of the variability of the immune response from one animal to another, polyclonal antibodies are difficult to standardize and can be used only in a limited number of immunoassays. Pooled antibodies made from many immunized animals of the same species are less likely to exhibit major batch-to-batch variations than pools made from only a few animals.

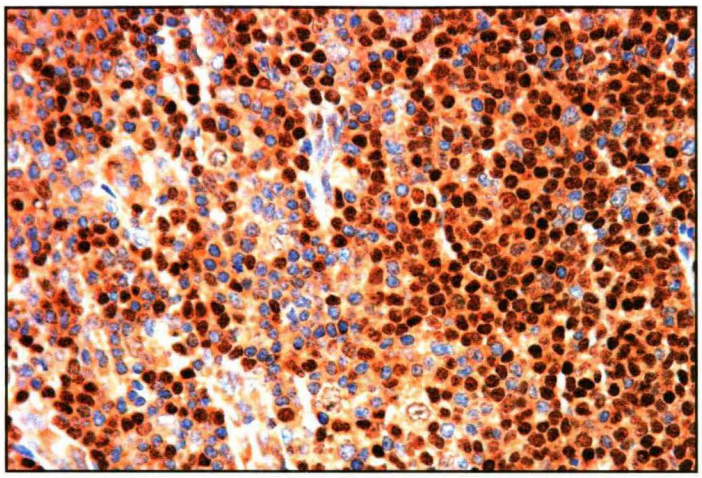
MONOCLONAL ANTIBODIES

The development of monoclonal antibody techniques revolutionized the whole science of immunology. Monoclonal antibodies are prepared by injecting mice with an antigen. B lymphocytes are fused with nonsecreting myeloma cells (nonsecreting plasma

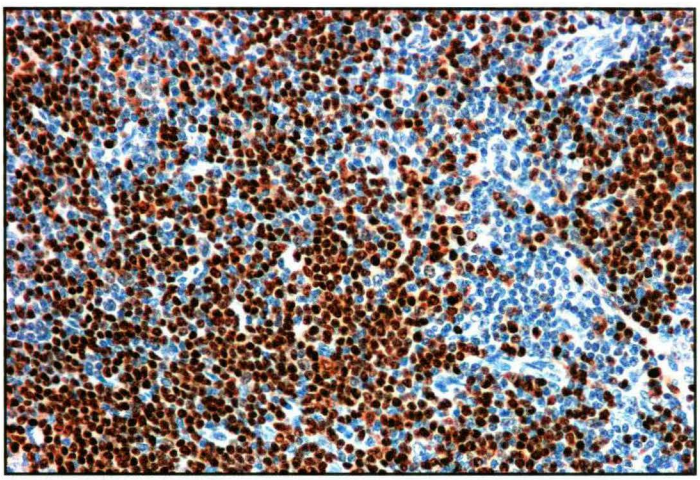
cell tumor). This in vitro fusion yields hybrid cells (hybridoma) that retain the antibody secretion capability of the B cell and the immortality of the tumor cells. Hybridoma cells can be cloned, and a single clone is capable of producing antibody that is identical in molecular structure to the original. The antibody can be produced in unlimited quantities by tissue culture or by transplantation into the peritoneal cavities of mice. Although other animal species can be used, monoclonal antibodies for the histopathology laboratory are produced most commonly in mice. Monoclonal antibodies can be characterized, standardized, and produced in unlimited quantities. The advantages of monoclonal antibodies include high homogeneity, the absence of nonspecific antibodies, and no batch-to-batch or lot-to-lot variability. Mouse monoclonal antibodies are purer than polyclonal antibodies, and monoclonal antibodies display the most desirable attributes (high affinity and selectivity) of polyclonals without displaying most of the undesirable characteristics (lack of chemical homogeneity and lack of continuous supply).

RABBIT MONOCLONAL ANTIBODIES

Rabbit monoclonal antibody production was first reported by Spieker-Polet [1995]. Since that initial report, the production of rabbit monoclonal antibodies is making its way into the clinical laboratory. This technology follows the same principal of monoclonal hybridoma production, but uses "rabbit fusion partner cells," which fuse with rabbit B cells. These antibodies have the potential to provide both the sensitivity of a rabbit antibody and specificity of targeting a single epitope **[i12.1]**, **[i12.2]**.



[i12.1] A section stained with CyclinD1 mouse monoclonal antibody demonstrating poor signal-to-noise ratio and nonspecific background staining.



[i12.2] A section stained with CyclinD1 rabbit monoclonal antibody demonstrating greater sensitivity and eliminating the nonspecific staining. Compare with **[i12.1]**

Tissue Handling

FROZEN TISSUE FIXATION AND PROCESSING

Before performing any immunologic procedure, the stability of the antigen must be considered. Because most antigens are soluble in aqueous solutions, the antigens must be fixed in situ when the technique permits.

Frozen sections of unfixed tissue constitute the classic preparation of tissue for immunofluorescence, because antigenic reactivity is least impaired and fluorescent antibody staining is strongest; however, in such sections, soluble antigens are lost [Sternberger 1979]. Formalin-fixed paraffin sections are rarely used in immunofluorescence because of inconsistent results; apparently, fixation and processing impair antigenic reactivity beyond the sensitivity of immunofluorescence [Sternberger 1979].

Dehydration, or drying, and fixation by chemical reagents are the 2 major categories of fixation. Dehydration unfolds and changes the solubility of the protein. Chemical fixation denatures and stabilizes proteins by coagulation, by forming additive compounds, or by a combination of the 2 actions. Acetone and alcohol are coagulating, nonadditive reagents; formaldehyde is a noncoagulating, additive fixative that cross-links proteins; and mercuric chloride is both a coagulating and additive fixative. The coagulant fixatives that do not form additive compounds permit good penetration of the antibody and do not block immunoreactive determinants. However, the treatment of cryostat sections with acetone, as currently practiced, does not achieve complete fixation. Extended immunohistochemical procedures may produce deleterious morphologic changes, including chromatolysis and loss of membranes [Farmilo 1989]. Allowing thin cryostat sections or cytospin preparations to dry for up to 48 hours before acetone fixation may improve morphologic preservation [Farmilo 1989]. It is my experience that frozen sections can be cut at 4-5 µm, placed in cold 10% neutral-buffered formalin for 5 minutes, then transferred to phosphate-buffered saline (PBS; pH 7.4) and held for routine immunohistochemical staining. Muscle sections should be cut, air-dried for 1 hour, and immediately stained with antibodies targeted for dystrophin or other muscular dystrophy-related proteins. Zenker and Bouin fixatives are also good for the preservation of some antigens, and alcohol-based fixatives such as methacarn (methanol-Carnoy solution) or alcohol-based proprietary reagents are reported to preserve antigens very well [Battifora 1986]. Elias [1985] states that there appears to be an

inverse relationship with regard to preservation of antigenicity and morphology. Glutaraldehyde preserves morphology best, but it irreversibly blocks tissue antigenic determinants.

FIXATIVES FOR PARAFFIN-PROCESSED TISSUE

Proper tissue handling, beginning with fixation and continuing through processing and microtomy, is pivotal to producing consistent results when performing immunohistochemical staining on paraffin sections. It is important for the immunohistochemist to understand the basics of how fixation, processing, temperature, and pH can affect the quality of a stain.

New regulations regarding predictive marker staining of tissue places responsibility on the clinical laboratory for documenting the time in fixative. For example, it is recommended that breast tissue from all invasive carcinomas be formalin fixed at room temperature for a minimum of 6 hours and a maximum of 48 hours [Wolff 2007] prior to HER2 testing. Tissue should be grossed and sectioned promptly to begin formalin fixation, and the time of fixation must be documented.

A fixative can positively or negatively influence the staining result with a given antibody. Not all antibodies are affected in the same way. The immunohistochemist should know which fixative was used before staining with an antibody. If the laboratory uses multiple fixatives, each antibody protocol should be validated with each type of fixative before staining patient tissue. If this is the case, it is desirable to have a multiple tissue control block containing tissue fixed with the different fixatives to ensure adequate results. It is also likely that different antibody dilutions and staining times may be required based on the tissue fixative.

The procedures commonly used in diagnostic laboratories are based on tissue fixed in 10% neutral-buffered formalin for an average of 8-12 hours. During fixation in neutral-buffered formalin, hydrogen bonds are formed that must be reversed to have successful antibody binding to the antigen site. In some cases, the epitope cannot be retrieved and frozen sections may be required. With the introduction of methods for unmasking or enhancing antigens, especially the microwave antigen retrieval methods, the time of exposure to formalin fixation is not emphasized as much as in the past. However, Taylor [1994] states that the first and perhaps most important thing that a laboratory can do is to standardize fixation, attempting as far as possible to limit the total time a specimen is in formalin to 24 hours.

In most tissues, antigenicity can be restored by using 1 or a combination of more than 1 epitope retrieval method. Using the mildest retrieval method with minimal damage to tissue morphology to achieve optimal staining is the goal. It is important to retain tissue morphology for diagnostic interpretation.

Herman [1988] demonstrated that zinc formalin preserved immunoreactivity remarkably well and that it can be used successfully on automated tissue processors. This report, and the search for better antigen retention and preservation, has led many laboratories to switch from 10% neutral-buffered formalin to some formulation of a zinc formalin solution for routine processing. Zinc formalin is discussed in Chapter 1, "Fixation," p16.

Less common fixatives such as mercuric chloride (B5) and Bouins are still used. B5 is noted for producing excellent nuclear morphologic detail in hematopoietic tissues such as bone marrow or lymph nodes. Because of concerns about mercury as an environmental hazard, it is being replaced with substitutes like acetic zinc formalin. Acetic zinc formalin is found to yield comparable results to B5 in immunohistochemical staining [Bonds 2005].

Bouin fixative, composed of glacial acetic acid, formalin, and picric acid, is not ideal for immunohistochemical staining. The complete removal of picric acid is difficult yet vital for adequate staining. Crisp staining detail is also lost with this fixative.

Because fixation varies markedly from laboratory to laboratory, vimentin provides an excellent way of determining when tissue has been overfixed. Vimentin staining is usually excellent in paraffin sections of tissue that have been optimally fixed in formalin but is progressively lost as the length of time in the fixative increases. When the vimentin stain is completely negative, the tissue is most likely overfixed and all antibody reactions should be interpreted with caution; if preservation of vimentin is uneven, then immunostains on parallel sections should be read in the area of most intense vimentin staining [Battifora 2002].

PROCESSING

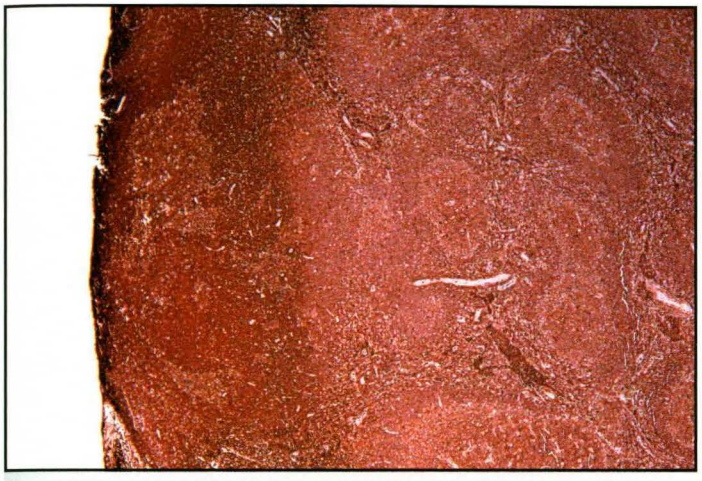
Complete fixation before processing is necessary to achieve optimal results for stains performed at either a protein or molecular level. Thorough dehydration and infiltration are necessary for consistent staining in the tissue section. False-negative or variable staining in the middle of a section in contrast to proper staining at the edges is a common artifact produced when the outer edge is formalin fixed and the center is alcohol fixed, the result of underfixation before processing **[i12.3]**.

The paraffin that is used in both the processor and embedding center should have a low melting point. Antigens are primarily proteins and they can be altered by high temperatures. Tissue exposed to high temperatures during processing can accelerate the damage. Reprocessing tissue may alter the epitope sites, causing irreversible damage and leading to false-negative results.

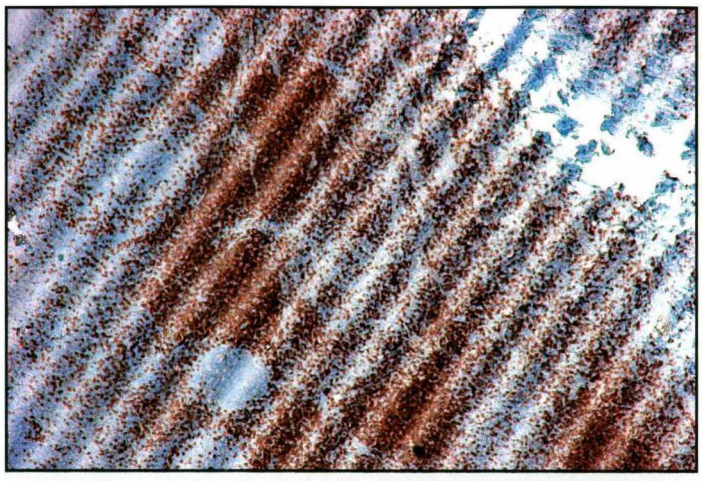
MICROTOMY

The water bath should contain clean deionized water for immunohistochemical slide preparation. Albumin and other water bath adhesives should be avoided because of potential nonspecific staining over the slide and tissue. Gloves should be worn to avoid squamous cell contamination. Positive charged slides are recommended to ensure tissue adhesion during the epitope retrieval and rinsing steps.

Staining quality is strongly affected by the quality of the tissue section. Sections should be free of wrinkles and folds. Good technique will decrease potential staining artifacts because of the reagents being trapped underneath the tissue or incomplete rinsing during staining **[i12.4]**, **[i12.5]**, **[i12.6]**.



[il2.3] A formalin-fixed lymph node stained with leukocyte common antigen, which demonstrates an alcohol-fixed center. This results from incomplete formalin fixation before beginning processing.

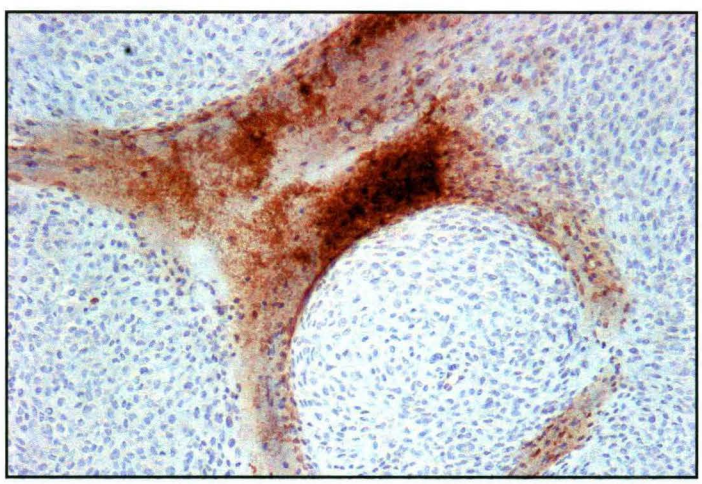


[il2.4] Chatter resulting from poor microtomy technique is demonstrated in this section.

Section thickness can affect staining intensity, so the patient tissue and control tissue should be the same thickness; 3-4 µm sections are recommended. Tissue sections routinely should be picked up in the same area of the slide, typically the center, to ensure proper coverage of reagents. Avoid placing the tissue on the edges of the slide because either this area is commonly missed by reagents or it dries out. Areas of incomplete coverage or drying may display false-negative, or nonspecific staining.

To preserve precious tissue when the block is returned to the chuck for additional sections, the technician should avoid further trimming by carefully realigning the previously sectioned block with the blade.

Air-drying overnight in front of a cool fan is optimal but is not realistic for a diagnostic laboratory. Once prepared, the tissue slide can be placed into a 55°C oven for 30 minutes. Avoid high-temperature ovens; many nuclear and cell surface markers are sensitive to dry heat in combination with high temperature, and false-negative or weak staining may result.

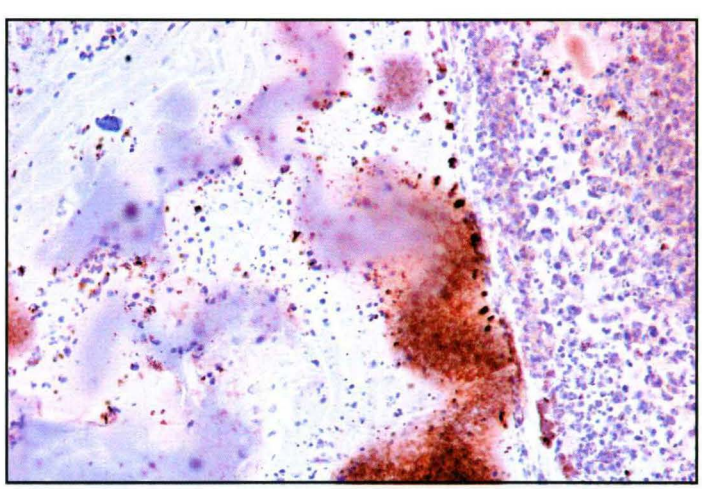


[il2.5] 3,3'-Diaminobenzidine chromogen is trapped underneath the tissue in this section.

EPITOPE ENHANCEMENT OR RETRIEVAL

There are 2 common categories of retrieval methods; the first is heat-induced epitope retrieval (HIER) and the second is enzyme-induced epitope retrieval (EIER). These methods are used when it is necessary to break down the hydrogen bonds that are formed during formalin fixation, because immunoreactivity can be compromised by some fixatives, especially aldehyde fixatives. Overfixation of tissue by formaldehyde can result in an antibody not having access to its epitope, thereby leading to a false-negative result. The detectability of many antigens in formalin-fixed tissue is greatly improved by epitope retrieval methods. Some of the advantages of epitope retrieval are [Lear 1995]:

- ability to further dilute antibodies
- exposure of epitope sites not previously detectable



[il2.6] 3,3'-Diaminobenzidine chromogen is trapped underneath the tissue in this section.

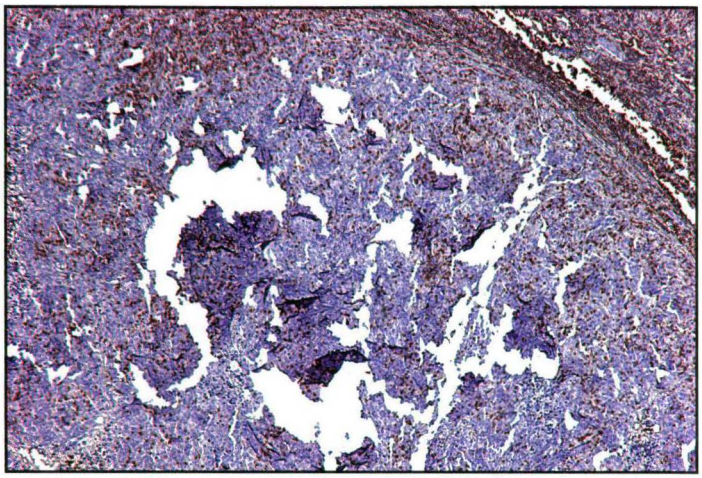
- more intense reactions with decreased incubation times
- more uniform staining
- decreased background staining
- day-to-day consistency of stains
- possibility of better standardization

■ *Heat-Induced Epitope Retrieval (HIER)*

The commercial antibody specification sheet typically states the recommended method for antigen recovery. According to Bancroft [2002], "major factors that govern the effectiveness of retrieval include the pH, volume of fluid, heating time, and temperature." Although there are many theories, it is not known exactly how and why the heating method works. The first method for antigen retrieval in formaldehyde-fixed, paraffin-embedded tissue sections was introduced by Shi [1991]; this revolutionized immunohistochemistry in the early 1990s. This method advocated immersing formalin-fixed tissue sections in a metallic salt solution and heating in a microwave oven to 100°C. Many articles have appeared in the literature since then, which address various antigen retrieval solutions, pH, length of heating, and methods of heating.

While high heat is the most important component of HIER, many studies have shown that the composition of the retrieval solution is also very important. The original retrieval solutions proposed by Shi [1991] (saturated lead thiocyanate and 1% zinc sulfate) have the disadvantage of potential toxicity, so numerous other less toxic solutions have been proposed. 2 of the most widely used are sodium citrate buffer (0.01M, pH 6.0) and ethylenediaminetetraacetic acid (EDTA; 1mM, pH 8.0). Other retrieval solutions that have been used are distilled water, glycine HCl (0.05M, pH 3.5), and 3M urea solution. Shi [1995] found that the pH value of the retrieval solution was more important than the composition, particularly for nuclear and cell surface antigens; they suggested that the critical influence of pH in antigen retrieval may provide an explanation of reported inconsistencies in HIER immunohistochemistry. They recommended that retrieval solutions of high pH be used for most of the antibodies in surgical pathology, and stated that Tris-hydrochloric acid or sodium acetate buffer solutions at pH 8-9 may be suitable for most antigens; however, certain nuclear antigens (estrogen receptor [ER], retinoblastoma protein [Rb], and MIB-1) show optimal staining at low pH. The disadvantage of a high pH is the extent of damage to the tissue sections after heating in a strongly alkaline solution. High pH can promote tissue detachment, resulting in holes in the tissue or, in extreme cases, complete removal from the slide **[i12.7]**. The observations of Shi [1995] further support the concept that the antigen- retrieval methods loosen or break the cross-linkages caused by formalin fixation. Many other methods of heating are also available, including modified pressure cooker, laboratory microwave oven, vegetable steamer or circulating water bath.

A standard method should be established in each laboratory, but more than 1 method should be available. A higher-volume laboratory will most likely require multiple retrieval solutions and methods, as the increased variety of antibodies will require different pH solutions and retrieval techniques.



[i12.7] The tissue has washed, or detached, in this section because of the antigen retrieval technique.

Technology has allowed for automated immunohistochemical stainers to perform the steps of heating and retrieval, reducing human error and ensuring that the temperature and exposure time are consistent each time a stain is performed, thus improving reproducibility.

■ *Enzyme-Induced Epitope Retrieval (EIER)*

In the EIER method, a proteolytic enzyme is used to expose epitope sites. Proteolytic enzyme digestion is the older of the 2 epitope enhancement methods, but is used much less frequently since the advent of the heat-induced epitope enhancement methods. As Elias [2003] stated, there is no universal proteolytic agent, so a number of enzymes have been used. Some of the solutions that have been used are 0.1% pronase in 0.5M Tris buffer, pH 7.5; 0.6% ficin; 0.1% protease in PBS, pH 7.4; and 0.4% pepsin in 0.01N hydrochloric acid. Different proteolytic enzymes may be needed for epitope enhancement of various antigens. Digestion times for these proteolytic enzymes vary from 1-60 minutes or more. It is important to know the concentration of the enzyme solution being used.

Although proteolytic enzyme digestion usually reduces nonspecific staining, it may increase nonspecific staining if not used carefully. It may weaken specific staining and create false-negative results, and also cause fragmentation or loss of tissue sections. The term "overdigestion" of the tissue is used to describe distorted tissue morphology and poor staining results. This can be caused when the incubation time is extended or concentration of the enzyme is high. Commonly the result is loss of cellular detail, in combination with nonspecific staining or no staining. Excessive digestion can promote loss of tissue from the slide during the staining process.

Measures should be taken to ensure that when EIER is performed manually, the digestion time is controlled for staining consistency. Temperature can influence the rate at which the digestion is achieved; the temperature should be the same each time an enzyme is used. A temperature of 37°C is commonly used for enzyme incubation. This step should be performed in a humidity chamber to avoid drying of the tissue.

■ *Combination of Both HIER and EIER Methods*

The use of enzyme digestion alone for epitope retrieval can reduce the quality of tissue morphology because of a lengthy exposure time. Therefore, it may be useful to combine both methods of epitope retrieval to minimize the extent of enzymatic pretreatment required. In general, HIER is performed first, followed by rinsing and then enzyme digestion for a short time (from 1-5 minutes).

Methods of Visualization

IMMUNOFLUORESCENCE

Immunofluorescence, the oldest of pathology's immunohistochemical techniques, is still widely used. This technique makes it possible to visualize antigens in tissue sections or in live cell suspensions.

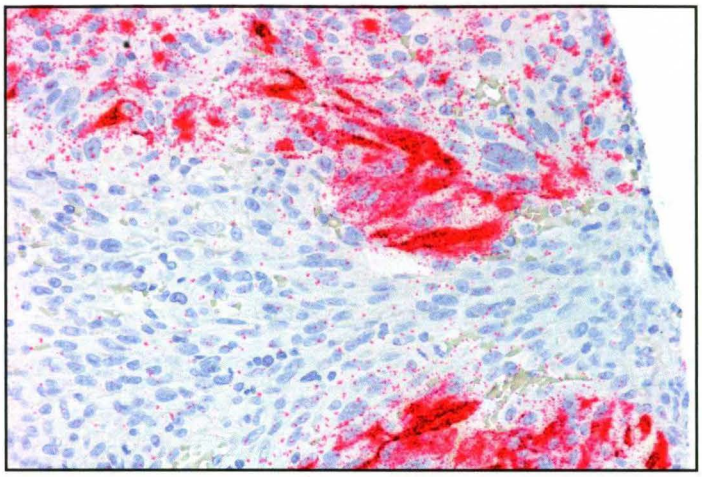
A fluorochrome is a dye that absorbs light and then emits its own light at a longer wavelength. This phenomenon of absorption and emission of light is called *fluorescence*. When the fluorochrome is attached or conjugated to antibody, the sites of reaction between antigen and the labeled antibody can be visualized easily.

The most commonly used fluorochromes in immunofluorescence techniques are fluorescein isothiocyanate (FITC) and rhodamine. Both of these dyes absorb light that is not visible to the human eye (UV) and emit light that is visible. Virtually any antigen can be detected with immunofluorescence. The combination of sensitivity, specificity, and simplicity makes the method very useful. In most routine histopathology laboratories, frozen sections of kidney and skin biopsy specimens are examined with immunofluorescence techniques, but differentiation of tumors is done with enzyme immunohistochemical techniques suitable for light microscopy.

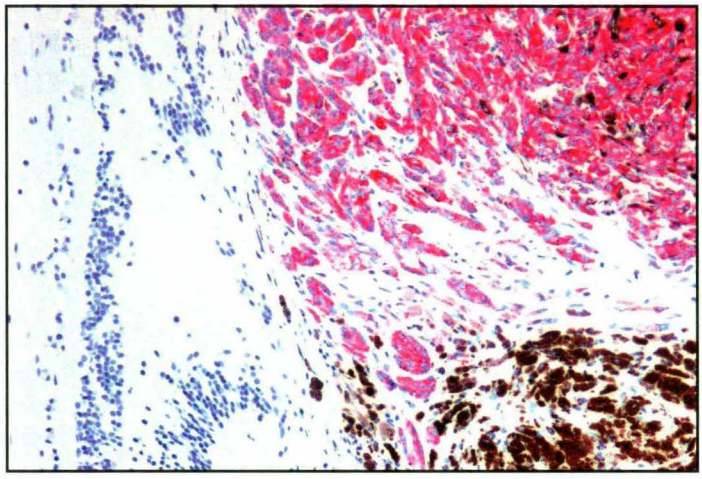
ENZYME IMMUNOHISTOCHEMISTRY

The fundamental principle of enzyme-labeled antibodies is similar to fluorescein-labeled antibodies. The enzyme, in the presence of a substrate and a chromogen (may be the same or separate reagents), provides the indicator system to visualize the location of the antibody. Various enzymes such as alkaline phosphatase, β-galactosidase, glucose oxidase, and horseradish peroxidase (HRP) are used as markers.

Horseradish peroxidase and alkaline phosphatase are the enzymes most commonly chosen for antibody visualization. HRP, in the presence of hydrogen peroxide (substrate) and a chromogen such as 3,3'-diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) will identify the sites of antibody binding by forming a colored compound. A HRP system routinely serves as a "work horse" in a clinical laboratory because it is consistent when producing large quantities of slides. AEC is soluble in organic solvents and requires an aqueous mounting medium, while DAB-stained slides may be mounted with synthetic resins.



[i12.8] The semipermanent alkaline phosphatase red chromogen is breaking down in this section.



[i12.9] A melanoma of the eye is seen in this section stained with HMB-45 using an alkaline phosphatase red chromogen to contrast with the melanin pigment.

Alkaline phosphatase is demonstrated by the use of naphthol-AS-phosphate (substrate) and a chromogen such as fast red-violet LB, fast-red TR or fast-blue BBN. Alkaline phosphatase is sensitive to heat and light which can lead to staining inconsistency. Semipermanent chromogens compatible with alkaline phosphatase allow the use of a permanent mounting media but require either no dehydration with air drying before coverslipping, or a quick dehydration to xylene. If not properly dehydrated, the alkaline phosphatase chromogen can break down and precipitate across the tissue [**i12.8**]. A red chromogen signal can assist in identifying antigen sites in those tissues containing melanin pigment which may obscure a brown signal [**i12.9**].

If hematoxylin is used as a counterstain, a formula such as Mayer hematoxylin is recommended because it does not contain alcohol. If Harris or another hematoxylin solution containing alcohol is used with AEC or the alkaline phosphatase chromogens, the reaction product will be dissolved and thus removed from the section. A false-negative result will be seen.

Immunohistochemical Staining Methods

Various techniques are used to detect the presence of antigen in the patient's tissue or the presence of antibody in the patient's serum. References to *direct* and *indirect* methods are typically used in conjunction with immunofluorescence techniques, whereas the *unlabeled*, or *soluble enzyme complex* and the *avidin-biotin-complex* (ABC) methods typically refer to visible light microscopic methods. The methods that will be described are:

- direct
- indirect
- unlabeled, or soluble enzyme immune complex
- avidin-biotin
- polymeric

DIRECT METHOD

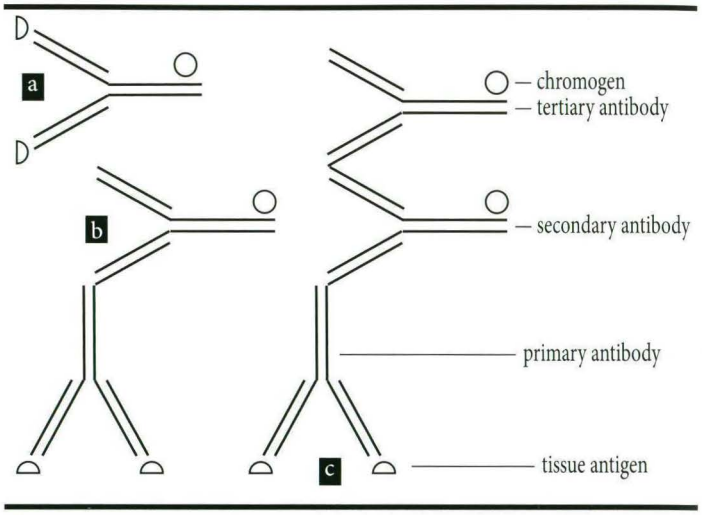
A labeled antibody of known specificity is used to identify antigens in the patient's tissue. An example is the detection of immune complex deposition in renal biopsy specimens using direct immunohistochemical techniques. In this method, the antibody may be labeled with fluorescein isothiocyanate (FITC) or other fluorescent dyes for fluorescence microscopy, or it may be labeled with an enzyme such as HRP, alkaline phosphate, or glucose oxidase for subsequent reaction with a chromogen and visualization with the light microscope **[f12.1]**.

INDIRECT METHOD

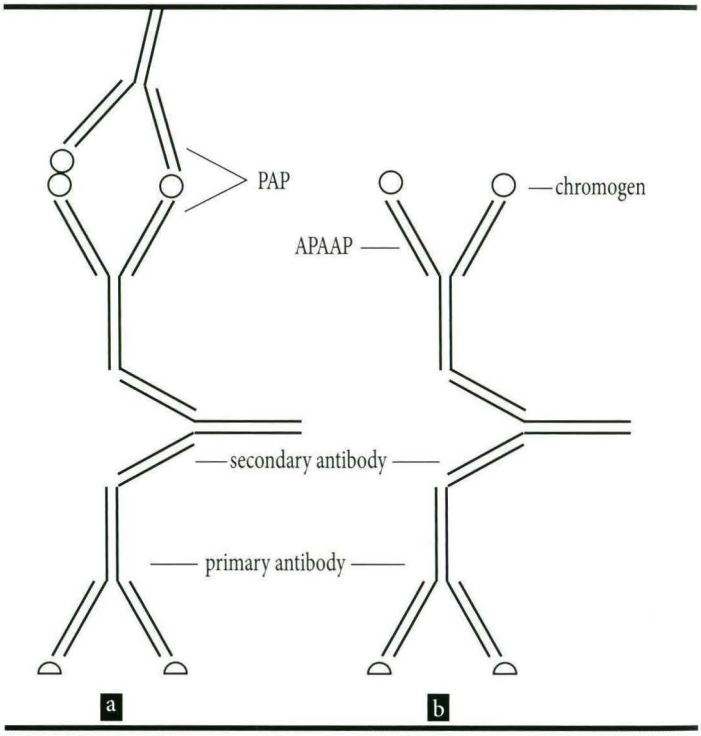
In the indirect method, the patient's serum is added to tissue sections containing known antigens to test the patient for the presence of antibodies to those antigens. An example is the test for the presence of antinuclear antibodies. The patient's serum may also be added to a known bacterium to detect the presence of bacterial antibodies in the patient. A labeled antibody (anti-human Ig) must be used to detect the bound antibodies from the patient's serum. These indirect methods most frequently involve immunofluorescence microscopy.

UNLABELED, OR SOLUBLE ENZYME IMMUNE COMPLEX, METHOD

This is a 3-step method using primary antibody, linking or secondary antibody, and soluble enzyme-antienzyme complex. The primary and the enzyme-antienzyme complexes must be made in the same animal species for the secondary antibody to link them together. These techniques are based on the original peroxidase-antiperoxidase (PAP) technique of Sternberger [1979]. The most common techniques in this category involve the use of either a PAP or alkaline phosphatase-antialkaline phosphatase immune complex **[f12.2]**.



[f12.1] A labeled antibody used in direct immunohistochemical techniques.

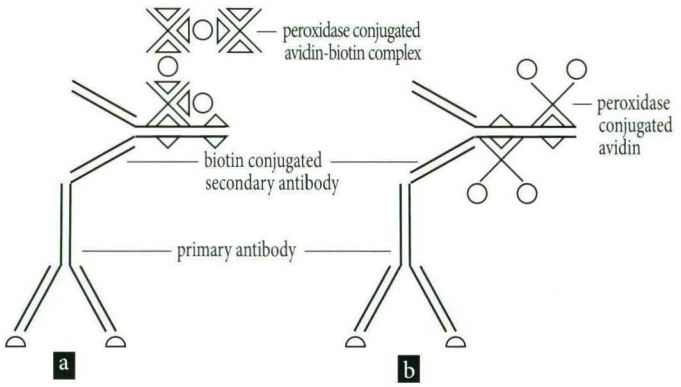


[f12.2] The peroxidase-antiperoxidase (PAP) immunohistochemical technique.

AVIDIN-BIOTIN METHODS

Avidin has a very high affinity for the vitamin biotin (over 1 million times greater than the affinity of antibody for most antigens) and the binding is essentially irreversible. Avidin isolated from egg white was used first in the ABC methods, but streptavidin, isolated from the microorganism *Streptomyces avidinii*, is more commonly used today.

In the avidin-biotin enzyme complex (ABC) method, the primary antibody is followed by a biotinylated secondary antibody (linking antibody). The third step in the ABC method is the application of a preformed avidin-biotin enzyme complex. This method has low background economy, and sensitivity reported to be up to 40 times that of other immunoperoxidase methods [Hsu 1981]. The antibodies may be used at higher dilutions than in other techniques **[f12.3]**.



[f12.3] The avidin-biotin-complex (ABC) immunohistochemical technique.

POLYMERIC DETECTION

The dextran polymer and monomer technology is the next generation of immunohistochemical staining. The polymer enzyme molecules such as HRP or alkaline phosphatase have been fused with the secondary antibody. Monomer technology uses a single strand, allowing for greater sensitivity and penetration at the antibody binding site. Turnaround times have been improved with the elimination of serum and avidin-biotin blocking steps. Staining steps would be: antibody, polymer secondary, and chromogen.

Controls

POSITIVE CONTROLS

A positive control must be run with each antibody stain each time it is performed. This applies to both manual and automated methods. While commercially prepared control slides can be used to check reliability of the reagents, these purchased slides should not be used as routine controls. The optimum control is one prepared in the laboratory under exactly the same conditions as the diagnostic tissue, including type and timing of fixation and processing. Because fixation varies and affects the reactions so dramatically, commercially prepared control slides are not ideal to determine if a negative reaction on the diagnostic slide is a true negative or if it is the result of overfixation. The method of preparing sausage controls is excellent [Battifora 1986], though somewhat time consuming and difficult to execute. With careful planning, the laboratory can make the most efficient use of available tissue to fulfill requirements of using controls containing both positive and negative tissue types. I have found that a tissue microarray technique such as that available from Beecher Instruments (Sun Prairie, WI) is a more effective means for constructing multitissue control blocks than previously reported manual methods.

NEGATIVE CONTROLS

In most laboratories, negative controls are run by substituting for the primary antibody either nonimmune serum from the same species as the primary antibody or the diluent used for the primary antibody. If diluent buffer is used, the negative control will not detect nonspecific binding of animal serum components to the tissue, and any staining observed will be the result of either endogenous peroxidase activity or binding of other antibody reagents to the specimen [Bourne 1989].

Antibody Evaluation and Validation

ANTIBODY SPECIFICATION SHEET

The antibody specification sheet provided with a commercial antibody contains valuable information regarding the antibody or antiserum. The immunohistochemist should be familiar with the specification sheet for many reasons.

The following are typically included, but are not always available:

- A monoclonal antibody will list the clone and the isotope (IgG or IgM) to ensure that the secondary antibody will bind to the primary antibody.
- The expiration date under recommended storage conditions.
- Long-term or short-term storage temperatures; some antibodies require long-term storage at –20°C. Measures to ensure that the antibody storage condition is optimal will assist with antibody stability and maximum reactivity for the life of the antibody.
- The protein concentration and suggested working dilution ranges.
- Pretreatment (antigen retrieval) solution, pH, and/or method, if recommended.
- Expected positive and negative tissue types, including normal and tumor.
- Reference to publications and applications for the antibody; these are useful if additional staining methods are needed.
- Antibody type: in vitro (IVD), research use only (RUO), or analyte specific reagent (ASR).

PREDILUTED AND CONCENTRATED ANTIBODIES

Prediluted antibody refers to a commercially available "ready to use" solution. The vendor has already taken a concentrated antibody and diluted it down to an "optimal" dilution. Because it has been diluted, the shelf-life is routinely shorter and it cannot be frozen to extend it, thus prediluted antibodies are less economical if not used before the date of expiration. Prediluted antibodies must be validated for reactivity before use on patient tissue. The laboratory cannot assume that the antibody is ready to use with that laboratory's method and must perform serial dilutions (1:2, 1:4, 1:8, and 1:16) to validate.

A concentrated antibody is one that is supplied in an "undiluted" form. Dilutions must be performed to find the optimal dilution for antigen detection, and the user has more control of fine-tuning the dilution to be compatible with the laboratory's fixation and processing protocols. These undiluted antibodies are more economical because they allow for the modification of titers used in staining. In addition, they routinely have a longer shelf-life when stored properly; most antibodies can be "snap" frozen using liquid nitrogen and stored at –70°C indefinitely.

If undiluted antibodies come in solution form, it is best to dilute only the amount of antibody needed for the current diagnostic slides or the smallest amount feasible when very high dilutions are needed. Dilutions are expressed as ratios, as in a 1:20 dilution. This is equivalent to 1 part antibody solution in a total of 20 parts, or 1 part antibody plus 19 parts of diluent. Pipettes capable of measuring in microliters (μL) are a must in the immunohistochemistry laboratory. Microliter (μL) and lambda (λ) are used interchangeably, with both equal to 0.001 mL. Pipettes that will cover a range, such as 0.5-10 μL, 10-100 μL, and 100-1,000 μL, are the most useful. For higher dilutions, it is best to prepare a more concentrated stock dilution, and then prepare further dilutions from this stock dilution.

A basic formula to use to make 1 mL (1,000 μL) of a 1:x dilution is [Chlipala 1985]:

$$\frac{1{,}000}{x} = \text{μL of raw antisera required}$$

$$1{,}000 - \frac{1{,}000}{x} = \text{μL of antibody dilution buffer required}$$

This formula can be changed easily by changing 1,000 μL (1 mL) to whatever volume is required.

ANTIBODY VALIDATION

Routinely a standard staining protocol will be used for most formalin-fixed tissue samples. This will also assist with laboratory reproducibility and consistency. Although it will be necessary to have unique protocols, the number of antibodies requiring unique detection, special pretreatments, and incubation times should be kept to a minimum.

An example of a standard laboratory protocol is as follows:

1. retrieval: EDTA, pH 8.0
2. heating method: modified pressure cooker (115°C for 25 minutes or 124°C for 40 minutes). Combination of time and temperature are based on the stringency of retrieval needed
3. peroxidase quenching: 3%, 10 minutes
4. detection system: universal polymer, HRP, DAB
5. staining times: antibody 30 minutes, polymer 10 minutes, and chromogen 5 minutes
6. counterstain: Mayer hematoxylin, 3 minutes

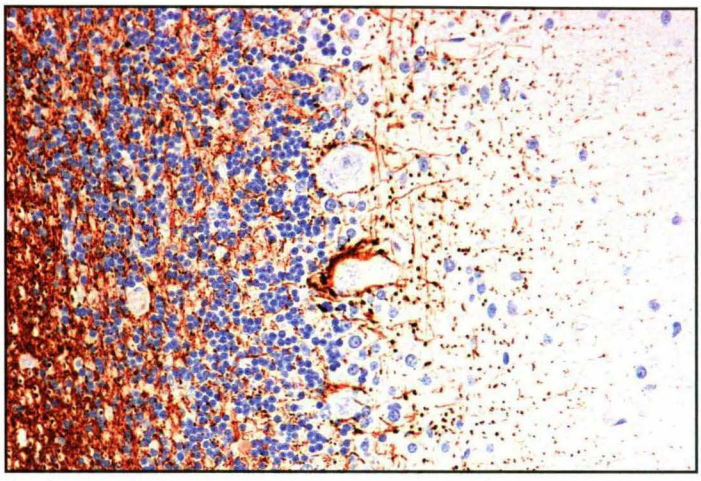
There are 2 approaches to new antibody validation, the first using no retrieval, multiple retrieval solutions, and multiple enzyme pretreatments; this approach may be necessary when validating a "homegrown" antibody or an antibody that has no previously documented use in the clinical laboratory. The second approach is researching the antibody and planning a validation protocol using the wealth of information available. Resources for a starting point are the antibody or detection vendor, antibody specification, journal articles, and textbooks [Dabbs 2006, Shi 2000]. These resources provide basic methods before staining, thus knowledge is gained of the recommended retrieval solution, pH and method, type of detection used, and positive and negative tissue types. Knowing expected staining patterns and location of expression, such as nuclear or cytoplasmic, can assist when reviewing staining results.

Upon receiving the new undiluted antibody, the laboratory will typically begin with a series of testing variables. First perform serial dilutions (1:100, 1:200, 1:400 and 1:800) of the antibody using a known positive and negative tissue control. Usually the manufacturer will indicate an approximate dilution; however, several different dilutions should be used with the standard laboratory protocol to determine the correct dilution for your laboratory. It is important to use a diluent that has carrier proteins and the proper pH for antibody stability.

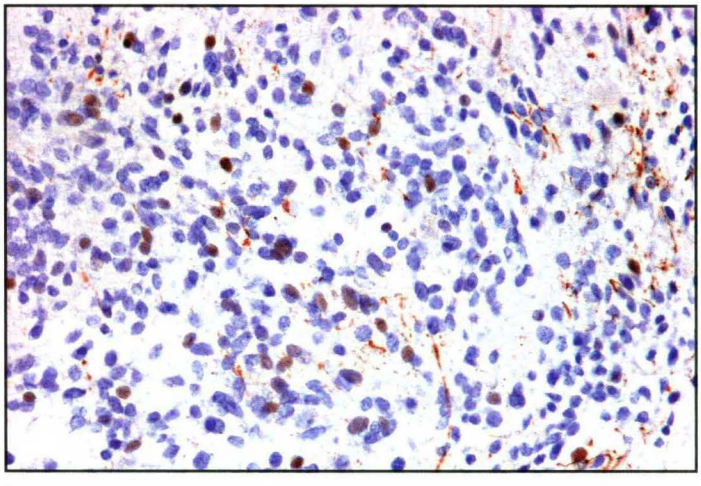
Review the results for an optimal dilution looking for the best signal-to-noise ratio. If the 1:100 targeted cells are staining faintly, with no nonspecific staining, then stain further with more concentrated dilutions of 1:25, 1:50, and 1:100. To ensure no errors in dilution preparation and reproducibility, always repeat in the new series the previous dilution that is closest to optimal. If the 1:800 appears overstained with high signal and high background, then start with 1:800, 1:1600, and 1:3200 on the next run.

If the initial staining yields negative or suboptimal results, then the laboratory should begin with changing only 1 variable at a time. Routinely changing the pH of the retrieval solution is first; if unsuccessful, the introduction of an enzyme step omitting the retrieval solution is recommended. The introduction of an enzyme will require not only a redetermination of the antibody concentration but also an evaluation of the time in the enzyme solution. It is recommended that when an enzyme is introduced, times of 5, 10, 20 and 30 minutes in the enzyme solution be evaluated to ensure that adequate retrieval is performed. Some antibodies do not require any type of retrieval, and using retrieval may introduce false-positive staining **[i12.10]**, **[i12.11]**, **[i12.12]**.

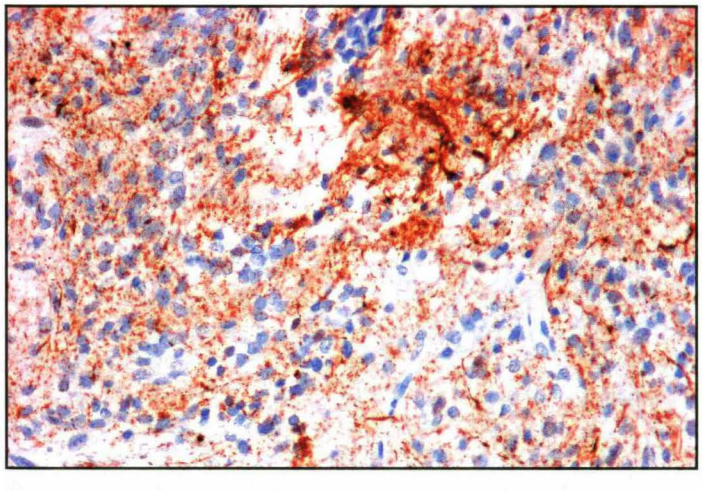
Once the appropriate protocol and dilution are determined for a given antibody, at least 5 or 6 different appropriate file cases should be run to validate sensitivity, specificity, accuracy, and reproducibility before using on patient tissue. After complete validation, the slides should be retained in a permanent file for reference. A written and/or electronic record of optimum antibody dilution and retrieval procedure, if applicable, should be maintained for each antibody **[f12.4]**.



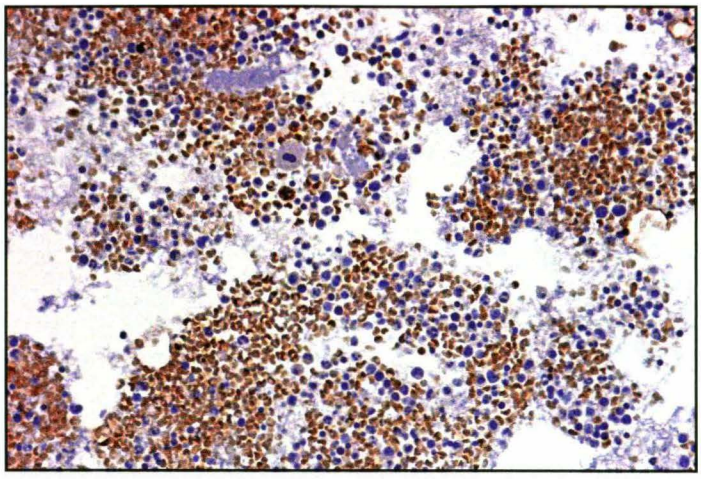
[i12.10] Expected staining patterns with SMI-31 antibody are seen on this positive control tissue from the cerebellum.



[i12.11] A brain biopsy specimen stained with SMI-31 antibody that demonstrates false nuclear staining because of unnecessary heat-induced epitope retrieval.



[i12.12] A brain biopsy specimen stained with SMI-31 antibody demonstrating expected staining patterns after no pretreatment of the sections. Compare with **[i12.11]**.



[i12.13] Red blood cells demonstrating positive staining after insufficiently blocked peroxidase activity.

STORAGE OF ANTIBODIES

Both the storage container and the temperature at which the antibody is stored are very important. Polypropylene, polycarbonate, or borosilicate glass is recommended for storage because these materials have low protein absorptive properties. If the antibody solution contains very low concentrations of protein, 0.1% to 1.0% bovine serum albumin may be added to reduce loss through polymerization and absorption on the container [Boenisch 2001].

The recommendations of the manufacturer as to the storage temperature should be carefully followed. Most prediluted antibodies and kits are stored at 4°C to 8°C to avoid repeated freezing and thawing. Once lyophilized antibodies have been diluted, they are usually divided into aliquots and stored at –70°C.

BLOCKING REACTIONS

Most immunoperoxidase methods include 2 blocking reactions. The first is the use of hydrogen peroxide, usually prepared in absolute methanol, to block tissue endogenous peroxidase activity. If the tissue contains many red blood cells, this blocking step is essential **[i12.13]**.

The second block is for nonspecific background staining which may occur as a result of antibody (protein) attachment to highly charged collagen and connective tissue elements [Bourne 1989]. If the first protein solution applied to the tissue is the primary antibody, nonspecific binding can occur. The secondary antibody will bind to this nonspecifically bound primary antibody, and when reacted with the substrate-chromogen, will give positive nonspecific staining. The best way to prevent this type of nonspecific staining is to add an innocuous protein solution to the tissue before the primary antibody is applied. The protein will bind to the charged sites, and then nonspecific binding of the primary will not occur. Nonimmune serum from the same animal species in which the secondary antibody is produced is the most common source of the blocking reagent. The nonimmune serum is applied just before the primary antibody. After 10-20 minutes of incubation, the excess is tapped off (do not rinse off) and the primary antibody is applied. Casein and non-dry fat milk have also been used as a blocking agent.

Validation Form for Antibodies and Tissue Controls

Date requested:_____

Purpose of development:

Antibody validation _____ Positive tissue control_____ Negative tissue control_____

Retrieval instrument: (circle one) Modified pressure cooker (125°) or (115°), Waterbath, Microwave, Steamer , or Other_____

Retrieval solution:_____

Lot#_____ Expiration_____ pH:_____

Enzyme pretreatment solution:_____

Lot#_____ Expiration_____ Time:_____

Detection: _____ Lot#_____ Expiration_____

Chromogen:_____ Lot#_____Expiration_____

Automated: y/n (if yes) instrumentation: _____

Antibody name	Antibody Lot#	Antibody exp. Date	Tissue Type	Control block ID	Dilution	Date stained	Comments/Results

Reviewed by (Medical Director or Designee)_____ Final approval date:_____

[f12-4] Antibody and tissue control validation form

MULTILINK BIOTINYLATED SECONDARY ANTISERA

Secondary antibodies that are typically called "multilink" or "universal" refer to cocktails of antibodies raised in different species. These secondary antibodies mixtures are commonly biotinylated anti-mouse IgG and IgM and anti-rabbit IgG. Other species such as goat can also be added. The laboratory technician should know the species in the secondary antibody mixture to ensure that the targeted antibody is compatible. One advantage of this type of secondary antibody is that it avoids the problem of having to stock a variety of specific link antibodies (eg, biotinylated goat antirabbit or goat antimouse IgG and IgM).

DAB REACTION PRODUCT INTENSIFICATION

Several methods have been used to intensify the DAB reaction product. Heavy metals can be used for intensification of the DAB reaction product by supplementing, or following, the incubation medium with reagents such as 1% cobalt chloride. However, there is a risk of increasing background staining with heavy metal intensification, and some of the metals should not be used with the immunohistochemical detection of nuclear antigens [Elias 2003], especially if weak hematoxylin counterstaining is used.

Imidazole is an effective intensification reagent. Elias [2003] states that the addition of 0.01M imidazole to the DAB incubation medium at pH 7.6 is superior to standard DAB and DAB-cobalt chloride-nickel sulfate or DAB-cobalt chloride procedures, both in sensitivity and detection efficiency. Imidazole also significantly inhibits the pseudoperoxidase activity of hemoglobin.

Osmium tetroxide may also be used to intensify the DAB reaction product, but unlike the other reagents mentioned, it is always used after the DAB reaction. The DAB reaction product is osmiophilic and will be darkened by mild poststaining osmification. This also helps prevent fading of the final reaction product during long-term storage; however, unless carefully done, this reagent has the possibility of darkening any background staining present as well as intensifying the reaction product.

Elias [2003] warns that increased sensitivity is not always commensurate with enhancement of staining intensity. For example, post-treatment osmium boosts staining intensity without increasing detection efficiency.

BUFFER SOLUTIONS

For reproducible results, the pH of the buffer used during staining must be maintained and checked often. For each new lot of commercially prepared buffer, or each time a buffer is prepared, the immunohistochemist should check and document its pH before use. Typically, 2 buffers are used during the staining process: PBS and Tris-base solution (TBS). Both can be used with a HRP detection system, but only TBS should be used with an alkaline phosphatase detection system. The addition of a wetting agent will assist with even distribution of the reagents during staining and improve rinsing steps; at the same time, it will slow down bacterial growth. Common wetting agents used are Tween-20, Triton-X and brij-35. The buffer should be used at room temperature during staining.

Commonly Used Antibodies and Their Applications

Antibody stains are routinely used by the pathologist to identify a neoplasm and define whether it is benign or malignant, and whether it is metastatic or primary. The pathologist identifies the extent to which cells in a neoplasm resemble normal cells (differentiation) both morphologically and functionally. The type of the neoplasm is defined by the germ cell layer (endoderm, mesoderm, ectoderm) from which it derives. Types of neoplasms include carcinomas, sarcomas, lymphomas, melanomas, mesotheliomas, neuroendocrine tumors, gliomas, and germ cell tumors.

NEOPLASTIC TERMINOLOGY

Neoplasm: A new growth of tissue in which cell multiplication is uncontrolled and progressive; can be either benign or malignant.

Tumor: Swelling; one of the cardinal signs of inflammation and morbid enlargement. Often used to mean "neoplasm."

Anaplasia: Lack of differentiation. Some features include variation in size and shape of cells and nuclei, dark nuclei, giant cells, and disturbed orientation.

Differentiation: The distinguishing of one thing from another and the extent to which neoplastic cells resemble comparable normal cells, both morphologically and functionally.

Benign: Shows a lesser degree of anaplasia than malignant tumors; often surrounded by a fibrous capsule; does not produce metastasis or significant invasion.

Malignant: A neoplasm that invades adjunct tissue and has the capacity to metastasize; typically shows degrees of anaplasia.

Primary: The site at which a neoplasm first arises.

Metastasis: Transfer of disease from 1 organ or part of the body to another not directly connected with it, because of transfer of either pathogenic microorganisms or cells (eg, malignant tumor cells).

The most common approach to the use of immunohistochemistry in diagnosis involves the use of panels of antibodies to differentiate one neoplasm from others under consideration. Examples of common panels are given in **[t12.1]**, **[t12.2]**, **[t12.3]**, **[t12.4]**

Quality Control

Measures of QC and thorough documentation are fundamental in a diagnostic laboratory. QC measures serve many roles in the laboratory and are especially useful for troubleshooting. Antibody and control tissue validation must be documented and maintained. Each laboratory should define a QC system that enables the immunohistochemist to have easy access to previous validation results and final protocols, especially in a laboratory where manual staining is performed. Tracking of lot numbers, expiration dates,

[t12.1] Anaplastic tumor workup (typical results)

	Cytokeratin AE1/AE3	Cam 5.2	CD45RB LCA	HMB-45/ MART-1	GFAP	Vimentin
Carcinoma	+	+	–	–	–	+/–
Lymphoma	–	–	+	–	–	+/–
Melanoma	–	–	–	+	–	+
Sarcoma	–	–	–	–	–	+
High-grade glioma	+	–	–	–	+	+

LCA, leukocyte common antigen; GFAP, glial fibrillary acidic protein

[t12.2] Small cell neoplasm panel

	Keratin (HMW)	NSE	Vimentin	LCA	Neurofilament	Desmin	WT-1	CD56
Small cell carcinoma	+	+	–	–	–	–	– (except ovarian)	+
Lymphoma	–	–	–	+	–	–	–	–
Neuroblastoma	–	–	–	–	+	–	–	+
Rhabdomyosarcoma	–	+	+	–	–	+	–	+
Wilms tumor	+	–	+	–	–	+	+	+/–

HMW, High-molecular weight; LCA, leukocyte common antigen; NSE, neuron-specific enolase

[t12.3] Adenocarcinoma subtypes

	CK7	CK20	TTF-1
Lung	+	–	+
Breast	+	–	–
Ovarian (non-mucinous)	+	–	–
Ovarian (mucinous)	+	+	–
Thyroid	+	–	+
Pancreas	+	–	–
Colorectal	–	+	–
Renal cell	–	–	–

CK, cytokeratin

[t12.4] Prostate carcinoma vs adenosis (benign hyperplasia)

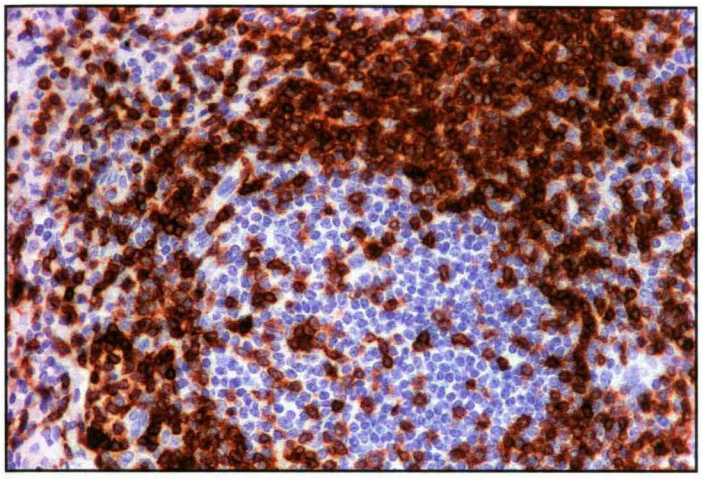
	PSA	PSAP	P63	Racemase PS540	HMW keratin, CK 903
Carcinoma	+	+	–	+	–
Hyperplasia	+	+	+ basal layer	–	+ basal layer

HMW, High-molecular weight; PSA, prostate specific antigen; PSAP, prostate specific alkaline phosphatase

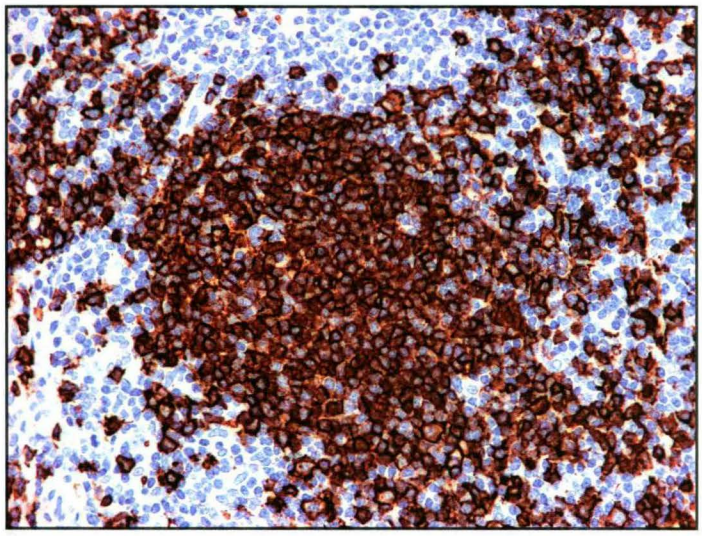
dilutions, protocols, pH monitoring, and validation of controls are basic QC in the immunohistochemistry laboratory. Laboratory accreditation agencies also have specific QC and documentation requirements. Both paper and electronic documentation are useful, but if a final protocol can be maintained electronically it assures the immunohistochemist that the most current information is saved and retrievable.

RECOMMENDED QC FOR AN ANTIBODY

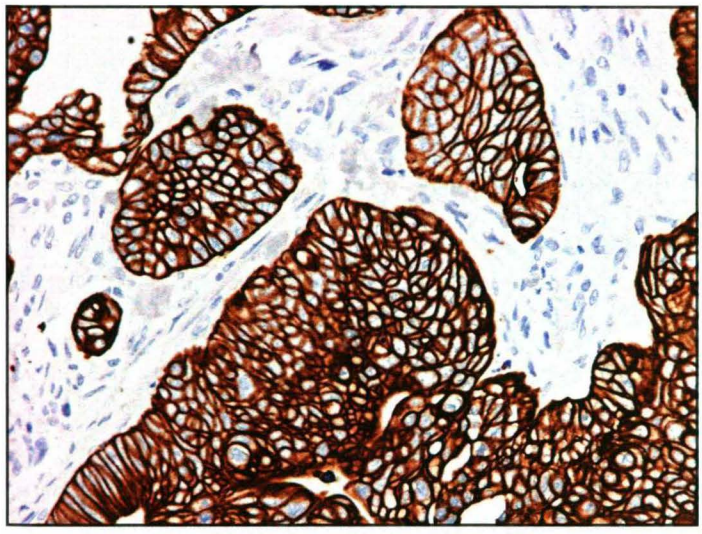
1. Verify and document on the bottle and the specification sheet the antibody name, receipt date, and expiration date.
2. Check storage temperature and conditions upon receipt.
3. Use a form to plan initial validation to include pretreatment, incubation times, detection system, control tissue, dilutions and date **[f12.4]**.
4. Document the date validation was performed and who approved the final procedure, such as the medical director.
5. Make a final copy of the procedure that can be accessed by the technician.
6. Keep all records and document and date any modifications to the procedure. Historical records may be useful when troubleshooting is required or revalidation of an existing antibody.



[i12.14] CD3 T-cell staining pattern is demonstrated in a normal lymph node control.



[i12.15] CD20 B-cell staining pattern is demonstrated in a normal lymph node control.

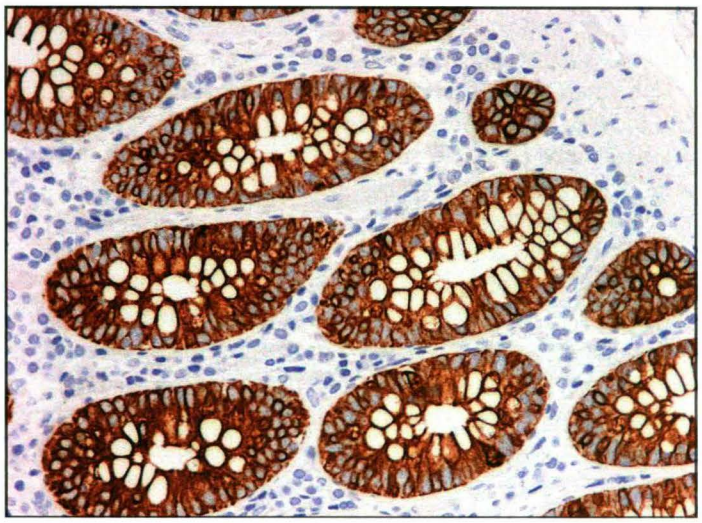


[i12.16] Cam 5.2 antibody staining of a colon adenocarcinoma control specimen.

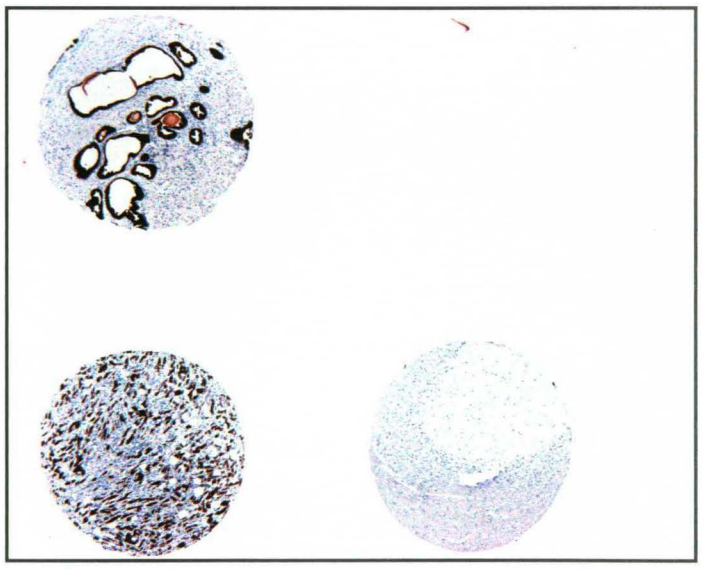
7. Maintain a separate slide file for each antibody lot and dilution validation. The date on the slides should correlate with the antibody records.

POSITIVE AND NEGATIVE TISSUE CONTROLS

Positive controls are those tissues that express the antigen in either normal tissue components or tumor cells **[i12.14]**, **[i12.15]**. Negative tissue controls are either normal tissue or tumor cells that do not contain that antigen. When validating an antibody, it is important to optimize the dilution using both positive and negative tissue types. This ensures that a low expressing tumor will not be missed and a false-positive result is not misinterpreted. Tissue blocks that contain several different normal and tumor tissues exhibiting positive and negative expression are very helpful to decrease the cost of validation. New technology such as the tissue microarrayer is making a constructive impact on QC measures with antibody validation **[i12.16]**, **[i12.17]**, **[i12.18]**.



[i12.17] Cam 5.2 antibody staining in normal colon control.



[i12.18] Tissue microarray cores demonstrating both positive and negative tissue controls.

RECOMMENDED QC FOR A TISSUE BLOCK

1. Verify the diagnosis of the tumor control tissue.

2. Using a validation form, document the control name, tissue type, antibody and lot number, results, and date stained **[f12.5]**.

3. Review the documentation of both positive and negative tissues after the results are validated. The laboratory medical director or designee must sign off of on the controls to be used.

4. With the assistance of the pathologist or pathologist assistant and based on the antibodies used, make a chart of tissues and create a tissue block of 2 or more tissues, including at least 1 positive and 1 negative tissue for each antibody for which it will be used.

DAILY QC OF IMMUNOHISTOCHEMISTRY

It is good laboratory practice to review and document the results of antibody staining for each day **[f12.5]**. At a minimum, one should document the antibody, lot number, dilution, control, date stained, and the results. Documentation of any repeats and why the repeat occurred is helpful in identifying any repetitive errors that may require retraining staff, a modification in protocol or automated instrumentation error.

A negative reagent control slide should be performed on each patient tissue to identify any nonspecific staining in the tissue that may be interpreted as positive staining in the antibody stained slide. If only 1 slide can be used for a negative reagent control and the case has multiple protocols being used, the negative control should be exposed to the condition most likely to produce artifacts, such as enzyme digestion, ABC detection system, or a combination of both.

STORAGE OF CONTROL SLIDES

Precut control slides are a way to be efficient with the technician's time, and serial sections allow for getting the most out of a block with minimal tissue loss. Conditions for long-term storage of precut slides are surprisingly important and frequently overlooked as a potential source of error in staining. Storing precut slides at room temperature is not recommended for long-term storage. The laboratory can benefit from having access to a –20°C freezer to store precut control slides. This temperature can stabilize antigenicity of most tissue types, but in my experience it is not good for all antigens. Placing the date cut on the slide is recommended as a reference to the length of storage time. If a control stops working, an appropriate troubleshooting step is to cut and stain a fresh section to verify that the antigenicity has not deteriorated on the precut slide.

A second method can be used if the laboratory does not have access to a –20°C freezer. Precut the slide, air dry overnight, and dip the slide into paraffin creating a seal over the tissue. This will require melting in the oven before starting the deparaffinization step. Place the slide in a low temperature oven for the minimum time needed to melt paraffin off the tissue.

Standardization

All immunohistochemical procedures for each antibody should be standardized, including epitope retrieval, time, and temperature and then done exactly the same way every time. Fixation should be standardized, both as to a reagent and time, but a reference laboratory will not have control of fixation, so it is important to have information such as type of fixation before staining. Automated immunostaining systems have increased the ability for standardization and reproducibility of procedures on a day-to-day basis within the same laboratory, and to some degree across different laboratories.

Since 1988, the Biological Stain Commission [Elias 1989] and the Food and Drug Administration (FDA) have been working to develop mechanisms to certify or otherwise regulate reagents and instruments. According to Taylor [1994], the pathologist must assume the ultimate responsibility for the laboratory test performance and the interpretation of that test. So that a single performance standard can be applied, Taylor [1994] advocates that every laboratory doing immunohistochemical staining participate in the immunohistochemistry quality assurance program developed by the College of American Pathologists (CAP). This program uses slides that are sent to each laboratory by CAP to be stained: antibody and reactive data are returned. This program tests the reagents used by the laboratory, the performance of the staining protocol, and the basic interpretation of the results. Unfortunately, the program will not test the procedures unique to that particular laboratory such as fixation and processing. However, the overall quality of the laboratory's fixation, processing, and staining procedures can be assessed at least in part by participation in an external quality assessment program such as the National Society of Histotechnology (NSH)/CAP Histology Quality Improvement Program (HQIP) available from CAP (http://www.cap.org). This program allows the participating laboratory to submit 5 variously stained slides, including 1 immunoenzyme-stained slide, for evaluation. These slides from designated surgical and control tissues should be handled routinely so that a true laboratory evaluation is obtained.

If a laboratory is accredited by CAP, it will then be required to undergo periodic onsite inspections by a group of peers. A laboratory will be expected to have the specific procedures and documentation in place. It is the responsibility of the laboratory to keep up with annual revisions of the checklist (http://www.cap.org).

The basic guidelines for CAP readiness in the immunohistochemistry laboratory, although not all inclusive, are as follows:

1. The procedure manual should be written using the guidelines recommended by the Clinical and Laboratory Standards Institute (formally known as NCCLS).

2. All methods and antibodies currently in use should have documentation of the validation of the staining procedures evaluated and selected for use. This should include items such as antibody name, lot number, receipt date, detection system,

Date stained: _____ **Page:** _____ **of** _____

	Antibody dilution	Tech initials	Antibody	AB lot#	Dilution	Control ID#	Results/ Comments	Corrective action
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								

Technical Review: _____ **Date:** _____ **Pathologist Review:** _____ **Date:** _____

Detection _____ **Lot #** _____ **EXP** _____ **Machine(s):** _____

Detection _____ **Lot #** _____ **EXP** _____ **Machine(s):** _____

Chromogen _____ **Lot #** _____ **EXP** _____ **Machine(s):** _____

Chromogen _____ **Lot #** _____ **EXP** _____ **Machine(s):** _____

Buffer - PBS: Lot# _____ **pH** _____ **Date prepared** _____ **Tech initial** _____

Buffer – TRIS: Lot# _____ **pH** _____ **Date prepared** _____ **Tech initial** _____

[f12.5] Daily antibody and positive control log

retrieval solution, method for retrieval, any temperatures used, dilution, incubation times, chromogen, and counterstain.

3. Procedures must be written to address specimens requiring nonstandard protocols because of fixation, frozen sections, imprints, or cytocentrifuge preparations. If the laboratory performs staining on frozen sections, then a detailed procedure for frozen specimens should be in the manual. A procedure for a cytology smear may require only a hydrogen peroxide quenching, omit any retrieval, and reduce incubation times; therefore, this specimen type requires a specific procedure addressing handling. These procedures should address postfixation, times, and temperatures. If the tissue requires retrieval, or if retrieval is omitted based on no formalin fixation, then the protocol must clearly identify each step. Proper quenching for staining with either the HRP or ABC detection systems must be included.

4. The procedure manual should be reviewed for changes in individual protocols and then signed off on by the medical director or designee, indicating that the manual is up to date. If a procedure changes, then handwritten changes on the permanent copy are acceptable, as long as the changes are noted with a date and the initials of the person making them. This also provides documentation that the procedure is kept up to date and has been reviewed by the user. Revision dates should be tracked on the procedure to keep records of changes.

5. When a procedure is discontinued, meaning no longer used, the laboratory must maintain a copy for 2 years, recording the initial date of use and the date discontinued.

6. Personnel should be trained and examined before performing patient testing. The laboratory testing personnel should review the procedure manual and sign off that they have reviewed it and are knowledgeable of the contents relevant to their scope of testing. The laboratory should perform competency testing of personnel who are handling patient materials. This can be accomplished in many different ways, such as a written examination and/or having testing personnel perform staining followed by review.

7. Methodology is critical for standardization; the laboratory should be consistent each day and every run. Steps in the processes should be monitored and documented. Tissue-processing temperatures throughout the process should be documented on either a chart or by a printout generated by automated instruments. An example is the HIER step: If the solution is to reach a specific temperature at a specific point, then it should be checked for that temperature and documented that the temperature was achieved. The chart should be reviewed every month; look for variance that may be responsible for weak or false-negative staining.

8. All reagent storage conditions should be checked upon receipt of the reagent. The reagent should be stored under the recommended conditions for maintaining its expected shelf-life. If a reagent is not stored properly, then it can deteriorate and the maximum use of the reagent may not be achieved or the quality of the staining may be affected (ie, weak or inconsistent staining). The laboratory may use paper or electronic documentation. Writing on the bottles can sometimes become hard to read if the paper becomes damp. My laboratory assigns a unique number for each new lot of antibody; the details such as date received, content, quantity, concentration or final dilution, storage requirements, and expiration date are all tied to the unique number. The bottle then contains the final concentration, the date it was made, and the antibody name. The unique number is placed on all QC documentation, thus allowing back-tracking when troubleshooting is required.

9. The pH of any buffer, commercial or in-house prepared, should be monitored; this should be documented before use. Once the pH is checked, the laboratory can document the pH, and the date checked, both on a chart and on the bottle, to ensure that all users know that the buffer meets staining requirements.

10. During a staining run, positive and negative controls should be used for each antibody involved; this can be achieved by using a tissue control that contains both positive and negative tissue types for a given antibody. Positive tissue controls are used to verify that the antibody reactivity is as expected and that the methods used were successful.

    - Fixation of the positive control should be the same as the patient tissue.

    - Ideally the control slide used contains more than 1 positive tissue. A possible combination would be a normal tissue control and a positive tumor control.

    - There are also internal positive controls in most but not all patient tissues; these are acceptable if their use is clearly defined in the procedure manual.

    - Negative reagent controls are necessary to confirm that the patient specimen is not demonstrating nonspecific staining that could be interpreted as "false positive."

    - Negative reagent controls are those that omit the antibody during the primary incubation step. All other steps must be exactly the same. This can be achieved by using different reagents, and should be defined by the laboratory. Commonly the antibody diluent in which the antibody is prepared is used for this purpose. A negative reagent control should be performed on the patient tissue for each type of detection (alkaline phosphatase or HRP); it is recommended that if multiple types of pretreatments are used, the harshest treatment be selected because it is the most likely to produce false-positive staining.

    - Negative controls verify that the pretreatment, heat, or pH of solutions is not introducing nonspecific staining that may be interpreted falsely as a positive.

    - Negative tissue controls primarily ensure the proper signal-to-noise ratio based on the antibody concentration.

- Both positive and negative reactions should be documented; it is preferred that the laboratory perform microscopic QC of all slides before releasing them from the laboratory.

11. Control slides should be stored at the proper temperature and for no more than the proper length of time based on laboratory standards and validation procedures. The goal is to store controls in such a manner as to maintain antigenicity of the tissue. The laboratory may choose to cut all control slides fresh, cut them weekly, or precut and store them at –20°C. The laboratory workflow and volume dictate what is reasonable. Whatever method the laboratory uses requires that a procedure be written giving specific instructions for storage. Before use, a control tissue should be validated for both positive and negative results. Documentation should be maintained regarding the reactivity of a given tissue for each antibody tested. It is good practice to have a backup control validated for use when the current control is exhausted.

12. Any reagent used by the laboratory must have a label. The name of the reagent, date received, date prepared or placed in use, concentration or dilution, expiration date, and recommended storage are minimal requirements. A laboratory should review reagents every month for those expiring the following month; purchasing a replacement reagent ahead of time will allow for receipt and proper validation before implementing into diagnostic staining.

13. The results should be reviewed for quality after each staining run. A document containing all the reagents used for staining, including lot numbers, ensures that the laboratory is recording and documenting the results. Some automated immunohistochemistry stainers can provide a printout of all the reagents used during a given run, and that printout can serve as the document, if the results are signed off as acceptable.

14. It imperative that the laboratory maintain both historical and current records for the validation of a new antibody or new lot. This includes a copy of the specification sheet for commercial antibodies. All attempts, whether successful or unsuccessful, are important documents to keep to serve as a reference to verify that no changes were made if troubleshooting is required. QC and documentation of steps and processes are necessary for proper slide quality to be accessed. The laboratory should maintain documentation of the review of slides to ensure that processes are monitored and corrective action is taken when technical errors arise. Although documents can be maintained in many ways, it is recommended that laboratories retain this information in paper as well as electronic forms.

    When antibody validation is finalized, the details should be documented and signed by the director or designee. The laboratory may find it helpful to create both a paper document and electronic record that lists all the variables such as the following:

    a. antibody name

    b. antibody vendor

    c. diluent for concentrates

    d. dilutions (for concentrate and prediluted antibody)

    e. pretreatment solution

    f. heat retrieval method

    g. retrieval time

    h. detection system

    i. buffer for staining (Tris or PBS)

    j. automated or manual staining times or standardized procedure

    k. chromogen and time

    l. counterstain

    m. results with comments for additional validation and a signature

15. Automated staining equipment is to be maintained in good working condition; the laboratory will need to keep documentation for daily, monthly, quarterly, or annual maintenance. This documentation should also allow for a record of instrument problems and can serve to track trends in technical errors or equipment malfunction. Corrective action documentation is needed to ensure that adjustments, training, or service calls for repair were made.

16. When the laboratory uses class I analyte-specific reagents (ASRs) the pathologist should be notified that they are interpreting staining results on an antibody that classifies as an ASR. CAP has specific recommendations regarding a memo and wording to be used.

17. For those antibodies that are "research use only" (RUO) the laboratory should document attempts to replace with an in vitro diagnostic antibody (IVD). The laboratory can maintain a spreadsheet or paper log to track replacement attempts. This should include the reason that an antibody cannot be replaced (eg, antibody clone is only available in RUO, and the laboratory will perform a search again in 1 year).

18. The laboratory should maintain antibody expiration dates either on paper or electronically. These dates should be reviewed every month to ensure that a stock of nonexpired antibody is available and that expired antibodies are not in use. Identifying antibodies as current, with back up volumes, allows for a quick check of those unexpired antibodies in use.

19. Each time a buffer is prepared, the pH should be documented. This can easily be achieved by preparing a paper log; included should be the buffer name, the vendor, the lot number, the pH, and whether corrective action is needed. It is recommended that the initial, and date of pH check be on the vial, bottle, or

storage container. The shelf-life of the buffer, once prepared, should be known; contact the vendor if using a commercial reagent or if preparing from buffer salts. Bacteria may grow quickly if a detergent is not added.

20. When an ABC or streptavidin-biotin complex detection system is used, the likelihood of false-positive staining in specific tissue types, such as liver and kidney, is high. Endogenous tissue biotin can create a staining artifact, so the laboratory should have blocking procedures in place to ensure that endogenous biotin does not cause interpretation problems. One method is to use avidin and biotin blocking before application of the biotin labeled secondary. Using a known tissue that is high in tissue biotin, such as kidney and liver, added to the positive tissue control can serve as a reference to ensure that proper blocking was achieved during each staining batch.

21. A high-volume laboratory may use batch controls to conserve control tissue or the number of controls run. The results of the positive tissue control should be documented each day and for each run if multiple batches of the same antibody are performed. It is not acceptable for the proper QC for 1 antibody to be stained and serve as a "daily" control, when the subsequent runs may have human or mechanical error.

    a. The positive control should be labeled with date and/or batch and then returned with the patient slides to the pathologist.

    b. If more than 1 pathologist may need access to the positive batch control slides, then the slides should be stored locally

Troubleshooting Immunoperoxidase Techniques
[Naish 1980]

STAINING RESULT

• Specimen	Unstained
• Positive control	Unstained

POSSIBLE CAUSES AND CORRECTIONS:

1. Primary antibody not added. Repeat procedure using checklist.

2. Substrate-chromogen improperly prepared. Repeat substrate-chromogen step with correctly prepared chromogen

3. Reagents used in wrong order. Repeat procedure using checklist.

4. Alcohol-based counterstain or mounting medium used with AEC, fast red, or tertrazolium salts. Repeat procedure using water-based counterstain and mounting medium.

5. Tissue sections were allowed to dry during staining procedure. Repeat procedure.

6. Wrong secondary antibody used (eg, antirabbit secondary with monoclonal primary). Repeat procedure, using correct secondary antibody.

7. Omission of labeled reagent. Repeat procedure using checklist.

8. Sodium azide in the buffer baths used. Remake buffers, avoiding sodium azide contamination.

STAINING RESULT

• Specimen	Weak staining
• Positive control	Weak staining

■ *Possible Causes and Corrections*

1. Substrate-chromogen improperly prepared. Review substrate-chromogen preparation and repeat procedure.

2. Primary antibody too dilute or defective. Replace defective antibody, and recheck antibody for correct dilution.

3. Insufficient incubation time. Determine optimal incubation time for antibody concentration used.

4. 1 or more defective reagents. Repeat procedure, replacing 1 reagent at a time.

5. Too much rinse buffer left on slides, diluting reagents excessively. Remove more of buffer during wiping step.

6. Epitope enhancement method incorrectly done. Check the reagents and all steps of the enhancement procedure.

STAINING RESULT

• Specimen	Weak staining
• Positive control	Stained

■ *Possible Causes and Corrections*

Antigen present in low concentration or masked during fixation. Repeat procedure using an epitope enhancement procedure or overnight incubation with the primary antibody.

STAINING RESULT

• Specimen	Excessive background
• Positive control	Excessive background

POSSIBLE CAUSES AND CORRECTIONS:

1. Paraffin incompletely removed. Deparaffinize for a minimum of 30 minutes.
2. Many cells containing endogenous peroxidase. Check blocking reagent. The blocking reagent and the link antibody should be from the same animal species.
3. Excessive adhesive used. Use less adhesive on slides.
4. Slides not well washed with buffer. Increase buffer washes.
5. Concentration of primary antibody, link reagent, or label reagent too high. Check dilutions, and decrease reagent concentrations (especially the primary antibody) if indicated.
6. Incubation time of either primary antibody or substrate too long. Repeat procedure, decreasing incubation times.

STAINING RESULT

• Specimen	Excessive background
• Control	No background

■ *Possible Causes and Corrections*

Free antigen in tissue because of necrosis, autolysis, or degeneration. Interpret in areas of less intense background staining.

Staining Techniques

Following are descriptions of representative techniques available for immunohistochemical staining for light microscopy. Techniques for immunofluorescence are not as widely used in routine histopathology and are beyond the scope of this chapter, whereas immunohistochemical staining of tumors has become routine. Almost every surgical pathology laboratory performs stains for the more common antigens. Only manual methods are given in this text, but automated staining is highly recommended because of the ability for increased standardization of the technique. The principle of the methods will be the same whether manual or automated.

BASIC PAP IMMUNOPEROXIDASE PROCEDURE [BOURNE 1989, STERNBERGER 1979]

■ *Purpose*

Localization of tissue antigens

■ *Principle*

This method utilizes 3 reagents: a primary antibody, a secondary antibody, and a PAP complex that is composed of the enzyme peroxidase and an antibody against peroxidase. The primary antibody is specific for the antigen. The secondary or "link" antibody is capable of binding to both primary antibody and to the PAP complex, because both primary antibody and PAP complex are produced in the same animal species.

■ *Fixative*

B-5, Zenker-, and Bouin-fixed tissues probably will not require epitope enhancement methods; however, some antigens are not well demonstrated after fixation in these reagents. Some formalin-fixed antigens may be better without the use of epitope enhancement methods, but most antigens are masked by formalin fixation and the tissue will require some type of epitope enhancement.

■ *Equipment*

Water bath maintained at 37°C, pH meter, moist chamber, drying oven maintained at 50°C to 55°C, Coplin jars, micropipettes.

■ *Technique*

Cut paraffin sections at 4-5 µm, mount on positive-charged, poly-L-lysine-coated or silanized slides (chapter 3, p70), and air-dry overnight or oven-dry at 50°C to 55°C for 30 minutes. Do not let the oven temperature exceed 60°C.

■ *Quality Control*

A section positive for the antigen must be used. A negative control substitution buffer or nonimmune serum from the same animal species as the primary should be run also.

■ *Reagents*

Modified PBS Buffer (Stock Solution)

Potassium phosphate, dibasic (K_2HPO_4)	188 g
Sodium phosphate, monobasic (NaH_2PO_4)	33 g
Sodium chloride	180 g

First, dissolve K_2HPO_4 in approximately 800 mL of distilled water in a 1-L beaker using heat and a stirring bar. Add NaH_2PO_4 and NaCl. Dissolve the salts completely, and dilute to 1,000 mL. Adjust pH to 7.4 if necessary, and store at room temperature.

Working PBS Solution

Modified PBS stock solution	40 mL
Distilled water	960 mL

Primary Antibodies

The appropriate dilution for each antibody must be determined. When preparing dilutions in the laboratory, dilute with 10% normal swine serum or the same animal species used for the link antibody.

Swine Antirabbit Linking Serum

Swine antirabbit immunoglobulins	100 µL
Normal swine serum, 10%	8 mL

Rabbit PAP

Rabbit PAP	100 µL
Normal swine serum, 10%	10 ml

AEC

3-amino-9-ethylcarbazole	15 mg
N-N dimethyl formamide	3.75 mL
0.05M acetate buffer (pH 5.2)	72 mL
3% hydrogen peroxide	0.75 mL

Dissolve 3-amino-9-ethylcarbazole in N-N dimethyl formamide. Add 0.05M acetate buffer (pH 5.2). Just before use, add 3% hydrogen peroxide.

Acetate Buffer (0.05M, pH 5.2)

Sodium acetate, anhydrous (4.05 g/L) or trihydrate (6.80 g/L)	700 mL
Acetic acid (1.43 mL/L)	300 mL

■ *Procedure*

1. Remove the slides from the oven, and immediately place them in xylene (3 changes for at least 5 minutes in each change).
2. Place the slides in absolute alcohol (2 changes for 3 minute each).
3. For Zenker- or B-5 fixed tissue, treat with iodine and sodium thiosulfate to remove mercury pigment, wash well with tap water; rinse with distilled water, and then rinse well with 95% and absolute alcohols.
4. Treat the slides with 1% hydrogen peroxide in absolute methanol (3.3 mL of 30% hydrogen peroxide, 96.7 mL of methanol) for 30 minutes at room temperature.
5. Wash the slides in 2 changes of 95% alcohol.
6. Wash the slides in distilled water.
7. If the need is indicated by prior tests, use the epitope enhancement method of choice.
8. Rinse well in distilled water.
9. Rinse the sections well with working PBS solution. Slides may stand in this solution while waiting for epitope enhanced sections.
10. Steps 10 through 15 are performed in a moist chamber with the slides flat.
11. Wipe around each section to remove excess buffer. Apply blocking serum (10% normal swine serum or the same animal species used for the link antibody). Leave on for 10 minutes.
12. Shake off excess blocking serum. Do not wash.
13. Flood with primary antibody. (For a buffer or negative control slide, leave the blocking serum on the slide during incubation of other slides with the primary antibody, or buffer may be added.) Incubate at room temperature for 60 minutes.
14. Wash well with working PBS solution using a wash bottle, then flood slides and leave for 10 minutes.
15. Wipe slides and apply the working dilution of the linking antibody (eg, swine antirabbit or goat antirabbit for polyclonal antibodies and rabbit antimouse for monoclonal antibodies. Multilink antibodies can also be used). Incubate 30 minutes.
16. Wash the slides well with PBS as in step 12.
17. Wipe the slides and apply the PAP. Incubate for 30 minutes
18. Wash well with PBS as in step 12.
19. Wash the slides thoroughly with distilled water. If they are not well rinsed at this point, there may be excess background staining.
20. Develop the sections with AEC for 30-40 minutes.
21. Wash the slides with several changes of distilled water.
22. Counterstain the sections with Mayer hematoxylin for 15 minutes.
23. Wash the sections in running water for 15 minutes.
24. Mount with an aqueous mounting medium or Crystal Mount.

■ *Results* [i12.19]

- Positive reaction — Brick red

■ *Technical Notes*

1. A multilink secondary antibody can be used.

2. DAB can be used as the chromogen instead of AEC (begin with step 19). This chromogen has the advantage of allowing the sections to be dehydrated, cleared, and mounted with a resinous mounting medium. The sections have greater clarity and are permanent [**i12.18**]. The procedure is as follows:

   a. Incubate sections in the dark for 3-8 minutes at room temperature in a freshly prepared solution of 0.01% hydrogen peroxide and 0.05% 3,3'-diaminobenzidine tetrahydrochloride in 0.05M Tris buffer, pH 7.2.

   b. Wash sections in distilled water, dehydrate, clear, and mount with synthetic resin.

3. The alkaline phosphatase-antialkaline phosphatase method is analogous to the PAP method presented above, except that in step 16, an alkaline phosphatase-labeled antibody is used instead of a peroxidase-labeled antibody. The substrate used in step 19 should contain naphthol phosphate and a chromogen such as fast red TR. The sections must be mounted with an aqueous mounting medium, because the chromogen is alcohol soluble.

4. Many of the chromogens are potential carcinogens and should be handled with caution.

5. Although troublesome to use, Crystal Mount allows AEC-stained sections to be subsequently coverslipped with a synthetic resin. The resulting clarity of the section is greatly enhanced.

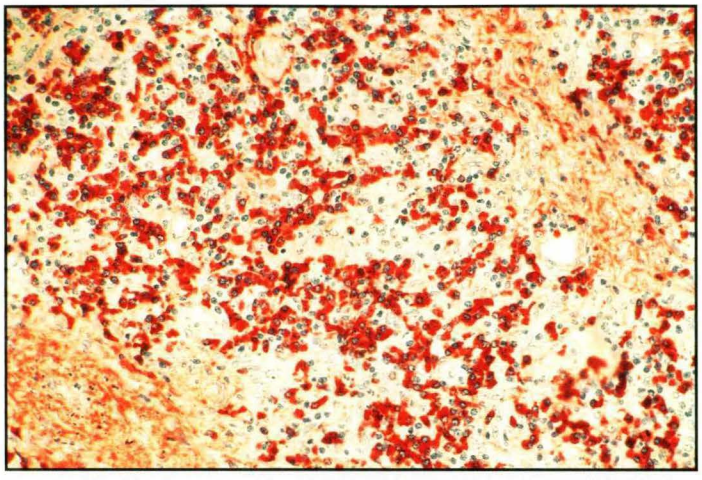
ABC-IMMUNOPEROXIDASE PROCEDURE

■ *Purpose*

Localization of tissue antigens.

■ *Principle*

This method uses 3 reagents: primary antibody, biotinylated secondary antibody, and ABC, which compromise the enzyme peroxidase conjugated to a complex of avidin and biotin. The primary antibody is specific for the antigen. The secondary or "link" antibody is conjugated with biotin and is capable of binding to the primary antibody and also binding the ABC. The free sites of the avidin molecule in the ABC allow binding to the biotin on the secondary antibody. Avidin is a protein with the ability to nonimmunologically bind 4 molecules of the vitamin biotin.



[i12.19] Cytoplasmic IgA in a lymphoma of the colon demonstrated on a paraffin section using the peroxidase-antiperoxidase method. Trypsin was used for epitope retrieval. The background staining is caused by the presence of immunoglobulin in the tissue fluids. The chromogen is 3-amino-9-ethylcarbazole.

■ *Steps for building an ABC-HRP complex*

1. **Peroxidase block:** A 3% solution of hydrogen peroxide in either deionized water or methanol to block tissue endogenous peroxidase. Make this solution fresh for each use.

2. **Protein block:** A blocking serum, normally a 10% solution used to block the normal (nonimmune, unlabeled) serum from the species in which the secondary antibody is raised.

3. **Primary antibody:** The antigen that is targeting the desired epitope to be demonstrated.

4. **Biotinylated secondary antibody:** A complex that reacts with the primary antibody to build a bridge between it and the staining complex. This solution is commonly referred to as a "link" in commercial kits.

5. **Enzyme label:** Complex conjugated to an enzyme. This large complex contains enzyme HRP, and is commonly referred to as "label" in commercial kits.

6. **Chromogen:** The chemical that reacts with the label so that the antibody complex can be visualized as a colored reaction product: an example is AEC, which is red 7.

7. **Counterstain:** The counterstain is used to demonstrate tissue morphology without interfering with the color reaction of the chromogen. Aqueous chromogens are not compatible with alcohol-based counterstains.

■ *Fixative*

10% neutral-buffered formalin, zinc formalin, B-5, Zenker, or Bouin-fixed tissue

■ *Equipment*

Water bath maintained at 37°C, pH meter, moist chamber, drying oven maintained at 50°C to 55°C, Coplin jars, micropipettes

■ *Technique*

Cut sections at 3-4 μm, mount onto positive charged slides, and air-dry overnight or oven-dry at 50°C-55°C for 30 minutes. Do not allow oven temperature to exceed 60°C.

■ *Quality Control*

A section positive for the antigen must be used. A negative control substituting buffer or nonimmune serum for the primary antibody should be run also.

■ *Reagents*

The following reagents are those not supplied with the usual commercial kits. This procedure is used with Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA); it may be used with other ABC kits by adjusting times to the manufacturer's specifications.

Modified PBS Buffer (Stock Solution)

Potassium phosphate, dibasic (K_2HPO_4)	188 g
Sodium phosphate, monobasic (NaH_2PO_4)	33 g
Sodium chloride (NaCl)	180 g

First, dissolve K_2HPO_4 in approximately 800 mL of distilled water in a 1-L beaker using heat and a stirring bar. Add NaH_2PO_4 and NaCl. Dissolve the salts completely, and dilute to 1 L. Adjust pH to 7.4 if necessary, and store at room temperature.

Working PBS Solution

Modified PBS stock solution	40 mL
Distilled water	960 mL

Primary Antibodies

Primary antibodies should be diluted with 1% to 2% bovine serum albumin to previously determined concentration.

AEC

AEC	15 mg
N-N dimethyl formamide	3.75 mL
0.04M acetate buffer (pH 5.2)	72 mL

Dissolve AEC in N-N dimethyl formamide, and add 0.04M acetate buffer

Acetate Buffer (0.05M, pH 5.2)

Sodium acetate, anhydrous (4.05 g/L) or trihydrate (6.80 g/L)	700 mL
Acetic acid (1.43 mL/L)	300 mL

■ *Procedure*

1. Remove the slides from the oven and immediately place them in xylene (3 changes for 5 minutes each).
2. Place the slides in absolute alcohol (2 changes for 3 minutes).
3. If fixed in Zenker or B-5 solution, treat the slides with iodine and sodium thiosulfate to remove the mercury pigment, wash well with tap water, rinse with distilled water, and then rinse well with absolute alcohol.
4. Treat the slides with 1% hydrogen peroxide in absolute methanol (3.3 mL of 30% hydrogen peroxide, 96.7 mL of methanol) for 30 minutes at room temperature.
5. Wash the slides with 2 changes of 95% alcohol.
6. Wash in distilled water. If indicated by previous testing, insert an epitope retrieval method at this point.
7. Rinse well with working PBS solution. Wipe around each section. Steps 8 through 14 must be done in a moist chamber with slides flat.
8. Apply blocking serum (the blocking serum is provided in the kit; prepare according to instructions). Allow to stand for 10 minutes.
9. Shake excess blocking serum off the slides. Do not wash.
10. Apply primary antibody and incubate for 60 minutes. (Leave the blocking serum on the negative control slide, or apply nonimmune serum or buffer to the negative control at this point.)
11. Wash the slides well with buffer; using a wash bottle, flood them with buffer, and then leave them for 10 minutes.
12. Wipe the slides, and apply diluted biotinylated antibody (prepare according to kit instructions). Incubate the slides for 30 minutes. Prepare reagent for step 14 at this time.
13. Wash sections well with buffer as in step 10.
14. Wipe the slides and apply Vectastain Elite ABC reagent (prepare according to instructions; this reagent should be prepared at least 30 minutes in advance). Incubate the slides for 30 minutes.

15. Wash sections well with buffer, as in step 10.

16. Wash slides thoroughly with distilled water. If not well rinsed at this point, there may be excess background staining.

17. Develop sections with AEC for 30-40 minutes,

18. Wash well in distilled water.

19. Counterstain with Mayer hematoxylin for 15 minutes.

20. Wash in running water for 15 minutes.

21. Mount with an aqueous mounting medium or Crystal Mount.

■ *Results* [i12.20], [i12.21]

• Positive reaction	Brick red

■ *Technical Notes*

See under the PAP procedure

HRP ENZYME-LABELED POLYMER PROCEDURE

■ *Purpose*

Localization of tissue antigens

■ *Principle*

This method uses 2 reagents: primary antibody, and HRP enzyme-labeled polymer secondary. The primary is specific for the targeted antigen, and the polymer has an affinity for the antibody. This complex is free of avidin and biotin and does not require blocking of proteins. It does require blocking for tissue peroxidase activity.

■ *Fixative*

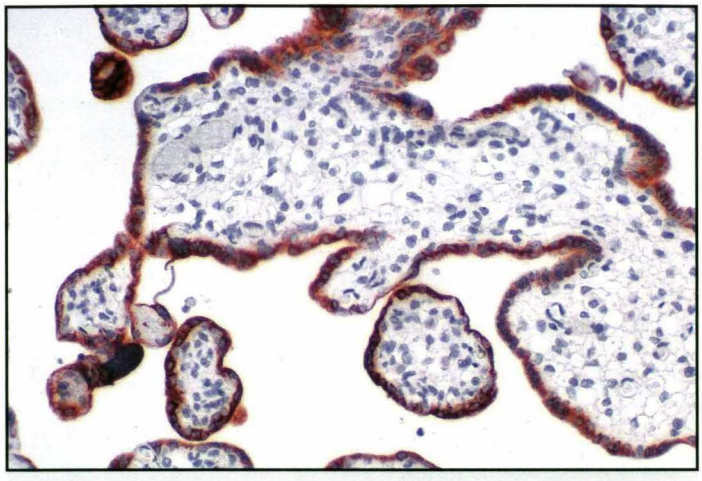
10% neutral-buffered formalin

■ *Equipment*

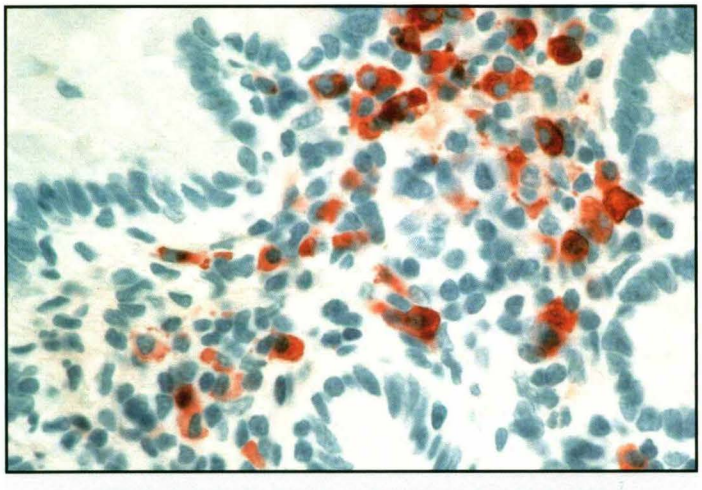
Water bath maintained at 37°C, pH meter, moist chamber, drying oven maintained at 50°C to 55°C, Coplin jars, micropipettes, laboratory pressure cooker

■ *Technique*

Cut sections at 3-4 µm, and mount on commercially prepared positive-charged slides. Remove any water from behind the section. Air-dry overnight or oven-dry at 50°C-55°C for 30 minutes and remove slides and place in front of a cool fan for 15 minutes.



[i12.20] A section of placenta stained with the peroxidase-antiperoxidase method using human chorionic gonadotropin antibody. The chromogen is DAB.



[i12.21] Plasma cells are seen in this section of gastrointestinal tract stained with lambda using the avidin-biotin-complex method. The enzyme used was peroxidase and the chromogen was 3-amino-9-ethylcarbazole.

■ *Quality Control*

Minimum of 2 tissue sections positive and negative for the antigen should be used to serve as a control slide. In addition, a negative patient control substituting antibody diluent or nonimmune serum for the primary antibody should be stained.

■ *Reagents*

The following reagents (with the exception of the primary antibody) are supplied in a commercial kit or part of a detection staining system. This procedure is used with Envision Plus Detection system (Dako Cytomation USA, Carpentaria, CA).

Tris-Buffered Saline Solution (with Tween-TBST), pH 7.6 Ready to Use

Use at room temperature. Discard after 1 week or if reagent becomes cloudy. Check and document the pH of each new lot received before staining patient tissue.

Primary Antibodies

Primary antibodies should be diluted in a diluent that contains proper carrier proteins to retain antigen stability after preparation. A specific pH may be recommended by the manufacturer or in a reference textbook. Polymer secondary reagent (containing both mouse IgG, IgM and rabbit IgG).

Chromogen Solution

Calculate the amount of buffer required to completely cover the quantity of slides being stained.

DAB (3,3'-diaminobenzidine), ready to use

Buffered substrate solution, pH 7.5, containing hydrogen peroxide and preservative, ready to use

Mix 1 drop of DAB with every 1 mL of buffered substrate solution in a conical tube

3% Hydrogen Peroxide

30% hydrogen peroxide	30 mL
Distilled water	70 mL

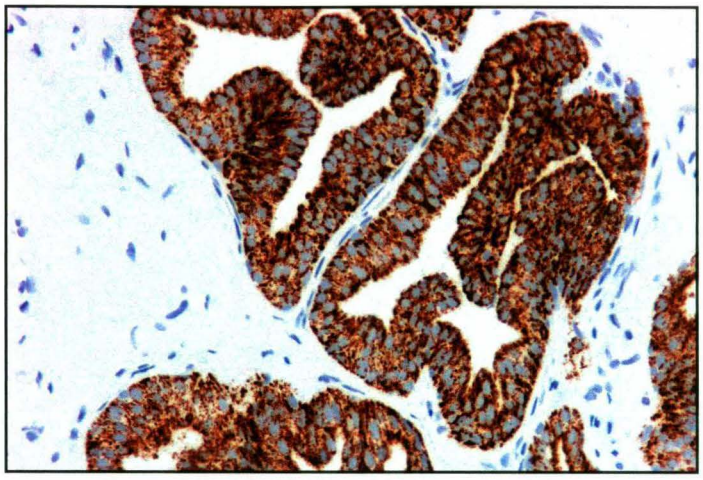
Prepare fresh for each batch of slides

Retrieval Solution (pH 6.0)

Ready to use

■ *Procedure*

1. Place slides into xylene (3 changes for 5 minutes each, using agitation).
2. Place slides in 100% alcohol (3 changes for 5 minutes each, using agitation).
3. Place slides in 95% alcohol (2 changes for 5 minutes each, using agitation).
4. Xylene and alcohol solutions should be changed after 40 slides.
5. Rinse slides in distilled or deionized water for 1 minute.
6. Place slides in 3% hydrogen peroxide for 10 minutes (specimen, such as bone marrow containing extreme amounts of red blood cells may require 20 minutes).
7. Rinse slides in distilled or deionized water for 1 minute.
8. Prepare pressure cooker according to the manufacturer's recommendations, and follow established protocol for performing antigen retrieval.



[i12.22] Prostate cancer stained with P504S antibody. The chromogen is 3,3'-diaminobenzidine.

9. Remove slides from pressure cooker, and allow to cool 20 minutes.
10. Rinse slides in distilled or deionized water for 1 minute.
11. Rinse slides in TBS solution, pH 7.6, and hold for staining.
12. Tap off excess buffer from slide. Do not allow tissue to dry out from this point forward.
13. Apply the primary antibody, and incubate in a moist chamber for 25 minutes.
14. Rinse slides well using TBS; use a wash bottle to remove excess antibody, and then place into a Coplin jar for 5 minutes.
15. Wipe off excess buffer, and apply the polymer secondary; place the slides into a moist chamber for 10 minutes.
16. Rinse slides well using TBS; use a wash bottle to remove excess antibody, and then place into a Coplin jar for 5 minutes.
17. Wipe off excess buffer and apply the DAB chromogen solution. Place the slides in a moist chamber for 5 minutes. Check the reaction. If necessary, repeat steps 16 and 17.
18. Rinse slides with distilled or deionized water.
19. Counterstain with Mayer hematoxylin for 1-3 minutes. Lighter counterstain is needed for nuclear antigens.
20. Coverslip using a permanent mounting medium.

■ *Results* [i12.22]

- Positive reaction — Brown to dark brown

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LEARNING ACTIVITIES

1. **Cut 3 frozen section slides of a lymph node at 4 µm:**
   a. Fix 1 slide cold acetone; 1 slide with 10% neutral buffered formalin; and air dry 1 section without fixative.
   b. Stain with CD45 (LCA)
   c. Compare the stained slides for different staining intensity and tissue morphology.
   d. Which procedure gives the best results?
2. **Cut 4 slides of a formalin-fixed paraffin-embedded prostate at 3 µm:**
   a. Pretreat each slide in a humidity chamber with pepsin enzyme with one of the following times:
      i. 1 minute
      ii. 5 minutes
      iii. 20 minutes
      iv. 1 hour
   b. Stain all slides together with AE1/AE3 cytokeratin antibody.
   c. Review slides and rank in order of signal-to-noise ratio.
   d. Do any of the slides demonstrate overdigestion or loss of morphology? If so, which one(s)?
3. **Cut 4 slides of CNS tissue at the following thicknesses: 3, 4, 6, and 10 µm**
   a. Perform a heat-induced epitope retrieval using citrate buffer, pH 6.0 in a modified pressure cooker. Cool down for 20 minutes.
   b. Stain with GFAP antibody.
   c. Review slides for quality, including tissue lifting and nonspecific staining.
   d. What artifacts do you see and why?
4. **Practice using micrometer pipettes to prepare 5 mL of the following dilutions:**
   a. 1:20
   b. 1:50
   c. 1:100
   d. 1:5000
   e. Write out the formula for preparing each dilution.

5. Stain a melanoma control tissue with HMB45 antibody.

a. Stain 1 slide using horseradish peroxidase and DAB chromogen, and the second slide using AEC chromogen.

b. What is the difference in the color of the chromogen?

c. What is different about the mounting media used for coverslipping?

d. What is the basis for using different mounting media?

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CHAPTER 13

Enzyme Histochemistry

OBJECTIVES

On completing this chapter, the student should be able to do the following:

1. Define
   a. enzymes
   b. oxidation
   c. reduction
   d. substrate
   e. hydrolase
   f. simultaneous coupling
   g. esterase
   h. phosphatase
   i. phosphorylase
   j. epimysium
   k. perimysium
   l. endomysium
2. Describe the histology of normal muscle
3. Differentiate between type I and type II muscle fibers
4. List 3 properties of enzymes
5. List 5 factors that influence enzyme demonstration
6. List an artifact that may be seen in unfixed frozen sections
7. Identify a storage solution for tissue to be used for enzyme studies
8. Identify the preferred method of freezing muscle tissue for enzyme studies
9. Describe the reactions for:
   a. α-naphthyl acetate esterase
   b. naphthol AS-D chloroacetate esterase
   c. ATPase
   d. acid phosphatase
   e. alkaline phosphatase
   f. NADH diaphorase
   g. SDH
   h. phosphorylase
10. Explain how the naphthol AS-D chloroacetate esterase stain differs from most enzyme techniques
11. Identify the results of the following stains:
    a. α-naphthol acetate esterase
    b. naphthol AS-D chloroacetate esterase
    c. ATPase
    d. acid phosphatase
    e. alkaline phosphatase
    f. NADH diaphorase
    g. SDH
    h. phosphorylase
12. Explain why the pH of the NADH diaphorase reaction is critical
13. Identify the disease indicated by a negative phosphorylase reaction
14. List 4 types of histochemical reactions for the demonstration of hydrolytic enzymes

This chapter is not intended as a compendium of enzyme histochemistry, but rather to introduce the student to this methodology. The techniques presented are a selection of those primarily used for the diagnosis of muscle diseases, and thus are commonly used in many surgical pathology laboratories.

Muscle Histology

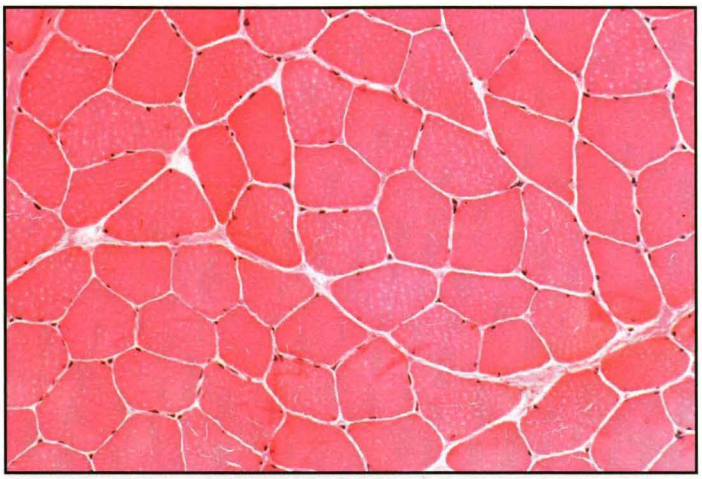
Most of the enzyme techniques presented in this chapter are used in the diagnosis of diseases affecting skeletal muscle, it is therefore appropriate to discuss the histology of normal skeletal muscle.

Individual skeletal muscle fibers (cells) normally range from 30 to 80 μm in diameter and may reach lengths up to 35 cm. Characteristics that distinguish skeletal muscle are the presence of multiple elongated nuclei located at fairly regular intervals just beneath the cell membrane (sarcolemma), and the presence of regular cross-striations that result from the particular arrangement of the contractile proteins, actin, and myosin **[i8.1, p161]**, **[i8.23, p181]**. Each muscle fiber is packed with myofibrils, cylindrical structures with prominent cross-striations. These myofibrils are arranged parallel to the sarcolemma and with the cross-striations in perfect alignment. With the use of the various histochemical reactions, individual myofibrils can be distinguished.

The space between the myofibrils (interfibrillar space) and beneath the sarcolemma is filled with the cytoplasm (sarcoplasm) of the muscle fiber. Mitochondria, sarcoplasmic reticulum, microtubules, intermediate filaments, free ribosomes, and the Golgi apparatus are organelles that are found in the sarcoplasm along with glycogen and lipid droplets.

Bundles of muscle fibers (fascicles) are surrounded by a dense layer of collagen called the *perimysium*, and an entire muscle is enclosed in a collagenous connective tissue sheath called the *epimysium*. Individual muscle fibers are polygonal in shape, fairly uniform in diameter, and separated from each other by a network of fine collagen fibers **[i13.1]**. This network is called the *endomysium*, and although it is not usually noted with the light microscope, it may become very striking in some pathological conditions. Nerves and blood vessels are usually seen in the perimysium and the endomysium, and capillaries may be noted in the endomysium.

Muscles vary in function; some, such as those involved in maintaining posture, are required to contract almost continuously, while others, such as the extraocular muscle, make rapid contractions of brief duration. In humans, these 2 types of muscle cannot be distinguished on the basis of the color of the muscle; however, in some species, such as domestic poultry, the 2 types can easily be distinguished in this manner. In poultry, thigh muscles are red and breast muscles are white. The "slow-twitch" red muscle fibers use predominantly an aerobic (oxidative) metabolism. They contain abundant mitochondria, lipid, and myoglobin (an oxygen-storing molecule), and have a good blood supply. These are type I fibers. Type II fibers, "fast-twitch" white fibers, have an anaerobic metabolism; they contain only a few mitochondria, little myoglobin, and have a relatively poor blood supply. These type II fibers are rich in glycogen and glycolytic enzymes, and have a high resistance to fatigue. On the basis of the adenosine triphosphatase (ATPase) histochemical reaction, type II muscle fibers have been further divided into types IIA, IIB, and IIC. Type IIC fibers are not frequently seen in normal adult muscle, because these fibers are undifferentiated and precursors of the other types. In humans, many muscles contain roughly 33% type I fibers and 67% type II fibers. The type II fibers are further divided into 50% type IIA fibers, and 50% type IIB fibers. In normal human muscle, type I, IIA, and IIB fibers are distributed in a checkerboard pattern. Various muscles contain a different distribution of the fibers, and charts of the normal distribution for the particular muscle studied by biopsy are commonly used when making a pathologic diagnosis.

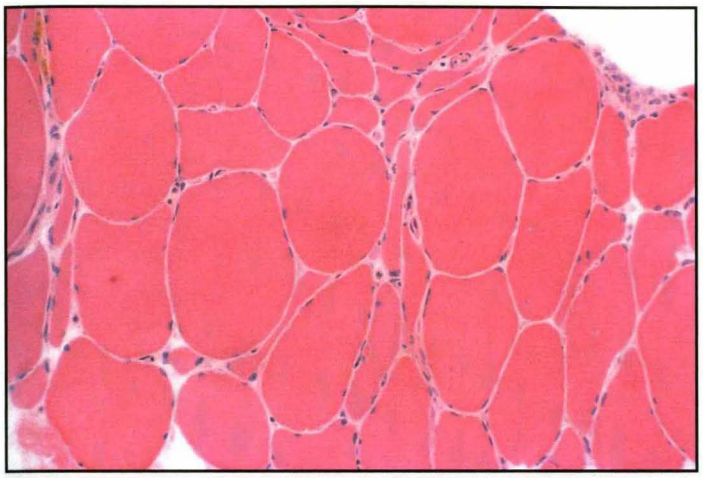


[i13.1] An H&E-stained section of normal skeletal muscle. Note that the fibers are fairly uniform in diameter and have peripherally located nuclei. Minimal freezing artifact can be seen.

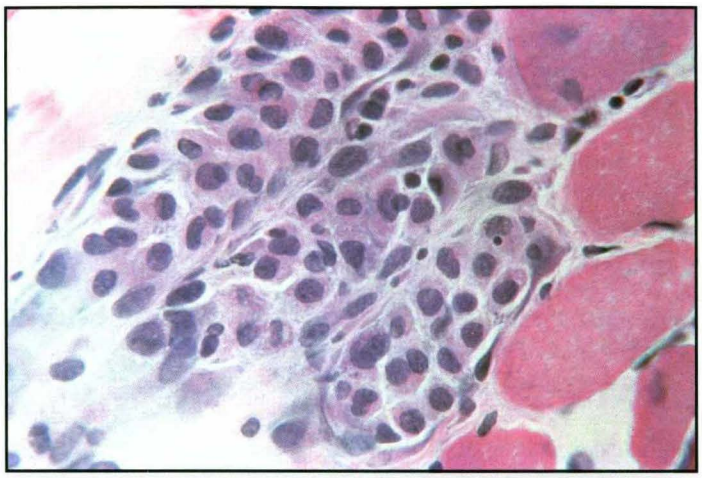
Pathologic Changes in Muscle

Some of the pathologic changes in muscle can be seen on the H&E stain. These include changes in the size (atrophy and hypertrophy) of both individual and groups of fibers, changes in the location and appearance of the nuclei, the presence of inflammatory cells, the presence of degenerating or regenerating fibers, and an increase in connective tissue **[i13.2]**, **[i13.3]**.

Histochemical techniques are required to demonstrate other changes such as type grouping, fiber type predominance, central cores, or target fibers. By using a panel of histochemical stains in conjunction with the hematoxylin-eosin (H&E) stain, most abnormal biopsy findings can be diagnosed as resulting from a primary muscle disease (myopathy) or secondary to denervation (neuropathy). A chart of the normal histochemical reactions is given in **[t13.1]**. The reader interested in more information on muscle and muscle diseases should refer to the book by Dubowitz [1985].



[i13.2] A marked variation in fiber size can be seen in his H&E-stained frozen section of skeletal muscle. Numerous atrophic fibers are seen in small groups, suggesting the presence of a neuropathic disease.



[i13.3] Three muscle fibers are undergoing phagocytosis in this H&E-stained frozen section, while more normal fibers are seen at the right of the photograph. Phagocytosis is a degenerative change seen in some inflammatory myopathies.

Enzyme Histochemistry

Beale first used enzymes for tissue digestion in 1861 by using gastric juice to digest excess tissue from the nerve fibers he was studying. Klebs in 1868 and Streuve in 1872 began the histochemical demonstration of enzymes in tissues by demonstrating that guaiac gives a blue color when applied to pus—the first demonstration of peroxidase. In 1900, Brandenburg demonstrated the presence of peroxidase in the granules of leukocytes.

Although there are more than 1,000 methods for demonstrating enzymes today, the primary use of enzyme histochemistry in the histopathology laboratory is in the diagnosis of muscle disease. Some of the same techniques may also be used in hematopathology for differentiation of the leukemias, and because of the overlap in techniques, frequently all enzyme histochemistry on tissue or smears is performed in the histopathology laboratory.

For most chemical reactions, the rate at which the reaction occurs can be changed by using either heat or a catalyst. If a reaction is heated, the rate increases, and conversely, when it is cooled, the rate of reaction slows down. Sometimes, heat or cold cannot be used in a reaction. Instead, a catalyst is used. A catalyst is a substance that changes the rate of the reaction of chemicals without being consumed by the reaction. A catalyst can either speed up (positive catalyst) or slow down (negative catalyst) the rate of the reaction. After the reaction, the catalyst is released to combine again with other chemicals.

Enzymes are proteins that catalyze chemical reactions occurring in biologic systems by temporarily combining with their specific substrate or the compound on which they act. Afterward, the enzymes are released unchanged, ready to combine with other substrate molecules.

$$\underset{\text{(enzyme)}}{E} + \underset{\text{(substrate)}}{S} \longrightarrow \underset{\text{(temporary combination)}}{ES} \longrightarrow \underset{\text{(product)}}{P} + \underset{\text{(enzyme)}}{E}$$

Many of these catalyzed reactions would occur without enzymes, but would be impossibly slow at biologic temperatures. Enzymes act alone or in combination with other nonprotein structures called cofactors to speed up the rate of reaction. Cofactors may be metal ions or complex organic molecules called coenzymes. Coenzymes, frequently vitamins, and metallic cofactors are bound to the enzyme with varying degrees of affinity. Coenzymes (CoE) frequently act as a cosubstrate; the chemical changes in the

[t13.1] Histochemical reactions of human muscle*

	Muscle Fiber Type			
Enzyme Technique	**I**	**IIA**	**IIB**	**IIC**
ATPase, pH 9.4-10.4	light	dark	dark	dark
ATPase, pH 4.2-4.3	dark	light	light	intermediate
ATPase, pH 4.6	dark	light	intermediate	dark
NADH	dark	light	light	light
SDH	dark	light	light	light
Phosphorhylase	light	dark	dark	dark

*Although these techniques show a difference between type I and type II fibers, only the adenosine triphosphatase (ATPase) technique will give a distinction between the various type II fibers. Sometimes some type II fibers will show an intermediate reaction with other techniques. The esterase, acid phosphatase, and alkaline phosphatase, are not used to differentiate between type I and type II fibers. NADH indicates reduced form of nicotinamide adenine dinucleotide, SDH, succinic dehydrogenase.

coenzyme exactly balance those taking place in the substrate (S). For example, with the dehydrogenases:

1 molecule S oxidized → 1 molecule CoE reduced
(dehydrogenated) (hydrogenated)

Oxidation and Reduction

Biological oxidation occurs with the:

1. addition of oxygen
2. loss of hydrogen
3. loss of electrons

Reduction occurs with the

1. loss of oxygen
2. gain of hydrogen
3. gain of electrons

Properties of Enzymes

Even a moderate amount of heat readily inactivates enzymes; this is an irreversible alteration of the enzyme. Enzymes are also sensitive to pH changes; some work best at an acid pH, others at an alkaline pH. The majority of enzymes are active in an environment that is approximately neutral, but an enzyme that works at an acid pH will be inactive in alkaline surroundings. Enzymes usually are very specific as to substrate. An enzyme recognizes its substrate by chemical groups on the substrate and by the spatial arrangement of these groups. The following factors influence enzyme demonstration [Barka 1965]:

1. Treatment of tissue before and during procedure. Many enzymes are removed or destroyed by fixation, while others are sensitive to freezing and thawing, so frequently compromises must be made. One may sacrifice some activity for better localization, or one may choose to maintain maximum activity and have considerable enzyme diffusion.
2. Nonoptimal substrate. Sometimes optimal substrate concentration cannot be obtained because of poor substrate solubility, or the substrate used may be hydrolyzed more slowly than the natural or other available substrates.
3. Nonoptimal temperature. There is an optimal temperature for enzyme activity, and sometimes, especially in azo dye simultaneous couple techniques, a different temperature must be used. Enzyme activity is usually destroyed at temperatures greater than 56°C.
4. Nonoptimal pH. Most enzymes are best demonstrated at a pH near 7.0; however, there are exceptions, as with acid and alkaline phosphatases. Again, some azo dye simultaneous coupling methods use a nonoptimal pH, which lowers enzyme activity.
5. Inhibitors. An excess of diazonium salts in the substrate, fixatives, heat, and some metallic ions may decrease or completely abolish enzyme activity.

Preservation of Enzymes

Ideally, tissue for enzyme demonstration should be fixed, because considerable diffusion artifact may be seen in unfixed frozen sections. When possible, the tissue should be fixed before freezing, because postfixation of the sections will not prevent the diffusion. Certain soluble enzymes require some fixation before freezing, or an insufficient amount of enzyme will be retained for demonstration; however, some enzymes will be inactivated by even the briefest fixation.

Cold (3°C-4°C) formalin, cold calcium formalin, and cold acetone have been used to fix tissue for some enzyme studies. After fixation, tissue may be stored in gum sucrose solutions (a 30% sucrose solution containing 1% gum acacia) at 4°C for several weeks.

If the technique to be used permits fixation, blood smears should be fixed as soon as they are dry, or the enzyme activity will decrease. Muscle biopsies are frozen unfixed, and whenever possible, the sections are postfixed. Although the cryostat freezer plate may be used for freezing tissue, a more rapid freezing technique is recommended. Because muscle is especially prone to ice-crystal artifact, isopentane suspended in liquid nitrogen is the preferred method of freezing. The isopentane is allowed to reach a temperature of –150°C before the muscle is placed in it. If liquid nitrogen must be used alone for freezing, the muscle should be dusted with talc. If the specimen is immersed directly in liquid nitrogen, some gaseous nitrogen will surround the specimen and freezing will be impeded.

Classification of Enzymes

Enzymes are specific as to the type of reaction catalyzed. Except for the oldest known enzymes (eg, trypsin, pepsin), enzymes are usually named by adding "ase" to the specific substrate or molecule on which the enzyme acts, or to a stem indicating the type of reaction. There are 6 basic reaction classifications: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Lyases add chemical groups to double bonds, while an isomerase rearranges chemical groups on a substrate. Ligases combine 2 substrates.

These enzymes are used in other reactions in the body, but are not enzymes for which we stain in the muscle system. The groups of major importance in histopathology and hematopathology are the oxidoreductases, hydrolases, and transferases. These classes will be defined later.

HYDROLASES

The hydrolases act on various substrates, usually through the addition of water; however, in some instances water may be removed. This class of enzymes includes the broad subclasses:

1. esterases, which catalyze the hydrolyses of ester linkages
2. phosphatases, which hydrolyze esters of phosphoric acid
3. peptidases, which attack peptid bonds

There are 4 types of histochemical reactions for the demonstration of hydrolytic enzymes:

1. Simultaneous capture or coupling. This is the most frequently used technique and involves the release of a reaction product by the action of the enzyme on the substrate. The reaction product is rapidly captured or coupled either with a diazonium salt (to give an insoluble azo dye) or with a metallic ion. In the azo dye methods, both the diazonium compound and substrate are present in the incubating medium. In the metallic precipitation methods of Gomori, the initial coupled reaction product is frequently not visible, so metal substitution methods are used to obtain a visible reaction product.
2. Postincubation coupling. This technique depends on the formation of an insoluble reaction product, which must remain at the reaction site for the entire incubation period. The sections are then treated with the coupler in a separate step.
3. Self-colored substrate. The reaction product must be both insoluble and colored in the technique, use a soluble colored substrate, and no coupler or capture agent is necessary.
4. Intramolecular rearrangement. The substrate used is soluble, and hydrolysis causes a molecular rearrangement, resulting in an insoluble colored reaction product.

■ *Esterases*

Esterases are enzymes capable of breaking the bond between carboxylic acid and alcohols, phenol, or naphthols. Esterases may be divided into "specific" and "nonspecific" esterase, the classification is not always straightforward, and each group comprises many closely related enzymes. There is considerable overlap in substrate specificity, optimal pH, substrate concentration, and response to activators and inhibitors. The majority of specific and nonspecific esterases are capable of hydrolyzing α-naphthyl acetate.

A diazonium compound is used in the substrate as a coupling reagent, resulting in the formation of an insoluble azo dye. Most rapid coupling occurs at pH 6.0 to 6.5, and freshly hexazotized pararosaniline, formed by the action of sodium nitrate on pararosaniline (basic fuchsin), is among the best diazonium (contains an –N=N- group linked to 1 hydrogen atom) compounds.

$$\alpha\text{-naphthyl acetate} + \text{water} \xrightarrow{\text{esterases}} \alpha\text{-naphthol} + \text{acetic acid}$$

$$\alpha\text{-naphthol} + \text{hexazotized pararosaniline} \rightarrow \text{azo dye} \downarrow$$

Burstone states that diazonium salts exhibit varying degrees of instability and should be stored in the refrigerator. Solutions of these salts should be used immediately, and storage of solutions is not recommended.

■ *Phosphatases*

Phosphatases are hydrolytic enzymes that break the bond between an alcohol and a phosphatase group; they are present in a wide variety of plant and animal tissue. Some phosphatases such as ATPase act specifically on a single substrate; others act with less substrate specificity and are divided into 2 groups: those exhibiting optimal activity at an alkaline pH (alkaline phosphatases) and those exhibiting optimal activity at a low pH (acid phosphatases). ATPase is found in mitochondria and bound to myosin, a contractile muscle protein. Acid and alkaline phosphatases are found in lysosomes. The general reaction may be expressed thus:

$$\begin{array}{l} \text{OH} \\ | \\ \text{R} - \text{C} - \text{P} = \text{O} + \text{water} \\ | \\ \text{OH} \end{array} \xrightarrow{\text{phosphatases}} \underset{\text{(alcohol)}}{\text{ROH}} + \underset{\text{(phosphoric acid)}}{\text{H}_3\text{PO}_4}$$

The released phosphate or the remaining organic residue is made visible by various techniques. The Gomori-type metal precipitation techniques precipitate the orthophosphoric acid with calcium or lead. A complex series of reactions eventually leads to the formation of a colored metallic sulfide:

$$\underset{\text{(phosphoric acid)}}{\text{H}_3\text{PO}_4} + \underset{\text{(calcium chloride)}}{\text{CaCl}_2} \longleftrightarrow \underset{\text{(calcium phosphate)}}{\text{CaHPO}_4} + 2\text{H}^+ + 2\,\text{Cl}^-$$

$$\underset{\text{(calcium phosphate)}}{\text{CaHPO}_4} + \underset{\text{(cobalt chloride)}}{\text{CoCl}_2} \longleftrightarrow \underset{\text{(cobalt phosphate)}}{\text{CoHPO}_4} + \text{CaCl}_2$$

$$\underset{\text{(cobalt phosphate)}}{\text{CoHPO}_4} + \underset{\text{(ammonium sulfide)}}{(\text{NH}_4)_2\text{S}} \longleftrightarrow \underset{\text{(cobalt sulfide)}}{\text{CoS}} + \underset{\text{(ammonium phosphate)}}{(\text{NH}_4)_2\text{HPO}_4}$$

Because $CaHPO_4$ is soluble at a low pH, the aforementioned reaction can be used only at an alkaline pH. It can be used for the ATPase or alkaline phosphatase reactions. The Gomori-type reaction for acid phosphatase uses lead to capture the phosphate ions.

Alternatively, the alcoholic residue of the substrate, after enzymatic hydrolysis, may be reacted with a diazonium salt to produce a highly colored insoluble azo dye. This usually involves either simultaneous coupling or postcoupling methods. The azo-dye method

is useful for both alkaline and acid phosphatase demonstration. Usually substituted naphthols are employed with naphthol AS-BI phosphate preferred for the acid phosphatase technique because of the extreme insolubility of the reaction product. Naphthol AS-M, AS-TR, AS-LC, and AS-BI phosphates are usually employed in the alkaline phosphatase techniques. With these naphthols, both the acid and alkaline phosphatase techniques are simultaneous coupling methods.

OXIDOREDUCTASES

This large group of enzymes includes:

1. oxidases, which can use molecular oxygen as the hydrogen acceptor with the resulting formation of water
2. peroxidases, which catalyze the oxidation of substrates by hydrogen peroxide
3. dehydrogenases, which remove hydrogen atoms (a proton and an electron) from an organic substrate

Dehydrogenases usually transfer the hydrogen atoms to a coenzyme; then from the coenzymes, the hydrogen is transferred to an orderly series of acceptors called the electron transport system. The electron transport system is very important in the production of cellular energy through coupling with oxidative phosphorylation. The ultimate acceptor for the hydrogen atoms is molecular oxygen, resulting in the formation of water:

$$4H^+ + 4e^- + O_2 \longrightarrow 2H_2O$$

■ *Diaphorases*

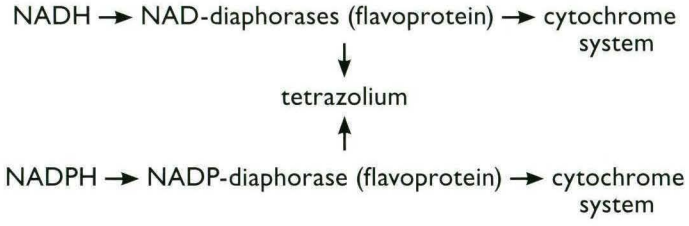
Most dehydrogenases are associated with a coenzyme that functions as a hydrogen and electron acceptor. The coenzyme, with rare exceptions, is nicotinamide adenine dinucleotide (NAD^+, or DPN in older literature) or nicotinamide adenine dinucleotide phosphate ($NADP^+$). As NAD^+ or $NADP^+$ accepts the electrons, they become reduced to NADH or NADPH.

Diaphorases are dehydrogenases, with the sole purpose of oxidizing NADH or NADPH:

$$NADH \xrightarrow{\text{diaphorase}} NAD^+ + H^+ + e^-$$

$$NADPH \xrightarrow{\text{diaphorase}} NADP^+ + H^+ + e^-$$

The diaphorases are flavoproteins containing flavine adenine dinucleotide; however, the precise identity has not been determined. The diaphorases are reoxidized by passing hydrogen and electrons to the cytochrome system. In the histochemical demonstration of diaphorases and NAD- or NADP-dependent dehydrogenases, a tetrazolium salt acts as a hydrogen acceptor, and the diaphorases are also known as tetrazolium reductases. Neither NADH nor NADPH can reduce the tetrazolium salt directly, but the reduction is attributed to the flavoprotein enzymes and is therefore called a *diaphorase* (to carry) reaction. Even in the presence of tetrazolium salts, some of the hydrogen atoms may escape the tetrazolium trap and be passed along the natural pathway into the cytochrome system:



The diaphorases, or tetrazolium reductases, are firmly bound in mitochondria and endoplasmic reticulum and are very important in muscle enzyme histochemistry. This enzyme is frequently used as a marker of mitochondria.

TRANSFERASES

Transferases are enzymes that transfer a functional group from one compound to another. There are a number of transferases, but phosphorylase is the only one that will be discussed in this chapter.

■ *Phosphorylase*

Phosphorylases are transferases that transfer phosphate groups. They are widely distributed in animal and plant tissues, and catalyze a number of reversible reactions leading to the synthesis and decomposition of saccharides. Glycogen phosphorylase is the best known phosphorylase, catalyzing the following reaction:

$$\text{glycogen} + n(H_3PO_4) \longleftrightarrow n(\text{glucose 1-phosphate})$$

In vivo, phosphorylase is a cytoplasmic enzyme functioning only in the degradation of glycogen; however, in vitro, muscle phosphorylase catalyzes the synthesis and hydrolysis of an unbranched polysaccharide of amylose type. The length of the unbranched chains formed is proportional to the amount of phosphorylase activity present and, when stained with iodine, will vary in color depending on the length of the chain. A negative reaction is yellow, and variations of brown, lavender, purple, and intense blue-black are seen with increasing chain length.

Freezing Muscle Biopsy Specimens

Muscle tissue is extremely subject to ice crystal artifact. If pronounced, the artifact can make the muscle biopsy stains uninterpretable **[i2.24]**, **[i2.25]**. The method of freezing is critical, and the following is a recommended procedure:

1. Orient the fresh unfixed muscle tissue so that cross-sections will be obtained.

2. Prepare a piece of cork by writing the patient's name on the side of the cork with a waterproof marker. Punch a hole in an edge of the cork and run a piece of thread through it.

3. Mount the oriented muscle tissue on the cork with gum tragacanth **[i13.4]**. The tragacanth will keep the muscle standing up in the correct orientation. Do not allow the tragacanth to cover the entire section. Use only enough to orient the biopsy. The gum tragacanth is prepared as follows:

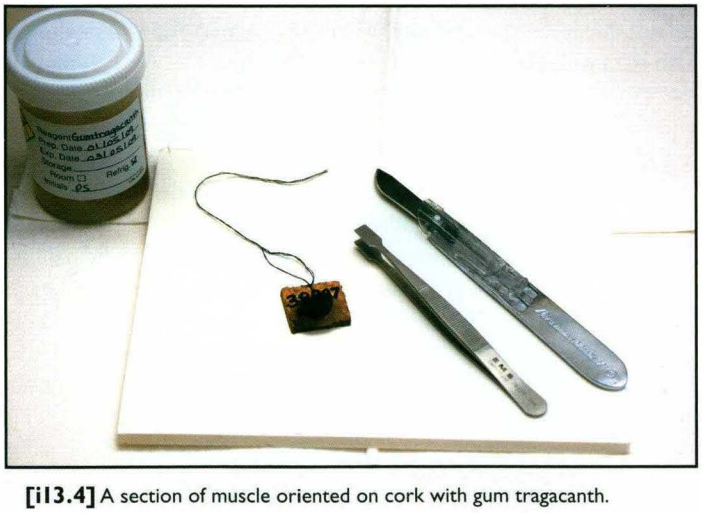
Gum tragacanth	1.5 g
Glycerine	0.6 mL
Distilled water	15 mL
Thymol or phenol	1 crystal

Make at least 2 days before use. Mix all ingredients well with a glass or wooden rod. Let stand overnight and mix 3 or 4 times during the next day. Improper mixing causes softening on cutting and makes sectioning impossible. Store in the refrigerator.

4. Suspend a steel beaker of isopentane (2-methylbutane) in a Dewar flask containing liquid nitrogen **[i13.5]**. Allow the temperature of the isopentane to reach –150°C. *(Measure temperature by thermometer. Do not guess* **[i13.6]***).* This is the most important step in preventing ice-crystal artifact (holes) in the tissue.

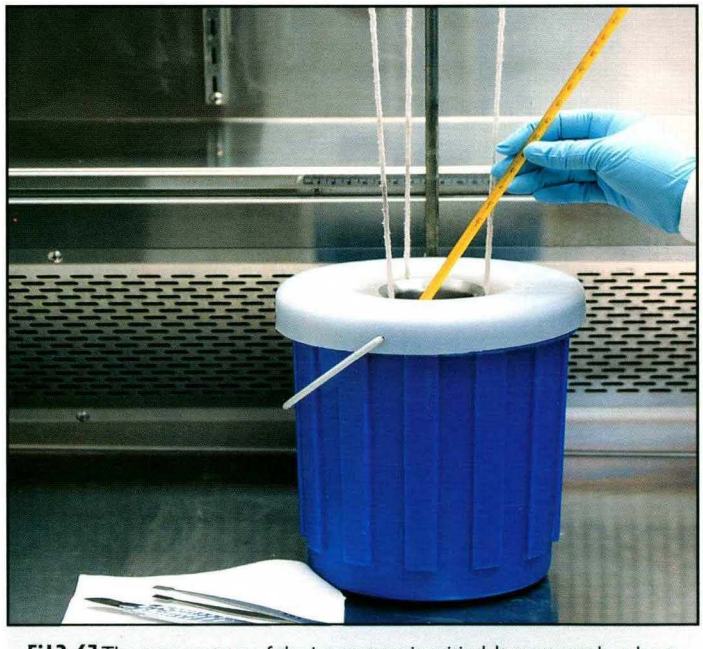
5. When the temperature reaches –150°C, place the cork containing the muscle biopsy in the isopentane. Allow to remain for about 15 seconds **[i13.7]**, then put the specimen directly into liquid nitrogen.

6. The specimen may be left in liquid nitrogen until ready to cut **[i13.8]**. The thread placed in the cork may be taped on the outside of the nitrogen container. Move the specimen to the cryostat, mount the cork on a disk with a freezing medium or water, and allow a 10-minute warm-up for the tissue to reach

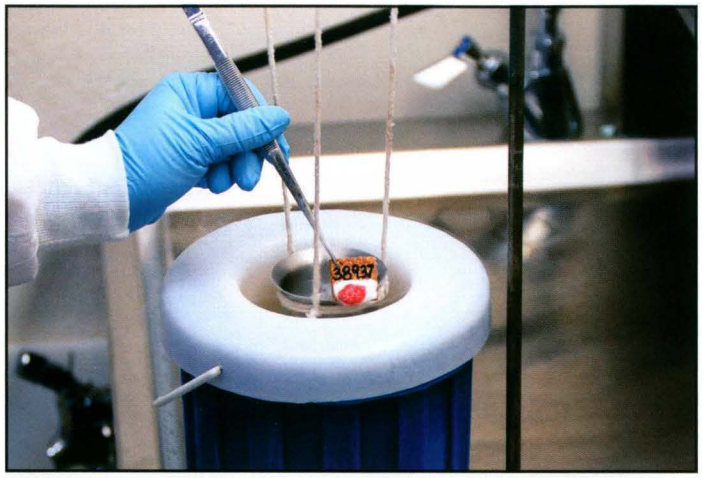


[i13.4] A section of muscle oriented on cork with gum tragacanth.

[i13.5] The equipment for freezing a specimen is shown. The small beaker contains isopentane; it will be lowered into the container of liquid nitrogen.



[i13.6] The temperature of the isopentane is critical. It must reach at least –150°C before the muscle is placed in the syrupy liquid for freezing.



[i13.7] After the temperature of the isopentane reaches –150°C, the cork containing the muscle biopsy should be submerged in the isopentane for 12 to 15 seconds. The biopsy specimen can then be placed directly into liquid nitrogen, and allowed to remain there until ready to section, or mounted on a metal cryostat, and placed in the cryostat.

[i13.8] The beaker of isopentane can be suspended in a Union Carbide liquid nitrogen container. If the string is threaded through a hole made in the cork before freezing, the frozen specimen can be left suspended in liquid nitrogen until ready to section, or after sectioning until ready for long-term storage. If necessary, the string can be taped to the outside of the container for easy retrieval.

[i13.9] After freezing, the cork containing the muscle biopsy specimen is attached to a metal cryostat disk. After allowing time for the specimen to reach cryostat temperature and the isopentane to evaporate, sectioning can be performed.

cryostat temperature (–20°C) **[i13.9]**. Failure to allow time for the tissue to reach –20°C will yield poor sections; the tissue will be too cold, and therefore too hard to section. Also time must be allowed for excess isopentane to evaporate from the tissue.

7. After sectioning, remove the cork from the disk, wrap in plastic wrap and then in foil, identify with labeled tape, and store at –70°C. Work quickly (you might precool the plastic wrap and foil) and place the wrapped specimen in liquid nitrogen to transport to the freezer if it is any distance from the work area.

8. Sections should be collected in a slide box and stained as soon as possible. Sections may be kept in the freezer for several days, provided they are kept in a closed container. A slight reduction in some enzyme activity may be noted with storage.

α-NAPHTHYL ACETATE ESTERASE STAIN FOR MUSCLE BIOPSIES [BARKA 1965, CARSON 1983]

■ *Purpose*

This nonspecific esterase stain is most useful for differentiating between type II atrophy and neurogenic atrophy, because denervated muscle fibers stain dark and type II atrophic muscle fibers do not. Motor end-plates and lysosomes in inflammatory cells are also demonstrated.

■ *Principle*

Esterases are enzymes that are capable of hydrolyzing aliphatic and aromatic ester bonds. Depending on their preference for substrates, these esterases have been classified as specific or nonspecific. Histiocytes will stain with this method, as will accumulations of acetylcholinesterase in denervated fibers. The hydrolyzed alcoholic residue will couple with the hexazotized pararosaniline solution to give an insoluble brightly colored azo-dye as the reaction product at the enzyme site.

pararosaniline
(basic fuchsin) + sodium nitrate → hexazotized pararosaniline

α-naphthyl acetate + water $\xrightarrow{\text{esterases}}$ α-naphthol + acetic acid

α-naphthol + hexazotized pararosaniline → azo dye ↓

■ *Fixative*
None

■ *Technique*
Frozen sections of unfixed tissue cut at 10 μm and mounted on slides (at the bottom).

■ *Quality Control*
A section of skeletal muscle containing inflammatory cells or motor end-plates should be used.

■ *Reagents*

0.2N Phosphate Buffer, pH 7.2

Sodium phosphate, dibasic, anhydrous	11.36 g
Potassium phosphate, monobasic	2.70 g
Distilled water	Dilute to 500 mL
Check the pH, and adjust with 1N sodium hydroxide or 1N hydrochloric acid (HCl) if necessary. Store in refrigerator.	

Pararosaniline Stock Solution

Pararosaniline, acridine free	1 g
Distilled water	20 mL
Concentrated HCl	5 mL
Warm solution gently, cool to room temperature, filter, and store in the refrigerator. The solution is stable for weeks.	

Sodium Nitrite, 4% Solution

Sodium nitrite	0.4 g
Distilled water	10 mL
Make fresh every week, store in refrigerator	

α-Naphthyl Acetate in Acetone, 1% Solution

α-Naphthyl acetate	1 g
Acetone	100 mL
Store in refrigerator	

1N HCl

HCl, concentrated	37 mL
Distilled water	963 mL
Very slowly add the acid to the water. Store at room temperature.	

1N Sodium Hydroxide

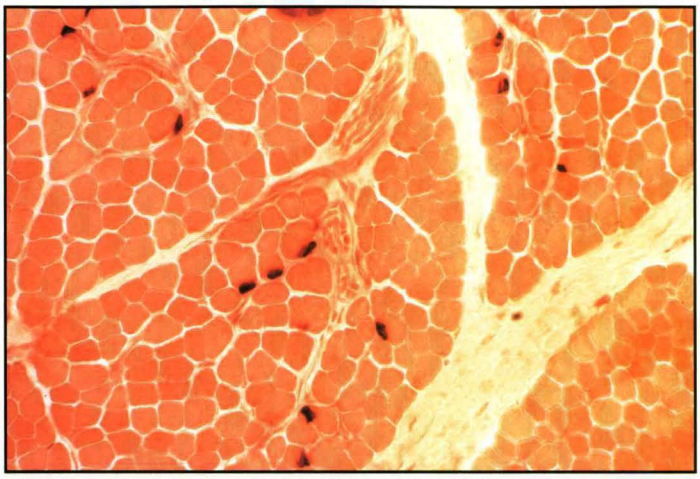
Sodium hydroxide	4 g
Distilled water:	Dilute to 100 mL
Store at room temperature	

Incubation Solution (prepare just before use)

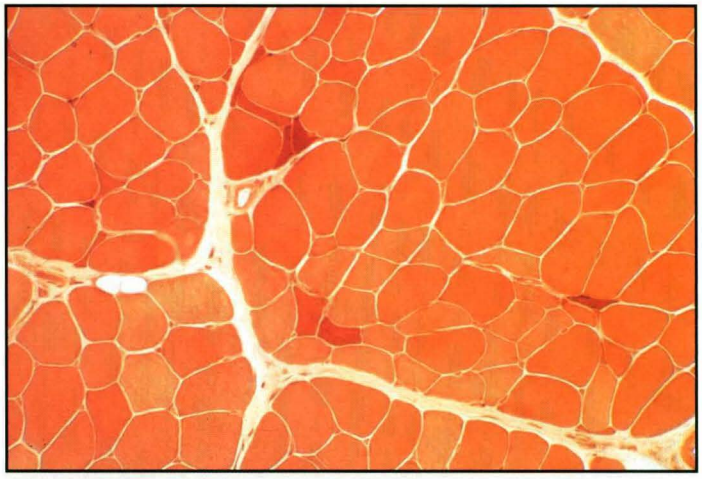
Add in the following order, mixing after each addition:	
Pararosaniline stock solution	0.8 mL
Sodium nitrite, 4% solution	0.8 mL
Allow to stand for 2 to 3 minutes and then add:	
0.2M Phosphate buffer, pH 7.4	20 mL
α-naphthyl acetate, 1% solution	0.5 mL
Adjust the pH of the solution to between 6.8 and 7.2 with either 1N NaOH or 1N HCl, whichever is indicated. Filter	

■ *Procedure*

1. Incubate sections in the incubation solution for 1 to 2 hours at 37°C in a water bath or incubator oven.
2. Wash sections in running water for 1 hour.
3. Dehydrate sections through graded alcohols, clear in xylene, and mount with a synthetic resin.



[i13.10] A section of muscle stained with the α-naphthyl acetate esterase technique for nonspecific esterase. The very dark red-brown areas are motor endplates.



[i13.11] A section of skeletal muscle stained with the α-naphthyl esterase technique. The dark staining of the small angular fibers indicates that these fibers have been denervated and that a neuropathic disease process has occurred.

■ *Results* [i13.10], [i13.11]

• Motor endplates:	Brick red due to staining of acetylcholine esterase
• Normal muscle fibers	Very pale yellow
• Denervated muscle fibers	Dark red-brown
• Macrophages and lysosomes	Dark red-brown

■ *Technical Notes*

1. The incubation medium will turn very cloudy during the incubation and may cause a slight precipitate to appear on the tissue sections if they are not washed very well after the incubation.

2. Type I fibers stain slightly darker than type II fibers, but this reaction cannot be used for fiber typing.

NAPHTHOL AS-D CHLOROACETATE ESTERASE TECHNIQUE [LEDER 1961, CARSON 1983]

■ *Purpose*

To identify granulocytes in the classification of leukemias or in chloromas, because esterase is found in the lysosomes of some granulocytes.

■ *Principle*

Esterases are enzymes that are capable of hydrolyzing aliphatic and aromatic ester bonds. Depending on their preference for substrates, these esterases have been classified as specific or nonspecific esterases. This is a specific esterase stain and is unique in that it is 1 of the rare enzyme techniques that can be performed on paraffin-embedded tissue. As in the α-naphthyl acetate esterase technique, the hydrolyzed alcoholic residue couples with hexazotized pararosaniline to give a brightly colored azo-dye reaction produce at the site of enzyme activity.

■ *Fixative*

Smears: 2 minutes in absolute methanol/formaldehyde:

Absolute methanol	40 mL
Formaldehyde, 37%	5 mL
Wash well with distilled water	

Tissues: Any well-fixed tissue except bone (only ethylene-diamine tetraacetic acid [EDTA]–decalcified bone can be used). Pick paraffin sections up on the bottom of the slide and air-dry overnight.

■ *Equipment*

pH meter, Coplin jars, small beakers, filter paper, glass pipettes

■ *Quality Control*

Most aspirate or buffy coat smears will have an internal control, or normal aspirate smears can be obtained from hematology. Spleen is used as a control for paraffin sections.

■ *Reagents*

Esterase Solution A

HCl, concentrated	1.6 mL
Distilled water	8.4 mL
ρ-rosaniline, acridinfrei	0.4 g
Store in the refrigerator	

Esterase Solution B

Sodium nitrate	0.4 g
Distilled water	10 mL
Make fresh every week, and store in the refrigerator	

Esterase Solution C

0.1N HCl	14.6 mL
0.1M Sodium barbital	15.4 mL
Make fresh	

0.1N HCl

HCl, concentrated	4.18 mL
Distilled water	495.82 mL
Store at room temperature	

0.1M Sodium Barbital (Sodium Diethylbarbiturate)

Sodium barbital	2.06 g
Distilled water	Dilute to 100 mL
Refrigerate	

Working Esterase Solution

Prepare immediately before use, using clean, dry glassware:

Part I:

Esterase solution A	1 drop
Esterase solution B	1 drop
Mix in a 50-mL beaker and let stand 1 minute, add:	
Esterase solution C	30 mL

Mix well. Using the pH meter, adjust the pH to 6.3 by adding drops of 0.1N HCl if above 6.3, or by adding drops of 0.1M sodium barbital if below 6.3. The solution should be colorless and clear.

Part II: *(Use only glass beakers and pipettes for this solution)*

Naphthol AS-D chloroacetate	10 mg (0.01 g)
N, N-dimethylformamide (Sigma)	1 mL

Dissolve in a 50-mL beaker. Add part I to part II, and mix well. The solution should be faint pink and cloudy. Filter into a Coplin jar.

Mayer Hematoxylin

See chapter 6, p111

■ *Procedure*

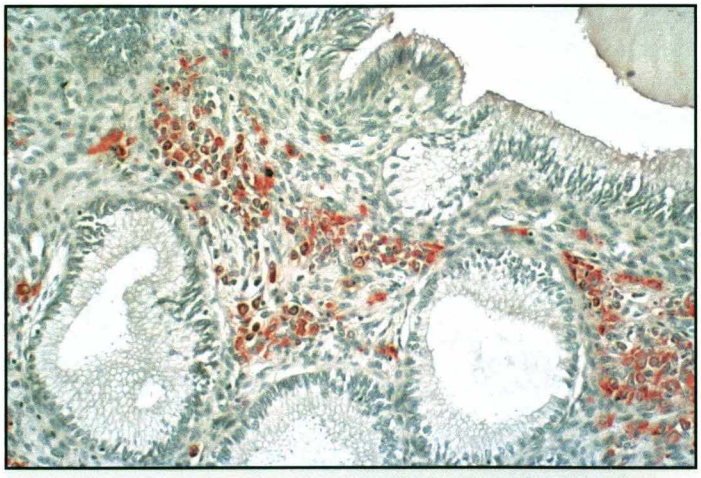
1. Deparaffinize and hydrate sections.
2. Stain in freshly prepared and filtered working esterase solution for 30 minutes.
3. If, after 30 minutes, the reaction is not complete, refilter the solution and stain the slides for 30 minutes more.
4. Wash the slides in running water for 5 minutes followed by distilled water.
5. Counterstain for 5 minutes in Mayer hematoxylin.
6. Wash in running water for 5 minutes to blue the nuclei.
7. Rinse in distilled water.
8. Blot sections completely dry. Let air-dry for several minutes.
9. Dip in xylene and mount with synthetic resin.

■ *Results* **[i13.12]**

• Positive cells (granulocytes and mast cells)	Bright red
• Nuclei	Blue
• Background	Unstained

■ *Technical Notes*

1. If the air-dried slides are not placed in xylene before mounting, a drying artifact will be created in the sections. Refractile areas will be seen in the nuclei, and a brown, granular, pigmentlike artifact may be seen scattered throughout the section.



[i13.12] A paraffin section of cervix stained with the naphthol AS-D chloroacetate esterase technique shows a marked infiltrate of myelogenous leukemic cells (red). This is a specific esterase stain and is a rare enzyme technique that works well on paraffin-embedded sections.

2. *N, N*-dimethylformamide reacts with plastic, so only glass pipettes and beakers should be used in this procedure.

3. Sodium barbital is a narcotic. It may be harmful if swallowed, inhaled, or comes in contact with the skin. It can be addictive, and may cause sensitization by inhalation and skin contact. Thorough washing is indicated after handling, and it should be used with adequate ventilation. Wear protective eyeglasses or chemical safety goggles, gloves, and appropriate protective clothing to prevent skin exposure. N,N-dimethylformamide is harmful if swallowed, inhaled or absorbed through skin. It has a severe contact rating, and a threshold limit value of 10 ppm (time-weighted average [TWA]). Protective clothing also should be worn when handling this chemical.

ATPASE STAIN [DUBOWITZ 1985, CARSON 1983]

■ *Purpose*
The ATPase stain, with preincubation at various pHs, will differentiate type I, type IIA, and type IIB fibers and help in separating myopathic from neuropathic processes.

■ *Principle*
Muscle ATPase is dependent on the influence of pH, and this pH effect allows subtyping of the fibers. The method involves a Gomori-type metal precipitation of the hydrolyzed orthophosphoric acid with calcium. A complex series of reactions eventually leads to the formation of a colored metallic sulfide:

$$ATP + H_2O \xrightarrow{ATPase} ADP + H_3PO_4 + energy$$

H_3PO_4 (orthophosphate)

Because of the presence of calcium ions in the incubating solution, the phosphate immediately combines to form calcium phosphate. At an alkaline pH, calcium phosphate is insoluble and is therefore deposited at the site of enzyme activity:

$$H_3PO_4 + CaCl_2 \longleftrightarrow CaHPO_4\downarrow + 2H^+ + 2Cl^-$$

Calcium phosphate is not a colored compound and is not visible with the light microscope, so the calcium is exchanged for cobalt. Cobalt phosphate is still not visible, so the phosphate is exchanged for sulfide with the resultant formation of black, insoluble cobaltous sulfide.

$$CaHPO_4 + CoCl_2 \longleftrightarrow CoHPO_4 + CaCl_2$$

$$CoHPO_4 + (NH_4)_2S \longleftrightarrow CoS + (NH_4)_2HPO_4$$

■ *Fixative*
None

■ *Equipment*
Liquid nitrogen, isopentane, cryostat, Dewar flask, pH meter, Coplin jars, pipettes

■ *Technique*
Frozen sections of unfixed tissue cut at 10 µm and mounted on slides (at the bottom). Cut 3 slides, 1 labeled 4.3, the second 4.6, and the third 9.4.

■ *Quality Control*
The sections of skeletal muscle have an internal control. No other control is necessary.

■ *Reagents*

Barbital Acetate Buffer Stock Solution A

Sodium acetate·$3H_2O$	1.94 g
Sodium barbital (sodium barbital is a narcotic and must be handled as such)	2.94
Distilled water	100 mL
Store in refrigerator	

Barbital Acetate Buffer Stock Solution B (0.1N HCl)

HCl	8.3 mL
Distilled water	991.7 mL
Store in refrigerator	

Barbital Acetate Buffer Working Solution

Just before use, mix:	
Solution A (handle as narcotic)	10 mL
Solution B	20 mL
Distilled water	16 mL
Adjust the pH to 4.6 with 1N NaOH or 0.1N HCl. Pour half of the solution into a Coplin jar labeled 4.6. Adjust the remaining half of the solution to 4.3 and pour into a jar labeled 4.3.	

Sodium Barbital Solution (use to make 10.4, 9.4, and incubation solutions)

Sodium barbital, 0.1M (2.06 g/100) (handle as narcotic)	30 mL
Calcium chloride, 0.18M (13.23 g/500)	30 mL
Distilled water	90 mL
Prepare fresh and save 75 mL for incubating solution	

Incubating Solution

Sodium barbital solutions (handle as narcotic)	75 mL
Adenosine-5-triphosphate	100 mg

Calcium chloride, 1%

Calcium chloride	5 g
Distilled water	500 mL
Store in the refrigerator	

Cobaltous Chloride, 2%

Cobalt chloride	5 g
Distilled water	250 mL
Store in the refrigerator	

Ammonium Sulfide Solution

Ammonium sulfide (light)	15 drops
Distilled water	25 mL
Make fresh each time. Prepare and use under a hood.	

Procedure

1. Prepare solutions:

a. Prepare working barbital acetate buffer solution at pH 4.6 and 4.3 (acid preincubation).

b. Make sodium barbital solution.

c. Adjust pH of approximately 20 mL of the sodium barbital solution to pH 10.4 (alkaline preincubation).

d. Adjust pH of approximately 55 mL of sodium barbital solution to pH 9.4

e. Prepare incubation medium. Separate into 2 jars.

2. Start slides pH 4.6 and 4.3 (solution 1a). Leave in solution 5 minutes.

3. Transfer to a jar of solution 1d (pH 9.4). Use separate jars. Leave slide for 3 minutes.

4. Place above slides together in the first jar of incubating medium. Incubate at 37°C for 30 minutes.

5. 2 minutes into the incubation period, start the 9.4 slides in solution 1c (pH 10.4). Leave for 14 minutes.

6. Place the pH 9.4 slides in the second jar of incubating medium at 37°C for 14 minutes.

7. Combine all slides and rinse in three 30-second changes of 1% calcium chloride.

8. Place in cobaltous chloride for 3 minutes.

9. Wash slides well in distilled water, 2 changes of 1 minute each.

10. Transfer to ammonium sulfide solution for 20 to 30 seconds. This step should be done under a hood.

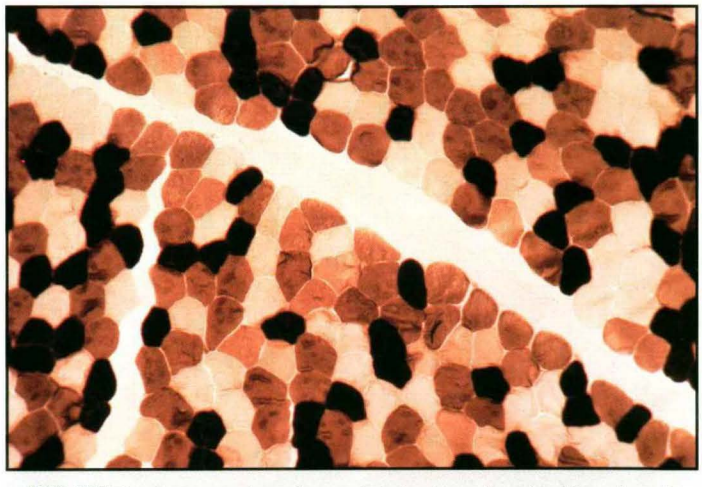
11. Wash in running water for 5 minutes. Dehydrate, clear, and mount with synthetic resin.

■ *Results* **[i13.13], [i13.14], [i13.15], [i13.16]**

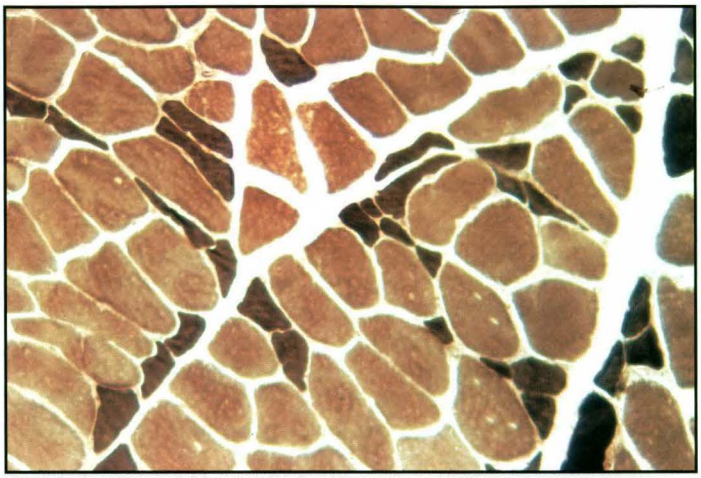
- pH 9.4 slide — Type II fibers are dark and type I fibers are light to unstained.
- pH 4.3 slide — Type I fibers are dark and type II fibers are light to unstained.
- pH 4.6 slide — Type I fibers are dark, type IIA fibers are light, and type IIB fibers are intermediate.

■ *Technical Notes*

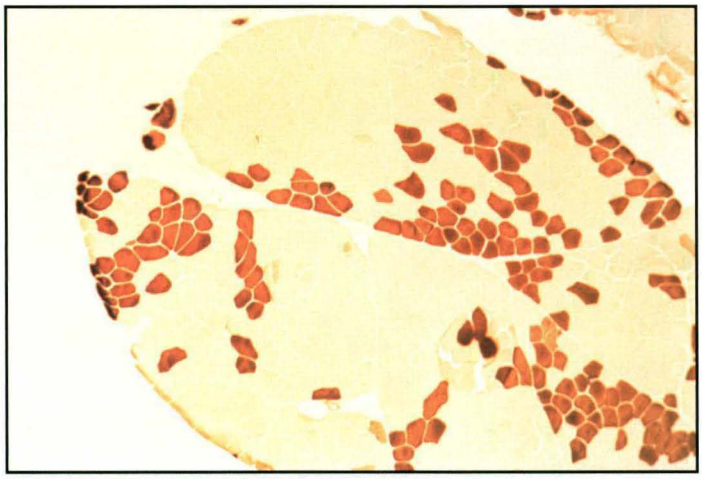
1. Depending on particular buffers, pH meters, and electrodes, the reaction is sometimes better if the pHs of the barbital acetate buffers (acid preincubation) are lowered to pH 4.1 to 4.2 and pH 4.4 to 4.5. If problems in differentiation occur, different pHs should be tried.



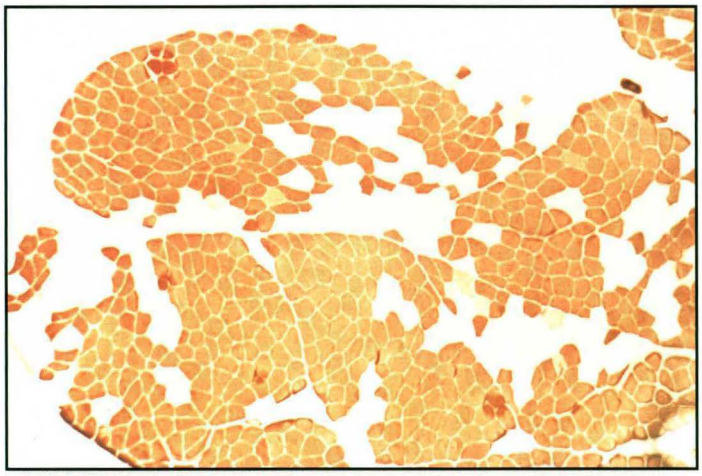
[i13.13] This frozen section of normal skeletal muscle stained for adenosine triphosphatase (ATPase) activity at pH 4.6 shows 3 distinct types of muscle fibers. Type I fibers are very dark, type IIA fibers are very light, and type IIB fibers are intermediate. Normal muscle contains approximately one-third of each fiber type distributed in a checkerboard pattern.



[i13.14] A frozen section of muscle stained for adenosine triphosphatase activity at pH 9.4. At this pH, type I fibers will be light and type II fibers will be dark. Note that the atrophic (small angular) fibers are scattered throughout the section and are all type II fibers. This is type II atrophy, a nonspecific finding.



[i13.15] The adenosine triphosphatase reaction on this section of muscle was done at pH 9.4, and the grouping (large type grouping) of the dark (type II) fibers and light (type I) fibers is diagnostic of neuropathy.



[i13.16] The adenosine triphosphatase reaction on a section of muscle adjacent to that seen in **[i13.15]** was done at pH 4.3. The results are the reverse of those seen in **[i13.15]**; the dark fibers are type I and the light fibers are type II.

1. This is the preferred stain for fiber typing. In normal muscle, approximately a third each of types I, type IIA, and type IIB fibers are present.

2. This stain will fade with time.

3. Rarely, type IIC fibers can be seen on the pH 4.3 slide stained intermediate between the dark type I and the light type II fibers.

4. ATPase is found in the mitochondria in all cells or attached to myosin in the muscle. ATPase in mitochondria is activated by magnesium and is inhibited by calcium. Muscle ATPase, on the other hand, is activated by calcium and inhibited by magnesium. The calcium used in the incubating solution therefore activates the muscle ATPase while inhibiting the mitochondrial ATPase.

5. Two very hazardous chemicals are used in this procedure. Sodium barbital is a narcotic. It may be harmful if swallowed, inhaled, or comes in contact with the skin. It can be addictive, and may cause sensitization by inhalation and skin contact. Thorough washing is indicated after handling, and it should be used with adequate ventilation. Wear protective eyeglasses or chemical safety goggles, gloves, and appropriate protective clothing to prevent skin exposure. Ammonium sulfide may be fatal if swallowed or inhaled. It is harmful if absorbed through the skin and will cause burns to any area of contact. Poisonous and flammable hydrogen sulfide gas may be liberated. Concentrations above 50 ppm may cause headache, insomnia, nausea, sore throat, dizziness, and pulmonary edema; above 600 ppm for 30 minutes can cause death.

ACID PHOSPHATASE IN MUSCLE BIOPSIES [BANCROFT 1975, CARSON 1983]

■ *Purpose*

The acid phosphatase stain indicates the presence of inflammatory cells in the biopsy; acid phosphatase is considered a marker enzyme for lysosomes. Muscle fibers in acid maltase deficiency also show an increase in acid phosphatase.

■ *Principle*

Acid phosphatases are phosphomonoesterases that hydrolyze esters of orthophosphoric acid at an acid pH, varying from 4.5 to 6.0. After enzymatic hydrolysis, the alcohol residue of the naphthol AS-BI phosphate is reacted with hexazotized pararosaniline to produce a highly colored insoluble azo dye. This involves the simultaneous coupling method in which the diazonium compound is incorporated into the incubating medium along with the substrate.

$$\text{napthol-AS-BI-phosphate} + \text{water} \xrightarrow{\text{acid phosphatase}} \text{napthol-AS-BI} + \text{orthophosphate}$$

$$\text{naphthol-AS-BI} + \text{hexazotized pararosaniline} \rightarrow \text{red azo dye} \downarrow$$

■ *Fixative*
None

■ *Equipment*
Liquid nitrogen, isopentane, cryostat, Dewar flask, pH meter, Coplin jars, glass pipettes, small glass beakers

■ *Technique*
Frozen sections of unfixed tissue cut at 10 µm and mounted on slides (at the bottom)

■ *Quality Control*
The sections of skeletal muscle have an internal control, because neutrophils and histiocytes will stain positive. No other control is necessary.

■ *Reagents*

2N HCl

HCl, concentrated	8.3 mL
Distilled water	50 mL
Store at room temperature	

Pararosaniline Hydrochloride

Pararosaniline hydrochloride	1 g
2N HCl	25 mL
Heat gently, cool to room temperature, and filter. Store in the refrigerator.	

Sodium Nitrite, 4% Solution

Sodium nitrite	2 g
Distilled water	50 mL
Make fresh every week	

Veronal Acetate Buffer

Sodium acetate·3H_2O	9.71 g
Sodium barbiturate (must be handled as a narcotic)	14.71 g
Distilled water	500 mL
Store in refrigerator	

Incubating Medium

Just before use, mix:	
Pararosaniline hydrochloride	0.8 mL
Sodium nitrite, 4%	0.8 mL
Mix and let stand for a minimum of 2 minutes, add substrate solution	

Substrate solution (do not use any plastic)

Naphthol AS-BI phosphoric acid	10 mg (0.01 g)
N, N-Dimethylformamide	1 mL
Veronal acetate buffer	5 mL
Distilled water	13 mL
Mix well, check pH (4.7-5.9), adjust with 0.1N NaOH if necessary and filter.	

Methyl Green

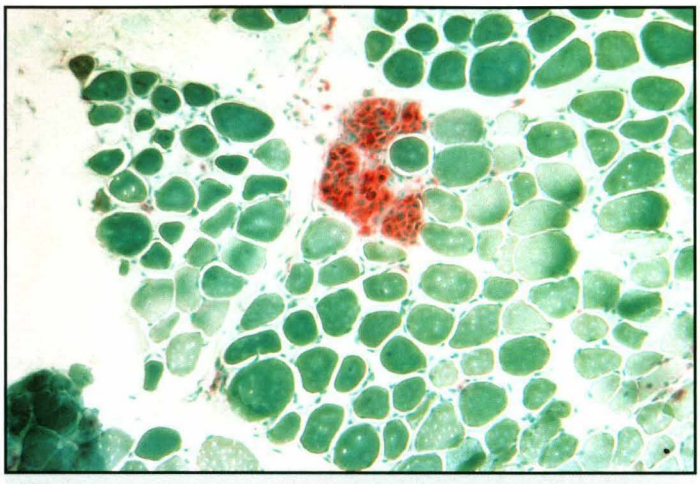
Methyl green	2 g
Veronal acetate buffer (handle as narcotic)	100 mL
Bring pH down to 5.0 with HCl, then lower to 4.0 with acetic acid. Filter before use.	

■ *Procedure*

1. Incubate sections in the incubating mediums for 60 minutes at 37°C.
2. Rinse very well in distilled water.
3. Place slides in 2% methyl green for 2 minutes.
4. Dehydrate very rapidly in 95% and 100% alcohol and clear in xylene.
5. Mount with synthetic resin.

■ *Results* **[i13.17]**

- Sites of acid phosphatase activity — Red
- Background — Green



[i13.17] An acid phosphatase reaction is seen on a frozen section of muscle showing a necrotizing or inflammatory myopathy.

■ *Technical Notes*

1. Acid maltase (type II glycogenosis) deficiency will give a positive reaction. This is also known as Pompe's disease and is a lysosomal storage disease.

2. If the dehydration step is not done rapidly, too much methyl green will be removed from the section.

3. *N, N*-dimethylformamide reacts with plastic, so only glass pipettes and beakers should be used in this procedure.

4. Sodium barbital is a narcotic. It may be harmful if swallowed, inhaled, or comes in contact with the skin. It can be addictive, and may cause sensitization by inhalation and skin contact. Thorough washing is indicated after handling, and it should be used with adequate ventilation. Wear protective eyeglasses or chemical safety goggles, gloves, and appropriate protective clothing to prevent skin exposure. *N,N*-dimethylformamide is harmful if swallowed, inhaled, or absorbed through skin. It has a severe contact rating, and a threshold limit value of 10 ppm (TWA). Protective clothing also should be worn when handling this chemical.

ALKALINE PHOSPHATASE STAIN FOR MUSCLE BIOPSIES [BURSTONE 1962, CARSON 1983]

■ *Purpose*

To detect regenerating muscle fibers

■ *Principle*

Alkaline phosphatases hydrolyze esters of orthophosphoric acid at an alkaline pH, approximately 8.6 to 8.8. The alcohol residue of the naphthol AS-BI phosphate, after enzymatic hydrolysis, is reacted with fast red violet to produce a highly colored insoluble azo dye. This involves the simultaneous coupling method in which the diazonium compound is incorporated into the incubating medium along with the substrate.

$$\text{naphthol-AS-BI-phosphate + water} \xrightarrow{\text{alkaline phosphatase}} \text{napthol-derivative + orthophosphate}$$

$$\text{naphthol derivative + fast red violet} \rightarrow \text{red azo dye} \downarrow$$

■ *Fixative*

None

■ *Equipment*

Liquid nitrogen, isopentane, cryostat, Dewar flask, pH meter, Coplin jars, glass pipettes, and small glass beakers

■ *Technique*

Frozen sections of unfixed tissue cut at 10 μm and mounted on slides (at the bottom)

■ *Quality Control*

The sections of skeletal muscle have an internal control, because small amounts of alkaline phosphatase are present in blood vessel walls. No other control is necessary.

■ *Reagents*

0.2M Tris

TRIS (Trizma base)	24.2 g
Distilled water	1,000 mL
Mix well; store in refrigerator (4°C)	

0.1M HCl

HCl	3.7 mL
Distilled water	996.3 mL
Slowly add the HCl to the water, and mix well; store at room temperature	

Tris buffer, pH 8.74

0.2M Tris	10 mL
0.1M HCl	7 mL
Distilled water	26 mL
Make fresh just before use each time. Adjust the pH to 7.4 if necessary by using either the 0.2M Tris or the 0.1M HCl. Discard any unused buffer (approximately 18 mL).	

Incubating Medium

Naphthol AS-BI phosphate	5 mg (0.005 g)
N, N-Dimethylformamide	0.1 mL
Dissolve, and then add:	
Tris buffer	25 mL
Distilled water	25 mL
Fast red violet	30 mg (0.03 g)
Mix well, and filter into a Coplin jar	

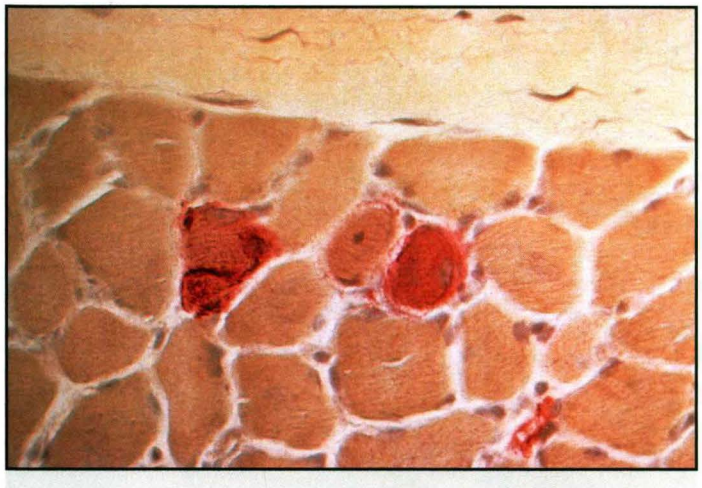
Mayer or Harris Hematoxylin (see chapter 6, pp110-111)

■ *Procedure*

1. Immerse frozen sections in the incubating medium and incubate at room temperature for 30 to 60 minutes.
2. Remove slides and rinse well with distilled water.
3. Counterstain for 30 seconds to 1 minute in hematoxylin (optional step).
4. If counterstained, wash sections well with several changes of distilled water.
5. Mount sections with an aqueous mounting medium.

■ *Results* [i13.18]

- Sites of enzyme activity — Pink-red to red
- Nuclei — Blue



[i13.18] An alkaline phosphatase reaction on a frozen section of skeletal muscle shows several degenerating muscle fibers. [Image courtesy of Wenk P, William Beaumont Hospital]

■ *Technical Notes*

1. With alkaline phosphatase, staining of the perifascicular and endomysial connective tissueis abnormal in patients with inflammatory myopathy. According to Armbrustmacher, this abnormal staining may be the most important histologic clue to the diagnosis of this disorder in the absence of inflammation. Regenerating muscle fibers are selectively positive with alkaline phosphatase techniques.

NADH DIAPHORASE [FARBER 1956, CARSON 1983]

■ *Purpose*

The NADH technique demonstrates abnormalities in mitochondria, Z-band material, and sarcoplasmic reticulum.

■ *Principle*

In the histochemical demonstration of diaphorase and NAD-dependent dehydrogenase, a tetrazolium salt acts as hydrogen acceptor. NADH cannot reduce the tetrazolium salt directly; the reduction is attributed to the flavoprotein enzymes and therefore is called a "diaphorase" (to carry) reaction.

$$\text{NADH (reduced)} \xrightarrow{\text{diaphorase}} \text{NAD+ (oxidized)} + \text{hydrogen}$$

$$\text{Nitro blue tetrazolium (NBT)} + \text{hydrogen} \rightarrow \text{formazan} \downarrow$$

■ *Fixative*

None

■ *Equipment*

Liquid nitrogen, isopentane, cryostat, Dewar flask, pH meter, Coplin jars, pipettes

■ *Technique*

Air-dried frozen section cut at 10 µm and mounted on slides (at the bottom)

■ *Quality Control*

The sections of skeletal muscle have an internal control. No other control is necessary.

■ *Reagents*

Saline Solution

Sodium chloride	0.85 g
Distilled water	100 mL
Store in refrigerator	

Phosphate Buffer, pH 7.4

Sodium phosphate, monobasic (0.69 g/50 mL, distilled water)	19.5 mL
Sodium phosphate, dibasic (1.41 g/100 mL distilled water)	80.8 mL
Store in refrigerator	

NBT Solution

NBT	100 mg (0.1 g)
Distilled water	50 mL
Make up only 50 mL at a time. Store in the refrigerator.	

Incubating Medium

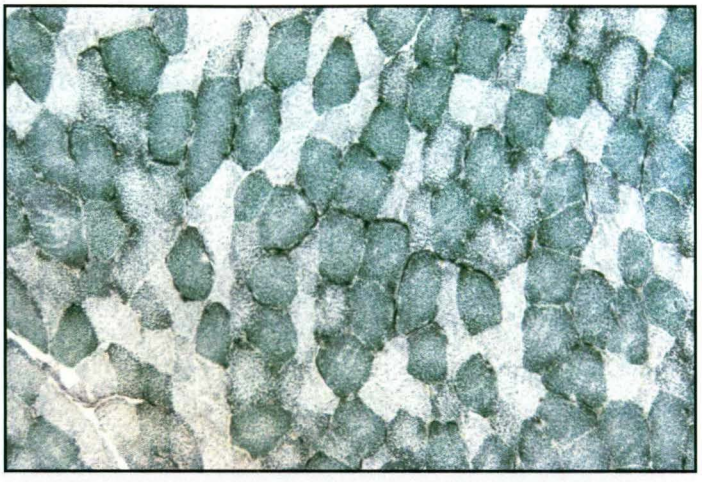
Just before use, mix:	
NADH (β-Nicotinamide adenine dinucleotide, reduced form, disodium salt, grade III, Sigma No. 340-110, 10 mg/vial)	1 vial
Distilled water	4 mL
Saline solution	2 mL
Phosphate buffer	2 mL
NBT solution	10 mL

■ *Procedure*

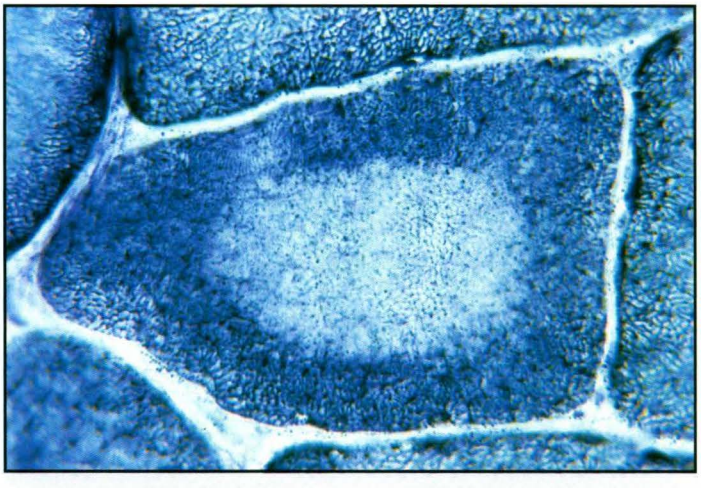
1. Incubate sections in incubating medium at room temperature for 30 minutes.
2. Rinse slides well in distilled water.
3. Mount with Immu-Mount or glycerine jelly.

■ *Results* **[i13.19], [i13.20], [i13.21]**

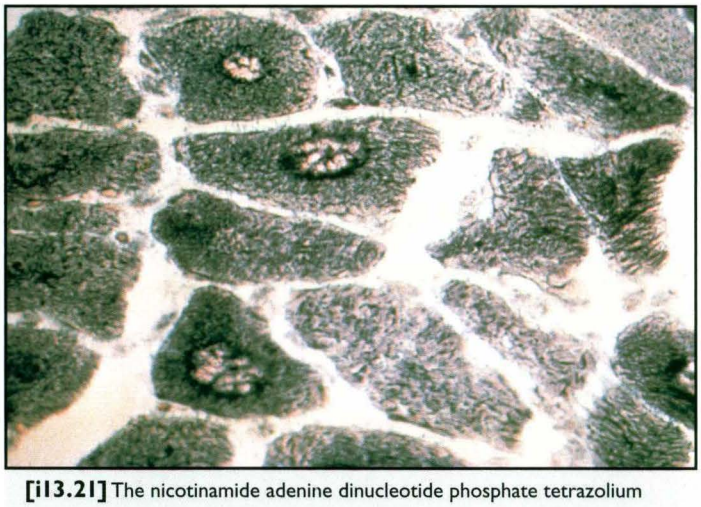
- Site of enzyme activity — Dark purple deposits (Z-band material, sarcoplasmic reticulum, and mitochondria all react strongly)
- Type I muscle fibers — Dark purple
- Type II muscle fibers — Light



[i13.19] The nicotinamide adenine dinucleotide phosphate tetrazolium reductase reaction on a frozen section of normal skeletal muscle shows a checkerboard pattern of type I fibers (dark) and type II fibers (light).



[i13.20] This muscle fiber, stained for nicotinamide adenine dinucleotide phosphate tetrazolium reductase activity, shows the central area to be devoid of mitochondria and oxidative enzyme activity, in marked contrast to the normal peripheral zone. The presence of many of these central cores is usually diagnostic of central core disease, but sporadic cores in occasional fibers are difficult to assess.



[i13.21] The nicotinamide adenine dinucleotide phosphate tetrazolium reductase stain on this frozen section of muscle reveals many target fibers. These are diagnostic of a neuropathic disease process. [Image courtesy of Wenk P, William Beaumont Hospital]

■ *Technical Notes*

1. According to Pearse [1972], if the sections are dehydrated in alcohols, cleared in xylene, and mounted in a synthetic medium, aggregates and crystals of the diformazan or of a diformazan-protein complex are commonly observed. These, unfortunately, bear a chose resemblance in size and shape to mitochondria but can be produced in model systems in which mitochondria are not present.

2. The pH of the reaction is critical; and must be kept close to neutral, or according to Troyer:

   a. Mitochondria may swell and enzyme solubility may be affected.

   b. NAD^+ may become unstable.

   c. At a higher pH (above 8.0), reduced coenzymes may be able to reduce the tetrazolium salts directly and form a precipitate. Troyer calls this "nothing dehydrogenase." The phenomenon increases progressively with an increase in pH, and it may be a major source of error if the pH is too high.

3. Architectural changes in muscle are indicated with this procedure. These changes include central cores, target fibers, nemaline rods, and targetoid fibers.

SUCCINIC DEHYDROGENASE (SDH) [NACHLAS 1957, CARSON 1983]

■ *Purpose*

To further identify the source of NADH diaphorase activity, because only mitochondria show positive SDH activity.

■ *Principle*

SDH is a mitochondrial enzyme belonging to the oxidase system, which together with the cytochromes, form a chain. This enzyme participates in the Kreb's cycle and is a useful index of the activity of this cycle. SDH is a coenzyme-independent dehydrogenase that catalyzes the reaction:

$$\text{succinate} \xrightarrow{\text{succinic dehydrogenase}} \text{fumarate} + \text{hydrogen}$$

This enzyme is demonstrated by incubating the fresh frozen sections with the succinate substrate in the presence of a tetrazolium compound such as NBT. The hydrogen released from the substrate is transferred to the tetrazolium:

$$\text{NBT} + \text{hydrogen} \rightarrow \text{formazan} \downarrow$$

■ *Fixative*

None

■ *Equipment*

Liquid nitrogen, isopentane, cryostat, Dewar flask, pH meter, Coplin jars, pipettes

■ *Technique*

Frozen sections of unfixed tissue cut at 10 μm and mounted on slides (at the bottom)

■ *Quality Control*

The sections of skeletal muscle have an internal control. No other control is necessary.

■ *Reagents*

Phosphate Buffer, 0.2M, pH 7.6

0.2M sodium phosphate monobasic (2.78 g/100 mL)	13 mL
0.2M sodium phosphate dibasic (5.37 g/100 mL)	87 mL
Check pH and adjust to 7.6 with 1N NaOH or 1N HCl if necessary. Store in the refrigerator.	

Sodium Succinate Solution, 0.2M

Sodium succinate ($NaOCOCH_2CH_2COONa{\cdot}6H_2O$)	2.70 g
Distilled water	50 mL
Prepare fresh	

NBT Solution

NBT	100 mg (0.1 g)
Distilled water	50 mL
Make up only 50 mL at a time. Store in the refrigerator.	

Incubation Medium

Just before use, mix:	
Phosphate buffer, 0.2M	5 mL
Sodium succinate solution	5 mL
NBT solution	5 mL
Distilled water	5 mL

Physiological Saline

Sodium chloride	8.5 g
Distilled water	1,000 mL
Store at room temperature	

10% Formalin-Saline Solution

Physiological saline	45 mL
Formaldehyde, 37% to 40%	5 mL
Prepare fresh each time	

■ *Procedure*

1. Incubate sections for 30 minutes at 37°C in the incubation medium.
2. Rinse sections in physiological saline.
3. Fix sections in 10% formalin-saline solution for 10 minutes.
4. Rinse in 15% alcohol for 5 minutes.
5. Mount with an aqueous mounting medium.

■ *Results* [i13.22]

- Sites of SDH activity — Blue

[i13.22] A frozen section of normal muscle stained with the succinic dehydrogenase (SDH) reaction. Type I fibers are dark and type II fibers are light. Only mitochondria show a positive reaction with this technique.

PHOSPHORYLASE STAIN FOR MUSCLE [ERÄNKÖ 1961, CARSON 1983]

■ *Purpose*

The muscle phosphorylase procedure is useful in the diagnosis of McArdle disease, which has a single enzyme defect.

■ *Principle*

In vitro, muscle phosphorylase produces an unbranched polysaccharide of amylose type from glucose-1-phosphate. The length of the unbranched chains formed is proportional to the amount of phosphorylase activity present and when stained with iodine, will vary in color, depending on the length of the chain. If the branching enzyme present in muscle is allowed to act, it will form 1,6 glucosidic linkages or the branched polysaccharides of glycogen or amylopectin. Alcohol is usually added to the incubation medium to prevent the action of branching enzyme. Glycogen is added to the incubating medium as a primer and insulin is an activator.

■ *Fixative*

None until ready to stain, then cold acetone for 5 minutes

■ *Equipment*

Liquid nitrogen, isopentane, cryostat, Dewar flask, pH meter, Coplin jars

■ *Technique*

Frozen sections of unfixed tissue cut at 10 μm and mounted on slides (at the bottom)

■ *Quality Control*

Sections of skeletal muscle, except in cases of McArdle disease, have an internal control. No other control is usually necessary. In the rare case in which a negative stain is obtained, then a slide from a previous normal muscle case must be used as a control and the test repeated. If McArdle disease is suspected, use a control known to contain phosphorylase activity.

■ *Reagents*

Acetate Buffer, pH 5.9

0.2M acetic acid (1.16 mL/100 mL)	2.5 mL
0.2M sodium acetate·3H_2O (13.6 g/500 mL)	97.5 mL

Incubating Solution

Acetate buffer, pH 5.9	100 mL
α-D-glucose-1-phosphate	1 g
Adenosine-5-phosphate (mono)	100 mg (0.1 g)
Glycogen	20 mg (0.02 g)
Sodium fluoride	1.8 g
Polyvinyl pyrrolidone (PVP)	9 g
Insulin (40 IU/mL)	10 drops

Do not filter immediately, but do filter before using. This solution keeps for several months at 0°C to 4°C.

Gram Iodine

Iodine	3 g
Potassium iodide	6 g
Distilled water	900 mL

Place the iodine and potassium iodide in approximately 150 mL of the water. Stir until completely dissolved, then add the remaining water. Store at room temperature.

Dilute Gram Iodine

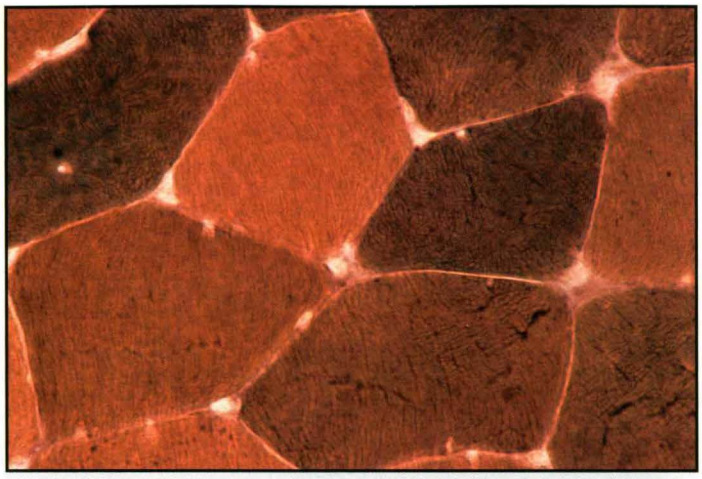
Gram iodine	5 mL
Distilled water	45 mL
Make fresh each time	

Iodine-Glycerine Mounting Medium

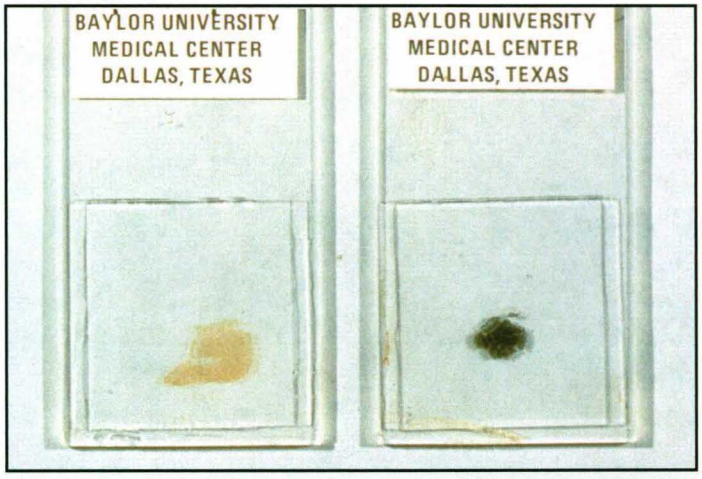
Gram iodine	4 mL
Glycerine	40 mL
Store in the refrigerator	

■ *Procedure*

1. Air-dry sections and leave at room temperature for 10 minutes.
2. Fix in cold acetone 5 minutes.
3. Incubate at 37°C in capped jar for 1 hour in:
   a. Filtered stock incubating solution 15 mL
   b. Absolute ethyl alcohol 3 mL



[i13.23] This frozen section of muscle has been stained for phosphorylase activity. The color formed when the section is stained with iodine is determined by the length of the unbranched chains formed or the amount of phosphorylase activity present. A negative reaction is yellow, and an intense blue-black color is displayed by the longest chains. Type II fibers usually have the most intense reaction, and the darker fibers seen in this sections are type II.

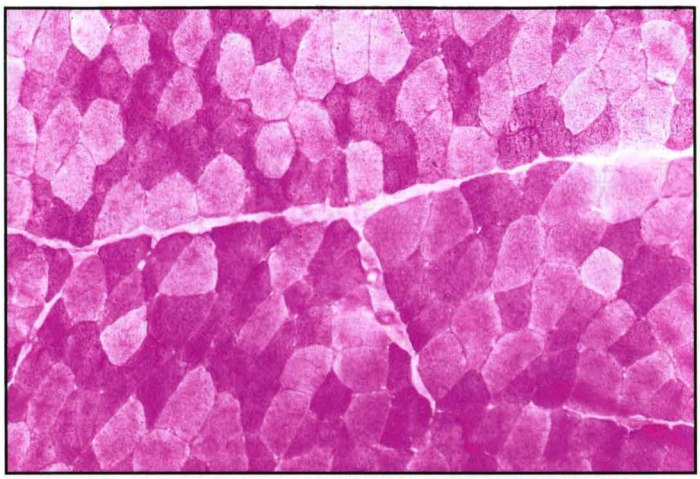


[i13.24] The sections in this photograph have been stained for phosphorylase activity. The negative (yellow) reaction on the left indicated a phosphorylase deficiency diagnostic of McArdle disease. The control slide on the right shows normal activity.

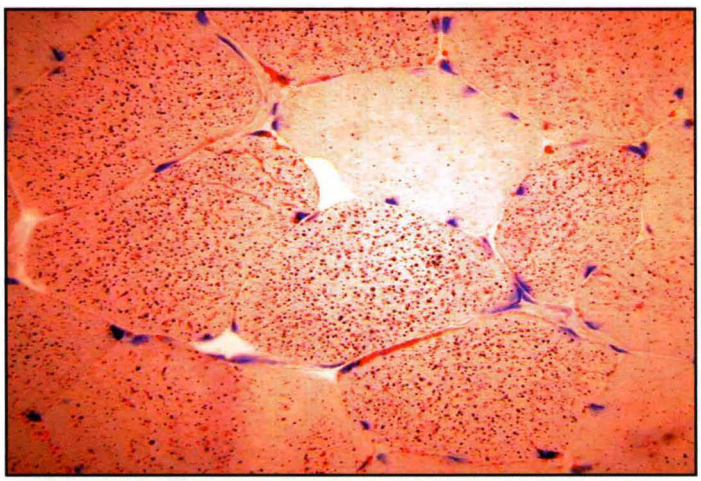
4. Shake off excess medium and wash briefly in 40% alcohol. Air-dry sections.
5. Fix in absolute alcohol for 20 minutes. Air-dry sections.
6. Stain in dilute Gram iodine for 6 to 70 minutes.
7. Mount in glycerine-iodine.

■ *Results* [i13.23], [i13.24]

• Phosphorylase activity	Varying shades of brown, blue and purple
• Total absence of phosphorylase activity	Indicates McArdle disease



[i13.25] A frozen section of a normal muscle has been stained with the periodic acid-Schiff technique. The normal checkerboard pattern of fiber types can be seen.



[i13.26] A frozen section of a normal muscle has been stained with the oil red O technique.

■ *Technical Notes*

1. The slides must be read immediately, because they begin to fade rapidly. If the slides fade before they are read, they can be restained in dilute Gram iodine.

2. McArdle disease is 1 of 7 glycogen storage diseases. Each involves the absence of an enzyme involved in glycogen metabolism. 4 of the glycogen storage diseases involve muscle [Dubowitz 1985].

Nonenzymatic Procedures for Muscle Disorders

Because this chapter deals primarily with techniques used in the diagnosis of muscle diseases, it is appropriate to mention that there are several nonenzymatic procedures included in the panel of stains used for this purpose. These stains commonly include periodic acid-Schiff (PAS), oil red O, and Gomori trichrome. The PAS is used for the detection of glycogen storage diseases, and the oil red O for lipid storage disorders. While the PAS **[i13.25]** and oil red O **[i13.26]** do not differ in the procedures used and results obtained, the results obtained with the Gomori trichrome differ radically from that seen in paraffin sections, therefore that procedure is included here.

MODIFIED GOMORI TRICHROME [SARNAT 1983]

■ *Purpose*

This stain is used for the detection of changes in the normal morphology of both the muscle fibers and the surrounding connective tissue.

■ *Procedure*

This is an excellent stain used for striated muscle and peripheral nerve. Architectural alteration in muscle fibers, such as nemaline rods or "ragged red" fiber change, are readily seen because of the differential staining of pathological inclusions and the sarcoplasm. Proliferation of endomysial connective tissue is also demonstrated. Intramuscular nerve twigs stand out in sharp contrast to muscle fibers and connective tissue because of the chromotrope staining of their myelin sheaths.

■ *Fixative*

None

■ *Equipment*

Liquid nitrogen, isopentane, cryostat, Dewar flask, pH meter, Coplin jars

■ *Technique*

Frozen sections of unfixed tissue cut at 10 µm and mounted on slides (at the bottom)

■ *Quality Control*

This stain generally does not require an external control; if desired, slides from another case can be run as an external control.

■ *Reagents*

Gomori Trichrome Solution

Fast green FCF	0.6 g
Chromotrope 2R	1.2 g
Phosphotungstic acid	1.2 g
Acetic acid, glacial	2.0 mL
Distilled water	200 mL

Mix well until all ingredients are dissolved. Adjust the pH of the stain with NaOH or 1.0 M acetic acid to pH 3.4. Filter the staining solution prior to use each time to remove any dye precipitate. Store at 4°C. Prepare fresh solution every 2 to 3 weeks.

■ *Procedure*

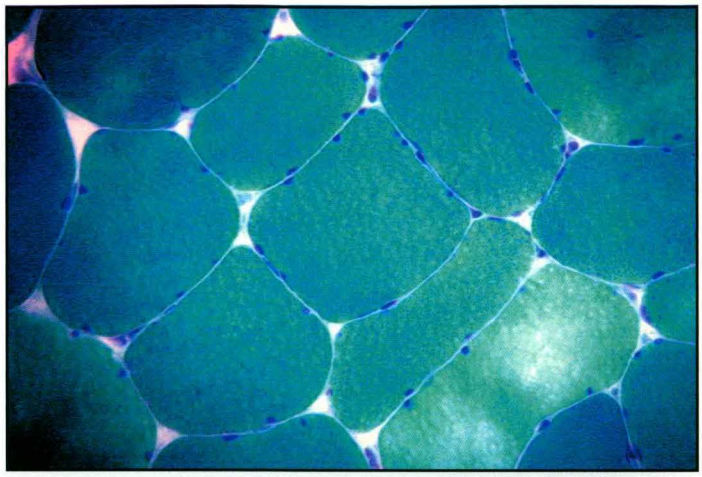
1. Allow frozen sections to air-dry at room temperature. *Do not fix.*
2. Stain in Harris hematoxylin for 5 minutes.
3. Wash well in distilled water to remove excess hematoxylin.
4. Stain in trichrome solution for 10 to 30 minutes.
5. Differentiate by a few dips in 0.2% acetic acid.
6. Dehydrate in 95% and absolute alcohol; clear in xylene, and mount with synthetic resin.

■ *Results* [**i13.27**]

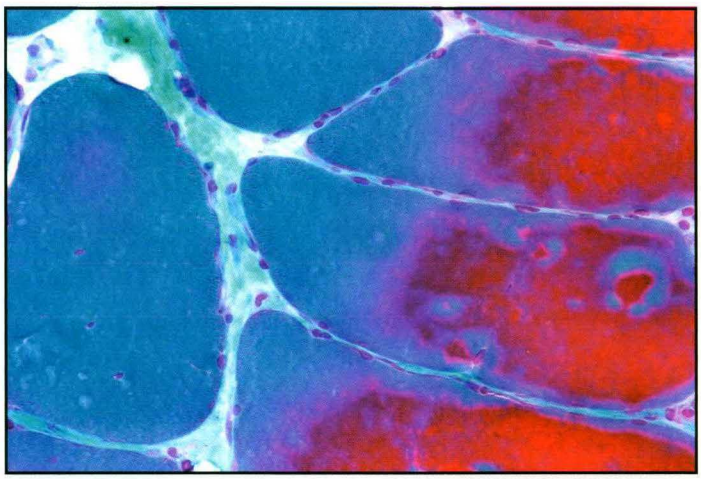
• Nuclei	Red-purple
• Myofibrils	Bluish-green
• Intermyofibrillar sarcoplasm	Red
• Interstitial connective tissue	Light green
• Myelinated nerve twigs	Red
• Nemaline rods and abnormal mitochondria	Red

■ *Technical Notes*

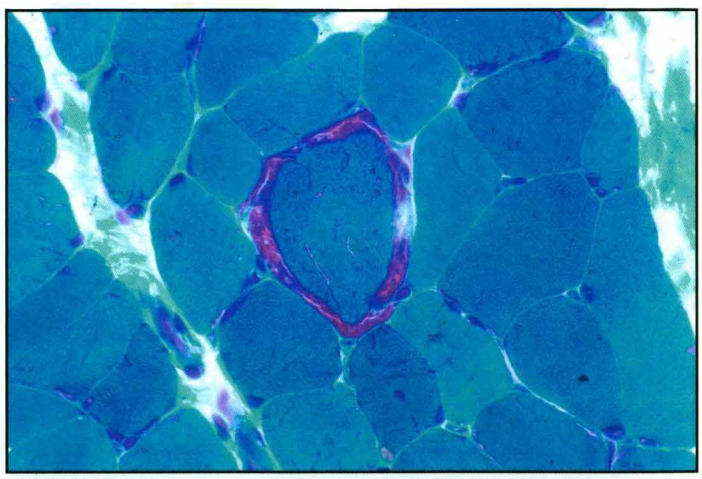
1. The muscle must not be fixed before performing this stain. Even brief periods in fixative will change the stain results [**i13.28**].
2. Ragged red fibers suggest mitochondrial disease, but may be seen in other disorders [**i13.29**].
3. Chromatrope 2R does not have a stable shelf-life. It must be replaced periodically as needed to make good trichrome stain.



[i13.27] The modified Gomori trichrome has been used on this frozen section of muscle. Note that the muscle is stained greenish-blue and not the usual red seen on paraffin sections.



[i13.28] This muscle biopsy specimen stained with the Gomori trichrome was put in formalin briefly and the staining pattern of the muscle has been affected.



[i13.29] The ragged red fiber seen in the center of this Gomori trichrome-stained frozen section may indicate a mitochondrial disease.

Acknowledgment

Thanks are due to GJ Race, MD, and Lippincott/Harper & Row for permission to use much of the material from Race GJ, ed. *Laboratory Medicine*. Philadelphia, Harper & Row, 1983;vol 3:chap 23 (out of print).

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LEARNING ACTIVITIES

1. **Obtain a piece of skeletal muscle, cut a cross section, and freeze in the cryostat as usual. Cut a section and stain with H&E.**

2. **Cut a second cross section and freeze by the fastest method available, preferably isopentane in liquid nitrogen. Cut a section and stain with H&E.**

3. **Compare the 2 different H&E-stained sections. Which is better? Why?**

4. **Cut 2 more sections from the rapidly frozen muscle and:**

    a. Perform the one-step trichrome as given in this chapter, p328. How does this trichrome compare with the trichrome done on paraffin sections?

    b. Perform a NADH diaphorase stain. Observe the different staining of type 1 and type 2 fibers. Which fiber type is stained darker?

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CHAPTER 14

Electron Microscopy

OBJECTIVES

On completing this chapter, the student should be able to do the following:

1. Describe 6 ultrastructural criteria for determining good fixation
2. List 2 advantages of primary osmium fixation
3. List 3 disadvantages of primary osmium fixation
4. List 6 advantages of primary aldehyde fixation
5. List 3 disadvantages of primary aldehyde fixation
6. List 3 advantages of primary buffered picric acid-formaldehyde (PAF) fixation
7. List 3 disadvantages of primary PAF fixation
8. Define dual-purpose fixative
9. Explain why paraformaldehyde needs to be heated and alkalinized when preparing solutions
10. Identify how long tissue can remain in each of the following solutions without harm:
    a. osmium tetroxide
    b. glutaraldehyde
    c. formaldehyde
    d. PAF
    e. formaldehyde-glutaraldehyde
11. Compare and contrast processing for epoxy resin embedding with processing for paraffin embedding
12. Define transitional solvent
13. Describe 6 factors that influence fixation of specimens for electron microscopy
14. State the function of each of the chemicals in Epon, Araldite, and Spurr embedding media
15. List 7 steps that will make sectioning of resin-embedded tissue easier
16. Identify the type of knife used for cutting thin sections
17. Identify the purpose of 0.5-μm sections
18. Identify 8 sectioning problems and the appropriate corrective action for each
19. Describe how section thickness is related to color
20. State the preferred thickness in nanometers and in color for thin sections
21. Describe 2 methods of staining 0.5-μm plastic sections
22. Identify 2 heavy metals used for staining thin sections
23. State the reason that air and breath should be excluded from the lead citrate staining solution
24. Define electron lucent and electron dense
25. State the reason glass bottles should not be used for the lead citrate solution
26. Identify the parts of the cell as seen with the electron microscope (see **[i6.1]**, **[i6.3]**)

Fixation

Just as fixation is probably the most important step in light microscopy, it is without a doubt the most important step in electron microscopy. It is at the cellular level that the effects of fixation become so apparent, and many common histological fixatives do not preserve tissue adequately for electron microscopic examination. The quality of fixation can be determined by examining various parts of the cell **[i6.1]**, **[i6.3]**. Good fixation will show the following ultrastructural characteristics:

1. **Plasmalemma**. Complete, with no breaks in the membrane.
2. **Nuclear envelope**. Uniform, undilated space between the inner and outer nuclear membranes.
3. **Mitochondria**. No swelling or disruption. Mitochondria are very sensitive indicators of the quality of fixation.
4. **Endoplasmic reticulum**. Regular width and regular arrangement of cisterns or channels.
5. **Cytoplasm**. Finely precipitated and not too obvious in most cells. When well preserved, it appears more complex than when poorly preserved.
6. **Nucleus**. Varies with the type of fixative from finely granular with osmium to aggregated chromatin masses with aldehydes.

FIXATIVES

Only 2 categories of fixatives have proved to be of general use. The first of these, osmium tetroxide, has been used since the early days of electron microscopy because it has long been recognized as a superior preservative of cytologic detail. Since the early 1960s interest in aldehyde fixatives has been increasing because this type of fixation also permits histochemical studies to be done. Aldehydes, especially formaldehyde, are of particular value to the histopathology laboratory because specimens can remain in the fixative for long periods with no deleterious effects. This is not true with osmium tetroxide, which begins to leach out proteinaceous material with prolonged fixation. Lipids are not rendered insoluble by the aldehydes, so a brief secondary fixation with osmium tetroxide is recommended. This double fixation gives a final result that is very similar to original osmium tetroxide fixation, but more proteinaceous material is preserved.

Zamboni fixative [Zamboni 1967], a phosphate-buffered picric acid-formaldehyde (PAF) solution, was introduced in the late 1960s. It is not widely used today, but it can be used for both light and electron microscopy. This is a stable fixative solution; proteins are not precipitated by picric acid in a neutral solution, and tissue may remain in the solution at room temperature indefinitely. Specimens are usually postosmicated following this fixative.

■ *Advantages of Primary Osmium Tetroxide Fixation*

1. Provides excellent preservation of cytological detail.
2. Renders lipids insoluble, giving excellent membrane preservation.

■ *Disadvantages of Primary Osmium Tetroxide-Fixation*

1. Specimens cannot be left in fixative more than 2 to 4 hours, preferably less.
2. Penetration is poor; specimens must be minced to approximately 1-mm cubes.
3. Histochemical studies cannot be performed.

■ *Advantages of Primary Aldehyde Fixation*

1. Allows better penetration of fixative.
2. Histochemical studies can be performed.
3. If formaldehyde is used, electron microscopy can be performed on specimens that have been in the fixative for long periods.
4. Formaldehyde and some formaldehyde-glutaraldehyde mixtures can be used as dual-purpose fixatives (satisfactory for both light and electron microscopy).
5. Can be used easily for perfusion of tissues.
6. When followed by postosmication (secondary fixation in osmium), optimum preservation of cellular detail can be achieved.

■ *Disadvantages of Primary Aldehyde Fixation*

1. Lipids are not preserved unless secondary osmium tetroxide fixation is used. Up to 93% of lipids can be extracted after formaldehyde fixation, but only 7% can be extracted after secondary osmium fixation.
2. Membrane-bound cavities have a tendency to be slightly enlarged beyond desirable limits.
3. Membranes are electron lucent unless secondary osmium tetroxide fixation is used.

■ *Advantages of Primary Buffered PAF Fixation*

1. Specimens can remain in the fixative solution at room temperature indefinitely.
2. Penetrates tissue rapidly and stabilizes cellular proteins.
3. Can be used to fix tissue for both light and electron microscopy.

■ *Disadvantages of Primary Buffered PAF Fixation*

1. Lipids are not well preserved unless secondary osmium tetroxide fixation is used.

2. Some cytoplasmic granules and lysosomes may not be preserved.

3. Some background substances may not be well preserved.

FACTORS INFLUENCING FIXATION

1. **pH.** Fixative solutions for electron microscopy are usually buffered between pH 7.2 and 7.4 because that is the approximate pH range of most animal tissues. Commonly used buffers are phosphate, cacodylate, s-collidine, and veronal acetate.

2. **Temperature.** Traditionally, fixation was done at 4°C but some swelling of the perinuclear membrane [Carson 1972] and destruction of microtubules may occur with cold fixation. Today, fixation for routine electron microscopic examination is usually carried out at room temperature.

3. **Tonicity.** The tonicity of blood plasma is approximately 300 mOsm, and many electron microscopists believe that this is the most appropriate tonicity for fixatives. With most fixatives, it is the tonicity of the buffer salts that is important. The fixatives themselves are not osmotically active. Dextrose and sucrose are frequently used to adjust the tonicity of fixatives.

4. **Length of fixation.** When using formaldehyde, buffered PAF, or formaldehyde-glutaraldehyde (4CF-1G) for fixation, tissue may remain in the fixative indefinitely; however, some extraction of various tissue components may occur over time. If glutaraldehyde is used, the tissue should be fixed no longer than overnight, preferably 2-4 hours, and then held in buffer for future processing. If osmium tetroxide is used as a primary fixative, the tissue should be minced to 1-mm cubes and processed after a 1-2 hour fixation period.

FIXATIVE SOLUTIONS

Formulas for the preparation of several fixative solutions will be given. Extensive studies have shown modified Millonig formaldehyde solution to be an excellent dual-purpose fixative for use in the surgical pathology laboratory [Carson 1973]. With this fixative, electron microscopy can be performed on any stored specimen, and preselection of specimens for electron microscopy prior to fixation is no longer necessary. Other dual-purpose fixatives are a combination of commercial formaldehyde and glutaraldehyde, and a buffered PAF solution.

All these dual-purpose fixatives provide easy and satisfactory tissue preservation for routine light preparations, and still preserve tissue suitably for electron microscopic studies even after prolonged storage at room temperature. The fixatives that are used only for electron microscopy require preselection of tissue; that is, a small portion of the fresh tissue must be placed in the electron microscopy fixative as soon as possible after interruption of the blood supply, and the remainder of the tissue is placed in the fixative used for light microscopy.

Paraformaldehyde with Cacodylate Buffer

1. Prepare a 0.4M sodium cacodylate buffer solution by placing 8.56 g of sodium cacodylate in a graduated cylinder. Dilute to 100 mL with distilled water. Adjust the pH to 7.3 to 7.4 with concentrated hydrochloric acid.

2. Place 4 g of paraformaldehyde in an Erlenmeyer flask with 50 mL of distilled water. Warm to 60°C and depolymerize by adding 0.1N sodium hydroxide (40 g NaOH/1L distilled H_2O) drop by drop to the warm solution, stirring mechanically until the solution clears.

3. Add 25 mL of 0.4M cacodylate buffer solution and 2 mL of 0.5M calcium chloride (5.55 g $CaCl_2$/100 mL distilled water) to the depolymerized solution. This gives a 4% paraformaldehyde solution with 0.1M cacodylate buffer and 0.01M calcium chloride. The pH should be 7.3 to 7.4. Cacodylate buffer contains arsenic and should be handled carefully.

Paraformaldehyde or Glutaraldehyde with Phosphate Buffer [Pease 1964]

1. In a clean Erlenmeyer flask, place the following:

26% monobasic sodium phosphate solution	83 mL
52% sodium hydroxide solution	17 mL
Mix well	

2. Add 4 g of paraformaldehyde or 18 mL of 25% glutaraldehyde.

3. The paraformaldehyde must be heated to depolymerize.

4. Adjust the pH to 7.2 to 7.4 as necessary.

Formaldehyde with Phosphate Buffer (Modified Millonig Fixative) [Carson 1973]

Formaldehyde, 37% to 40%	0 mL
Distilled water	90 mL
Sodium phosphate monobasic	1.86 g
Sodium hydroxide (NaOH)	0.42 g
Mix well. The pH should be 7.2 to 7.4	

This solution may be prepared conveniently in large quantities for routine use as follows:

1. Fill a 6.5-gallon container (previously calibrated to 22,000 mL) approximately half full with tap water.
2. Add a 1-lb bottle of monobasic sodium phosphate and stir until dissolved.
3. Add 1,000 mL of stock NaOH solution (411.2 g per 4,000 mL deionized water) and mix well. Be sure the stock NaOH solution is well mixed.
4. Dilute to the 22,000 mL mark with tap water.
5. Add 2,440 mL 37% to 40% formaldehyde and mix well.

This volume is convenient because only 1 weighing (stock NaOH solution) is required for every 4 times the solution is prepared.

Formaldehyde-Glutaraldehyde (4CF-1G) [McDowell 1970]

Dissolve in 50 mL of water:	
Monobasic sodium phosphate (NaH_2PO_4)	1.16 g
Dissolve in 38 mL of water:	
Sodium hydroxide (NaOH)	0.27 g
Combine the above 2 solutions, then add:	
Formaldehyde, 40%	10 mL
Glutaraldehyde, 50%	2 mL
Mix well and store at 4°C	

Buffered PAF (Zamboni Fixative) [Zamboni 1967]

1. Place 20 g of paraformaldehyde and 150 mL of double-filtered, saturated solution of picric acid in a 2-L flask. Heat solution for 2 hours in a 60°C water bath to dissociate the paraformaldehyde into formaldehyde.
2. Add 2.52% sodium hydroxide drop by drop, with mixing, until the solution clears.
3. Filter the solution and allow to cool.
4. Dilute the solution to a volume of 1 L with phosphate buffer prepared as follows:

Sodium phosphate monobasic (NaH_2PO_4)	3.31g
Sodium phosphate dibasic ($Na_2HPO_4 \cdot 7H2O$)	33.77 g
Add distilled water to make 1 L. Mix well. The PAF solution should have a final pH of 7.3 and an osmolality of 900 mOsm.	

Osmium Tetroxide with Cacodylate Buffer

1. Place 1 g of osmium tetroxide and 50 mL of distilled water in an amber dropping bottle. Mix until dissolved.
2. Add the following, mixing after each addition:

0.4 M cacodylate buffer (see paraformaldehyde with cacodylate buffer)	20 mL
Sucrose	2 g
0.5M $CaCl_2$ (5.55 g per 100 mL)	1.6 mL

4. Check pH and adjust to 7.2 to 7.4 if necessary.
5. Store at 4°C.

Osmium tetroxide solutions should be used under a fume hood because the vapor is very irritating and will fix the conjunctiva, cornea, nasal mucosa, or other exposed tissues it contacts to a depth of several cell thicknesses. Cacodylate buffer contains arsenic and should be handled carefully.

Osmium Tetroxide with Phosphate Buffer

1. Place 1 g of osmium tetroxide in a 125-mL amber dropping bottle.
2. Add the following, mixing well after each addition:

2.26% sodium phosphate, monobasic	83 mL
2.52% sodium hydroxide	17 mL
Distilled water	10 mL
Dextrose	0.54 g

3. Check the pH and adjust to 7.3 to 7.4 if necessary.
4. Store at 4°C.

Osmium tetroxide solutions should be used under a fume hood because the vapor is very irritating and will fix the conjunctiva, cornea, nasal mucosa, or other exposed tissues it contacts to a depth of several cell thicknesses.

Immediate fixation is very important, and small blocks of tissue should be placed in fixative as soon as possible. It cannot be emphasized enough that proper and immediate fixation is absolutely necessary for optimal morphologic preservation, and is the most important step in electron microscopy. As with light microscopy, the results of improper fixation cannot be corrected in the later steps of specimen processing, microtomy, and staining. Although the use of aldehyde fixatives allows one to use stored specimens for electron microscopic examination, the preservation

will be best near the cut surfaces; therefore, sections for electron microscopy should be taken near these surfaces if possible. As with light microscopy, when immunoelectron microscopy is being considered, overfixation can be a problem.

Processing

Processing tissue for routine electron microscopy is similar to processing for light microscopy. Because the commonly used embedding media are not miscible with water, the tissues must be dehydrated and frequently carried through a transitional solvent before being placed in the embedding medium.

DEHYDRATION

As in light microscopy, ethyl alcohol is most commonly used for dehydration. To prevent contamination with atmospheric moisture, reagent grade ethanol should be purchased in small containers that can be used rapidly.

Other dehydrating agents have been used with varying success. These include acetone, dioxane, 2-ethoxyethanol, and dimethyl formamide.

TRANSITIONAL SOLVENTS

Transitional solvents are necessary with most epoxy and polyester resin embedments because alcohol will mix only very slowly with epoxy resins and not at all with polyester resin mixtures. They correspond to clearing agents in processing for paraffin embedding.

Propylene oxide is used with epoxy resins and may also be used with the polyester resins, but styrene is the transitional solvent of choice with the polyester resins.

EMBEDDING MEDIA

Methacrylate embedding was among the earliest methods used in electron microscopy. It has the chief advantage of partially volatizing in the electron beam and thus enhancing specimen contrast to such a degree that no "stain" is necessary on tissue fixed in osmium tetroxide (and the disadvantage of having to clean the electron microscope every week instead of every several months). However, some fine cellular detail is lost because of the decomposition in the electron beam, with the subsequent formation of transient liquid interfaces. This embedding medium was widely used until effective electron "stains" were developed in the late 1950s.

Vestopal W is a polyester resin that is used for embedding. It sections easily, and the sections stain well; however, it must be obtained from foreign sources. Epon, Araldite, and Spurr epoxy resins probably are the most widely used embedding media for electron microscopy. Luft [1961] studied the cutting qualities of epoxy resins at various anhydride: epoxide (A:E) ratios, and many mixtures in use are based on his work.

PROCEDURE FOR ROUTINE PROCESSING AND SPURR EMBEDDING

Spurr epoxy resin is a low-viscosity embedding medium that infiltrates specimens readily after any one of several dehydrating fluids such as ethanol, isopropanol, dioxane, and propylene oxide have been applied. This epoxy resin is miscible with ethanol, and therefore no transitional solvent is necessary. Sections are easily cut and are tough under the electron beam.

A good procedure for routine use is as follows:

1. Place the fresh tissue blocks of approximately 0.5 cm maximum thickness in fixative. Use a volume of fixative that is at least 20 times that of the specimen. Allow to fix, preferably overnight. This may be done at room temperature. If glutaraldehyde fixative is used, it is best to fix the tissue (thin sections are essential because glutaraldehyde does not penetrate well) for 2 to 4 hours and then transfer it to buffer solution. The tissue can remain in buffer solution indefinitely. The buffer solution is the same as the fixative but omits the glutaraldehyde.

2. Take a core biopsy specimen of the fixed tissue (ie, remove the mechanically and osmotically damaged and/or contaminated outer 1 to 2 mm of the tissue), place in a pool of fixative or buffer, and mince to 1-mm cubes with a carefully washed (oil- and wax-free) razor blade. Dental wax provides a good surface for mincing **[i14.1]**. Be very careful not to squeeze or mash the tissue.

3. Carefully transfer the minced specimen to a small vial containing some of the fixative or buffer that came from the original specimen bottle. This solution will have already exerted the maximum osmotic effect. Cork the vial. Stop here if it is not convenient to continue processing at this time **[i14.2]**. The remainder of the procedure should be carried out without interruption.

4. Decant the aldehyde fixative, and add approximately 2 mL of cold osmium tetroxide fixative solution. (Not inserting a buffer wash after formaldehyde fixation has not created a problem in our laboratory; however, most laboratories probably prefer to insert a buffer wash between the aldehyde fixative and osmium tetroxide. If the buffer wash is desired, prepare the buffer using the fixative recipe but omit the aldehyde.) Allow the osmium fixative containing the specimens to come to room temperature, and fix for 45 minutes, swirling occasionally so that there is continuous contact of the fresh fixative with the surface of the tissue. While the specimen is in osmium tetroxide:

    a. Insure that the glassware to be used in the preparation of the resin is clean and dry.

    b. Prepare the labels and place them in the embedding capsules. Usually the labels contain the year, specimen number, and block number for easy retrieval.

[i14.1] Wax provides a good surface for mincing fixed tissue into 1-mm cubes for processing. A clean sharp blade is necessary if mechanical trauma and possible specimen contamination are to be avoided.

[i14.2] Minced tissue may be held in a vial of fixative until ready for processing.

5. Add an equal amount of 50% ethanol (USP) to the specimen in the osmium tetroxide fixative and then carefully decant the supernatant liquid. If this is done quickly, no obvious precipitation of the osmium occurs.

6. Quickly add a second portion of 50% ethanol to the specimen, agitate momentarily (1-2 minutes), and again carefully decant. Add another volume of 50% ethanol, and allow to stand for 10 minutes.

7. Decant and add 70% alcohol. Allow to stand for 10 minutes.

8. Decant and add 95% alcohol. Allow to stand for 10 minutes.

9. Quickly add freshly opened 100% ethanol, decant, and then refill with 100% ethanol and allow to stand for 10 minutes. Wash with 1 more change of 100% ethanol, allowing to stand for 10 minutes.

10. Decant, add a small aliquot of pure epoxy mixture prepared as described below, swirl to mix, and allow to stand for 15 minutes. Weigh rather than measure the following components in a dry plastic beaker, adding each in turn:

Vinyl cyclohexane dioxide (ERL 4206)	10 g
Diglycidyl ether of propylene glycol (DER 736)	6 g
Nonenyl succinic anhydride (NSA)	26 g
Dimethylaminoethanol (DMAE)	0.2 to 0.4 g (approx 24 drops)

Mix thoroughly with an applicator stick after the DMAE is added, stirring for 1 to 2 minutes. Keep the components at room temperature. A plastic pipette can be used to dispense the final amount of each component so that no excess is added. Do not use glass Pasteur pipettes because they chip easily and small fragments of glass can be embedded with the specimen.

11. Let the specimen sink to the bottom of the vial in the first change of pure epoxy resin, then decant and gently transfer the specimen to a clean, dry vial.

12. Immediately add another aliquot of the epoxy mixture, and let stand for 30 minutes. Decant.

13. Fill the dry, labeled capsules with the embedding mixture.

14. Transfer the specimen to the capsules containing the catalyzed Spurr resin, carefully matching the specimen number to the label. The transfer may be done with forceps if care is taken not to mash the tissue, or the specimen may be transferred conveniently with an applicator stick that is slightly pointed or slanted **[i14.3]**. Multiple sections can be embedded in plastic bottle caps. If needed, these may be cut out and glued to an epoxy block with a fast-drying epoxy glue.

15. Close the capsules, and polymerize in a 60°C oven for approximately 12 hours.

■ *Technical Notes*

1. ERL 4206 is an epoxy resin, DER 736 is a flexibilizer, NSA is the hardener, and DMAE is the accelerator or catalyst.

2. The chemicals used in this procedure are toxic and rapidly absorbed through the skin, so protective clothing should be worn. Epoxy resins can also cause dermatitis. If any areas of skin are contacted by these components or by the mixture, the affected areas should be washed with soap and water. Do not wash with a solvent. Use the resin only in adequately ventilated areas, preferably under a fume hood, and avoid repeated contact with the liquid.

[i14.3] Crushing of the tissue is avoided by using a pointed applicator stick for transfer to the embedding capsules. Extra sections or sections in which orientation is critical are conveniently embedded in a plastic bottle cap. The desired section may be cut out and glued on a block.

PROCEDURE FOR ROUTINE PROCESSING AND EPON EMBEDDING

This procedure is not recommended in very humid areas. Luft [1961] studied the cutting qualities of epoxy resins at various A:E ratios. A:E ratios of 0.90 to 1.0 (recommended for industrial use) produce blocks that are very highly cross-linked but very difficult to cut. At A:E ratios of about 0.60 to 0.65, there is a marked deterioration of the cutting properties. An A:E ratio of 0.70 was selected as optimal. In the past, the appropriate proportions of resin mixture needed to be calculated for each lot of Epon 812. Epon 812 was manufactured and marketed by the Shell Chemical Co (Division of Shell Oil Co, New York, NY). It is no longer available from Shell, but substitutes may be obtained from Electron Microscopy Sciences (Hatfield, PA [Embed 812]); Ladd Research Industries Inc (Williston, VT [LX-112]); Ernest F. Fullam Inc (Clifton Park, NY [Epox 812]); and Ted Pella, Inc. (Redding, CA [Eponate 1]). With the kits now on the market, the calculations are no longer necessary. A good procedure for use with Epon is as follows:

1. Follow steps 1 through 7 of the Spurr procedure.
2. Decant and add 95% alcohol. Allow to stand for 10 minutes. Repeat once.
3. Decant and add freshly opened 100% ethanol, decant, and then refill with 100% ethanol and allow to stand for 10 minutes. Repeat this step twice more for a total of 4 washes with 100% ethanol and for slightly longer than 30 minutes.
4. Decant the last wash of absolute ethanol, and add 100% propylene oxide to the specimen. Let stand 15 minutes. Repeat this step once.
5. Prepare the catalyzed resin mixture according to the instructions provided with the product used. In a small vial, prepare a mixture of 50:50 propylene oxide and catalyzed resin. Most resin mixtures consist of Epon 812, 2 hardeners (dodecenyl succinic anhydride [DDSA] and methyl nadic anhydride [MNA]), and an accelerator or catalyst (tridiethylaminomethyl phenol [DMP30]).
6. Decant the last wash of propylene oxide from the specimen, removing as much as possible. Immediately add a small amount of the 50:50 propylene oxide and catalyzed resin mixture. Allow to stand for 15 minutes with intermittent agitation.
7. Decant and cover the specimen with a small amount of fresh catalyzed embedding resin. Let stand until specimens sink to the bottom of the vial. The specimens are now ready to embed.
8. Follow steps 13 through 15 of the Spurr procedure.

■ *Technical Note*

Epoxy resins can cause dermatitis; therefore, protective clothing should be worn when handling the chemicals used in this procedure. Adequate ventilation should also be available.

PROCEDURE FOR LR WHITE PROCESSING FOR ELECTRON MICROSCOPY IMMUNOLABELING [LOTT 1995]

LR White (London Resin Co Ltd, Hampshire, England; may be obtained from EM Sciences, Hatfield, PA) is a "hydrophilic" (water-loving) acrylic monomer that is easily penetrated by aqueous reagents. This means that the reagents used for labeling will readily penetrate the resin to reach the tissue antigenic sites. It is very important that these antigenic sites be well preserved, with maximum reactivity retained. Specimens should be fixed with an aldehyde fixative immediately after the blood supply is interrupted; however, fixatives that cross-link excessively (eg, Karnovsky or glutaraldehyde in concentrations above 0.1%-0.5%) should be avoided. Fixation should be limited to about 4 hours for 1- to 2-mm cubes and should never exceed 12 hours. Ideally, for consistent immunolabeling results, the time of fixation should be consistent with that which gives maximum immunolabeling and yet maintains adequate ultrastructural preservation. LR White is stable in the electron beam, and the same specimen can be used for both light and electron microscopy if the fixation is appropriate. The following protocol is an example for processing tissue with LR White acrylic monomer when electron microscopy immunolabeling is desired:

1. Fix 1-mm cubes of tissue for 1 to 4 hours. Do not allow the specimens to fix for more than 12 hours.
2. Decant the aldehyde fixative, and wash with cacodylate or phosphate buffer for 10 to 15 minutes.
3. Decant the buffer solution, and begin the dehydration with 2 changes of 60% ethyl alcohol for 10 minutes each.
4. Decant and dehydrate with 2 changes of 80% ethyl alcohol for 10 minutes each.

5. Decant and dehydrate with 2 changes of 95% ethyl alcohol for 10 minutes each.

6. Decant and place in a 2:1 mixture of LR White (2 parts LR White to 1 part ethyl alcohol) for 1 hour at room temperature, with agitation. This mixture must be clear; excessive alcohol will produce a cloudy, turbid solution that will result in unsatisfactory polymerization.

7. Replace with fresh resin, and allow to remain for 1 hour at room temperature with agitation.

8. Replace with another fresh change of resin, and refrigerate overnight at 4°C. The specimen should be agitated in this solution of resin.

9. The following morning, replace with another fresh change of resin, and allow to remain for 1 hour at room temperature with agitation.

10. Embed into BEEM (Better Equipment for Electron Microscopy, West Chester, PA) or gelatin capsules containing fresh resin. Seal and polymerize at 50°C to 55°C for 20 to 24 hours. Do not allow the temperature to vary more than ±5°C, because the proteins affecting antigenicity may be denatured above 62°C. Limit the oxygen exposure, and use a vacuum oven if possible.

■ *Technical Notes*

1. Specimens are not postfixed in osmium tetroxide because of this reagent's harsh, deleterious effect on tissue antigenicity.

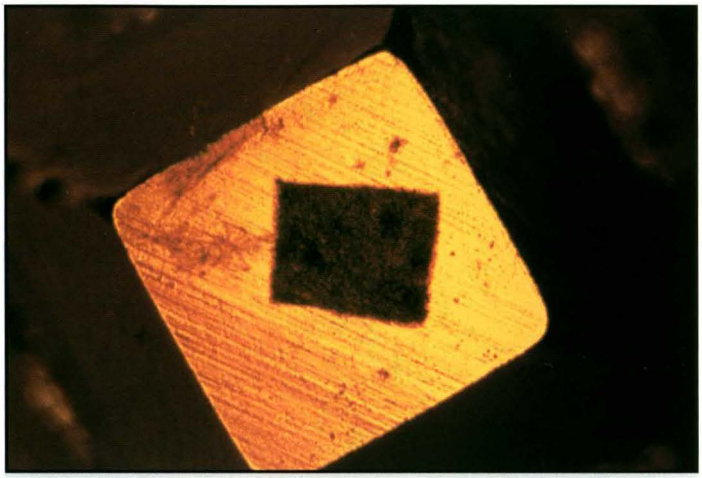
2. Acetone should be avoided in this procedure because it acts as a free radical scavenger and could interfere with polymerization of the resin. DMP30 generates acetone, so it should also be avoided.

3. Drain or blot tissue between solution transfers.

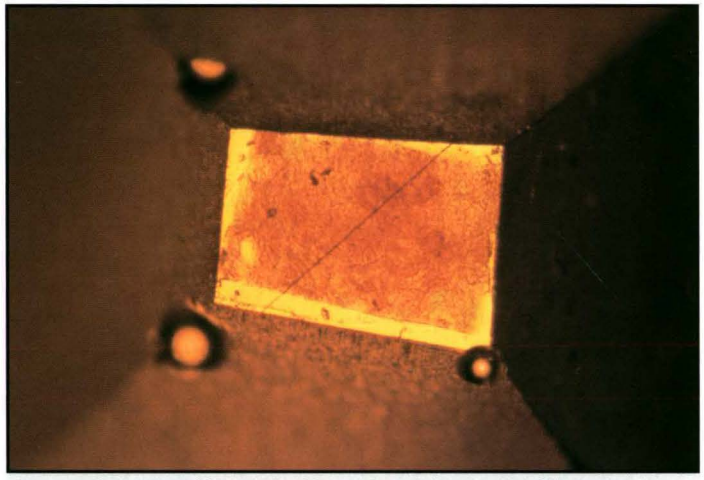
4. The resin may be used straight from the refrigerator. Tissue first introduced to the resin should float. As the tissue absorbs the resin, it will gradually sink to the bottom of the container.

5. The resin will tolerate partial dehydration, accepting tissue from 70% ethanol. This results in improved antigenicity.

6. For immunolabeling, LR White must be thermally cured and not accelerator cured. Thermal cure is done at 50°C to 55°C for 20 to 24 hours. Contact with oxygen should be limited; therefore, BEEM capsules should be tightly capped. If oxygen is not limited, some blocks may remain wet on top and may not cut well (they may seem soft when thin sectioned).



[i14.4] An untrimmed epoxy resin block containing tissue. The face shown is the surface to be trimmed before sectioning.



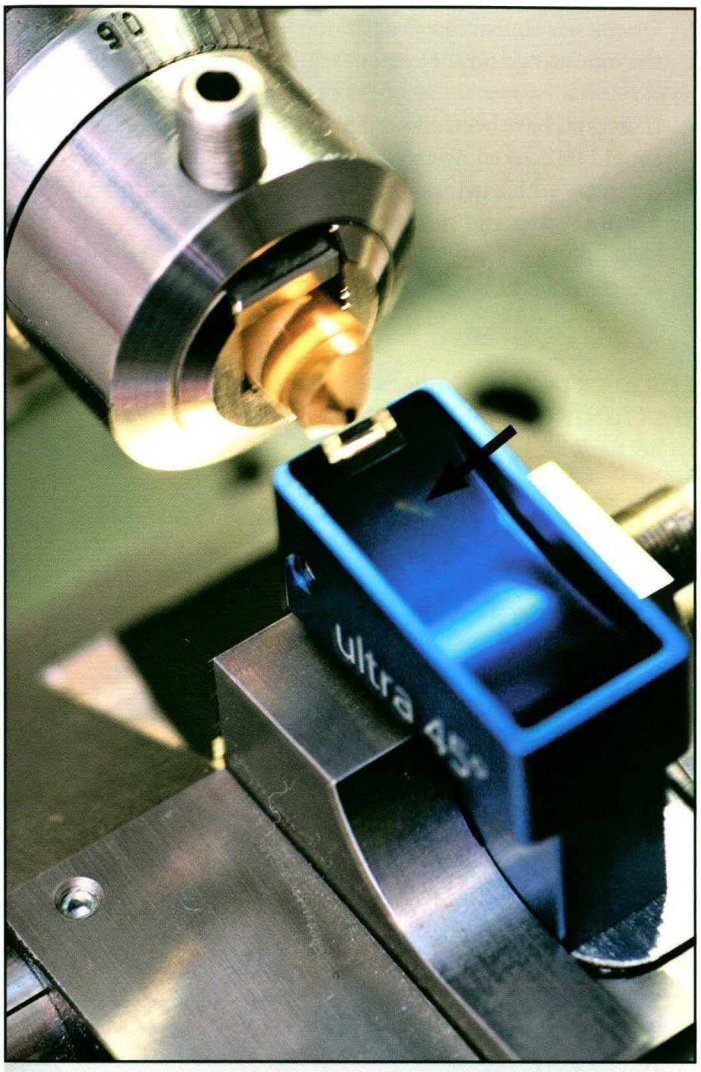
[i14.5] All excess plastic has been trimmed from around a block of kidney tissue. 2 glomeruli can be seen in the block, which is now ready for sectioning. Note that the block has been trimmed in a trapezoidal shape; the larger of the parallel edges should be placed so that it is at the bottom and strikes the knife first. The block may have to be trimmed further when ready to cut thin sections.

Sectioning

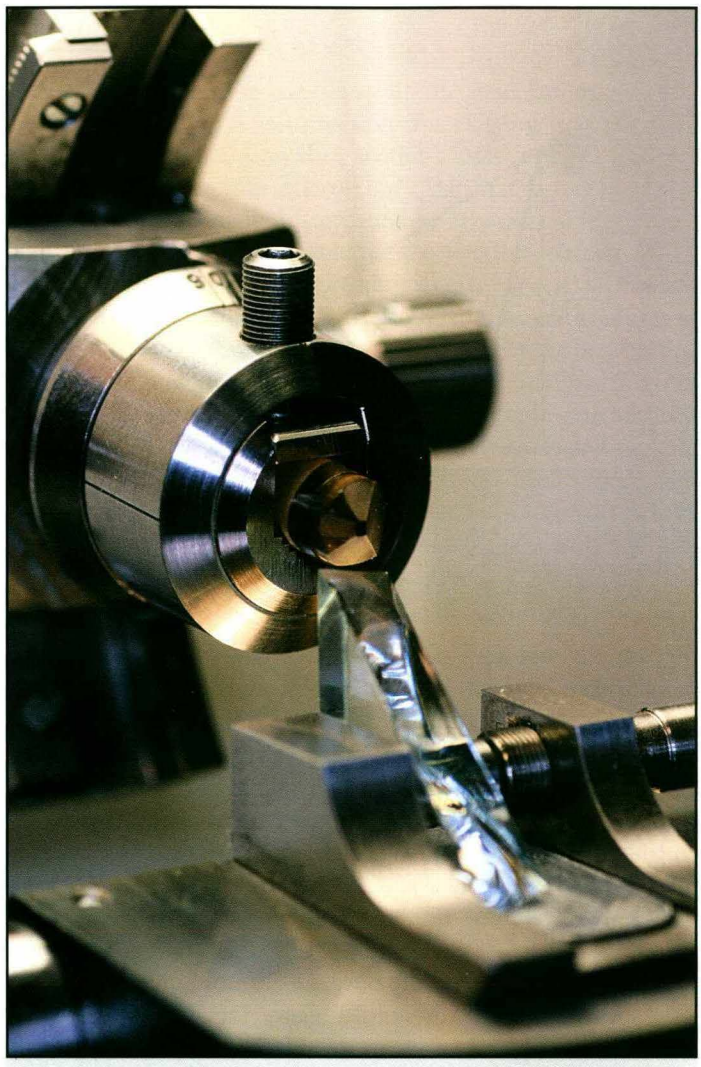
Sectioning is the most difficult of electron microscopic techniques, and it must be taught in a laboratory by experienced personnel; therefore, no effort will be made here to describe specific techniques of sectioning or the various microtomes that are available. Instead, a few hints that will make sectioning easier are provided as follows:

1. Have a microtome table that is free of vibration and located in a draft-free area. It is impossible to obtain chatter-free sections if there is vibration, and sections will not form a ribbon in a drafty location. These factors are most important when choosing a site for the electron microscopy laboratory.

2. Trim the block so that there is as small a face to section as possible [**i14.4**], [**i14.5**].



[i14.6] A diamond knife is usually used for cutting thin sections. A ribbon of sections (arrow) can be seen on the water in the trough.



[i14.7] Glass knives have been used for many years to cut 0.5-µm sections for microscopic examination.

3. Trim the face to a trapezoid shape with the longer of the parallel edges at the bottom. As each section is cut, it pushes all of the preceding sections from the knife edge.

4. Use lens paper to keep the trough liquid of your diamond knife clean when in use. Carefully pull it across the surface of the liquid. Avoid contact with the knife edge.

5. Do not let the fingers touch the knife, forceps, or any equipment that will be in contact with the trough liquid or thin sections because oil or cellular debris will contaminate the sections.

6. Do not hurry, because in electron microscopy "haste makes waste." Patience and steady, well-coordinated hands are probably the most desirable traits of an electron microscopy technician.

7. Use oil-free blades for electron microscopy work. Oil may be removed by rinsing the blade with acetone and then with water.

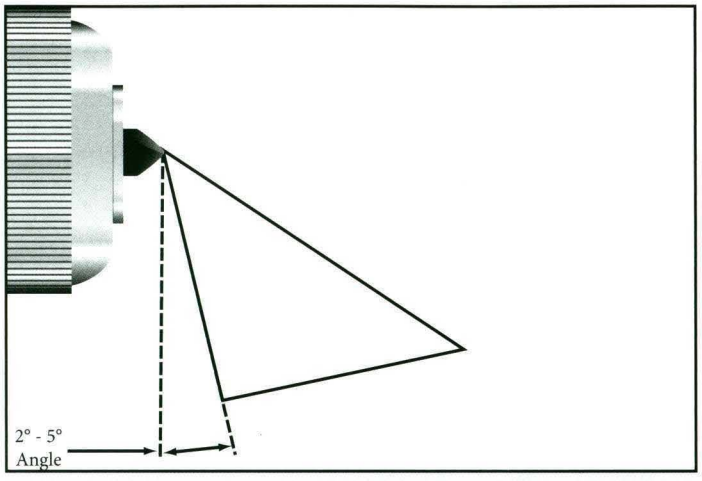
SECTION THICKNESS

Thin sections vary from 50 to 90 nm. The thickness can be accurately determined from the interference colors shown by the sections as they float in the collecting trough. It is crucial that the illumination and viewing system be adjusted so that the bright reflection from the fluid surface is seen through the microscope. Fairly thick sections show bright colors, such as purple, blue, green, and yellow. Gold sections are approximately 90-nm thick, and silver sections are approximately 50-nm thick. Dull gray sections are too thin for use. Interference between rays of light reflected from the upper and lower surfaces of the sections give rise to the colors, and the color varies very precisely with the distance between the 2 surfaces.

KNIVES

Diamond knives **[i14.6]** are used for most thin sectioning and should be handled very carefully and cleaned after each use. It is ideal if each technician can have at least 1 knife to use and be responsible for.

Glass knives **[i14.7]** have been used for many years for cutting 0.5-µm sections to check for proper tissue orientation and content. Glass knives must be broken shortly before use, because



[f14.1] The angle between the block face and knife (clearance angle) should be 2° to 5° as indicated. It has been exaggerated for the purpose of illustration in this figure.

they rapidly lose their edges. Although glass knives were originally broken by hand, mechanical glass breakers are used today in laboratories that still use this type of knife. Many electron microscopy technicians today use low-grade diamond *histo* knives for this purpose. These knives are cost effective, yield better sections, and save technician time. A *histo* knife has a 5-mm cutting edge, and with proper care, one of these knives can be used for approximately 2 years without resharpening. Although glass knives were also used originally for cutting thin sections, diamond knives are preferred because they do not lose their edges as rapidly and usually have wider, more uniform cutting edges. An angle (clearance angle) measuring 2 to 5 between the block and the vertical face of the knife is used commonly **[f14.1]**.

■ *Use and Care of Diamond Knives*

With proper use and care, diamond knives can be used for long periods. The following are recommended practices for achieving the maximum use from the knife:

1. Avoid touching the edge of the knife with any solid objects (grids, tweezers, or loops).

2. Avoid using solvents in the trough of the knife, because the sealing material between the knife and the boat may be dissolved, or the cellular materials of the specimen may be dissolved or destroyed.

3. Do not allow sections to dry on the cutting edge of the knife.

4. Immediately after picking up the sections, remove all unused sections with a hair or an eyelash, and clean the knife.

5. Use only a cleaning rod specifically designed for cleaning diamond knives. Material such as balsa wood, toothpicks, bamboo rods, orangewood sticks, plexiglass, Teflon rods, and Tigon tubing should not be used to clean the diamond knives, because any of these materials can easily chip the edge of the knife.

6. Do not use sonication to clean the knife, because it can loosen the mounting and the cement sealing of the sides of the knife.

7. If sections have been allowed to dry on the knife edge, the knife should be soaked overnight in a dilute solution of a nonionic detergent with a neutral pH. After soaking, rinse the knife with distilled water and clean with the cleaning rod.

8. Use different knives for different types of specimens.

CORRECTING PROBLEMS ENCOUNTERED IN SECTIONING

1. Sections are of varying rather than uniform thickness.
   a. Check tightness of specimen block, knife holder, and knife.
   b. Knife may be dull; change to a different area or have resharpened.
   c. Try a faster or slower cutting speed.
   d. Block may be soft; heat at 60°C for 24 hours.
   e. Check for drafts and air conditioning that may be causing temperature variation.
   f. Check microtome and table for vibration; keep a steady cutting rhythm.

2. Sections are skipped or not cut at all.
   a. Reset microtome advance.
   b. Knife may be dull; change to a different area or have resharpened.
   c. Tighten knife and specimen block firmly.
   d. Block face may be wet; dry with lens paper.
   e. Block may be soft; heat at 60°C for 24 hours.
   f. Check microtome and table for vibration; keep a steady cutting rhythm.

3. Chatter or undulations in sections.
   a. Reduce cutting speed.
   b. Reduce knife clearance angle.
   c. Reduce the size of the block face.
   d. Check microtome and table for vibration; do not touch unless manually operated.

4. Sections crumble or stick to knife edge.

a. Raise meniscus level of trough fluid.

b. Clean knife edge. (Glass knives cannot be cleaned and must be discarded.)

c. Increase knife clearance angle.

d. Block face may be dirty; clean with lens paper and alcohol.

5. Section lifted by specimen block.

a. Lower meniscus level of trough fluid.

b. Dry block face with lens paper.

c. Increase clearance angle.

d. Clean knife edge.

e. Block face may be electrified: increase room humidity or touch block face with wet lens paper.

f. Check back of knife facet for fluid droplet, dry with lens paper.

6. Sections are split or there are lengthwise lines in sections.

a. There may be a nick in knife cutting edge; move knife to new area or replace.

b. Clean knife edge.

c. Block may contain glass or dirt; discard block or use an old knife.

7. Ribbon is curved.

a. Upper and lower block edges may not be parallel; retrim.

b. Block sides may be unequal in length; retrim.

8. Face of specimen block gets wet.

a. Lower meniscus level of trough fluid.

b. Dry the back of knife cutting facet.

c. Clean knife edge and block face.

d. Dry the block face with filter paper

e. Increase the room humidity.

Staining

Two types of staining are done in the electron microscopy laboratory: staining 0.5 µm sections for viewing with the light microscope, and staining thin sections (500 Å or 50 nm) for viewing in the electron microscope. The first is true staining familiar to those in the histopathology laboratory; the second uses heavy metal "stains" to enhance the electron contrast. Stains will not penetrate epoxy resins readily, so many special techniques are not possible without going through laborious procedures to remove the resin before staining. In my laboratory the best routine stain was found to be a toluidine blue-basic fuchsin mixture originally marketed as Paragon Multiple Stain for Frozen Sections [Martin 1966]. This is a polychromatic stain that is easy and rapid to perform. This stain has given superior results and can be used with both Epon- and Spurr-embedded sections, whereas the Paragon Epoxy Stain will not stain Spurr-embedded sections well. Other useful, rapid techniques for staining 0.5 µm or "thick" sections are alkaline toluidine blue and basic fuchsin-methylene blue.

Staining 0.5-µm Sections

TOLUIDINE BLUE-BASIC FUCHSIN PROCEDURE [MARTIN 1966]

Staining Solution

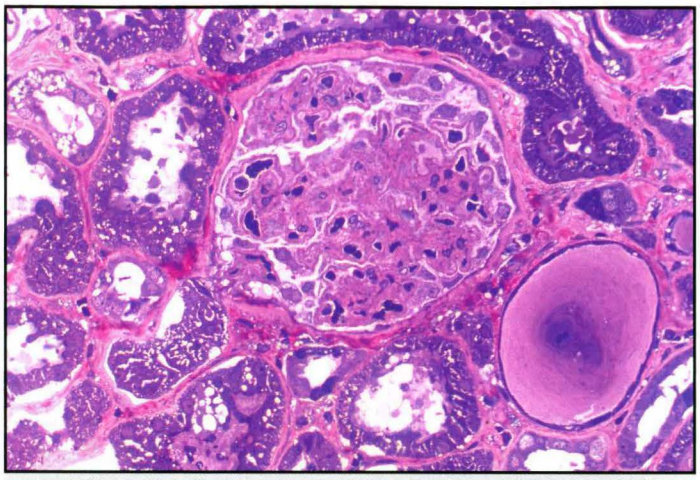
Toluidine blue, CI 52040	0.73g
Basic fuchsin, CI 42510	0.27g
Ethyl alcohol, 30%	100 mL
Mix well and keep tightly capped	

■ *Procedure*

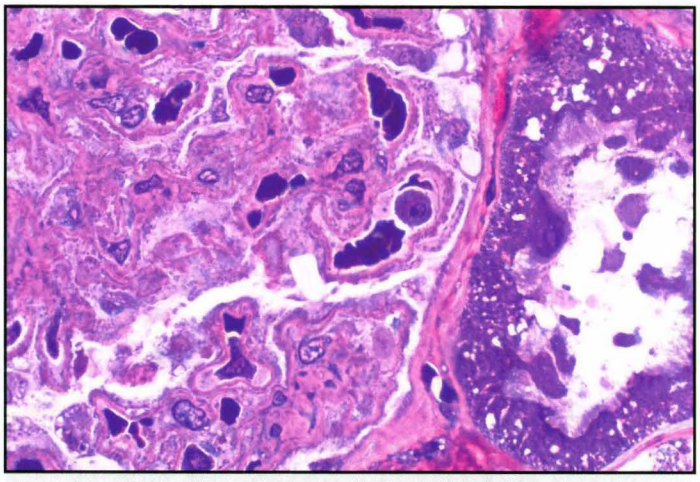
1. Cut thick sections (0.5 to 2.0 µm) of the epoxy embedded material, and place them on a drop of distilled water on a clean glass slide. A true green-colored section is ideal. If the section is too thick, it tends to wash off.

2. Place the slide with the sections on a hot plate at approximately 200°C. Allow the water to evaporate and the sections to dry.

3. Drop several drops of the toluidine blue-basic fuchsin stain on the dried sections. Add a minute amount (pinch) of powdered sodium borate to the heated stain on the specimen. The borate cannot be added to the stock solution, because it will cause the stain to become unstable. Continue heating for about 30 seconds or until the stain begins to steam and assumes a metallic green scum over the surface.



[i14.8] A 0.5-μm section from the block shown in **[i14.5]** stained with toluidine blue-basic fuchsin stain. The tissue was fixed in modified Millonig formalin solution. Examination with the light microscope will indicate the area of primary interest so that further trimming of the block can be done. The disease demonstrated in this section of kidney is membranous glomerulonephritis.



[i14.9] A higher magnification of the stained section shown in **[i14.8]**. A part of a kidney glomerulus is shown in the photograph.

4. Using a wash bottle with distilled water, gently wash off all excess stain.

5. Blot dry with bibulous paper.

6. Allow to continue air drying (drying in the oven oxidizes and decolorizes the sections) for several minutes, and then apply a coverslip with a commercial low-viscosity toluene-based acrylic resin.

■ *Results* **[i14.8], [i14.9]**

• Nuclei	Dark purple
• Cytoplasm	Pink to lavender
• Fat	Gray-green to gray-blue
• Red blood cells	Magenta

■ *Technical Notes*

1. Fresh distilled water should be used. Any bacteria or trash in tap water or distilled water that has been standing may adhere to the section, will stain, and will interfere with photography as a superimposed artifact.

2. Keep the stain capped. As soon as precipitate shows in the sections and is not a result of overheating the slide while staining, discard and prepare fresh stain.

TOLUIDINE BLUE STAINING

■ *Reagents*

Toluidine Blue, 2%

Toluidine blue	2 g
Distilled water	100 mL

Sodium Borate 2%

Sodium borate	2 g
Distilled water	100 mL

■ *Procedure*

1. Cut and dry sections as in steps 1 and 2 under the toluidine blue-basic fuchsin procedure.

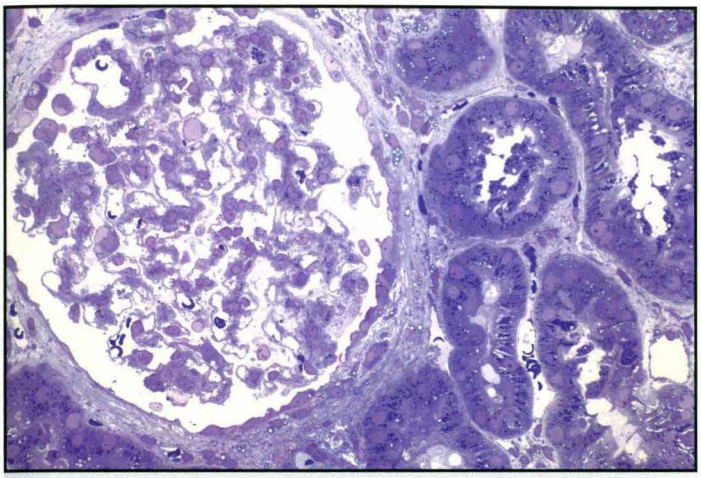
2. Mix equal parts of 2% toluidine blue and 2% sodium borate, and filter through Whatman #1 filter paper.

3. With the slide on the hot plate at approximately 65°C to 95°C, flood the area over and around the sections. Stain for 1 to 2 minutes.

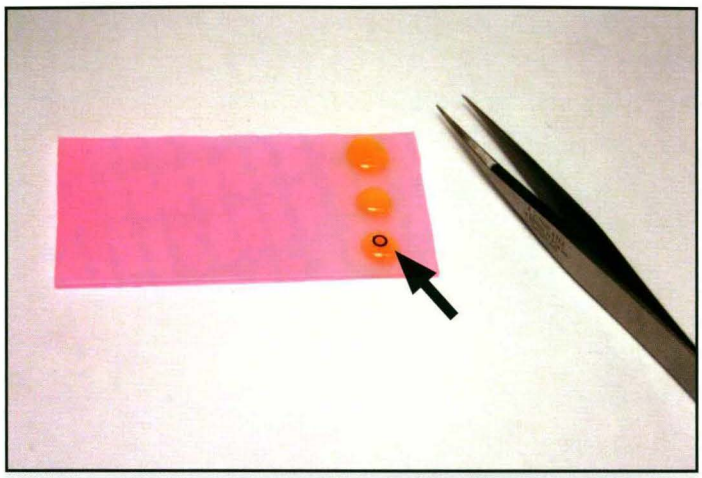
4. Rinse well with a stream of distilled water, and then dip the slide in absolute alcohol. Allow the slide to air-dry thoroughly, and coverslip with a low-viscosity toluene-based acrylic resin.

■ *Results* **[i14.10]**

• Nuclei	Dark purple
• Cytoplasm	Lavender
• Fat	Gray-green to gray-blue
• Red blood cells	Deep blue to purple



[i14.10] A 0.5-µm section of kidney stained with toluidine blue. The tissue was fixed in Millonig formalin solution. [Image courtesy of Lott R, Birmingham, AL]



[i14.11] Single grids are stained with uranyl acetate by floating (arrow) on the solution, section side down. Uranyl acetate staining may also be done in-block during processing.

Staining Thin Sections

Lead Citrate Solution [Reynolds 1963]

1. Boil 100 mL distilled water for 30 minutes.

2. To 30 mL of the freshly boiled water in a plastic bottle add:

Lead nitrate [$Pb(NO_3)_2$]	1.33g
Sodium citrate [$Na_3(C_3H_5O_7)\ 2H_2O$]	1.76g

3. Shake solution vigorously for 1 minute. Allow to stand for 30 minutes with intermittent shaking (10 seconds each minute). This ensures complete conversion of lead nitrate to lead citrate.

4. To 18.5 mL of the boiled distilled water, add 1.5 mL Acculute 1N sodium hydroxide (Anachemia Chemicals Ltd, Rouses Point, NY).

5. Add the sodium hydroxide solution to the lead citrate solution. Invert once. Avoid further agitation of the stain when using. The solution keeps well and may be used for several months. Any precipitate will settle, and only the supernatant solution should be used.

■ *Procedure*

1. Pick up sections on the dull side of 200-mesh copper grids.

2. Place the grids containing the sections on a clean piece of filter paper, section side up. Allow them to air-dry for several minutes under a Petri dish. Any dust must be avoided if possible.

3. While the grids are drying, prepare a saturated solution of uranyl acetate in 50% ethanol. Put approximately 0.2 g of powdered uranyl acetate in a clean glass vial and add approximately 3 mL of absolute ethanol and 3 mL of distilled water. Wear protective clothing. Use a stopper, mix well, and filter some of the saturated uranyl acetate solution into the depressions of a color reaction plate that has been scrubbed with scouring cleanser, rinsed with distilled water, and dried, or drop on a piece of wax.

4. Stain sections in the uranyl acetate for 1 to 3 minutes depending on the section thickness (stain thinner sections longer) by gently floating the grid on the surface of the stain, specimen side down [**i14.11**].

5. Dip the grid for 1 to 2 seconds in a 50% solution of methanol and distilled water to help wash the grid free of any remaining uranyl acetate crystals (optional step).

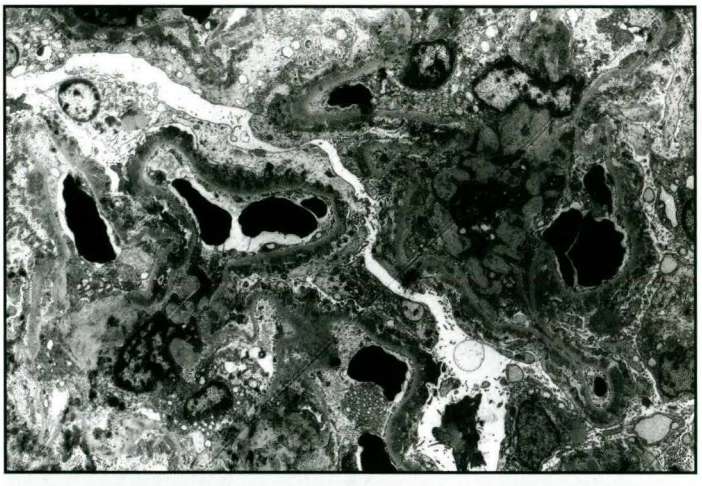
6. Rinse the grid in at least 6 changes of distilled water, at least 10 dips in each change.

7. Dry the grid by placing a piece of filter paper between the tines of the forceps and advancing it toward the grid. Gently place the grid back on the filter paper to dry. Cover with a Petri dish to avoid dust contamination.

8. Take a clean Petri dish lid, invert, and place a small container of sodium hydroxide pellets and a piece of wax underneath. The sodium hydroxide will absorb any carbon dioxide from the air, and aid in obtaining precipitate-free stained sections.

9. Place a couple of drops of lead citrate (more if indicated by the number of grids) on the wax, fload the grids on the drops section side down for 1 to 3 minutes, depending on the section thickness and stain intensity desired. *Do not breathe on the grids or stain during this step because your breath contains a very high concentration of carbon*

[i14.12] Grids are stained with lead citrate in a carbon dioxide-free atmosphere to avoid stain precipitate. This may be done by placing a small container of sodium hydroxide pellets under a Petri dish for a few minutes before adding the drop of stain. The grid is placed on the drop of stain, section side down (arrow).



[i14.13] Electron micrograph of the kidney shown in **[i14.9]** and **[i14.10]**. The specimen was fixed in modified Millonig formalin solution and postfixed in osmium tetroxide.

dioxide, which will cause staine precipitate ($PbCO_3$) on the grids **[i14.12]**.

10. Wash in 6 changes of distilled water, at least 10 dips in each change.

11. Dry the grid with filter paper as described before.

12. When the grid is dry, it is ready to be placed in the electron microscope.

■ *Results* **[i14.13]**

Tissue structures will either deflect electrons (electron dense) and will appear as dark structures on the fluorescent screen, or they will transmit electrons (electron lucent) and will be pale to invisible. The heavy metal stains have been taken up by the structures that appear electron dense (ie, membranes).

■ *Technical Notes*

1. Most glassware is slightly soluble at the alkaline pH of this "stain," and the carbonate $(CO_3)^{2-}$ ion contained in the glass can thereby combine with the Pb^{2+} ion to form the relatively insoluble lead carbonate salt. This will contaminate the sections on the grids. For this reason, plastic containers are more desirable when preparing the lead solution.

2. Use chemicals of analytical reagent grade because divalent lead salts of volatile acids tend to decompose with the formation of lead oxides on storage. If old lead salts are used, the final staining solution will be turbid and tend to stain poorly.

3. A carbon dioxide-free sodium hydroxide solution should be used to prepare the lead solution. Acculute 1N NaOH (40.0 mg/mL NaOH), carbon dioxide free, is recommended.

4. This method is good when only 1 or 2 grids are to be stained. When multiple grids are to be stained, an electron microscopy multiple grid holder should be used. 24 grids can be stained at 1 time using approximately 10 mL of stain.

5. Lead salts are potentially highly dangerous substances because lead accumulates in the body and can easily reach toxic levels. If old solutions are allowed to dry or stains are spilled, very fine powders are apt to result, which can be picked up easily on the hands. Protective clothing should be used when handling lead solutions. Uranium acetate is highly toxic with a radiation hazard from inhalation of fine particles. Radioactivity is blocked by nearly all materials (glass containers, gloves, and clothes); however extreme care should be used when handling the powder. Gloves, goggles, and an apron should be worn. Hands should be washed thoroughly after handling.

Special Techniques

While the aforementioned techniques are basic to any electron microscopy laboratory, occasionally special problems arise that preclude routine handling. Techniques for handling some of these unusual or nonroutine specimens follow. It is not intended as an exhaustive compendium of special techniques, but focuses on those found to be of special help in surgical pathology.

BLOOD CELL PREPARATION

If ultrastructural studies are desired on platelets, leukocytes, or erythrocytes, sections can be easily obtained with the following procedure [Anderson 1965]:

1. Using a syringe, withdraw approximately 7 mL of venous blood, and gently transfer to a 10 mL centrifuge tube containing 12 mg of ethylenediamine tetraacetic acid or 35 to 45 U of heparin.

2. Mix anticoagulant and blood by very gentle inversion of the tube.

3. Centrifuge at 1,000 rpm for 10 minutes.

4. Using a plastic pipette, carefully withdraw the supernatant and gently layer phosphate-buffered glutaraldehyde fixative (formaldehyde will not harden the buffy coat sufficiently for the next step) over the buffy coat.

5. Let fix for 30 minutes, and then lift the buffy coat disk out of the tube. This disk will have layered platelets, leukocytes, and some erythrocytes.

6. Cut the disk in thin slices, and place in phosphate-buffered osmium fixative. Fix and process by a routine method as previously described.

CELL SUSPENSIONS (FLUIDS, CULTURES, PARASITES, ETC)

The following procedure is for cell suspensions only when a firm cell pellet cannot be obtained. For cell cultures, material is available commercially on which cells can be grown and then processed as a routine specimen.

1. Centrifuge well-fixed cell suspensions at 1,000 rpm for 10 minutes. Decant the supernatant.

2. Resuspend cells in approximately 1 mL of fixative or buffer solution.

3. Withdraw cell suspension from tube with a tuberculin syringe.

4. Inject cells into a sealed bag prepared by heat sealing a Nuclepore filter (47-mm diameter, 5-μm pore size). Cut the sealed filter into 2 bags [**f14.2**].

5. For transmission electron microscopy, process bag as if it were a tissue section, using the routine procedure, beginning with osmium tetroxide fixation.

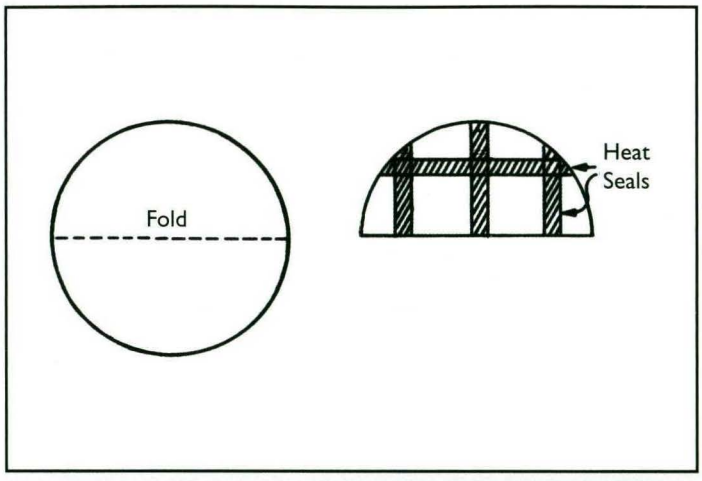
6. Flat embed specimens. After polymerization, use a microscope to locate cell clusters. Cut out and glue to block for sectioning.

7. The bag containing fixed cells may also be critical point dried, opened, and mounted cell side up for scanning electron microscopy.

Processing Tissues Previously Embedded in Paraffin

If the tissue was well fixed originally in a fixative suitable for electron microscopy, then this technique can prove to be of considerable value when all tissue has been processed in paraffin.

1. Using a sharp blade, carefully remove the desired tissue from the paraffin block. All excess paraffin should be removed, taking care not to break the tissue.



[**f14.2**] Method for making bags for processing cell suspensions.

2. Remove the paraffin with xylene, 2 changes for 2 hours each, then 1 change for 30 minutes.

3. Rehydrate with the following schedule:

• Absolute ethyl alcohol, 2 changes	10 minutes each
• Ethyl alcohol, 95%	10 minutes
• Ethyl alcohol, 70%	10 minutes
• Ethyl alcohol, 50%	10 minutes
• Phosphate buffered formaldehyde	1-2 hours

4. Place in fresh fixative and leave overnight. Rinse with phosphate buffer if desired.

5. Fix with osmium tetroxide and process as usual.

Processing Tissue from an H&E-Stained Paraffin Section

1. Remove the coverslip, and be sure all mounting medium is removed with xylene.

2. Rehydrate the slide through alcohols to water, and then place in phosphate-buffered formaldehyde overnight. Rinse with buffer if desired.

3. Using a glass cutting tool, cut the slide to smaller dimensions by cutting off the excess glass.

4. Place the slide, section side up, in a plastic container, and flood the section with osmium tetroxide. Allow to fix for approximately 30 minutes.

5. Proceed with processing as usual. Do not let section dry at any point.

6. To embed, place the slide, section side up, in a plastic container lid. Keep the section covered with fresh catalyzed resin. Pour a layer of resin over the slide and polymerize at 60°C for approximately 18 hours.

7. Remove the lid. The slide may be separated by immersing the slide-section-resin in liquid nitrogen for several seconds and then placing on the countertop. As warming occurs, the slide should pop free.

8. Cut out the portion of tissue desired and attach to a blank epoxy block with epoxy glue. If orientation is needed, the entire resin-embedded section can be placed on the warming plate and stained with toluidine blue-basic fuchsin stain as previously described.

9. Section carefully, as the tissue is very thin. (Please note that while this is not the most desirable technique, it does not allow electron microscopic studies on small lesions that have been totally embedded for viewing with the light microscope.)

Acknowledgment

I am deeply indebted to the American Society for Clinical Laboratory Science (formerly ASMT) for granting permission to reprint much of the material in this chapter from the original publication [Carson 1979].

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LEARNING ACTIVITIES

1. **Visit an electron microscopy laboratory and observe the following:**

   a. Mincing of tissue for processing, and compare the size to the sections cut for paraffin processing.

   b. Processing and embedding of specimens for electron microscopy, and compare the process to paraffin processing and embedding.

   c. Sectioning of blocks for electron microscopy and compare to paraffin microtomy.

   d. The image projected on the screen of the electron microscope, and locate the nucleus and some of the different cytoplasmic organelles.

2. **Practice the technique of mincing tissue to the appropriated size for electron microscopy processing.**

3. **Process and embed some of the minced tissue.**

4. **Cut a 0.5 micron section using a glass knife, and stain with the stain used in the laboratory. Examine microscopically.**

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CHAPTER 15

Cytopreparatory Techniques

Beth Cox, BS, SCT/HT(ASCP)

OBJECTIVES

On completing this chapter, the student should be able to do the following:

1. Define:
   a. diagnostic cytology
   b. liquid based cytology
   c. cross contamination
   d. cell block
2. Differentiate between gynecologic and nongynecologic specimens
3. Explain the use and composition of pre-fixatives
4. Explain the effect of pre-fixatives on cellular morphology
5. List acceptable fixatives for cytology
6. Summarize 3 slide smear methods
7. Describe methods to:
   a. handle sparsely cellular specimens
   b. reduce red blood cells in smears
   c. increase cellular adherence to slides
   d. remove excess mucus
8. State the effect on staining if Carbowax fixatives are not removed before staining
9. Describe methods for making cell blocks
10. State the purpose of the Pap stain
11. Outline the Papanicolaou staining technique, considering:
    a. primary reagents and/or dyes
    b. mode of action
    c. results of staining
    d. potential sources of error and appropriate corrective actions
12. Discuss how cross contamination occurs, and recommend techniques that can be used to prevent it.

Cytopreparation

Diagnostic cytology is the microscopic examination of cellular material for the diagnosis of disease, particularly cancer. Whereas histologic diagnosis relies on the architecture of the tissue to help determine the disease process, cytologic diagnosis relies on examination of individual cells and their morphology to give clues as to the cells' health and activity.

Cytologic specimens can be obtained from any body site, but are generally categorized as either *gynecologic* or *nongynecologic*. Gynecologic cytology, often referred to as "gyn" cytology, includes specimens from the female genital tract, notably from the cervix, endocervix, and vagina. Specimens from these sources, commonly called "Pap smears," were advocated by George Papanicolaou, MD in the 1940s. Nongynecologic, or "non-gyn," specimens, are from all other body sites. Common nongynecologic specimens include urine, sputum, cerebral spinal fluid, bronchial/gastric/esophageal washings or brushings, pleural/peritoneal/ascites/pericardial fluids, and fine-needle aspirations.

After cytology specimens are collected, they are brought to the laboratory for cytopreparation. Cytopreparation is the act of preparing and staining the microscopic slides to be examined for diagnosis. This step has a major impact on the final diagnosis, because cells that are poorly prepared, fixed, and stained can alter diagnoses. A false-positive or false-negative diagnosis can result from negligent cytopreparation. The preparation technique should be selected according to the characteristics of the specimen to ensure that a uniform, homogenous, and well-fixed sample is available for diagnosis. No single method will give the best results for all specimens. Although automated thin-layer preparation systems are available, simple manual techniques can provide optimal preparations.

Good cytopreparation requires an extensive understanding of fixation and staining. This makes the trained histotechnologist a good candidate to take responsibility for cytopreparation.

After cytopreparation and staining, the specimens are examined by the cytologist or pathologist. All cells on the slide are carefully screened to identify potential abnormalities. The cytologist's primary goal is to identify malignant and premalignant cells, but many other disease processes can be identified. These include bacterial and viral infections, and many nonmalignant conditions. Any potentially abnormal cells are noted and marked. The cytologist then uses all clues found on the slide to make a decision about the disease process present.

Collection

GYNECOLOGIC CYTOLOGY

Conventional gynecologic Pap smears are collected by the clinician; smeared in a thin, even layer on a slide; and fixed immediately while still wet. After fixation, the slides may be dried and sent to the laboratory for staining. Pap smears for liquid-based cytology techniques are collected with a cervical brush or broom, and the instrument is rinsed in a vial of commercial preservative to be forwarded to the laboratory. The BD SurePath Pap test (Becton Dickinson, Franklin Lakes, NJ) requires that the head of the brush is left in the preservative vial to be sent to the laboratory. The Cytyc ThinPrep PapTest (Cytyc Corp, Marlborough, MA) requires that the head of the brush is ***not*** included with the vial.

NONGYNECOLOGIC CYTOLOGY

It is preferable that all nongynecologic specimens are collected fresh, with no fixative or additive, and brought to the laboratory immediately. Realistically, short delays will not cause ill effects, and most fresh specimens can be held for 24 to 72 hours if refrigerated. They should never be frozen.

■ ***Body Fluids: Pleural, Peritoneal, Pericardial and Ascites***

The entire amount collected should be sent to the laboratory, rather than submitting a sample. Some laboratories choose to add heparin to their body fluids at the time of collection to prevent the specimen from clotting. This is not a requirement, because the clot often entraps the cells of interest and can be removed and wrung out; it makes an excellent cell block.

■ ***Breast/Nipple Discharges***

The clinician should prepare smears at the time of collection by using a circular motion to smear the material onto a labeled slide in an area the size of a nickel. The slides should be spray-fixed with cytology fixative immediately before any air drying can occur.

■ ***Cerebral Spinal Fluids***

The cells in these specimens deteriorate rapidly, so if the specimen cannot be brought to the laboratory for immediate preparation, use of a pre-fixative holding solution is recommended. To do this, dilute the specimen with an amount of alcoholic saline or Saccomanno fluid equal to the volume of the specimen.

■ ***Direct Scrapings for Viral Lesions (Tzank Smears)***

The clinician should prepare smears at the time of collection by using a circular motion to smear the material onto a slide in an area the size of a nickel. The slides should be spray-fixed with cytology fixative immediately before any air-drying can occur.

■ ***Washings: Bronchial, Esophageal, and Gastric***

Do not add fixative to the specimen. All specimens should be refrigerated until received by the laboratory.

■ ***Brushings: Bronchial, Esophageal, and Gastric***

Prepare direct smears at the time of collection by using a circular motion to smear the material onto a slide in an area the size of a nickel. The slides should be spray-fixed immediately, before any air-drying can occur. Alternately, the brush may be submitted in

physiologic saline for processing in the laboratory. Never place the brush in a fixative solution before cytopreparation, because this will significantly impair cellular adhesion to the slide and cell loss may occur.

■ *Urine*

A first morning urine specimen is not recommended for cytologic evaluation, because the cells may be degenerated after spending extended time in an acid environment in the bladder. Urine specimens are more fragile because of their acid pH, and do not tolerate long delays before cytopreparation; therefore, a pre-fixative solution may be added to the specimen to protect the cells. An amount of alcoholic saline or Saccomanno fluid equal to the volume of the specimen should be added.

■ *Fine Needle Aspirations*

Slides may prepared at the time of aspiration by placing 1 drop of the aspirated fluid on a slide, laying another on top and pulling the slides apart endways, then immediately fixing them (see the section on smear techniques for details). It is never recommended to do "feathered-edge" smears, as in hematology, because these will always show air-drying of the most significant cells at the feathered edge. After smear preparation, any material remaining in the needle may be rinsed in physiologic saline and sent to the laboratory. If a fixative is used to rinse the needle, the cells will not adhere to the slide well and cell loss may occur. If fluids are aspirated (cysts, etc), no fixative should be added and the specimen should be transported to the laboratory immediately for processing.

If the clinician needs an evaluation of the cellularity of the specimen to determine adequacy, either a toluidine blue wet film may be made, or a slide may be air-dried and stained with Diff-Quik, a Romanowsky-type stain.

Fixation

Rapid fixation in alcohol is an essential part of accurate cytologic interpretation. The fixative of choice for all cytology specimens is 95% ethyl alcohol (ethanol). The field of diagnostic cytology is based on the particular chromatin patterns created by this fixative. Other fixatives, especially formalin, are to be avoided because they create different chromatin patterns and may hinder diagnosis. When ethyl alcohol is not available, 95% denatured alcohol can be substituted successfully. Commercial cytology spray fixatives provide a convenient substitute. They generally contain the necessary alcohol, a small amount of acetone to facilitate fast drying, and polyethylene glycol to provide a thin protective coating. This polyethylene glycol coating must be completely removed by soaking the smears in alcohol for at least 15 minutes before staining, or the nuclear staining will be impaired.

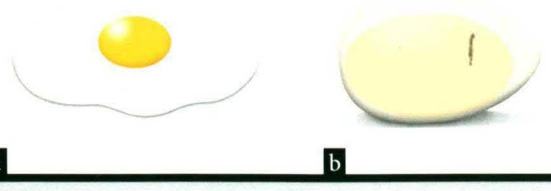
Alcohol is a dehydrating agent that causes cells to shrink as it removes or replaces water. This sharpens the nuclear membrane, resulting in the nuclear detail characteristic of cytologic preparations. All alcohols do not cause the same amount of shrinkage; therefore, it is necessary to understand the effects of the various alcohols so that the proper concentration can be chosen as a fixative. Methyl alcohol causes less shrinkage than ethyl alcohol; therefore, 100% methyl alcohol is a substitute for 95% ethyl alcohol. Isopropyl alcohol causes more shrinkage; therefore, 80% isopropyl alcohol can be substituted for 95% ethyl alcohol [**t15.1**].

[t15.1] Substitutes for 95% ethyl alcohol for cytologic fixation

95% denatured alcohol
Commercial cytology spray fixatives
80% isopropyl alcohol
100% methyl alcohol

Optimally, cytology smears are prepared with fresh unfixed specimens and the material is wet-fixed on the slide. This method promotes adhesion of the cells to the slide. It also causes them to spread out and flatten, allowing better visualization of the cellular morphology. Sometimes it is necessary to pre-fix specimens while they are still in a liquid state. Pre-fixed specimens produce cells that are "rounded-up"; they appear smaller and denser, and the fine morphologic details are more difficult to visualize. Cytologists must adjust their criteria when diagnosing cells that were fixed before making the smear. To understand this, think of the difference in appearance between a fried egg and a boiled egg. The fried egg, like the cells fixed onto the slide, flattens out significantly, increasing the diameter and thinning out the egg white/cytoplasm [**f15.1a**]. The boiled egg and rounded-up cell appear to have much smaller diameters, and the yolk or nucleus inside can barely be seen [**f15.1b**].

Fixation should occur immediately, while the specimen is still wet on the slide. Unfixed cells smeared on a slide will begin to air-dry very rapidly; therefore, application of the fixative must occur within 1 to 2 seconds of the cells' contact with the slide. Air-drying cells causes nuclear swelling, distortion, and loss of cytoplasmic density. Nuclear chromatin patterns are lost, and cytoplasm becomes eosinophilic, losing the metabolic differentiation. The effects caused by air-drying of cells can make diagnosis impossible. Therefore, any chance of air-drying is to be strictly avoided. When using spray fixatives, the slide should be flooded with the liquid. A light misting of the smear will not provide adequate fixation, and air-drying artifact will occur.



[f15.1] a, Cells fixed onto a slide flatten out like a fried egg, spreading out the cytoplasm/egg white and making the nucleus/yolk easily visible; **b**, cells fixed in a liquid remain rounded up like a boiled egg, and appear smaller and denser.

Single or thin layer cell preparations fix quite rapidly in alcohol, generally within 1 to 2 minutes. Thick smears or those containing abundant mucus could take twice as long. Cells in a liquid suspension can take much longer to fix, because the alcohol will be diluted by the liquid in the specimen.

Specimens that are collected fresh should be brought to the laboratory and prepared immediately. Short delays will not cause ill effects, and most fresh specimens can be held for 24 to 72 hours if refrigerated. Urine specimens are more fragile because they have acid pH and do not tolerate long delays. Also, the cells in cerebrospinal fluid are very delicate and are intolerant of long delays. Both of these types of specimens may require the use of a pre-fixative to preserve ideal morphology when cytopreparation is delayed.

PRE-FIXATIVES

The use of pre-fixatives has become a common practice to accommodate cytology specimens that must be transported distances or held for some time before cytopreparation. These are generally solutions with lower alcohol content that partially fix the cells to protect them from autolysis and putrifaction. This is not a substitute for final fixation in the appropriate-strength alcohol. Common pre-fixatives include Saccomanno fluid and alcoholic saline. There are many commercial pre-fixatives available under various names.

Saccomanno Fluid

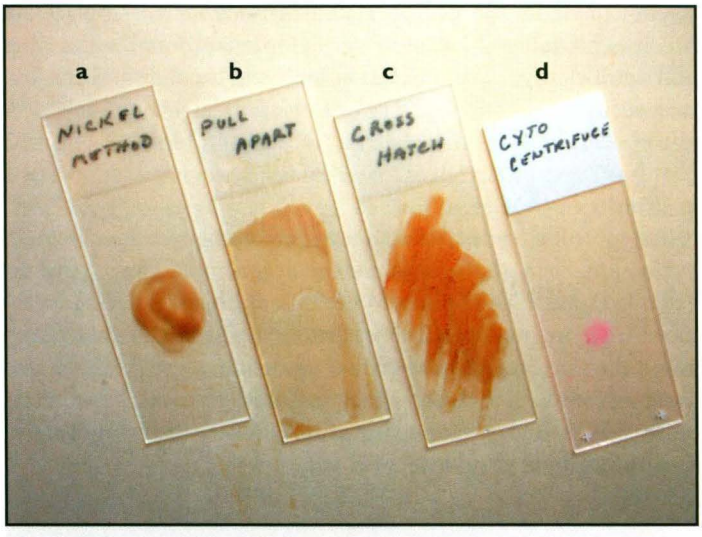
50% ethyl alcohol	980 mL
Carbowax, melted	20 mL

Alcoholic Saline

Saline	1 part
50% alcohol	1 part

Thin layer technologies have developed proprietary pre-fixative solutions containing weak alcohol and antimicrobial compounds. The Cytyc Corporation (ThinPrep) uses a weak methyl alcohol-based pre-fixative, and the SurePath system uses a weak ethyl alcohol-based solution. Cells from these preparations must still receive a final fixation in 95% alcohol after the smears are made.

Smears made from bloody fluids can be dropped into a Coplin jar containing Clark or Carnoy fixative (see chapter 1, "Fixation," p23) solution to help remove obscuring red blood cells (RBCs). The alcohol in these fixatives produces the necessary nuclear detail and the acetic acid lyses the RBCs. Fixation will occur in 2 to 4 minutes. Smears left in Carnoy or Clark solution for too long will lose chromatin detail. To prevent loss of nuclear detail, slides should be transferred to 95% ethyl alcohol (or equivalent) after 5 minutes in Clark or Carnoy solution.



[i15.1] Samples of smear methods: **a**, nickel method; **b**, pull-apart method; **c**, crosshatch method; **d**, cytocentrifuge preparation.

Smear Preparation

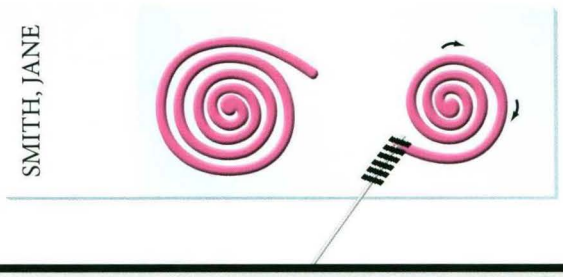
Many different methods are available to prepare cytology smears **[i15.1]**. However, certain techniques work best for particular specimen types. Understanding each technique will help determine the best method for individual specimens, avoiding methods that could produce suboptimal results and hinder diagnosis. Simple manual techniques can provide excellent preparations with a minimum of supplies and equipment. Basic equipment such as a cytocentrifuge can assist with problem specimens. Automated liquid-based methods can provide uniformity and standardization. Regardless of which method is chosen, the goal should always be to obtain an even monolayer of cells with excellent preservation of morphologic detail.

DIRECT SMEARS

Direct smears include scrapings, brushings, Tzank smears, viral inclusion smears. Often clinical personnel make cytology smears at the time of collection. This requires a method that is easy to communicate, simple to perform, and resists air-drying.

■ *Nickel Method*

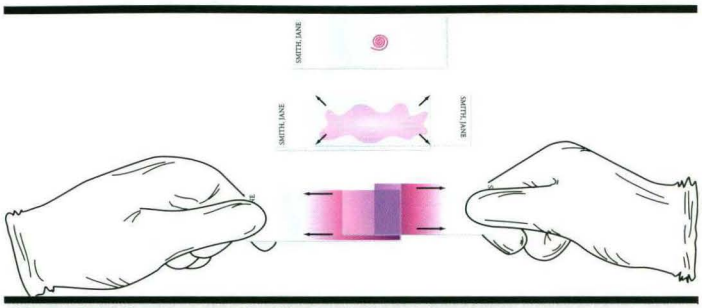
The *nickel method* is a successful technique for this purpose **[f15.2]**. It is easily explained and can be performed quickly. Spreading of the specimen in a tight circular pattern rather than laterally reduces the possibility of air-drying. Avoid cotton swabs to collect and smear cytology slides because the fibers absorb moisture from the specimen, causing air-drying and cellular distortion. Feathered-edge smears like those done for hematology should also be avoided because the diagnostic cells are pushed to the edge of the smear where they immediately air-dry and become useless for diagnosis.



[f15.2] Nickel method

Using a circular motion, smear the material from the scraper or brush onto a labeled slide in an area the size of a nickel.

Fix immediately by submersing in 95% ethyl alcohol, or by flooding the slide with cytology spray fixative.



[f15.3] Pull-apart method

Place 2-3 drops of sediment on a labeled glass slide.

Place another labeled glass slide upside-down on top.

Allow the sediment to spread naturally.

Gently pull the slides apart endways

Fix immediately to prevent air-drying, using 95% ethyl alcohol or a cytology spray fixative.

FLUIDS

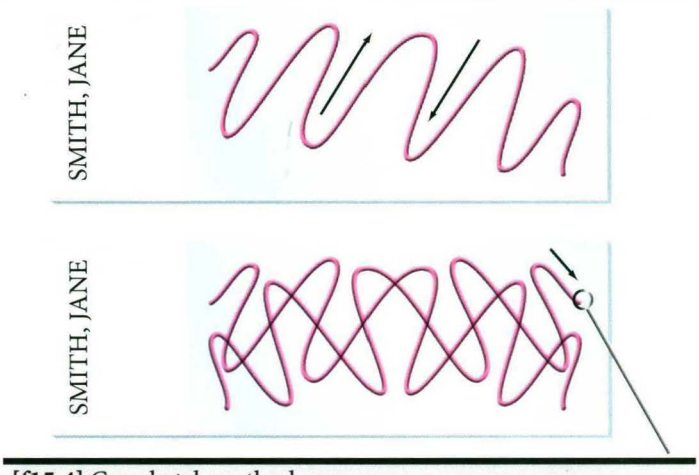
This includes cellular fluids such as pleural, peritoneal, pericardial, ascites, effusions, urine, cyst fluids, and washings (thin bronchial, esophageal, or gastric). Many cytology specimens are received in the laboratory as cells suspended in fluid. The first step is to concentrate the cells by centrifuging a representative sample of the specimen, and the resulting supernatant is poured or aspirated off, leaving the sediment intact at the bottom of the tube. Centrifugation for cytology is best performed at 2,000 rpm for approximately 10 minutes. Higher speeds will pack the cells too tightly, making it difficult to separate the clumpy sediment and produce an even monolayer on the smear. Lower speeds will not force the cells to the bottom of the tube, decreasing the chance of getting a representative sampling of the cells. A 50-mL conical tube is best; samples from 15-mL conical tubes generally do not produce an adequate specimen. A swinging-arm centrifuge will ensure that the cells are concentrated at the bottom of the conical tube rather than along the side. Once the cell button has been prepared, 1 of the following methods may be used to prepare the smear. If no cell button is visible in the conical tube after centrifugation, the specimen should be handled as a sparsely cellular specimen.

■ *Pull-Apart Method*

The pull-apart method is a good general purpose technique for cellular fluids without mucus. It is simple and gives an even monolayer of cells [**f15.3**].

■ *Crosshatch Method*

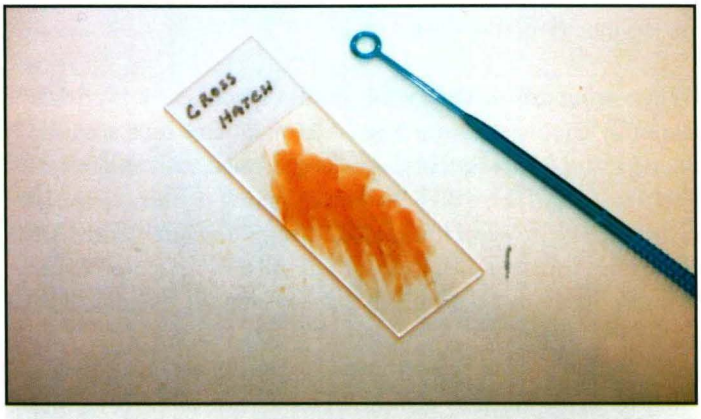
The crosshatch method is an excellent choice for cellular body fluids, especially those containing abundant blood [**f15.4**], [**t15.4**]. Spreading the sample back and forth deposits the larger, more diagnostic cells at the ends of the "arms" and successfully separates them from the blood cells. This method can be done with a small wire loop or a 10-µL disposable loop (the type used in microbiology). Practicing this technique a few times will help achieve a natural spreading pattern [**i15.2**].



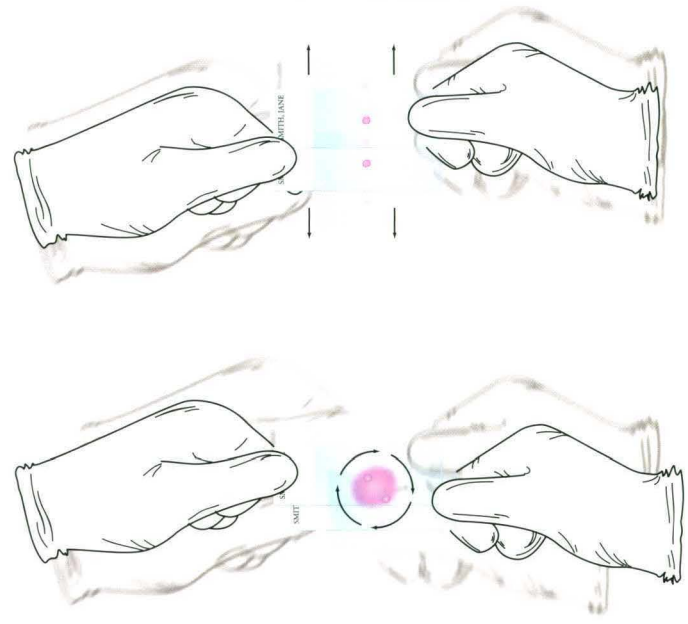
[f15.4] Crosshatch method

Using a disposable loop, place 2 or 3 loopfuls of sediment on a labeled glass slide. Working quickly, spread the specimen diagonally, going back and forth and corner to corner.

Fix immediately to prevent air-drying by quickly submersing in 95% ethyl alcohol or flooding with a cytology spray fixative. Ensure that a sufficient quantity of spray fixative is used. If the sediment beads form, spread it again with the wire loop, attempting to get a monolayer of cells.



[i15.2] A slide prepared by the crosshatch method, along with the wire loop used.

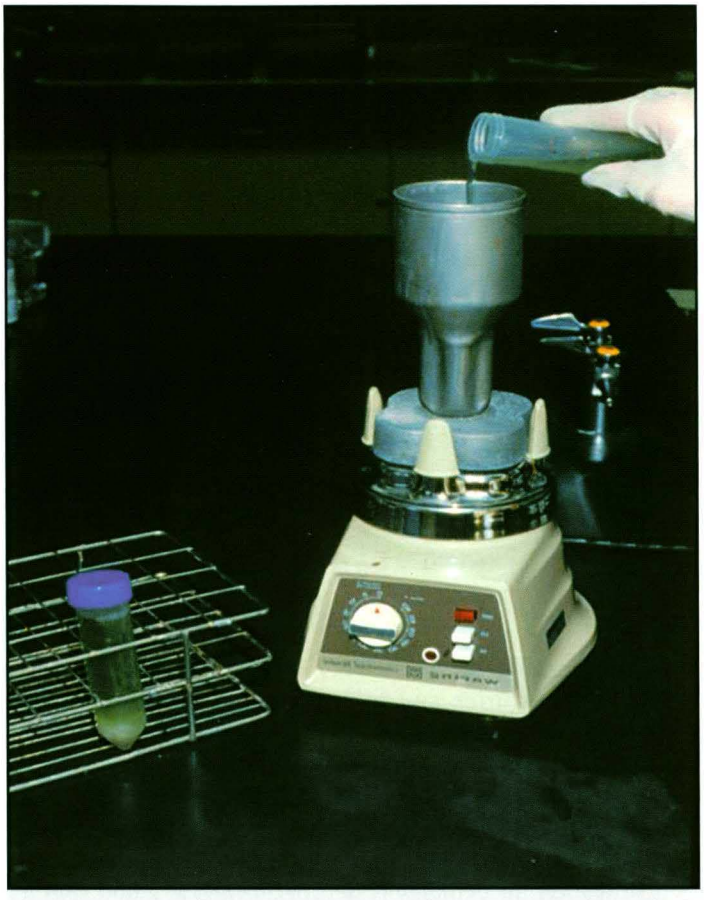


[f15.5] Crush (mash) method

Pick out any streaked or blood-tinged material and place on a labeled glass slide.

Place another labeled glass slide on top, and mash the material forcibly, but carefully, between the two slides, to yield a smear that is thin and evenly distributed over the entire slide. A quick "slap" back and forth motion of the slides is helpful to break apart chunks of material that may be in the specimen and thus allow a thin, even preparation.

Fix immediately to prevent air drying, using 95% ethyl alcohol or cytology spray fixative.



[i15.3] The Saccomanno method for sputum uses a microblender to homogenize the specimen and break up the mucus.

MUCOID SPECIMENS

This includes sputum and thick bronchial washings.

■ *Crush Method*

The crush method involves literally mashing the specimen between 2 glass slides using a forceful back-and-forth motion **[f15.5]**. Specimens with thick mucus will require more aggressive action to properly break up the mucus. This manual technique is very useful to effectively distribute the cells and produce an even smear. A key element in this method is to use sufficient force to break up all the mucus on the slide.

When using this method with sputum specimens it is critical to carefully choose the sampling area. Malignant cells are usually found clumped in select areas of the specimen, rather than spread out uniformly. Therefore, random sampling is likely to miss the diagnostic cells. Select thick, blood-tinged or white-flecked areas, which are usually rich in malignant cells. Buff-colored material is more likely to be purulent than to contain diagnostic cells.

Commercial products are available to assist in breaking up mucus in specimens, but they should be used with care. Any product that is harsh enough to destroy the mucus could harm the cellular morphology.

The Saccomanno method for sputum was developed in the 1970s to assist with screening programs for lung cancer. The method requires that sputum is collected into a Saccomanno (Carbowax) solution. In the laboratory, the specimen is homogenized in a microblender to break up all mucus present **[i15.3]**. Then the specimen is centrifuged at a slow speed (1,500 rpm) to differentially separate the mucus from the cells. At this speed, the cells will be forced to the tip of the centrifuge tube, while the broken-up strands of mucus stay in suspension. The supernatant with the mucus strands is poured off, and pull-apart smears are made from the cellular sediment.

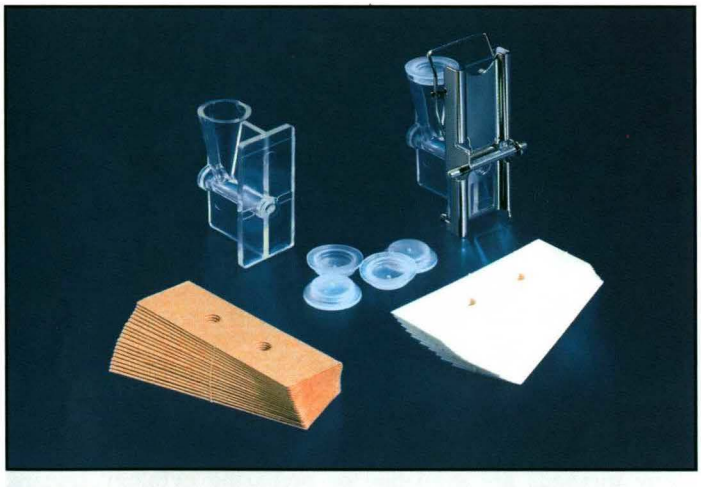
SPARSELY CELLULAR SPECIMENS

These include cerebrospinal fluid and sparsely cellular urines or body fluids. Sparsely cellular specimens provide a special challenge to harvest the maximum number of cells onto the smear. Simply performing conventional methods such as pull-apart or crosshatch smears will rarely provide an adequate number of cells for diagnosis.

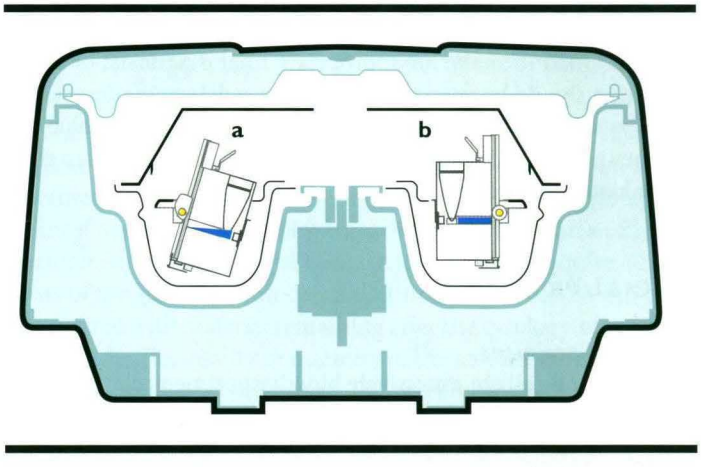
One of the first methods for handling sparsely cellular specimens was to filter the specimen using a cellulose or polycarbonate filter with a small (5 μm) pore size. Essentially all of the cells present in the specimen are captured. The cell-laden filters are then painstakingly stained and carefully mounted onto slides. Special staining protocols and mounting medium are required. This very cumbersome and time-consuming process has largely been abandoned in favor of newer technologies.

[t15.2] Cytocentrifuge preparations

1. Sparsely cellular specimens to be cytospun should be centrifuged, the supernatant poured off, and then the sediment resuspended in approximately 1 mL of cytospin collection fluid or Saccomanno fluid. (The exception to this is cerebrospinal fluids; the volume is usually too low to make centrifugation beneficial. Simply add an equal volume of cytospin-Saccomanno fluid.)
2. Assemble 2 funnel apparatus in the following order: metal or plastic back, labeled slide (face up), cytocentrifuge filter paper (smoothest side down), funnel device. Clip the holder into its slots. Assure that the filter paper, slide, and funnel are aligned with the bottom of the metal back and the top is clipped firmly in place.
3. Using a plastic pipette, place approximately 1 mL of sediment (in Saccomanno fluid) into each funnel. Keep the funnel apparatus tilted while handling so that the fluid does not prematurely contact the filter paper and slide. If the specimen is not sparsely cellular, less sediment will be required.
4. Place a cap on each funnel apparatus.
5. Place the 2 funnel apparatus opposite each other in the cytocentrifuge.
6. Spin the specimens for 7 minutes at 1,000 rpm, using the slow acceleration setting. If all of the liquid has not been absorbed after spinning, the specimens may be spun a second time.
7. Remove the funnel apparatus from the cytocentrifuge.
8. Release the clip of the apparatus, and gently remove the funnel.
9. Peel the filter paper away from the slide, taking care not to disturb the sediment that has been deposited through the window onto the slide.
10. Allow the slides to dry for a minimum of 10 minutes before staining. Cytocentrifuge preparations that have been fixed with a Carbowax solution (Saccomanno or cytospin collection fluid) require a minimum of 15 minutes soaking in 95% alcohol prior to staining to remove the Carbowax which would interfere with staining.



[i15.4] Cytocentrifuge funnel apparatus. [Image courtesy of Thermo Fisher Scientific]

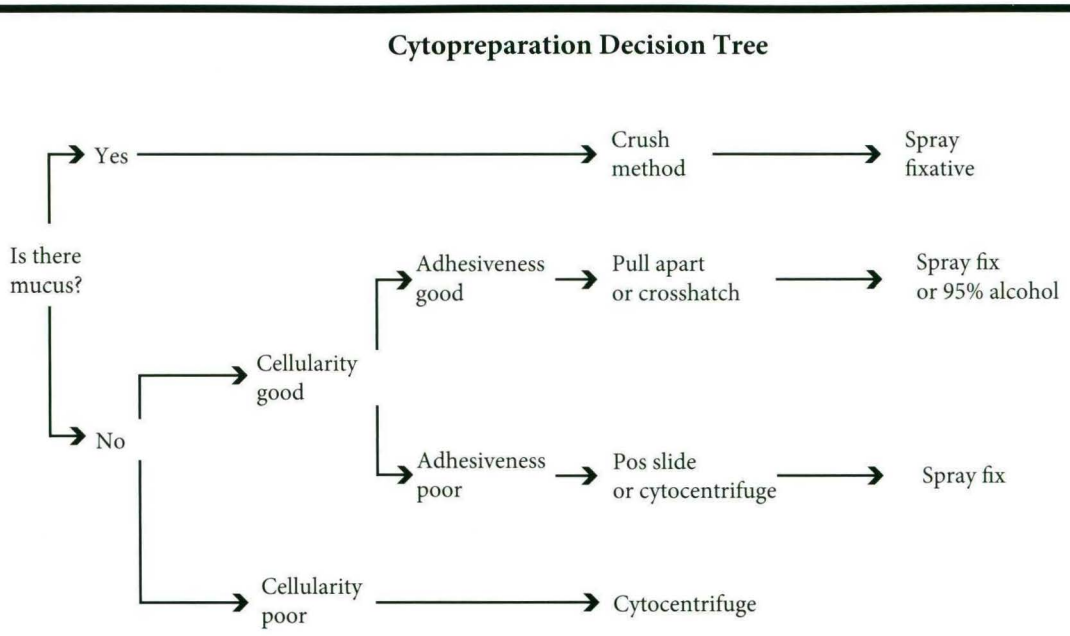


[f15.6] In the load position **a**, the cytocentrifuge funnel apparatus is tilted to prevent specimen contact with the slide. During operation **b**, the funnel apparatus straightens, allowing the specimen to move through the tunnel and be deposited on the slide. [Figure courtesy of Thermo Fisher Scientific]

Cytocentrifugation is the most common method of handling sparsely cellular specimens. The development of special centrifuges, called cytocentrifuges, allows the cells from a very small sample of fluid to be deposited directly onto a slide, while the residual fluid is absorbed away onto filter paper **[t15.2]**. There is very little cell loss with this method, and essentially all of the cells are recovered for diagnosis. A small amount of the cytology specimen is placed in a funnel type apparatus, which is attached to a filter card and a slide **[i15.4]**. This is placed in the cytocentrifuge. When the head of the cytocentrifuge spins, the specimen is forced from the funnel device through the tunnel, where the cells are deposited onto the slide and the fluid is absorbed into the attached filter paper **[f15.6]**.

FINE NEEDLE ASPIRATIONS

These include superficial organs, such as breast and thyroid, and deep organs, such as lung, liver, and pancreas. Aspiration cytology uses a fine-gauge needle (20-26 gauge) to remove a small amount of cell-laden fluid from the site for examination. This procedure is often performed in radiology under ultrasound or computerized axial tomography scan guidance. Usually only a couple of drops of fluid are obtained. The initial drops may be used to make pull-apart smears, and the needle can then be rinsed in physiologic saline to recover any residual specimen. This should not be confused with needle core biopsy, which produces a small sample of tissue and should be handled as a histology specimen.



[f15.7] Cytopreparation decision tree

The physician performing the needle aspiration may request an "adequacy determination" to be made at the time of the procedure. This will confirm if a satisfactory amount of cells have been obtained to make an appropriate final diagnosis, or if the aspiration should be repeated. An adequacy determination can be made by using a drop of the specimen to make a toluidine blue wet film or an air-dried Diff-Quik-stained smear. This slide can then be evaluated for adequacy of diagnostic cells.

SPECIAL PROBLEMS

■ ***Bloody Specimens***

The red blood cells in excessively bloody specimens can compromise the diagnosis by obscuring cellular morphology, or by overpopulating the specimen so that too few diagnostic cells are present on the slide. To overcome this, it is necessary to reduce the red blood cell population so that the diagnostic cells can be visualized; it is not necessary to remove all of the red blood cells. When a direct smear technique is indicated, the crosshatch method can assist in physically separating the red blood cells from the diagnostic cells. Using Clark or Carnoy fixative solution to fix prepared smears will lyse the red blood cells on the slide, making the diagnostic cells more visible.

Commercial products are available that will lyse the red blood cells in a specimen before the smears are made; however, great care must be taken because the lysing action may also cause damage to the cellular membrane of the diagnostic cells.

■ ***Poorly Adhesive Specimens, Particularly Urine and Breast Fluids***

Urine and breast fluid specimens often adhere poorly to the slide, and cells may be lost into solutions during the staining process. Specimens inadvertently fixed in formalin also present this problem. Sometimes these specimens will totally "wash off" during staining, and the slide will be essentially acellular afterwards. This problem can be reduced by using coated or positively-charged slides for these smears. Poorly adhesive specimens prepared by cytocentrifugation are less likely to shed cells than when prepared by direct smearing. Spray fixing poorly adhesive specimens, rather than fixing in liquid, has also been shown to decrease the amount of cell loss from the slide.

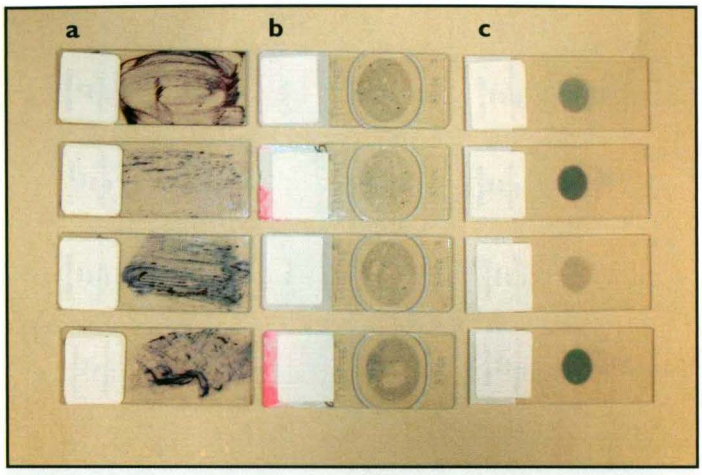
CHOOSING THE BEST METHOD

Many methods are available for preparing cytology smears, but it is very important to choose the right method based on each specimen's characteristics: consistency, cellularity, and volume. There is no single universal method that makes the best cytopreparations for all specimens. For example, not all body fluids will have the same characteristics, and therefore not all body fluids should be prepared by the same method. Specimens that contain mucus require a method to prevent the mucus from obscuring cellular detail. Very low-volume specimens or those with low cellularity require choosing a technique that will retain maximum cellularity onto the slide. High-volume specimens require smears to be made from a true homogeneous representative sample. In very bloody specimens, the red blood cells will outnumber and obscure the diagnostic cells, impairing diagnosis, so a method may be chosen to reduce or remove blood from the specimen. The *cytopreparation decision tree* can assist in those choices [**f15.7**].

Liquid-Based Cytology

One of the greatest obstacles in the development of automated computer image analysis of Pap smears was the poor quality of conventional Pap smears. To overcome this, preparation systems were developed using liquid-based specimens. This resulted in improved slide preparations for both manual evaluation and computerized image analysis.

Two liquid-based preparation systems are most commonly used: the ThinPrep Pap test and SurePath liquid-based Pap test. Although both systems produce a high-quality monolayer slide, they use vastly different methods to achieve this **[i15.5]**.



[i15.5] **a**, Conventional Pap smears; **b**, ThinPrep preparations; **c**, SurePath preparations.

The ThinPrep system uses a filtration method to produce monolayer preparations. Specimens to be prepared by the ThinPrep system are collected into vials containing a preservative solution with a weak methyl alcohol content. The vials are placed into the ThinPrep processor, where the sample is homogenized and blood and mucus are broken up. Next, a negative pressure draws a portion of the fluid through a special filter that collects a thin, even layer of cellular material. The filter is pressed against a labeled slide to deposit the collected cells, and the slide is dropped into a 95% alcohol fixative bath. Slides prepared in this manner are ready to be stained using traditional methods. Both gynecologic and nongynecologic specimens can be prepared using the ThinPrep system; however, nongynecologic specimens require additional suspension and centrifugation steps to remove blood and break up mucus. Consult the company's product literature for details on cytopreparation with its system.

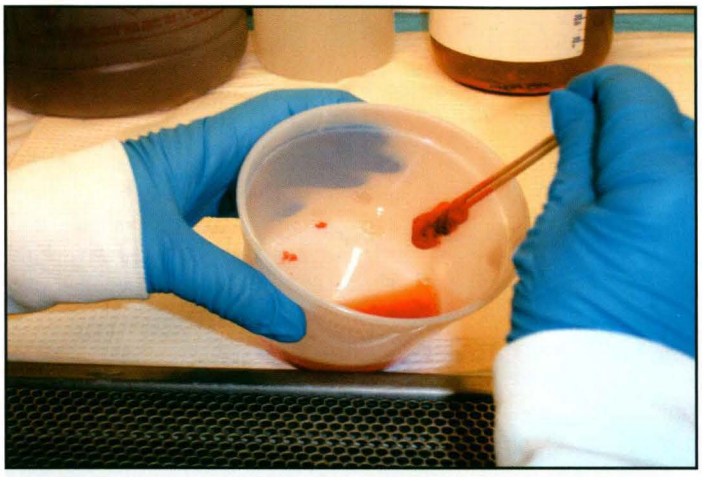
The SurePath system makes monolayer preparations by sedimentation. Specimens are collected into a preservative with very weak ethyl alcohol content. Before being loaded onto the Surepath PrepStain instrument, a cell enrichment process is performed. In this process, centrifugations are done using a density gradient that concentrates abnormal cells and removes up to 75% of the blood and mucus, increasing the ratio of abnormal cells on the slide. Following the cell enrichment process, the specimens are loaded onto the PrepStain instrument, where representative aliquots are placed into a chamber that sediments the cells onto the slide. The PrepStain processor also individually stains each slide, which assists in preventing cross-contamination problems. Both gynecologic and nongynecologic specimens can be prepared using the SurePath System; however, nongynecologic specimens require alternate suspension and centrifugation steps before being loaded onto the PrepStain instrument. The company's product literature should be consulted for details on cytopreparation with its system. A comparison of the 2 systems is given in **[t15.3]**.

Cell Blocks

A cell block is a method of preparing cytology material so that it can be processed, sectioned, stained, and viewed as a histology section. It can provide diagnostic information in addition to that obtained from cytology smears. Also, it is easier to do special stains if needed (including immunohistochemistry) on a cell block than on additional smears, because smears often require adaptations of the staining protocols and different controls. A cell block is prepared with material remaining after the cytology smears have been made. The quality or quantity of the smears should never be sacrificed to make a cell block. The method chosen is dependent upon the characteristics of the specimen. If a cytology specimen contains small fragments of tissue, the fragments should be carefully picked out and submitted as a cell block. Clots or heavy mucus in specimens should be used to prepare cell blocks. Loose cells are difficult to contain, embed, and section and therefore make poor cell blocks. A method should be used with all loose

[t15.3] Comparison of ThinPrep and SurePath systems

Characteristic	ThinPrep	SurePath
Preservative	Weak methanol-based	Very weak ethanol-based
Mono-layer method	Sample withdrawn onto filter; filter pressed to slide, deposits cells	Sample placed on slide to sediment, excess aspirated off
Slide specimen	Homogenized specimen, blood reduced	Homogenized specimen, blood and inflammation reduced, ratio of diagnostic cells increased
Specimen area per slide	20 mm circle	13 mm circle
Cells per slide	53,000 average	73,000 average
Sampling brush head left in vial	No	Yes



[i15.6] The clot from a body fluid can be wrung out using applicator sticks to make a cell block.

cellular material to "block" it together so that it can be handled "en masse." There are several methods for doing this including thrombin-prothrombin clot, albumin, and agar.

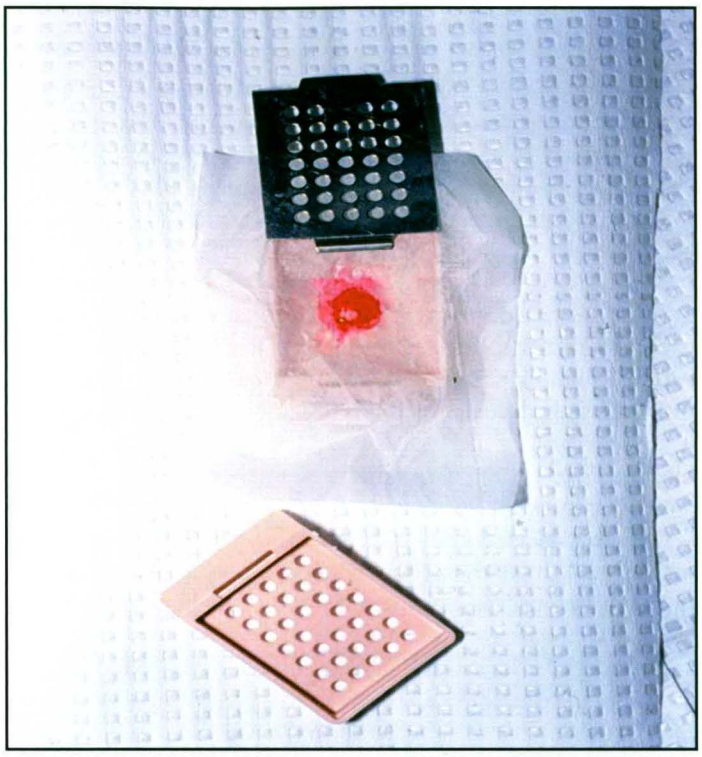
METHODS

■ *For Specimens with Spontaneous Clots* [i15.6]

1. Squeeze all of the extra liquid out from the clot with wooden applicator sticks until a solid mass is achieved.
2. Place this mass into a cassette labeled with the cytology accession number. If the mass does not hold together well, it should be wrapped in lens paper. If the clot is large, submit a representative portion.
3. Place the cassette in formalin for processing as tissue.

■ *For Cellular Material Entrapped in Mucus*

1. After all of the smears have been made, mix up the remaining portion of the sediment.
2. Add approximately 25 to 30 mL of 95% ethyl or denatured alcohol to the centrifuge tube and mix by inverting several times. Alternately, 10% NB formalin may also be used.
3. Allow this to sit until the specimen is completely fixed (usually 15-30 minutes).
4. Centrifuge for 10 minutes at 2,000 rpm.
5. Pour off the supernatant fixative.
6. Place the remaining mass in a cassette labeled with the accession number. If the mass does not hold together well, it should be wrapped in lens paper. If it is large, submit a representative portion.
7. Place the cassette in formalin for processing as tissue.



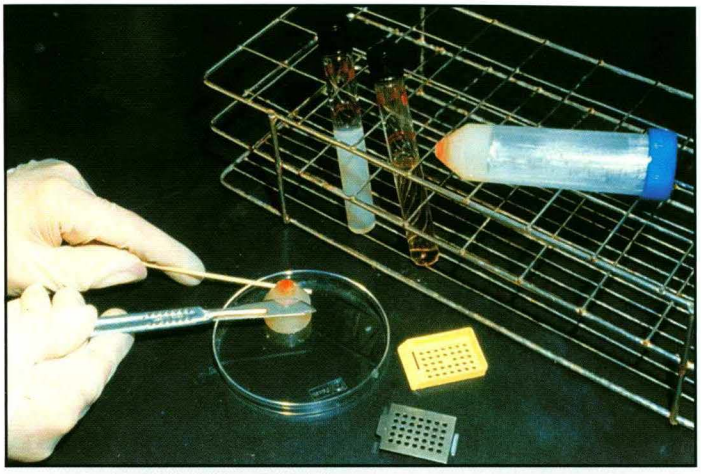
[i15.7] Loose cells can be blocked together using albumin. This cell block has eosin added to increase visibility.

■ *For Loose Cellular Material: Albumin Method* [i15.7]

1. After all of the smears have been made, mix up the remaining portion of the sediment.
2. Add 2 to 3 drops of 30% bovine albumin. (*Ask the blood bank for outdated albumin.)*
3. Mix gently with an applicator stick.
4. Add 95% alcohol to fix. Specimen should form a soft ball.
5. Place this mass into a cassette labeled with the cytology accession number. If the mass does not hold together well, it should be wrapped in lens paper. If the clot is large, submit a representative portion.
6. Place the cassette in formalin for processing as tissue.

■ *For Loose Cellular Material: Agar Method*

1. After all of the smears have been made, mix up the remaining portion of the sediment.
2. Add approximately 4 mL of completely melted 4% agar to the sediment in a centrifuge tube. Mix gently.
3. While the agar is still hot, immediately centrifuge for 10 minutes at 2,000 rpm.
4. Place in the refrigerator until the agar is completely solidified.



[i15.8] When making an agar cell block, the tip containing the sediment should be cut away from the excess agar, then bisected and submitted in a cassette for processing.

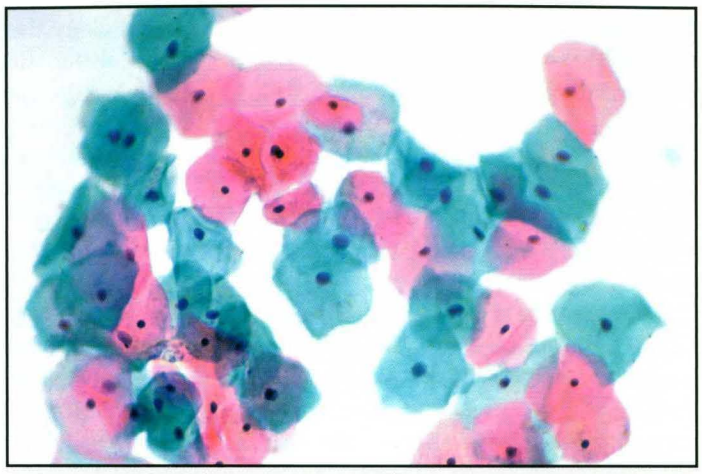
5. Gently pry the agar button from the tube with a wooden applicator stick. Do not disturb the cell button at the tip.

6. Using a scalpel blade, carefully cut the tip of the agar cone containing the cellular material away from the base of the cone with no cellular material [**i15.8**].

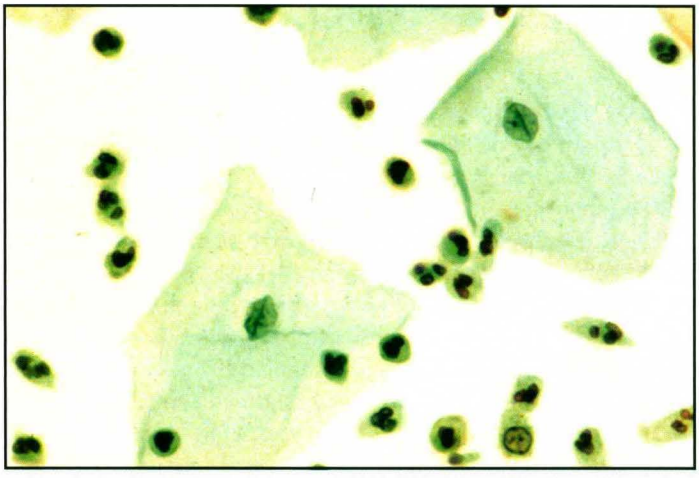
7. Slice the cellular button in half to form 2 semicircles.

8. Place the pieces in a cassette labeled with the cytology accession number. The cassette is then placed in formalin for processing as tissue.

9. The 2 semicircles must be embedded with their common cut side down, so that a section is cut through all layers of the centrifuged button.



[i15.9] Papanicolaou-stained smear demonstrating various tones of pink and blue-green in the cytoplasm.



[i15.10] Papanicolaou-stained smear showing nuclear detail in an intermediate cell and in polymorphonuclear neutrophils.

Cytology Staining

Cytology smears are routinely stained by the Papanicolaou technique [Papanicolaou 1954], commonly called the Pap stain. This stain provides optimal microscopic visualization of the nuclear and cytoplasmic morphology. It is not specific for any compound related to malignancy; instead, it allows good visualization of cellular morphology so that cellular health and disease processes can be evaluated. Five dyes in 3 different solutions make up the Pap staining protocol. Hematoxylin is used as the nuclear stain. The first counterstain, OG-6, consists of orange G, which stains keratinized cells. The second counterstain, EA, contains light green SF yellowish, eosin Y, and Bismarck brown Y. EA differentially stains the cytoplasm of cells, allowing the cytologist to distinguish between different cell types. Both counterstains use phosphotungstic acid as a mordant.

Well-stained cells are transparent, allowing the observer to interpret any underlying cells. Differentially stained cytoplasm, exhibiting a spectrum of colors, demonstrates the health status and activity level of that cell. Chromatin patterns are crisp and distinct [**i15.9**]. The 3 dye solutions in the Papanicolaou staining technique are used for their ability to accomplish these goals.

HEMATOXYLIN

Papanicolaou staining uses either Harris or Gill hematoxylin formulas to stain the nucleus. Whichever formula is used, a well-stained cytology smear should demonstrate nuclear detail that is crisp and distinct. Harris hematoxylin may be used either progressively or regressively. Gill hematoxylin is used progressively, and is the most widely used hematoxylin for cytology preparations because of its reputation for not overstaining. The Gill II (double strength) formula is used for routine staining. Gill I (single strength) and Gill III (triple strength) are useful for special applications. Hematoxylin staining quality can be evaluated by viewing the nucleus of an intermediate squamous cell; the granular intranuclear detail should be well defined. Alternately, examine a polymorphonuclear leukeocyte; the detail inside the nucleus and the threads between the lobes should be clearly visible [**i15.10**]. The

same staining principles regarding hematoxylin in histology also apply to Pap staining. This includes formula, pH, acid alcohol for decolorization, bluing, and troubleshooting.

OG-6

The first counterstain, OG-6 is an alcoholic solution of orange G (orange gelb) dye and the mordant phosphotungstic acid. OG-6 is the least complex of the stains in the Papanicolaou technique, and therefore shows the least variation and the least breakdown over time. OG-6 stains the tonofiliments in keratin a bright orange, helping to distinguish keratinizing neoplasms. It is important to note that many cytologic specimens will contain no keratinized cells and therefore no orange color is expected to be seen when reviewing these smears. Many laboratories will add glacial acetic acid to the standard OG-6 formula to enhance specificity and drastically decrease the staining time required.

EA

■ *EA-36, EA-50, EA-65, Modified EA [Gill 1991]*

EA is a polychrome cytoplasmic stain containing eosin Y, light green SF yellowish, Bismarck brown, and phosphotungstic acid. The phosphotungstic acid acts as a dye excluder, enabling eosin and light green to stain differentially. Proper staining demonstrates a spectrum of colors in the cytoplasm of the cells. Metabolically inactive cells show various shades of pink, while the metabolically active cells stain various shades of blue-green. The eosin in the formula will stain superficial squamous cells, nucleoli, erythrocytes, and cilia. Light green will stain intermediate squamous cells, parabasal and columnar cells, histiocytes, leukeocytes, large- and small-cell undifferentiated carcinomas, and cells deriving from adenocarcinoma. EA-36 contains a larger proportion of light green dye, approximately twice as much light green as EA-65. Commercial formulations of EA-50 are similar to those of EA-65, which is recommended for specimens with significant amounts of mucus present. The various stains penetrate the cells at different rates, making it essential to properly regulate both the stain time and the rinse time afterwards. Eosin Y is a smaller molecule and penetrates the cells at a faster rate. Light green takes longer to physically penetrate the cells, but replaces the eosin Y in the metabolically active cells. Eosin Y also rinses out of the cells faster than light green. As a result, staining in EA for too short a time will produce falsely eosinophilic cells, and rinsing for too long will produce false blue-green cells.

The light green in the formula is very light sensitive, and therefore EA should be kept in a dark, opaque container to extend the stain's shelf-life.

The Bismarck brown in the EA formula is notorious for linking with the phosphotungstic acid mordant and precipitating out of solution, making the stain too weak for good use. Gary Gill [1991] formulated a modified EA, which eliminates the Bismarck brown and provides a more stable stain.

PAPANICOLAOU STAIN [PAPANICOLAOU 1954, GILL 1991]

■ *Purpose*

To distinguish cellular components by obtaining highly detailed chromatin, differential counterstaining, and cytoplasmic transparency.

■ *Fixative*

Any alcohol-fixed cytologic specimen

■ *Equipment*

Graduated cylinders, Erlenmeyer flasks

■ *Reagents*

Orange G, 10% Stock Solution

Orange G	10 g
Distilled water	100 mL

Orange G, Working Solution

Orange G, 10% stock solution	20 mL
Phosphotungstic acid	0.15 g
95% ethyl alcohol	980 mL
Glacial acetic acid (optional)	10 mL

Eosin Y, 20% Solution

Eosin Y (water soluble)	20 g
Distilled water (70-80c)	100 mL

Light Green SF, 3% Solution

Light green SF yellowish	3 g
Distilled water (70-80c)	100 mL

Modified EA Solution

Eosin Y, 20% solution	20mL
Light green SF, 3% solution	10 mL
Phosphotungstic acid	4 g
95% ethyl alcohol	700 mL
Absolute methyl alcohol	250 mL
Glacial acetic acid	20 mL

■ *Procedure*

1.	95% ethyl alcohol	10 minutes
2.	Running water	10 dips
3.	Hematoxylin: Harris or Gill II	1-6 minutes
4.	Running water	Until clear
5.	Ammonia water, 0.25% or Scott tap water substitute	30 seconds
6.	Running water	30 seconds
7.	70% ethyl alcohol	10 dips
8.	95% ethyl alcohol	10 dips
9.	OG-6	30 sec-1 min
10.	95% ethyl alcohol, two changes	10 dips each
11.	Modified EA, EA- 50, or EA-65	1-3 minute
12.	95% ethyl alcohol, 2 changes	10 dips each
13.	100% ethyl alcohol, 3 changes	10 dips each
14.	Xylene, 3 changes	1 minute each

■ *Results*

• Chromatin	Blue
• Keratin	Orange
• Superficial squamous cells	Variable shades of pink
• Nucleoli, cilia, RBCs	Variable shades of pink
• All metabolic cell cytoplasm	Variable shades of blue-green

■ *Technical Notes*

The following hints are useful for achieving good Pap staining regardless of the method used:

1. All solutions before the first dehydration step should be filtered or replaced daily to prevent cross-contamination.
2. Clean alcohol rinses after the counterstains are essential for optimal cytoplasmic staining. If the counterstain colors look "dirty," it is probably because of overused alcohol rinses.
3. It is necessary to presoak all specimens that have been fixed with spray fixatives or Saccomanno fluid before staining to remove the Carbowax coating on the cells, which would interfere with hematoxylin staining. A 10-minute presoak in 95% alcohol at the beginning of the staining circuit will normally remove the Carbowax.
4. When preparing stock solution, adjust for total dye content.
5. Store all solutions in light blocking containers.

TOLUIDINE BLUE WET FILM [NAYLOR 1985]

■ *Purpose*

Toluidine blue wet films are a rapid method to determine cellularity during procedures, such as fine-needle aspirations, and to evaluate specimens for cross-contamination potential.

■ *Principle*

Toluidine blue gives metachromatic staining in unfixed cells.

■ *Fixative*

Any unfixed cytologic specimen

■ *Equipment*

Graduated cylinders, Erlenmeyer flasks, pipettes, filter paper

■ *Reagents*

Toluidine Blue

Toluidine blue	0.5 g
95% ethyl alcohol	20 mL
Distilled water	80 mL

Dissolve toluidine blue in alcohol. Add distilled water, and bring the final volume to 100 mL. Filter.

■ *Procedure*

1. Place a drop of specimen on a slide.
2. Place a drop of toluidine blue beside the specimen drop.
3. Mix the drops with a corner of a coverslip, then place the coverslip on top of the mixture.
4. Scan the slide under a microscope for cellularity.

CROSS CONTAMINATION

Because cytology specimens consist of loose cells smeared on the slides, there is a significant possibility of cells detaching from 1 slide during staining, floating away in the solutions, and attaching to a different slide. It is possible for this to cause a false diagnosis. To prevent this, specimens that have a high potential for cross-contamination must be stained separately from other nongynecologic specimens and the solutions filtered between batches. To determine if a specimen has potential for cross contamination, a toluidine blue wet film can be done. The wet film will show if the specimen contains large numbers of cells and cell clusters, indicating that the fluid may be abnormal and should be stained separately.

Occasionally, cytology smears will be air-dried and stained with a Diff-Quik or rapid Giemsa stain to provide better visualization of particular cellular elements.

SPECIAL STAINS

Essentially all special stains done on tissue sections can also be done on cytology smears, simply by omitting the deparaffinization steps and beginning the stain procedure from the 95% alcohol fixative. The smears should be air-dried if fat stains are requested. Most immunohistochemical procedures can be performed; however, it may be necessary to adjust epitope retrieval and incubation steps. Formalin-fixed tissue control sections may not provide optimal controls for special staining procedures done on cytology smears.

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LEARNING ACTIVITIES

1. **Prepare 3 smears of urine sediment (alternately, buccal smears may be used). Fix 1 smear in 95% alcohol, fix 1 smear in formalin, and allow 1 smear to air-dry. Stain with the Papanicolaou technique. Microscopically examine all slides and note the differences in nuclear and cytoplasmic detail, and in staining characteristics.**

2. **Prepare several smears of urine sediment (alternately, buccal smears may be used). Stain using the Papanicolaou technique as follows:**

   a. Standard times in all stains.

   b. Standard times in hematoxylin and OG-6, halved time in EA.

   c. Standard times in hematoxylin and OG-6, doubled time in EA.

Microscopically examine all slides and note what differences, if any, were caused by the timing changes.

Glossary

Absorption: When tissue is penetrated by, or absorbs, a dye solution and becomes colored without any other change or chemical reaction occurring. An example is the absorption of oil red O or Sudan black B by fat.

Achromatic: Without color, or not easily stained. In microscopy, achromatic lenses are corrected for two colors, red and blue. This helps produce images free of chromatic aberrations.

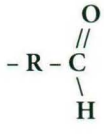
Acid mucosubstances: A term used to include both the acid mucopolysaccharides (connective tissue mucins) and the acidic glycoproteins (epithelial mucins). Most acid mucopolysaccharides contain hyaluronic or hexuronic acid, and the glycoprotein contains sialic acid. Acid mucopolysaccharides and acidic glycoproteins do not stain the same with all mucin techniques.

Acidophilic: A basic (cationic, positively charged) substance that is easily stainable with acid dyes. An example is cell cytoplasm, which is readily stainable with the acid (anionic, negatively charged) dye eosin.

Additive: A chemical or substance that adds on to, or combines with, another substance, usually improving, strengthening, or altering it. Many fixative molecules add on to tissue proteins, altering and stabilizing them in the process.

Adsorption: The accumulation of stain by the surface of a tissue component. This phenomenon is influence by the affinity of oppositely charged ions for each other (electrostatic attraction or bonding).

Aldehyde: An organic compound containing carbon, oxygen, and hydrogen arranged as in the following group:



Aliphatic hydrocarbon: Organic chemical compound composed of carbon and hydrogen, in which the carbon atoms are linked in an open chain, rather than in a ring arrangement. Aliphatic hydrocarbons can be used as clearing agents.

Amphoteric: Describes a substance that is capable of acting as either a base or an acid, depending on the pH of the solution.

Anaplasia (of neoplasms): Lack of differentiation. Some features include variation in size and shape of cells and nuclei, dark nuclei, giant cells and disturbed orientation.

Angstrom (Å): A unit of length equal to 100 millionths (10^{-8}) of a centimeter, or 10^{-10} meters. This unit has been replaced by nanometer (nm) as the official unit of measurement. One nanometer is equal to 10 Å or 10^{-9} meters.

Anion: A negatively charged dye or tissue component. Anionic substances are also referred to as acidic, although it has nothing to do with pH.

Anthracotic pigment: An accumulation of carbon from inhaled industrial pollution, cigarette smoke, or coal dust. The pigment is seen most frequently in the lungs or in lymph nodes from that area.

Antibody: Commonly known as immunoglobulins, antibodies are proteins that are produced by B lymphocytes in response to antigenic stimulation.

Antigen: Any substance that can induce a detectable immune response.

Antigen enhancement: Epitopes can be exposed or "unmasked" by either heat or enzymes, thus enhancing the antigen-antibody reactivity.

Antigenic determinant: The area of an antigen that determines the specificity of the antigen-antibody reaction.

Apochromatic: Corrected for both spherical and chromatic aberration. Apochromatic microscope objectives have been corrected for three colors and other lens aberrations.

Aqueous: A solution prepared in water.

Argentaffin: A reaction in which certain tissue components have the ability to bind or be impregnated with silver ions and then reduce the silver to its visible metallic form.

Argyrophilic: The ability to bind or be impregnated with silver ions: however, a reducing agent is required to reduce the silver to its visible metallic form.

Aromatic hydrocarbon: Organic chemical compound composed of carbon and hydrogen, in which the carbons are linked in a ring arrangement. Aromatic hydrocarbons are used as clearing agents.

Artifact: A structure or substance not normally present but produced by some external force or action. Examples are mercury pigment, tissue floaters, knife lines, and air bubbles.

Autolysis: Destruction or digestion of tissues and cells by the enzymes normally present in the cells.

Auxochrome: The chemical group present in a dye that causes it to bind to certain tissue elements. This group can develop a charge (+ or –) and thus bind to oppositely-charged groups present in the tissue. The amino ($-NH_2$) and carboxyl (–COOH) groups are frequently occurring auxochromes.

Axon: The nerve process that conducts impulses away from the cell body of the neuron. Neurons have a single axon. Axons are included along with dendrites in the term "nerve fibers"; axons have also been referred to as the axis cylinder.

Bacilli (singular, bacillus): Rod-shaped bacteria.

Bacteria (singular, bacterium): Single-cell microorganisms.

Basophilic: An acidic (anionic, negatively charged) substance that is easily stainable with basic (cationic, positively charged) dyes.

Bevel angle: The angle made when the two cutting facets of a microtome knife intersect. On most American-made knives, the bevel angle is 28° to 32°.

Binocular: Having two oculars. A binocular microscope has two eyepieces, or oculars.

Birefringence: Splitting of a light wave into two waves that are refracted in different directions. This phenomenon is also referred to as anisotropism or double refraction.

Buffer: A solution containing acid and alkaline components in the desired concentration so that the pH will be maintained when small amounts of other acids or bases are added.

Carbohydrate: Compounds containing carbon, hydrogen, and oxygen only. Included are sugars, starches, and cellulose.

Catalyze: To modify, speed up, or slow down a reaction without being consumed in the process. Enzymes catalyze biological reactions.

Cation: A positively charged dye or tissue component. Cationic substances are also referred to as basic.

Cell block: A concentration of cells from a cytology specimen prepared so that it can be processed, sectioned, stained, and viewed as a histology section.

Chelation: Forming a ring compound by joining a chelating agent to a metal ion. Chelating agents are organic compounds. An example is ethylenediaminetetraacetic acid (EDTA), which decalcifies by binding calcium ions.

Chromatic aberrations: The distortion of color in an image produced by a microscopic lens.

Chromatin: Usually used to refer to the stainable material (a complex of nuclei acids and protein) in the nucleus; however, the nucleus contains two types of chromatin: (1) dispersed chromatin (euchromatin), or that part of the DNA that is active in RNA synthesis; and (2) condensed chromatin (heterochromatin), or that portion of DNA that is not active in RNA synthesis. Hematoxylin stains only heterochromatin.

Chromagen: A benzene derivative containing a color-bearing group, or chromophore.

Chromophore: The chemical grouping that bestows the property of color on a compound.

Cirrhosis: Diffuse fibrosis or sclerosis of any organ, especially the liver.

Clearance angle: The angle formed by the intersection of the block face and the adjacent cutting facet of the knife. This angle should be approximately 3° to 8°.

Clearing: Process of replacing the alcohol (dehydrating agent) with a reagent that is miscible with paraffin. Most clearing agents leave the tissue transparent, hence the name clearing agent.

Clone: A group of cells that are all derived from a single progenitor cell, all having the same characteristics as one another.

Coagulation: Cloudiness, flocculation, and clot formation are stages or grades in the process of coagulation. Several reagents coagulate protein sols during the process of fixation.

Cocci (singular, coccus): Spherical or spheroid-shaped bacteria.

Decalcification: The process of removing calcium from bone or tissue, thus allowing routine microtomy on paraffin-embedded material.

Decolorization: The removal of color, or excess color, from tissue. Only some tissue components are decolorized in a process known as regressive staining. Common decolorization methods involve the use of acidic or basic solutions, excess mordant, or oxidizers.

Dehydration: The removal of water from tissue.

Denaturation: To change the nature of. Fixatives denature proteins by changing the soluble (liquid) contents of the cell into insoluble substances so that those substances are not lost during the subsequent processing steps. This change can be caused by either chemical (fixative solutions) or physical (heat, desiccation) means. Denaturation causes the protein molecule to unfold and the internal bonds to become disrupted.

Denatured alcohol: Ethyl alcohol containing another reagent that has been added to make the alcohol undrinkable. Frequently methyl or isopropyl alcohol are used to denature ethyl alcohol.

Differentiation: Process by which excess stain is removed from a tissue section, so that only the desired element is left stained and may be visualized easily against the colorless or counterstained background.

Differentiation (of neoplasms): The distinguishing of one thing from another, eg, the extent to which neoplastic cells resemble comparable normal cells, both morphologically and functionally.

Endogenous: A term used to indicate pigments or minerals that arise or are produced from within the body.

Enzyme digestion: The use of enzymes to digest certain tissue components. Examples are the use of diastase to remove glycogen, or hyaluronidase to remove some types of connective tissue mucin.

Enzymes: Proteins that catalyze chemical reactions in biological systems.

Epitope: The simplest form of antigenic determinant present on a complete antigenic molecule.

Esterases: Hydrolytic enzymes that act on carboxylic acid esters of alcohols, phenol, and naphthols.

Exogenous: A term used to indicate pigments or minerals that originate from outside the body.

Fire Triangle: Fuel, oxygen, and an ignition source form what is known as the fire triangle. All three of these elements are necessary for a fire.

Fixation: The stabilization of protein.

Fixative: A chemical that alters tissue by stabilizing protein in such a way that the tissue is resistant to further changes.

Flash point: The lowest temperature at which a liquid produces enough vapors near the surface of the liquid or within the container used to form an ignitable mixture with air.

Fungi (singular, fungus): Primitive plants that possess no roots, stems, leaves or chlorophyll.

Glia (neuroglia): Cells that provide the supporting network for the central nervous system. There are 4 types of glial cells: oligondendroglia, astroglia, microglia, and ependymal cells.

Gliosis: A condition marked by excessive proliferation of glial cells and/or processes.

Glycogen: The chief storage polysaccharide in animals. It is especially abundant in muscle and liver tissue. Glycogen is readily hydrolyzed by α- and β-amylase to form glucose and maltose.

Gynecologic cytology: Includes specimens from the female genital tract, notably from the cervix, endocervix, and vagina.

Hematein: The oxidation product of hematoxylin and the active staining ingredient in hematoxylin solutions. Hematein is the active dye formed by the action of either light and air or a chemical oxidizing agent on hematoxylin.

Hematin: Granular, brownish-black crystalline deposit occurring in tissue. Hematin is the term commonly used to denote formalin pigment, formed by the action of acidic formaldehyde on blood-rich tissue, but malarial pigment and acid hematin are also hematins.

Hematogenous: Most commonly used to denote pigments derived from blood.

Hematoidin: A yellow-brown, crystalline, bile-related pigment, probably a heme breakdown product. It is found primarily within old hemorrhagic foci and frequently within old splenic infarcts. It is demonstrated by the techniques for bile.

Hemochromatosis: Disease characterized by excessive deposition of hemosiderin in tissues, often interfering with the function of the organs of deposition.

Hemofuscin: A brown pigment derived from hemoglobin.

Hemosiderin: A loose complex of ferric iron (Fe^{3+}) and protein; hemosiderin is the storage form of iron. It is a yellowish-brown pigment and is stored primarily in the bone marrow.

Hydrolases: Enzymes that act on various substrates by either adding or removing water.

Hydroxyapatite: A major and essential ingredient of normal bones and teeth. Hydroxyapatite makes up bone mineral and the matrix of teeth, and gives bone and teeth their rigidity.

Hyphae (singular, hypha): Vegetative, tubular fungal structures that may or may not possess cross walls (septa). Many hyphae together compose a mat-like fungal colony known as the mycelium.

Hypo: A term commonly used to refer to a sodium thiosulfate solution.

Hypotonic: A solution that will cause cells to swell. Sodium chloride solutions of less than 0.9% concentration are hypotonic to animal tissue cells.

Immunogen: An antigen, or a substance that can induce an immune response.

Impregnation (metallic): The deposition of silver or gold on or around, but not in, the tissue component to be demonstrated.

Incubation: To maintain sections at optimal environmental conditions for the desired reaction to occur.

Infiltration: Permeation, as in paraffin permeating (spreading or flowing throughout) tissue. Infiltration is also known as impregnation.

Ion-exchange resin: A substance that exchanges one ion for another. Ion-exchange resins are used in decalcification and exchange ammonium ions for the calcium ions removed from the tissue. The formic acid used for decalcification is rapidly cleared of calcium ions, and thus daily solution changes are avoided.

Isotonic: Fluids into which normal animal cells can be placed without causing either swelling or shrinkage of the cells. A 0.9% solution of sodium chloride (saline) or a 5% glucose solution is approximately isotonic.

Lake: The combination of a mordant (eg, aluminum) with a dye (eg, hematein). The dye-lake can bind to tissue components and form an insoluble colored deposit.

Lipid: Any of numerous fats and fat-like materials that are generally insoluble in water but soluble in common organic solvents.

Lipofuscin: Commonly referred to as "wear and tear" pigments, these yellow-brown pigments are probably breakdown products resulting from the oxidation of lipids and lipoproteins.

Magnification: The enlargement of an optical image. The magnification of a microscopic image is calculated by multiplying the magnification of the ocular and the objective used.

Magnification, empty: An enlargement or magnification of a microscopic image without any further useful information being obtained.

Meniscus: The curved upper surface of a nonturbulent liquid in a container. If the surface of the container is wet by the liquid, the curve will be concave.

Metachromasia: A change of color. Certain tissue elements stain metachromatically with certain dyes in that they give a color that differs from that of the stain used. Mast cell granules stain metachromatically with toluidine blue in that they give a rose to violet color against the normal blue (orthochromatic) staining of the background.

Metallic impregnation: The deposition of silver or gold on or around, but not in, the tissue component to be demonstrated. The metal is then reduced to its visible metallic form either by the tissue or by a reducing agent.

Microincineration: Heating microscopic sections at a very high temperature (about 650°C). All organic material is burned off and the inorganic residue can be studied.

Micrometer (μm, formerly micron): One millionth (10^{-6}) of a meter.

Micrometry: The measurement of minute distances with the microscope.

Microtomy: The cutting of thin sections of tissue for microscopic examination.

Milliliter (mL): A unit of volume measurement. One milliliter is equal to 10^{-3} liters. Milliliter is used interchangeably with cubic centimeter (cc).

Mineral: Any naturally occurring, homogeneous, inorganic substance having a definite chemical composition and characteristic crystalline structure. Minerals can be studied by microincineration, in addition to the frequent demonstration by special stains.

Miscible: Capable of mixing or being mixed.

Monoclonal: Derived from a single clone of cells, such as an antibody.

Mordant: A reagent used to link the stain, or dye molecules, to the tissue. Many mordants in histology are metals, especially those linking hematein to tissue (ie, aluminum, tungsten, iron, and chromium).

Mycelium: A fungal colony composed of a mat of intertwined hyphae.

Myobacteria (singular, mycobacterium): A genus of slender, rod-shaped bacteria. Included are the causative organisms of tuberculosis and leprosy.

Myelin: A white fatty material encasing and providing a protective sheath for some nerve fibers.

Nanometer (nm): One billionth (10^{-9}) of a meter.

Neuron: A nerve cell.

Nissl substance: Clumps of rough, or granular, endoplasmic reticulum. Because of the RNA content, Nissl substance stains sharply with basic aniline dyes, such as thionin and cresyl echt violet.

Noncoagulation: Absence of clot formation.

Non-gynecologic cytology: Includes specimens from all body sites with the exception of those from the female genital tract.

Objective: The lens at the lower end of the barrel, or body tube, of the microscope. Several objectives, yielding different magnifications, are found on most microscopes.

Ocular: The eyepiece of the microscope, or the lens found at the upper end of the barrel or body tube.

Orthochromatic: Dyes that do not stain metachromatically are said to be orthochromatic; they stain tissue components as expected or the same color as the dye solution.

Osmolality: The concentration of osmotic solutions expressed in terms of numbers of particles in solution. One osmol is the number of particles in a gram molecular weight of undissociated solute. If the solute dissociates into two ions, 1 gram molecular weight of the solute equals 2 osmols.

Oxidation: Occurs by combining with oxygen, by the loss of hydrogen, or by the loss of electrons. Hematoxylin is oxidized to hematein by the loss of hydrogen.

Oxidizing agent: A substance that causes oxidation. Hematoxylin is oxidized to hematein by either chemical means or by light and air. For some staining techniques, certain tissue components are oxidized to a more reactive state, such as the oxidation of some carbohydrates to aldehydes by either periodic acid or chromic acid prior to the Schiff reaction.

Parfocal: In same plane of focus. Microscope objectives are said to be parfocal when they can be changed without the need to refocus.

Permissible exposure limit (PEL): The maximum allowable 8-hour time-weighted average of exposure to a substance as defined by OSHA. The PEL is expressed in parts per million (ppm).

Phosphatase: A hydrolytic enzyme that breaks the bond between an alcohol and a phosphate group. Phosphatases are present in a wide variety of plant and animal tissue. Some phosphatases, such as adenosine triphosphatase (ATPase), act specifically on a single substrate; others act with less substrate specificity, and are divided into two groups: those exhibiting optimal activity at an high pH (alkaline phosphatases), and those exhibiting optimal activity at a low pH (acid phosphatases).

Phosphorylase: A transferase that transfers phosphate groups. Phosphorylases are widely distributed in animal and plant tissues, and they catalyze a number of reversible reactions, leading to the synthesis and decomposition of saccharides. Glycogen phosphorylase is the best-known phosphorylase.

Pigments: A heterogeneous group of substances that contain enough natural color to be visible without any further staining. Examples are melanin, a brownish-black pigment found normally in skin, hair, and eyes; and hematin (formalin pigment), a brownish-black pigment found in blood-rich tissues fixed in acidic formalin solution.

Polarization: The examination of slides by using a polarizer and an analyzer to obtain light vibrating in only one plane. Anisotropic or birefringent material will be bright against a dark background if the optical paths of the polarizer and analyzer are crossed.

Polychromatic: Describes a single dye solution that stains tissue components different colors by a phenomenon other than metachromasia. One example is a solution prepared from a dye that is not pure but contains several dyes. The variety of color in the tissue is due to the selective adsorption of the different dye components by various tissue elements.

Polymer: A covalently-bonded chain of individual building blocks called monomers.

Polymer detection system: A polymer with an enzyme label chemically attached in the place of biotin, typically used in a two-step detection system.

Polymerization: A chemical reaction in which 2 or more small molecules join together to form a larger molecule containing repeating structural units of the original molecules. Formaldehyde tends to polymerize into paraformaldehyde; methanol is added to stock formaldehyde solutions to retard polymerization.

Polysaccharide: A group of 9 or more monosaccharides joined by glycosidic bonds. Starch and cellulose are examples.

Precipitate: To separate from solution, usually caused by chemical action. For example, in the Prussian blue reaction, iron is dissolved by the hydrochloric acid, and then immediately precipitated by potassium ferrocyanide. The ferric ferrocyanide precipitate is known as Prussian blue.

Pre-fixative: A solution used in cytology to partially fix cells to protect them from autolysis and putrifaction during prolonged transportation, or holding. Common pre-fixatives include Saccomonno fluid and alcoholic saline.

Preservative: A fluid that will neither shrink nor swell tissue, will neither dissolve nor distort its constituent parts, will kill bacteria and molds, and will render enzymes inactive. But unlike a fixative, a preservative will not alter or modify tissue constituents in such a way that they retain their form during the subsequent processing steps.

Progressive staining: Staining to the desired intensity and stopping the stain. No differentiation step is used.

Protozoans (singular, protozoan): Single-celled animals that are simple structurally but complex functionally.

Pseudomelanin: A brownish-yellow pigment sometimes seen in the lamina propria of the colon, appendix, and, rarely, the small intestine. The pigment gives positive Fontana-Masson and Schmorl reactions.

Reduction: Accomplished through the loss of oxygen, or by the gain of hydrogen or electrons.

Refractive index: The ratio of the speed of light in a vacuum to the speed of light in the medium under consideration.

Regressive staining: The tissue is overstained and then decolorized, or differentiated, until the desired component contrasts sharply with the background. The Verhoeff elastic stain is an example of regressive staining.

Resolution: The optical ability to distinguish two objects a minimal distance apart as two objects. The light microscope has a resolving power of about 0.2 μm, ie, objects closer together than 0.2 μm can no longer be distinguished as two separate objects and will be seen as one object.

Ripening: Oxidation, as in the ripening or oxidation of hematoxylin to hematein.

Short-term exposure limit (STEL): The maximum allowable time-weighted exposure for any 15-minute period during an 8-hour work period.

Solute: The substance or chemical being dissolved.

Solvent: The liquid in which a substance is being dissolved.

Spirochetes: Spiral or corkscrew-shaped argyrophilic bacteria.

Substrate: The substance on which an enzyme acts, eg, naphthol AS-B1 phosphate serves as a substrate for acid phosphatase.

Supernatant: The liquid or fluid on top.

Time-weighted average: The amount of substance that a worker is exposed to, averaged over an 8-hour period.

Tophus: A deposit of urate crystals in tissue.

Tzank smears: Smears prepared by scraping the base of a skin ulcer to look for Tzank cells found in diseases such as chicken pox and herpes.

Universal solvent: Chemical reagent that can be used for both the dehydrating and clearing steps in tissue processing. Dioxane, tertiary butanol, and tetrahydrofuran are universal solvents.

Yeasts: True fungi, usually single-celled, oval-shaped fungi that reproduce by budding at the smaller end of the cell.

Validation: Establishing documented evidence that a process or system, when operated within established parameters, can perform effectively and reproducibly to meet the expected outcome.

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