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Histological Techniques for Marine Bivalve Mollusks

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This manual is dedicated to Dr. Aaron Rosenfield and to the staff of the Oxford Laboratory, past and present.

FOREWORD

During the mid-1950s, massive and widespread oyster mortalities, particularly in the Delaware and Chesapeake Bay areas, served as a stimulus to establish a Federal oyster mortality study program. Consequently, Congress appropriated funds to the then Bureau of Commercial Fisheries (BCF) within the U.S. Department of the Interior. These appropriations enabled Federal, state, and academic institutions and laboratories to conduct research and management programs. These programs, either by contract, interagency agreement, or direct grant, would be designed to recognize and understand symptoms of disease, disease processes, their cause(s), and, furthermore, would help in the development of strategies that would prevent or control shellfish mortalities and the spread of disease agents. At that time, the Annapolis Laboratory of the BCF, under Mr. James B. Engle, was housed in an apartment building in Annapolis, Maryland, and the disease study was staffed by one biologist, Mr. Richard Burton, and two temporary summer technicians. I was one of those technicians, and started my career with strictly on-the-job training in histological and, later, pathological methods. In 1960 the Laboratory was moved to Oxford, Maryland, where a larger disease research and ecology program was initiated.

Little was known at that time about oyster diseases, or even how to go about studying them. Standard vertebrate methods were used in the beginning, but it soon became obvious that many techniques needed modification and refinement. Because of the unique nature of invertebrate tissues, and their parasites, protocols were developed by Dr. Melbourne R. Carriker for the study of oyster disease in individual specimens and in populations.

Since that time, over 500,000 sections of oysters and, more recently, other mollusks, crustaceans, and fishes have been processed. New and revised histological protocols and methodologies have been developed by the cooperative effort of several Laboratory staff and from information published in the scientific literature and in pertinent manuals. Handwritten laboratory notes and diaries, etc., have been collected over the past 20 years and developed into this manual by the authors. The result is a working manual designed and written by technicians primarily for technicians, using very practical approaches and language which are comprehensible for someone just beginning, yet detailed and accurate enough to produce professional results in the hands of the experienced scientists should they elect to do the work themselves.

I am proud to have been able to work with the authors. Over the past several years, their efforts and those of the senior staff of molluscan, crustacean, and fish pathologists resulted in the development of this manual. I feel that the authors' diligent efforts, both in the production of excellent histologic material and of this manual, will result in substantially enhancing the field of invertebrate pathology worldwide.

C. Austin Farley

ACKNOWLEDGEMENTS

This manual is based upon the outlines, techniques, and guidelines of Virginia Liddell, Lucretia Krantz, and Helena Jones and we are grateful for their contributions.

We particularly acknowledge our resident molluscan pathologists, Austin Farley and Fred Kern, for their support and professional assistance in preparation of this manual, and our Laboratory Director, Dr. Aaron Rosenfield, for his guidance and encouragement.

We are grateful to Fred Kern for providing us with many of the photographs shown in this manual, as well as to Sara Otto for the use of her collection of specimens.

We particularly thank Dr. Phyllis Johnson and Martin Newman for their help and the supervision given us while working out special stains and techniques.

We appreciate the excellent anatomical illustrations created by Alice Jane Lippson.

Our special thanks to Helen Lang and Susie Hines for their assistance in the lengthy literature searches, and to Muriel McNelis for her patience and understanding during the stages of preliminary typing.

We are also grateful to Dr. Robert Lippson and Haskell Tubiash for their editorial assistance.

And we sincerely acknowledge the late Betty Sanders of the National Cancer Institute for her encouragement and inspiration in our field of study.

Last but certainly not least, we must give credit to the present and former staff members of the Oxford Laboratory. They provided us with ideas, techniques, approaches, and counsel. Their numbers are too numerous to mention but we acknowledge their contributions with gratitude.

SPECIAL ACKNOWLEDGEMENT

Due to the policy of this publication, it was not possible to recognize Mrs. Jane Swann as editor of this manual. We strongly feel that her talents and her editorial expertise should not be overlooked. We would like to express our appreciation to her through the following acknowledgement.

A very special thanks is given to our editor, Jane Swann, for the endless hours of dedicated work on this manual. She transformed collections of rough drafts, procedures, and notes into a viable publication. During the continual changes and reorganizing of this manual, Jane's patience and perseverance went well beyond her normal duties. We the authors are indebted to her for her diligent editing and consistent review over a period of several years. Working with her was a pleasure and we are sincerely grateful to her.

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Histological Techniques For Marine Bivalve Mollusks

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INTRODUCTION

Investigators at the Oxford Laboratory diagnose and study finfishes, crustaceans, mollusks, and a variety of other marine and estuarine invertebrates. However, this manual is intended to serve as a guide for histological processing of shellfish, principally bivalve mollusks. Basically, the techniques included are applicable for histopathological preparation of all marine animals, recognizing however that initial necropsy is unique to each species. Several of the procedures described are adaptations developed by the Oxford Laboratory staff. They represent techniques usually based on principles established for histopathologic study of mammalian and other vertebrate tissues, but modified for marine and aquatic invertebrates. Although the manual attempts to provide adequate information on techniques, it is also intended to serve as a useful reference source to those interested in the pathology of marine animals. General references listed in the back of the manual will provide histological information on species not included in the text.

The first chapter on handling live animals individually describes the procedures employed for oysters, clams, mussels, and scallops, from receipt to the point of fixation. Subsequent chapters provide information on the specific phases of histology with adaptions used by the Oxford Laboratory. The chapter on staining methods includes general tissue stains, special stains for specific tissue components, stains for frozen sections and for fresh and fixed blood smears. Variations in the duplicate stain protocols are given to correlate the pH of the stains with the fixatives and tissues. Most of the stains have been successfully used on tissues of mollusks, finfishes, and crustaceans. Special techniques and data sheets have been compiled in appendix form.

NOTES ON SAFETY

Safety cannot be overemphasized in a histology laboratory since volatile solvents and hazardous chemicals are used. Each employee should be made aware of the possible hazards involved. Laboratory supervisors must provide adequate instructions on the safe use of the chemicals involved, as well as storage and disposal, even though most manufacturers provide warning labels.

The first supervisory priority is to be sure your working facility is adequately equipped for safe operation. The working environment must be free of toxic vapors. Fume hoods are essential, and are utilized from the initial steps of preparing fixatives, solvents, and noxious reagents to the final steps of coverslipping and cleaning the dried, stained slides.

Protective stainless steel mesh gloves[1] are required for opening oysters and hard clams. Plastic gloves and disposable aprons should also be available and used as necessary. Pipette bulbs and disposable pipettes are used for contaminating and dangerous chemicals. Safety goggles and eyewashing facilities must be available. The laboratory should be equipped with a safety shower for emergencies.

Safe operation of a microtome is essential. Locking levers on the handwheel of the microtome should be kept in working order. When using the longer microtome knife, a piece of rubber hose is placed on the extended end as a safety guard.

Gas line hoses to bunsen burners and the Laboratory's fire extinguishers should be routinely checked, and the local fire marshal and Laboratory safety officer should perform periodic inspections.

Extreme care should be used with highly explosive anhydrous picric acid salts. Dried ammoniacal silver salt solutions are also extremely explosive. These chemicals are used in special stain techniques.

Technicians should become familiar with all aspects of the chemicals involved in histological procedures. Technicians who have not had the proper instruction in handling these dangerous chemicals risk serious injury to themselves and their co-workers.

[1]Whiting & Davis Co., Plainville, Mass.

CHAPTER I. HANDLING OF LIVE ANIMALS

A. SHIPPING INSTRUCTIONS FOR SHELLFISH

Live animals must be shipped and received in good physiological condition to ensure optimal tissue analysis. If specimens cannot be shipped live in good condition, see Chapter III for instructions on using specific fixatives prior to shipment. The following instructions provide for shipment of live shellfish to ensure viable samples on arrival:

- Pack live shellfish (e.g. oysters or clams) (sample of 50) in ambient salinity, seawater-soaked burlap, with cold packs, in a well-insulated, well-constructed waterproof shipping container. **Shipment should be made early in the week (Monday or Tuesday) to avoid arrival during the weekend period, with possible loss of samples because of improper storage conditions.**
- On outside of container, clearly label **"LIVE SPECIMENS, REFRIGERATE BUT DO NOT FREEZE."** For air freight, indicate **"HOLD AT AIRPORT ON ARRIVAL AND CALL LABORATORY"** (destination).
- Wire or phone, immediately after shipment has been made, indicating where and when sent, estimated time of arrival, name of originating and, if possible, anticipated delivering carrier, flight number and, most importantly, the waybill number.
- Ship by air freight (prepaid) directly to destination.
- Provide background information with each sample, such as: name of requestor; to whom report should be sent, including address and phone number; pertinent collection data, such as temperature, salinity, pH, species, origin, history, sampling location, number of specimens in sample, and date collected.

B. NECROPSY AND EXAMINATION OF SHELLFISH SPECIMENS

Shellfish for histological processing should be sampled in the field by methods causing minimal, if any, damage to the specimens. They should be kept refrigerated, particularly in hot weather, until processed. Fixation should be as soon as possible after collection to reduce physiological or postmortem changes.

1. Processing Steps

- Record field data and history of sample
- Assign code
- Scrub animals to remove fouling organisms
- Measure shells
  - Bill to hinge for oysters, mussels
  - Across shell, side to side, for clams, scallops
- Open shellfish
  - Pry hinge open and cut adductor muscle to avoid damaging tissues
- Record the following:
  - Gaping bivalves that do not close
  - Weak muscle or loss of muscle adhesion
  - Lack of mantle reaction
  - Any unsual odor
  - Stabbing specimen when opening
  - Incision (cut)
  - Laceration (tear). (Reaction to stabbing can cause acute inflammatory infiltration of hemocytes to localized areas.)
- Record macroscopic information. (Shell abnormalities, parasites, and physiologically related conditions included represent only a few of the multitude associated with marine shellfish. The purpose of these examples is to show a spectrum of conditions seen during an examination of specimens. Any observations noted during the macroscopic examination will aid the investigators in their analyses.)
  - Shell abnormalities. (Experience has shown that certain conditions are predictable from samples of a certain geographic area. Each condition and degree of prevalence has an effect on the animal and how the animal has adjusted to it.)
    - Mantle recession (Fig. 1a)
    - Recovery (Fig. 1b)
    - *Cliona* sp. (boring sponge) penetrations, including estimated degree of infestation (Figs. 2a, b)
    - *Polydora* sp. (mud worm) tunnels, including estimated degree of infestation (Figs. 3a, b, c)
    - "Maladie du pied" (Fig. 4)
    - Shell blisters (Fig. 5)
    - Shell pustules (Fig. 6)
    - Calcareous malformations (Fig. 7)
    - Drill cases and/or drill holes (Fig. 8)
  - Obvious parasites
    - *Pinnotheres* sp. (gill area) (Fig. 9)
    - *Mytilicola* sp. (gut area) (Fig. 10)
    - *Sphenophrya* sp. (gill area) (Fig. 11)
    - *Bucephalus* sp.
  - Physiologically related conditions
    - Fat (Fig. 12a)
    - Medium (Fig. 12b)
    - Watery (Fig. 12c)
    - Pale digestive diverticulum (Fig. 13a); normal digestive diverticulum (Fig. 13b)
    - Swollen tissue (edema); example, swollen heart (Fig. 14)
    - Color
      - Pigmentation (melanin)
      - Green deposits (diapedesis)
      - Other colorations
  - Other gross abnormalities
    - Watery cysts (Fig. 15)
    - Abscesses and tumors
    - Pearls (soft or hard)

2. Figures and Explanations

a. Shell Abnormalities

1) Mantle Recession (Fig. 1a)

Heavy fouling of the inside shell margin indicates prolonged mantle recession. This condition usually indicates a sick oyster (Farley, 1968).

2) Recovery (Fig. 1b)

The oyster has recovered from previous recessive growth (mantle recession), overlaying the fouled inner shell margin with new periostracum. "The periostracum is a film of organic material (scleroprotein called conchiolin), secreted by the cells located near the very edge of the mantle. . . the conchiolin appears as amorphous, viscous and transparent material which hardens shortly after being deposited" (Galtsoff, 1964).

3) *Cliona* sp. (boring sponge) penetrations (Figs. 2a, b)

According to Galtsoff (1964), "Small round holes on the surface of mollusk shells indicate the presence of the most common animal associated with the oyster, the boring sponge." The sponge reaches the oyster tissue itself only in cases of old, heavy infestation. Usually, the holes made by the sponge are rapidly covered by a deposition of conchiolin. "However, should the deposition of shell material be delayed by adverse conditions, the sponge makes direct contact with the mantle and produces lysis of the epithelium and underlying connective tissue. Dark pigmented pustules form exactly opposite the holes in the shell" (Galtsoff, 1964).

4) *Polydora* sp. (mud worm) tunnels (Figs. 3a, b, c)

"*P. websteri* invades the shell cavity of the oyster, settles on the inner surface at a right angle to the edge, and builds a U-shaped mud tube with both orifices external. The structure is soon covered by a layer of conchiolin deposited by oyster and becomes a semitransparent blister" (Galtsoff, 1964).

5) "Maladie du pied" (foot disease) (Fig. 4)

"The disease affects the area of the attachment of the adductor muscle, primarily on the lower concave (left) valve, and in certain cases the upper, flat valve. . . In advanced cases the muscle becomes detached from the valve and forms irregular cysts of horny and slightly elastic material. Later on when the cyst extends beyond the area of the muscle attachment, the cyst walls become covered with calcareous shell deposit" (Galtsoff, 1964).

6) Shell blisters (Fig. 5)

Blisters frequently are found on the inside of the shells near the adductor muscle. "Chambers found in *C. virginica* consist of irregular cavities containing mud or sea water. Such formations are called blisters. Blisters can be artificially induced by inserting a foreign object between the mantle and the shell. They are also caused by the invasion of shell cavity by *Polydora* or by perforations of the shell by boring sponges and clams" (Galtsoff, 1964).

7) Shell pustules (Fig. 6)

Related to Minchiniasis (MSX). "Oysters with raised yellow-brown conchiolin deposits on the nacreous surface of the shell. . . These deposits contained a creamy yellow fluid which consisted of moribund *M. nelsoni* plasmodia, hemocytes, and cellular debris. . . . when present it was a reliable gross indication of the disease" (Farley, 1968). Farley termed a stage of the MSX disease "remission," when the shell pustules appeared as described above.

8) Calcareous malformations (Fig. 7)

These abnormalities are pathological. "They are associated with the disturbance of calcium metabolism which manifests itself in an overcalcification of selected parts of the organism. . . They fall into two classes: 1) cysts and calcified cups; and 2) calcified tumors and pearl-like structures attached to the valve or formed inside the soft tissues. . . It is reasonable to assume that the described malformations represent a phenomenon analogous to the over-calcifications of various selected part of mammals described by Selye (1962) under the general term calciphylaxis" (Galstoff, 1969).

9) Drill cases and/or drill holes (Fig. 8)

The presence of oyster drill egg cases are recorded when attached to oyster shells in a sample since oyster drills are the most persistent predator of the oyster. The drill cases are tough, greenish leathery capsules in which oyster drill eggs are deposited. Small symmetrical holes in molluscan shells can be attributed to the action of oyster drills or other gastropods.

b. Obvious parasites

1) *Pinnotheres* sp. (gill area) (Fig. 9)

These small oyster or pea crabs invade the mantle cavity of oysters and other bivalve mollusks. The female crab settles chiefly on the surface of the gills, and grows with the growth of the host. ". . . the female crabs which have settled on the oyster erode its gills and impair their function. . . . Rapid regeneration of the damaged gills probably saves many oysters from death, but interference with the normal gill functions causes a relatively poor condition in many infested oysters" (Galtsoff, 1964).

2) *Mytilicola* sp. (gut area) (Fig. 10)

This "parasitic copepod is found in the intestinal tract of bivalves and is easily recognized by its red color and relatively large size which makes it visible to the naked eye" (Galtsoff, 1964).

3) *Sphenophrya* sp. (gill area) (Fig. 11)

These are ciliates which attack the gills of the oyster. "The disease is caused by a microparasite which has been tentatively placed in the genus *Sphenophrya*. The organism is a ciliate found in large cysts on the gill surface of diseased oysters" (Anonymous, 1965).

4) *Bucephalus* sp.

This trematode thrives in brackish water oysters and is inhibited by an increased salinity. "In cases of heavy infestation, the gonads and digestive diverticula are almost completely replaced by cercariae and by the long germ tubes of the sporocysts . . . " (Galtsoff, 1964).

c. Physiologically related conditions

1) Fat, medium, or watery (Figs. 12a, b, c)

During the initial necropsy, each shellfish is rated for its physiological condition as fat (Fig. 12a), medium (Fig. 12b), or watery (Fig. 12c). This condition reflects the oyster's general health and is affected by its environmental surroundings, stress, and seasonal and sexual changes. Fat oysters usually fill their shell; often they are sexually ripe and exhibit a creamy tan color. Medium oysters generally are smaller in their shell, flatter, and less rigid than fat oysters; there is more fluid inside the shell and their color is basically tan. Watery oysters are obviously bloated with water; they exhibit a grayish-tan color and the animal is slightly translucent.

2) Pale digestive diverticulum (Figs. 13a, b)

Oysters from the same location usually show uniform-colored digestive diverticula (reddish-brown, greenish-brown, yellowish-tan, or blackish-brown) since they have been exposed to the same environmental food column. Occasionally, a pale digestive diverticulum occurs, and is so recorded. Many times the pale color can be associated with a health problem, diagnosed only after microscopic examination.

3) Swollen tissue (edema), swollen heart ("cardiac vibriosis") (Fig. 14)

During a survey for parasite distribution in Chesapeake Bay oysters, Tubiash et al. (1971) noted sporadic cases of greatly enlarged and edematous pericardia. "Examination of aseptically aspirated pericardial fluid showed heavy concentrations of gram-negative motile rods which proved morphologically and culturally compatible with *Vibrio anguillarum*, an organism implicated in diseases of fishes and larval bivalve mollusks. Except for pericardial enlargement, the animals appeared to be grossly and histologically normal" (Tubiash et al., 1971).

4) Color

"Pigmentation may be considered as a certain phase of excretion. Green oysters of Long Island Sound develop dark green pigment as the result of absorption and storage of copper by the phagocytes. . . " (Galtsoff, 1964).

Other colorations sometimes associated with mollusks are the result of pigments from the microscopic plants (algae) that mollusks consume. Some pink pigmentation of stored shellfish may be the result of the growth of so-called pink yeasts or pigmented bacteria (McCormack, 1956; Boon, 1972; Paparella, 1973).

d. Gross abnormalities

1) Watery cysts (in the visceral mass, palp, or mantle) (Fig. 15)

"The cysts, seen in the live oyster, are large conspicuous bubble-like cavities generally distended by internal pressure of accumulated fluid. Sections show these cysts to contain a central granular material usually in concentric layers. . . . The cyst walls are formed of leucocytic cells which form themselves into an epithelium similar to that of the external epithelium of the mantle" (Mackin, 1962).

2) Abscesses and tumors

"Reports of tumors in bivalves are relatively common in comparison to other invertebrate groups. . . . a number of so-called tumors have been shown subsequently to be hyperplastic reactions to injury rather than true neoplasias" (Sparks, 1972).

"Systematic surveys of molluscan populations offer a good method of obtaining extensive numbers of naturally occurring lesions in an attempt to classify molluscan tumors and specify criteria for distinguishing among true neoplasia, hyperplasia, and injury responses in these invertebrates" (Pauley, 1969).

3) Pearls

"In the case of pearl formation, Boutan (1923) has shown that calcareous deposits are formed by amoeboid cells which crawl through the mantle epithelium, while the latter secretes the concentric layers of the organic matrix (conchiolin)" (Galtsoff, 1964).

C. PROCESSING OF SPECIMENS

A random sample (preferably 30-50 specimens) of the population is collected for histological processing and study. A code is assigned for further in-house reference to the sample and for permanent filing. All pertinent data furnished with the sample should be included with the new code, along with the name of the requestor and the date(s) of collection (see Appendix, Molluscan Pathology Report).

The initial steps employed in processing molluscan species are provided below. The discussion on processing oysters is presented completely in a stepwise manner.

A separate section is provided for special opening and processing techniques for clams, mussels, and scallops.

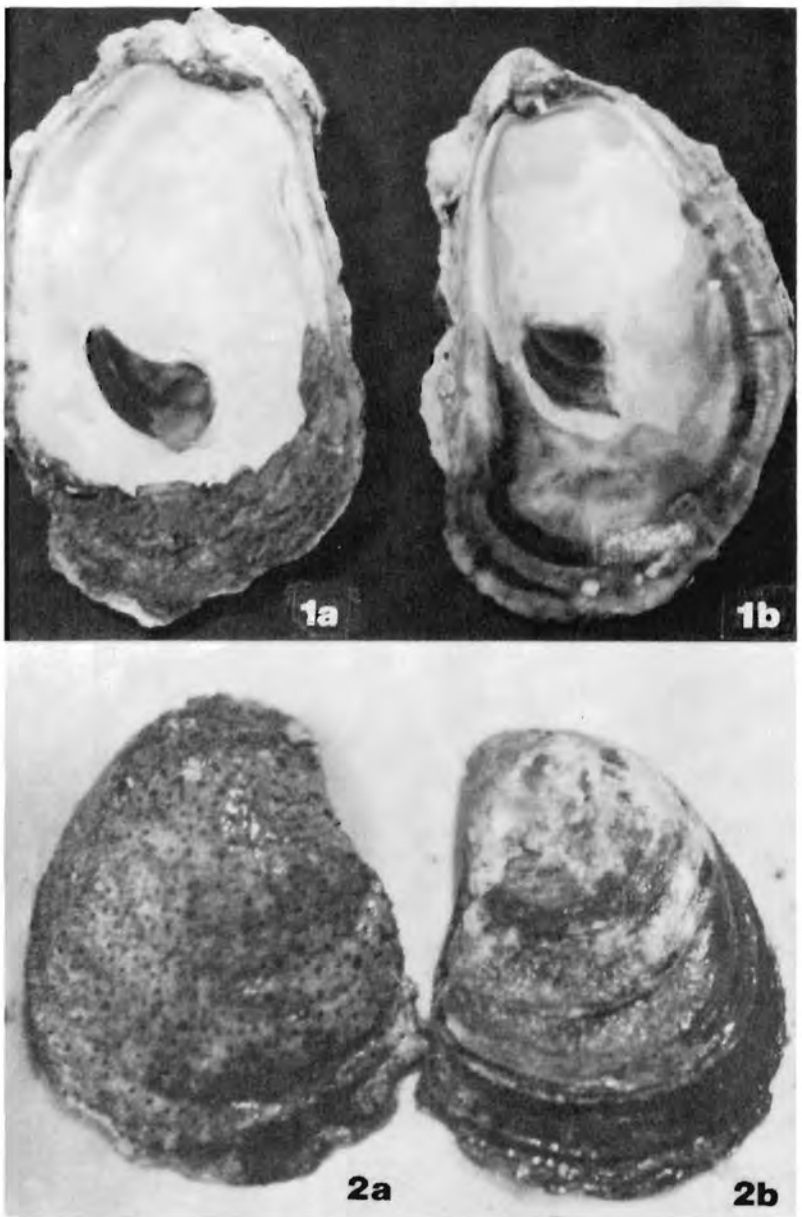
1. Oysters (*Crassostrea* and *Ostrea* spp.)

The oysters are scrubbed to remove sediment and fouling organisms, measured from the hinge to the bill (Fig. 16), and their size, in metric units, is recorded in numerical order. They are opened by inserting an oyster knife between the valves (Fig. 17) at the hinge arch and twisting to separate the hinge (Fig. 18). The adductor muscle is severed as close to the right valve as possible (Fig. 19), taking care not to injure internal tissue. The opened oysters are then examined (Figs. 20, 21) for

color, condition (fat, medium, or watery), macroparasites, and abnormalities. The body is removed from the shell by severing the adductor muscle as close to the left valve as possible. The rectum is excised with scissors (Fig. 22) and placed in a correspondingly coded thioglycolate culture tube (Fig. 23) for determining the presence of *Perkinsus* (*Labyrinthomyxa*) *marinus* (=*Dermocystidium marinum*) (Ray, 1952). The animal is then washed in clean, filtered, ambient sea water to remove sand and placed on a clean No. 14 rubber stopper for cutting a cross-section with a sharp razor blade (Fig. 24).

The first transverse cut is made where the palps and gills meet (see Figs. 25, 28 for location). The second cut is made 1-2 mm from the first cut, if frozen sections or tissue for electron microscopy study are desired. The third cut is made 4-5 mm beyond the second cut for histologic sectioning (on a very small specimen it may be just above the adductor muscle). Figure 29 illustrates oyster tissues which will be included in a 4- to 5-mm transversely cut cross-section. A confusing point may be the crystalline style which is rarely seen during the processing of oyster tissues. According to Galtsoff (1964), "The crystalline style is not a permanent structure. In oysters removed from the water and left in the air the style dissolves in a short time."

The anterior portion containing the palps is placed in a 2-oz screw-top jar containing Millipore[1]-filtered sea water, correspondingly coded, and used for fresh squash examination (most parasites can be diagnosed at this point). The remaining 6- to 7-mm-thick digestive diverticula section is placed in a Technicon[2] autopsy tray with its identifying code label (Fig. 26). The tray is immersed in a petri dish of sea water so the tissue will not dry out before fixing. The portion below the fourth cut from each animal is frozen for future reference, or discarded. The 1- to 2-mm section of tissue to be used for frozen sectioning is placed in a labeled petri dish with the just-cut surface facing up, and is sectioned as described in the chapter on "Frozen Tissue Technique." When all the tissues in the sample are cut, the Technicon trays are placed in a jar of fixative (Fig. 27), coded, and processed as appropriate for the fixative. The scissors, razor blade, and stopper used for cutting should be rinsed between oysters to avoid carry-over contamination.



Shellfish abnormalities.
Fig. 1. a, Mantle recession; b, recovery (specimen courtesy S.V. Otto).
Fig. 2. a, *Cliona*; b, normal shell.

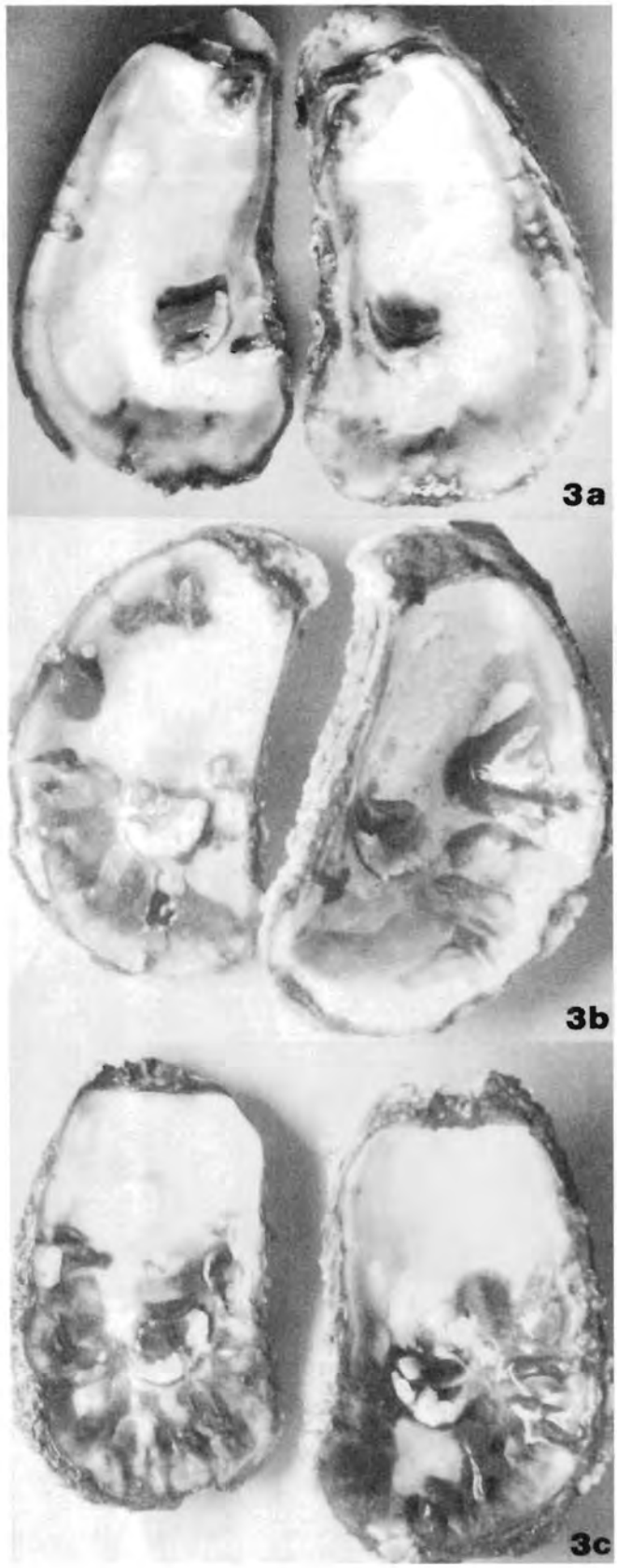
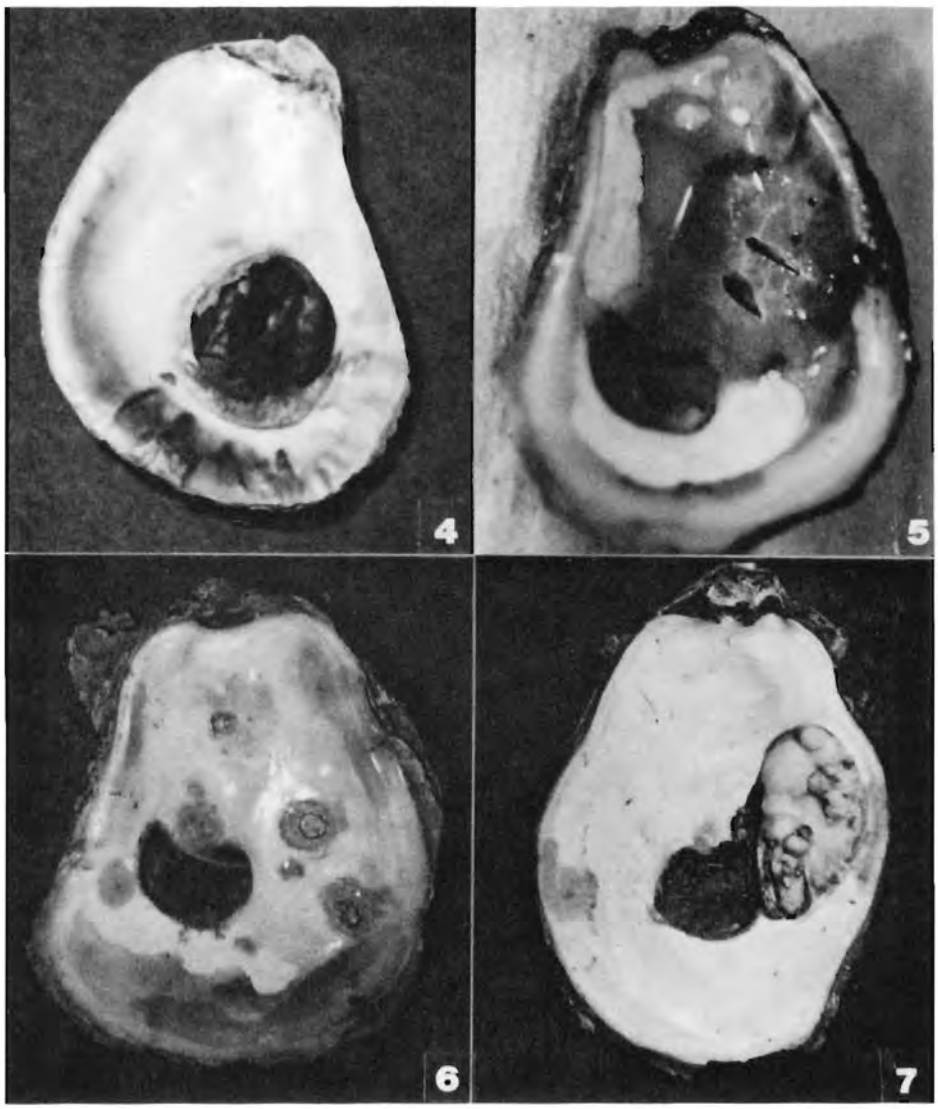


Fig 3. Shellfish abnormalities:
a, Light *Polydora* invasion;
b, medium *Polydora* invasion;
c, heavy *Polydora* invasion (specimen courtesy S.V. Otto).



Shellfish abnormalities.
Fig. 4. "Maladie du pied" (specimen courtesy S.V. Otto).
Fig. 5. Shell blisters with knife puncture marks.
Fig. 6. Shell pustules (specimen courtesy C.A. Farley).
Fig. 7. Calcareous abnormalities.

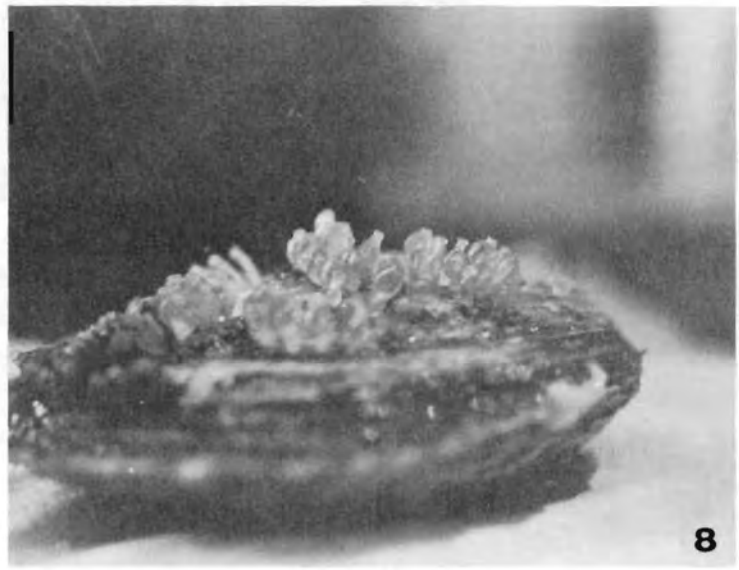
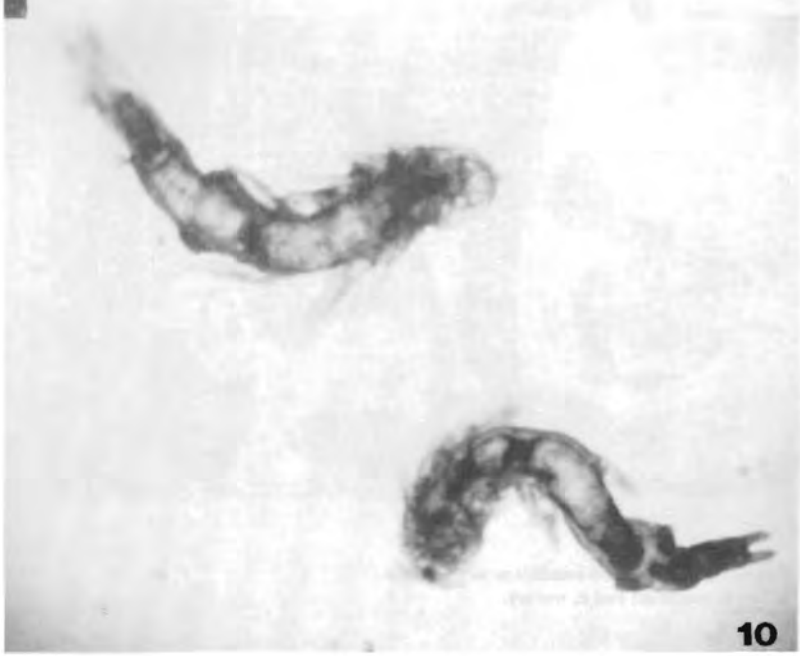
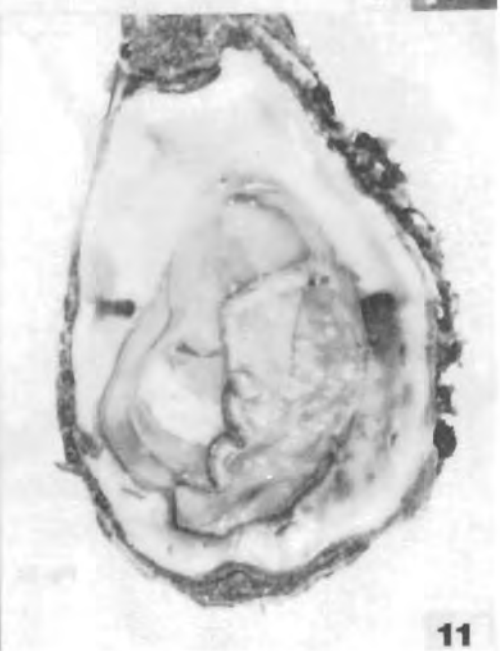


Fig. 8. Shellfish abnormality: drill cases.





Shellfish parasites.

Fig. 9. *Pinnotheres* **sp.**

Fig. 10. *Mytilicola* **sp. 10***X* **(photograph by C.A. Farley).**

Fig. 11. Multiple cysts on gill caused by *Sphenophrya* **sp. (photograph by C.A. Farley).**

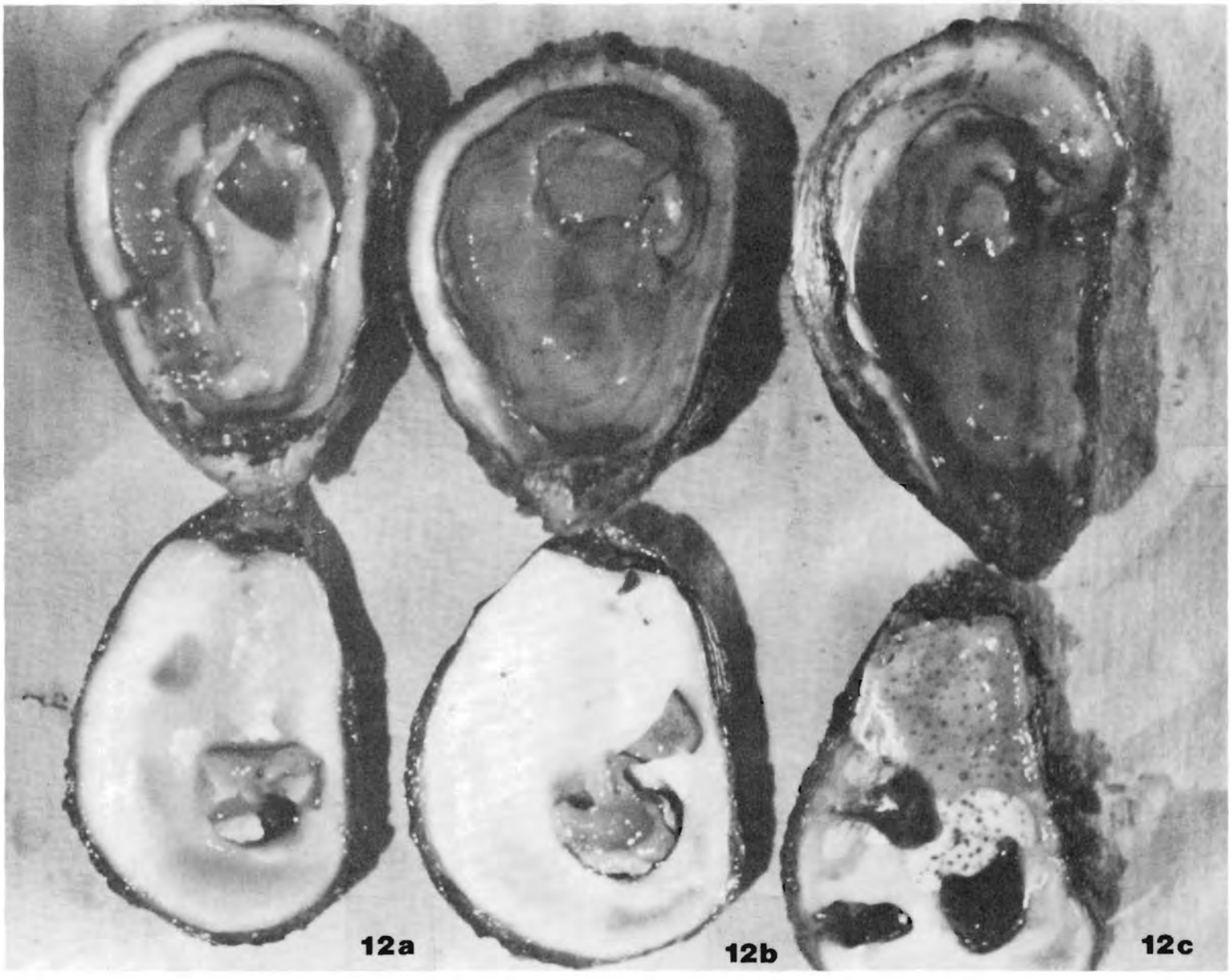
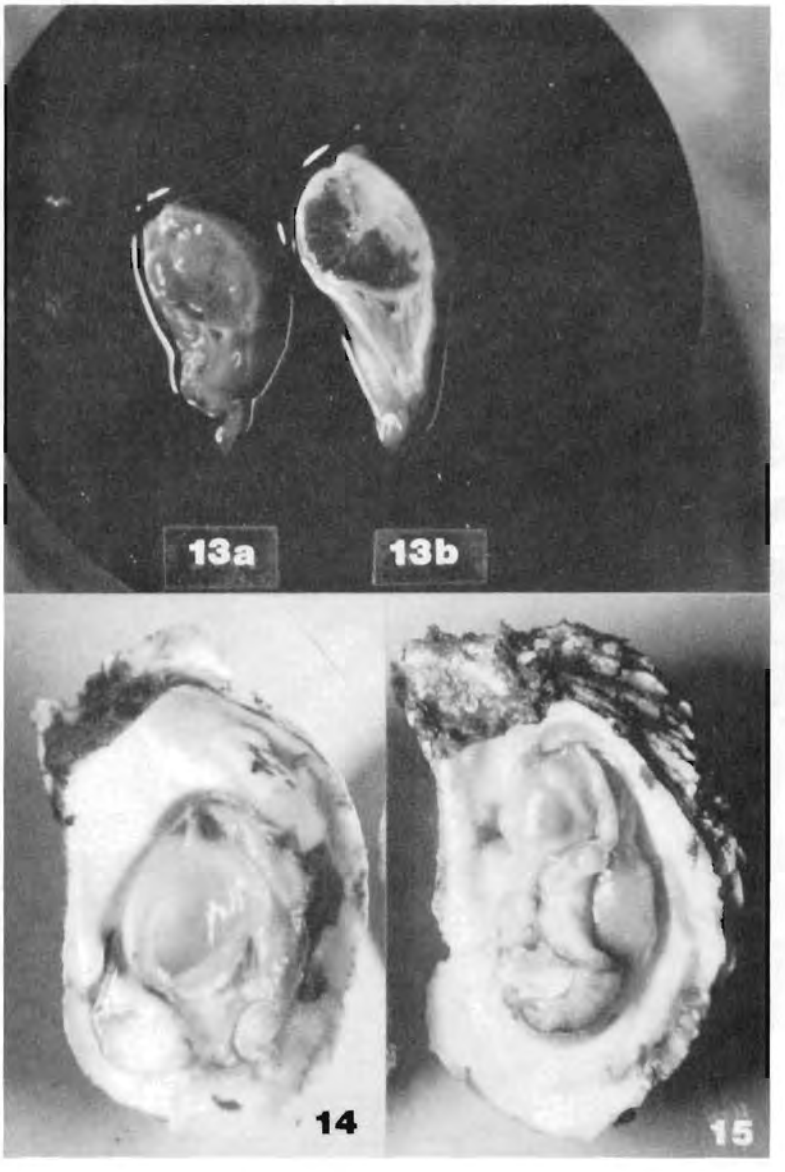


Fig. 12. Physiologically related conditions of shellfish: a, fat; b, medium; and c, watery.

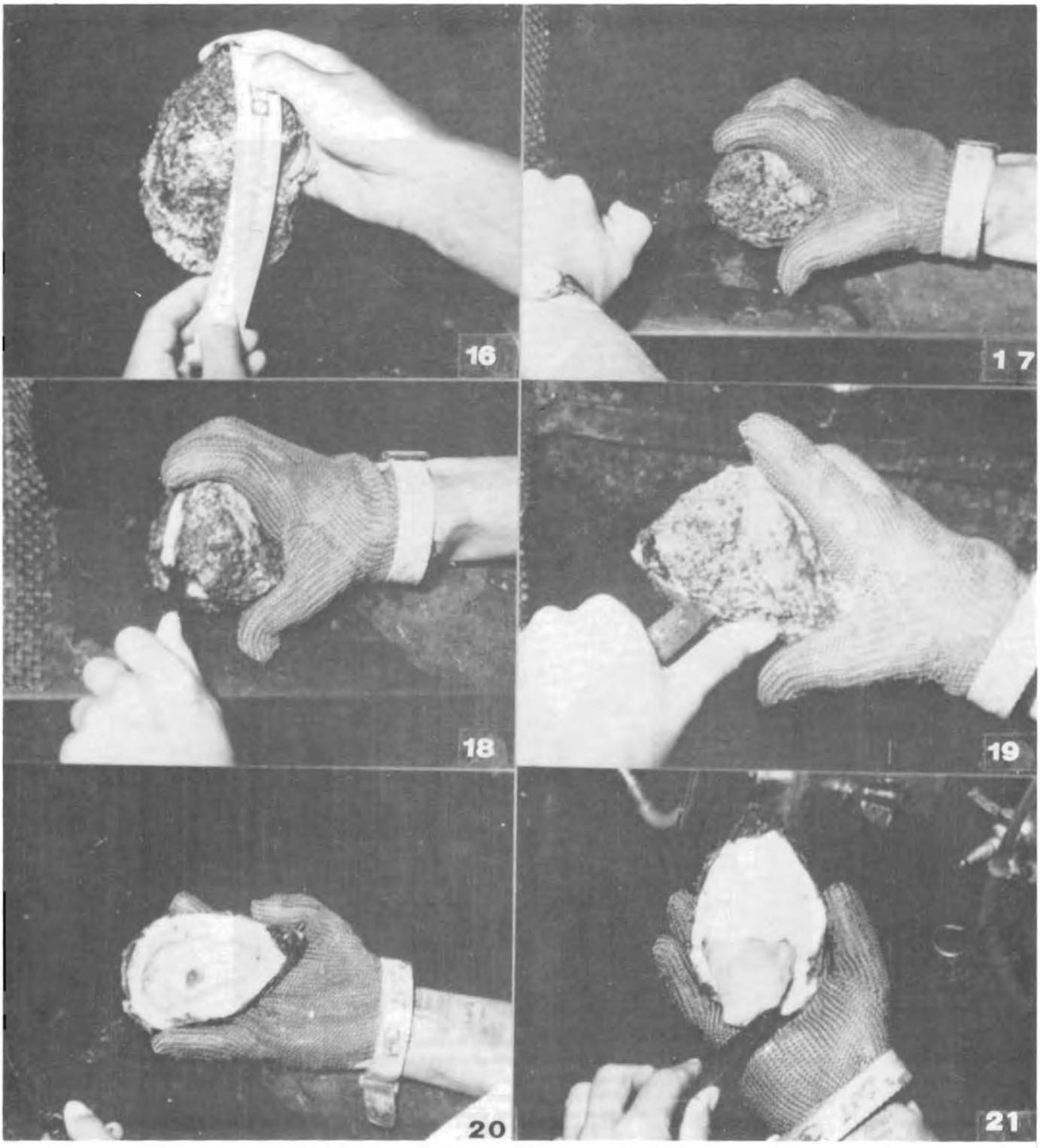


Physiologically related conditions of shellfish.

Fig. 13. a, Pale digestive diverticulum; and b, normal digestive diverticulum.

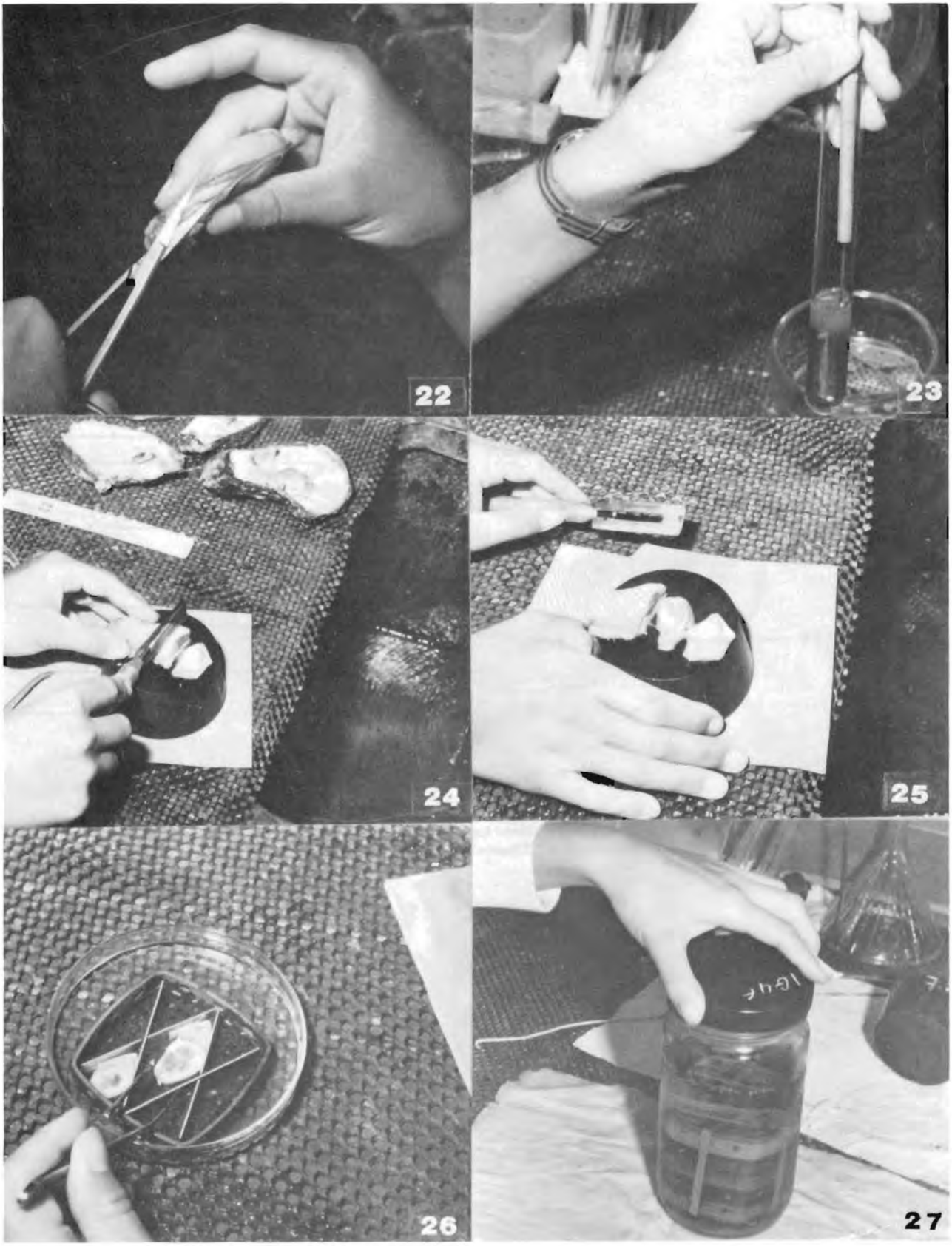
Fig. 14. Swollen heart (specimen courtesy S.V. Otto).

Fig. 15. Watery cysts (specimen courtesy S.V. Otto).



Processing of oyster, *Crassostrea virginica*.

Fig. 16. Measuring oyster from bill to hinge.
Fig. 17. Inserting knife in hinge.
Fig. 18. Twisting knife to "pop" hinge.
Fig. 19. Cutting adductor muscle.
Fig. 20. Removing top shell for inspection.
Fig. 21. Examining shell and oyster meat.



Processing of oyster.

Fig. 22. Excising rectum of oyster.

Fig. 23. Placing rectal tissue in thioglycolate for culture.

Fig. 24. Cutting cross-section.

Fig. 25. Oyster cut showing cross-section.

Fig. 26. Placing cross-section in tray with correspondingly coded label.

Fig. 27. Placing tray in fixative.

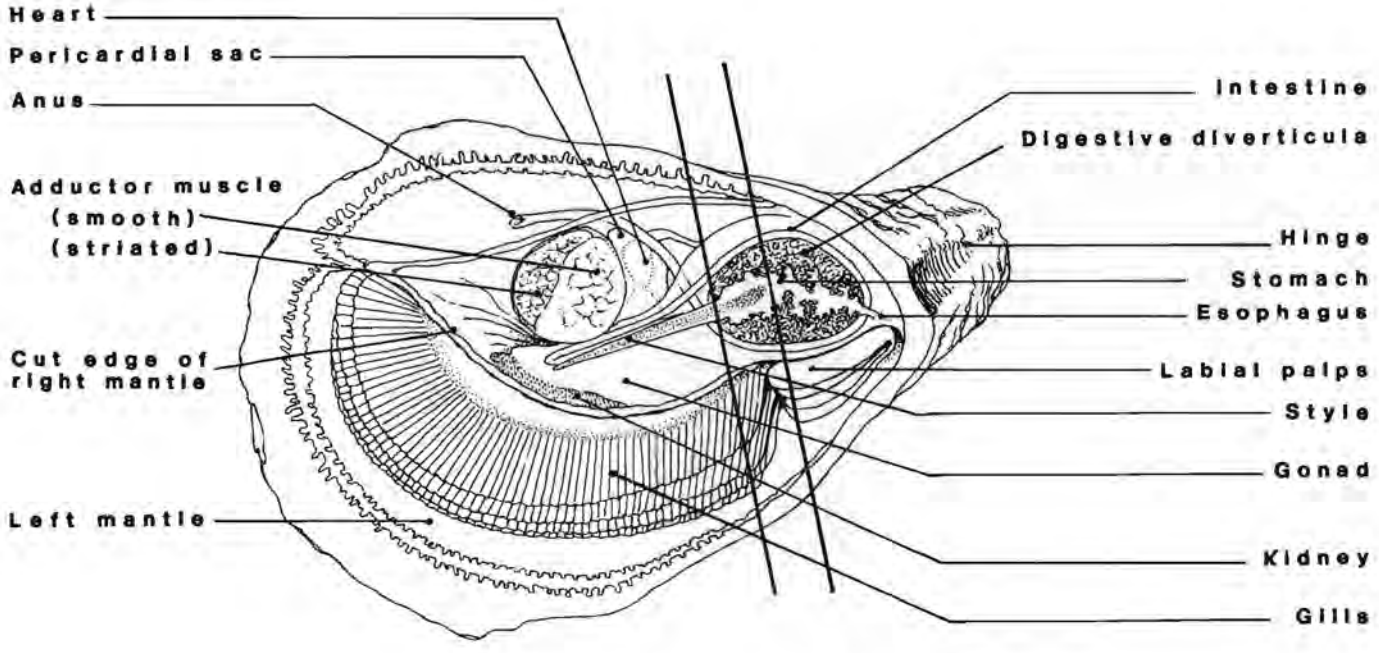


Fig. 28. **Gross anatomy of oyster. The bold parallel lines show the location where the tissue for cross-section should be taken (illustration by A.J. Lippson).**

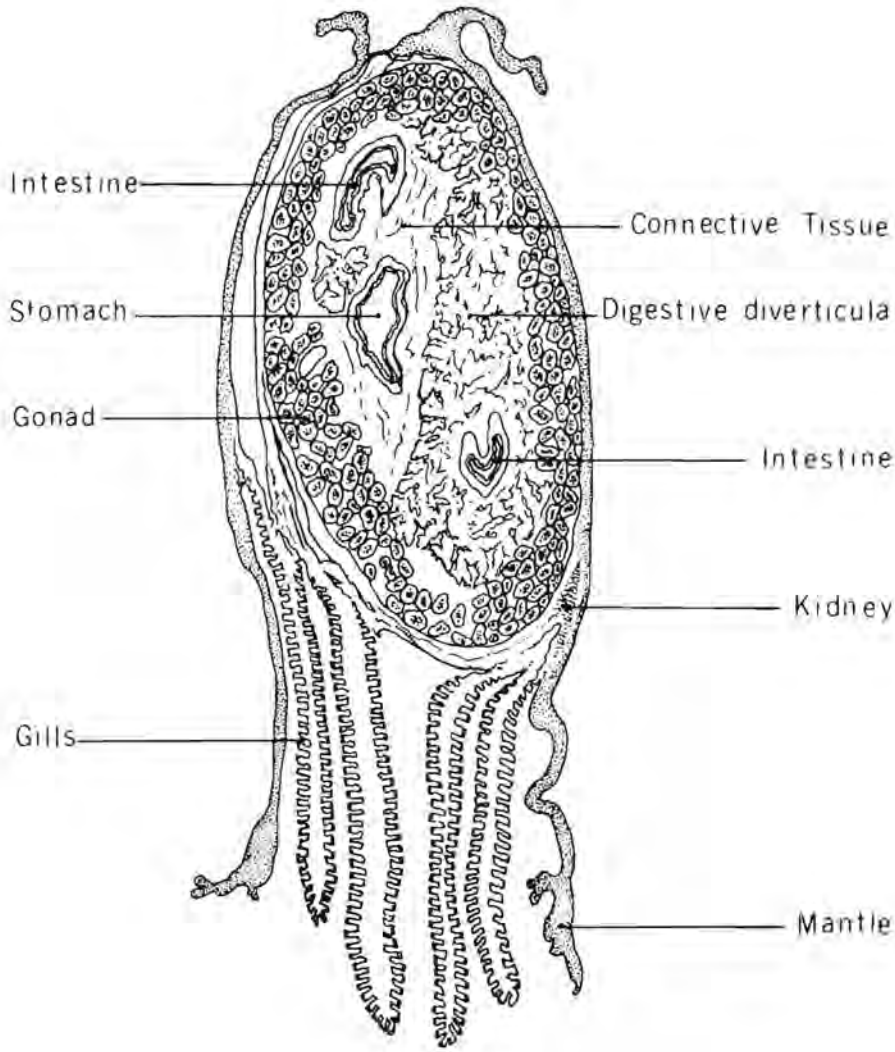


Fig. 29. Microscopic illustration of cross-section of oyster (illustration by †A.J. Lippson).

2. Clams

Clams can be successfully opened with relative ease when the right equipment and techniques are employed. Different species and various sizes will dictate the appropriate technique. The following methods have been utilized at the Oxford Laboratory.

The clams are held in a chilled environment until ready for processing. Chilling retards the metabolic rate and renders the clams sluggish and relaxed.

a. Surf clams (*Spisula solidissima*)

These large clams have an extensive muscular foot which prevents tight closure, and can be easily shucked. A long, sharp, flexible knife such as a fillet knife is used. Begin near the anterior adductor muscle, insert the knife behind the mantle next to the shell, and sever the muscle. Work the knife around the edge of the shell and cut the posterior adductor muscle. The same manipulation is used on the bottom shell. Immerse the clam in a bucket of ambient sea water and wash well, since these animals often contain much sand. The bucket of water should be changed frequently.

b. Soft-shell clams (*Mya arenaria*)

A disposable scalpel should be used when opening this species. The shells are fragile and brittle, so a light grip is recommended. Insert the blade along the shell just below the "snout" (siphon, Fig. 30), making sure the knife is positioned just below the mantle next to the shell, and cut around shell to hinge. Gently pry shell open and cut remaining tissue from the shell (Fig. 31). The longer it takes to insert the blade into the clam, the tighter the clam will close the shell (this is true for all bivalves). Therefore, insert the blade and cut the muscle as quickly as possible. Cut off the mantle and siphon (Fig. 32). Wash well and remove crystalline style if possible before making a cross-section (Fig. 33).

c. Duck clams (*Macoma balthica*)

These clams have paper-thin shells and are frequently damaged during collection. A quick and efficient method for opening these clams has been developed using a single-edged razor blade with a handle[1]. The blade is extended from the holder and wrapped for support. The blade is both thin and flexible and can easily be maneuvered into and around the shell (Fig. 34), separating the tissue and shell without damage to the tissue (Fig. 35). Excise rectum if thioglycolate cultures are anticipated (Fig. 36). After making the cut for the cross-section (Fig. 37), remove the crystalline style and process as usual.

d. Hard clams (*Mercenaria* and *Arctica* spp.)

Clams with thick hard shells are the most challenging bivalves to open. Measure from side to side (Fig. 38). Using an oyster or clam knife, insert the tip (Fig. 39) into the clam just below the hinge which, interiorly, is just above the adductor muscle. A hammer is necessary at times to penetrate the shell. Work the blade in for approximately one-half inch and twist. This will "pop" the hinge and occasionally the pressure may break the shell. However, the purpose is accomplished because the clam is exposed, making it easy to shuck without risking internal tissue damage. After the posterior adductor muscle is cut (Fig. 40), carefully slide the knife along the edge of the shell to free the attached mantle (Fig. 41). Continue around the shell and cut the anterior adductor muscle. Use the same cutting action on the lower shell to free the clam from the shell (Fig. 42).

Any variation of the above methods may be utilized as long as the clams are opened quickly, while avoiding any internal damage to the tissues.

Once the clam has been carefully shucked and rinsed thoroughly (Fig. 43), cut a cross-section approximately 3- to 5-mm thick (Fig. 45). Include as many major organs as possible. The anatomical drawing (Fig. 44) shows the different organs of a clam with a cutaway piece of mantle and gill section. For a successful cut, a very sharp knife blade is needed. Use a sawing motion, with very light pressure, to avoid displacing the soft tissues.

Larger clams are difficult to cut uniformly. When the first cut is made, the clam retracts the muscular foot so tightly that it is hard to make a straight, *thin*, second cut. "Thinly" cut tissue sections are emphasized because the texture of the internal clam tissue is so soft that good fixative penetration is difficult to accomplish.

The crystalline style in the clam is generally severed as the cross-section is taken. Remove the style when it is present, being careful not to damage the surrounding tissue. The crystalline style will harden during the embedment process, depending on the clam species, and may cause problems when sectioning (*Macoma balthica* is a good example).

Since clams may have sand in the folds of tissue, care should be taken to remove it all. Wash well with filtered ambient sea water and inspect the interfolds of the mantle and gills prior to making the cross-section. If the sand has not been successfully removed before the animal is fixed and embedded, it will be incorporated with the tissue and will present an obvious problem when sectioning. Sand will leave saw-tooth nicks on the knife-edge, and is extremely destructive to the tissue as well, creating lines and torn ridges throughout the entire section. Broken chips of the shell and pearls can cause similar problems, so wash the tissue well before making the cross-section. Allow the live clams to remain in ambient sea water as long as possible so as to purge the sand and debris from their tissues. This will alleviate many of the problems associated with sand in the gills and mantle tissue.

3. Mussels (*Mytilus* spp.)

Mussels from intertidal habitats often have many organisms within the byssus threads surrounding the shell; therefore, all initial processing begins with a thorough scrubbing. This removes the possibility of transferring contaminating artifacts to the inside tissue. The length is then measured from bill to hinge and recorded.

To open the mussel shell, insert a scalpel (Fig. 46) into the ventral byssal cavity and gently pry a small opening. Cut the mantle along the edge of the shell (Fig. 47). Take care to position the blade behind the mantle next to the shell, and cut in a clockwise motion around the bill to the hinge of the shell.

Return the knife to the byssal cavity and, in the same fashion, cut to the top of the shell. The meat condition is recorded, along with any pertinent macroscopic information. After the animal is washed in ambient sea water, the byssus is cut just into the byssal cavity tissue (Fig. 48). The mussel is then placed on a rubber stopper and a cross-section is taken (see Fig. 49), making sure all gill and mantle tissues are in place before the cut is made. Figure 50 shows the tissue orientation before the cross-section is placed in an autopsy tray (Fig. 51). The gross anatomy of the mussel is shown in Figure 52. The cross-section taken should include as many major organs as possible (see Fig. 53).

4. Scallops (*Argopecten* and *Placopecten* spp.)

Scallops are relatively delicate animals and should be handled carefully prior to processing. Their muscle system is weak and easily detaches from the shell. They tend to gape and dehydrate quickly when out of the water. They are usually collected by dredging methods, which leaves the scallops full of sand and grit. Optimum treatment of these animals would be to immerse them in holding tanks of ambient sea water cooled to 10°-15°C for 24 hr to purge the sand from the tissues. If this is not possible, they may be wrapped in soaked burlap or seaweed and kept cool and damp with ambient sea water until processing.

Begin by measuring the shell (Fig. 54). Then open with a scalpel on either side of the hinge (Fig. 55). Once the hinge is "popped," the scalpel is gently slipped into and around the shell and the muscle is cut (Fig. 56).

After the scallop is removed from the shell, wash thoroughly, but carefully, since fine sand sticks between the gill filaments (Millipore-filtered sea water is used to wash animals). When cutting the cross-section of the scallop as shown in Figure 57, the gills and mantle can easily detach from the animal and become disoriented in the cross-section. Figure 58 illustrates the gross anatomy of a scallop. If care is taken during processing, the organs seen in Figure 59 can be included in the cross-section taken for examination. For convenience, the piece of mantle may be eliminated unless specifically desired by the investigator, since the edge of the mantle is lined with sensory eyes which can cause extensive problems when sectioning the tissue.

5. Bivalve larvae

Most larvae are fixed in Davidson's fixative (see Table 1). The acetic acid aids in disintegrating the shell. Since the dissolved carbonate neutralizes the fixative, it should be changed frequently to maintain its effectiveness. Occasional agitation will enhance fixation. After the animals have been fixed, they should be washed and placed in versenate (disodium ethylenediaminetetraacetate) (Sanders, 1972) to complete decalcification of shells (see Appendix, A.2.). The time required for complete decalcification will vary with the size and species of animal. The versenate solution should be changed daily and the sample checked at that time. Once the end point of decalcification is determined, wash the larvae well and place in 70% ETOH until embedment. Bivalve larvae are processed by hand rather than mechanically with the Autotechnicon[4]. The solutions are pipetted onto and off the larvae at designated times, using processing schedules compatible to the size of the animals (see Table 3). The vacuum oven is often used to speed the process of dehydration and infiltration of paraffin into the larvae. Very small animals can be stained with a light solution of eosin after the 70% ETOH rinse to help the technician keep track of them when performing the embedding process

Seed-size bivalves, approximately 0.3-0.4 mm, are generally shucked out with a scalpel. Pry the hinge with a firm but light pressure to expose the muscle which, when cut, will allow inspection of the specimen.

Smaller shells tend to chip when opening; therefore, once shucked, the animals are completely immersed in ambient sea water to wash out any shell debris. Each animal is encircled by the correspondingly coded label and carefully placed into an autopsy tray for fixation and later processing on the Autotechnicon.

D. NOTES

[1]Millipore Filter Corp., Bedford, Mass.
[2]Technicon Instrument Corp., Tarrytown, N.Y.
[3]Edmond Scientific, Barrington, N.J.
[4]Technicon Instrument Corp., Tarrytown, N.Y.

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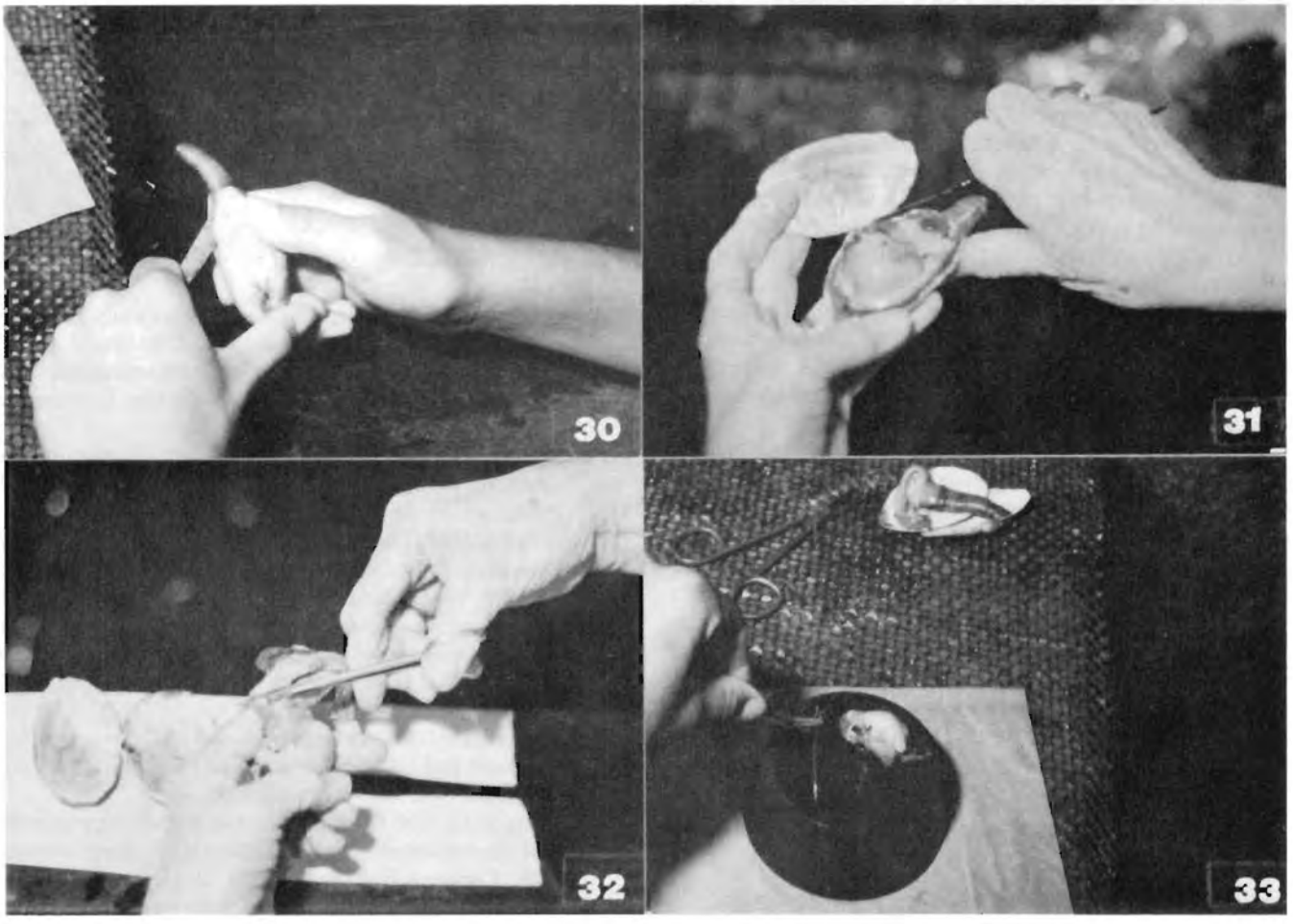
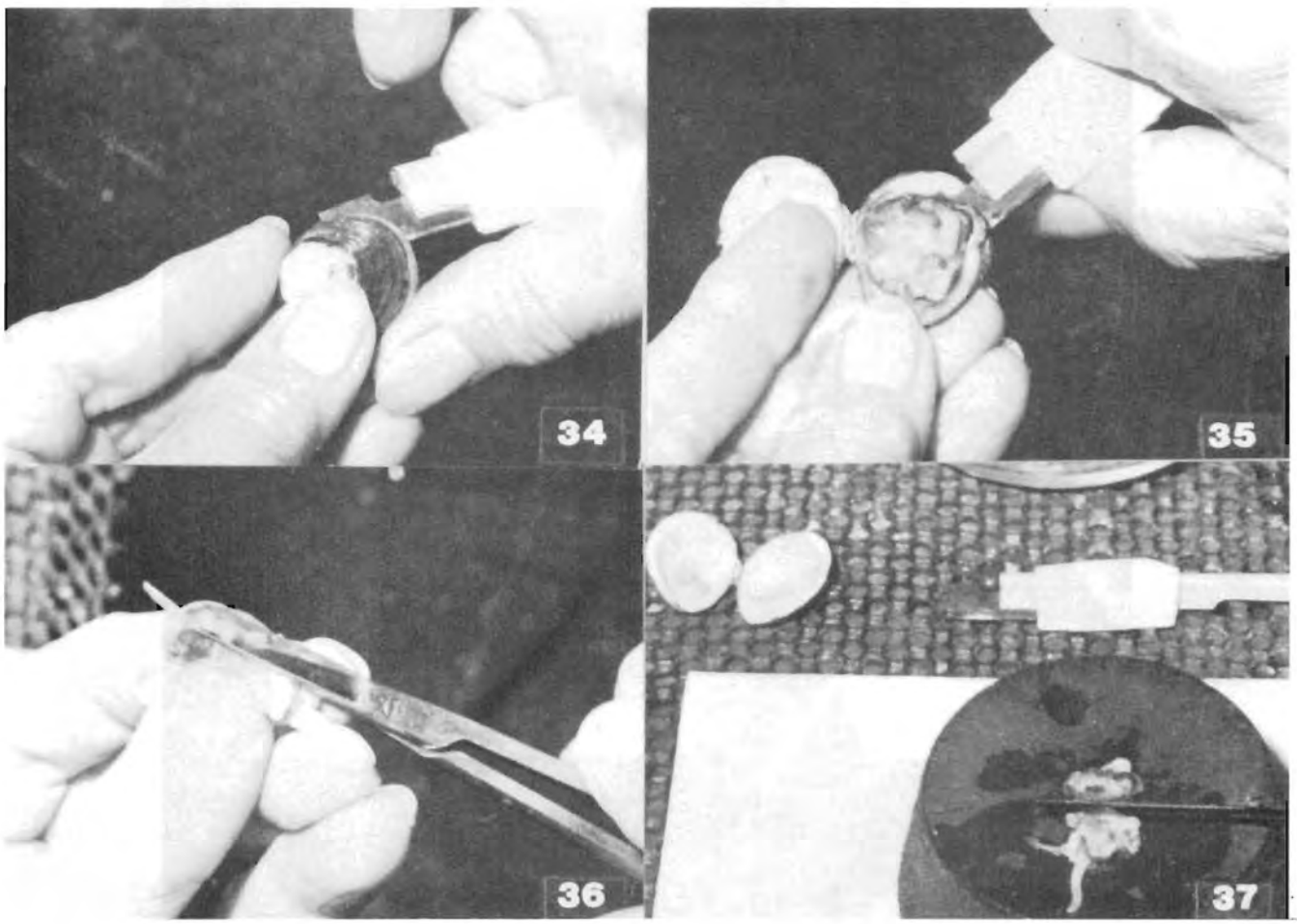
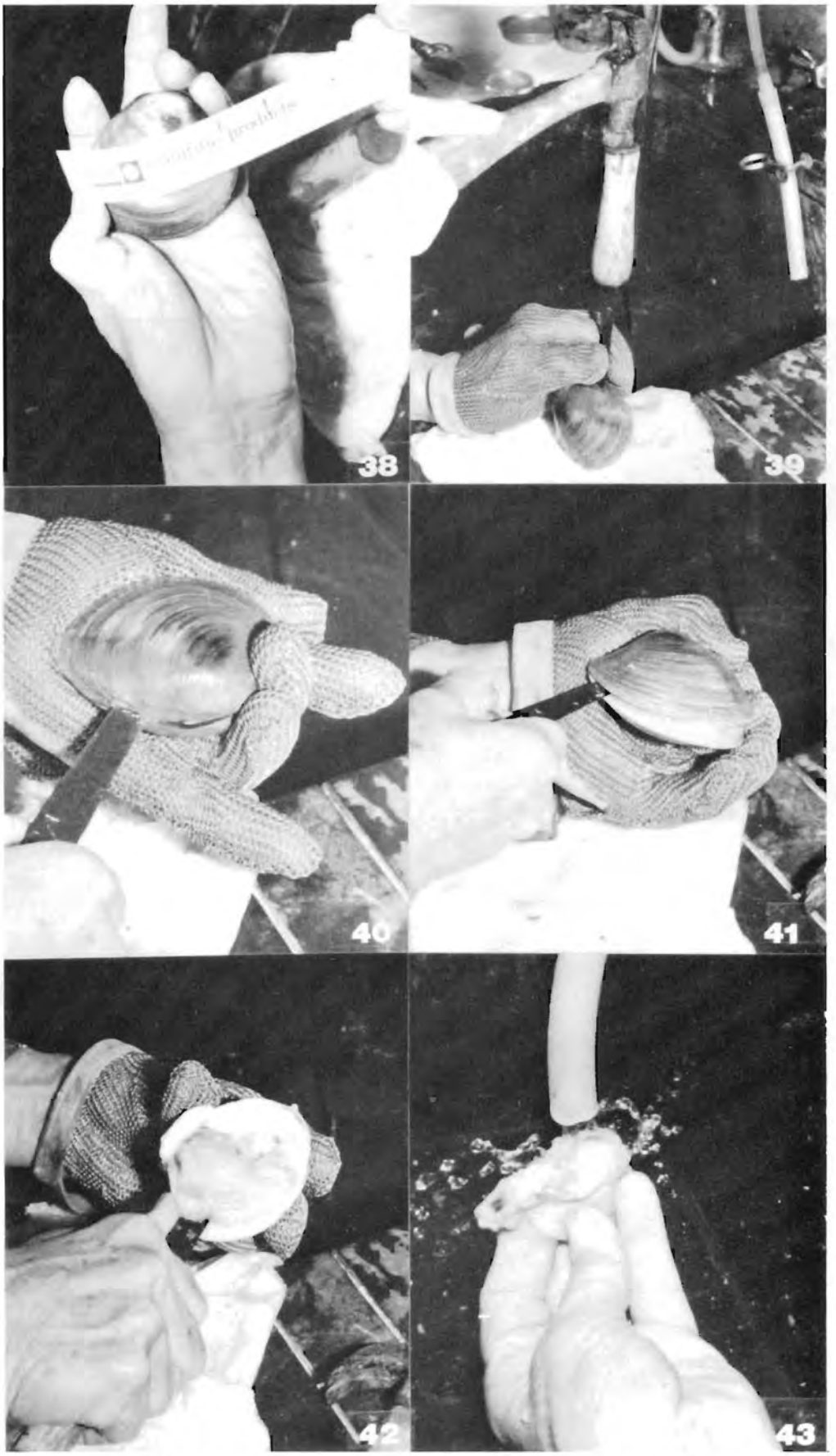


Processing of soft clam, *Mya arenaria*.
Fig. 30. Inserting knife.
Fig. 31. Carefully cutting clam muscles on lower shell.
Fig. 32. Cutting off siphon and connecting tissue.
Fig. 33. Removing crystalline style.



Processing of duck clam, *Macoma balthica*.
Fig. 34. Inserting knife.
Fig. 35. Cutting lower adductor muscle.
Fig. 36. Removing (excising) rectum.
Fig. 37. Taking cross-section.



Processing of hard clam, *Mercenaria mercenaria.*

Fig. 38. Measuring clam, side to side.
Fig. 39. Inserting knife.
Fig. 40. Cutting adductor muscle.
Fig. 41. Cutting muscles around edge of mantle.
Fig. 42. Removing clam from shell.
Fig. 43. Rinsing in ambient sea water to remove excess sediment in gills and mantle

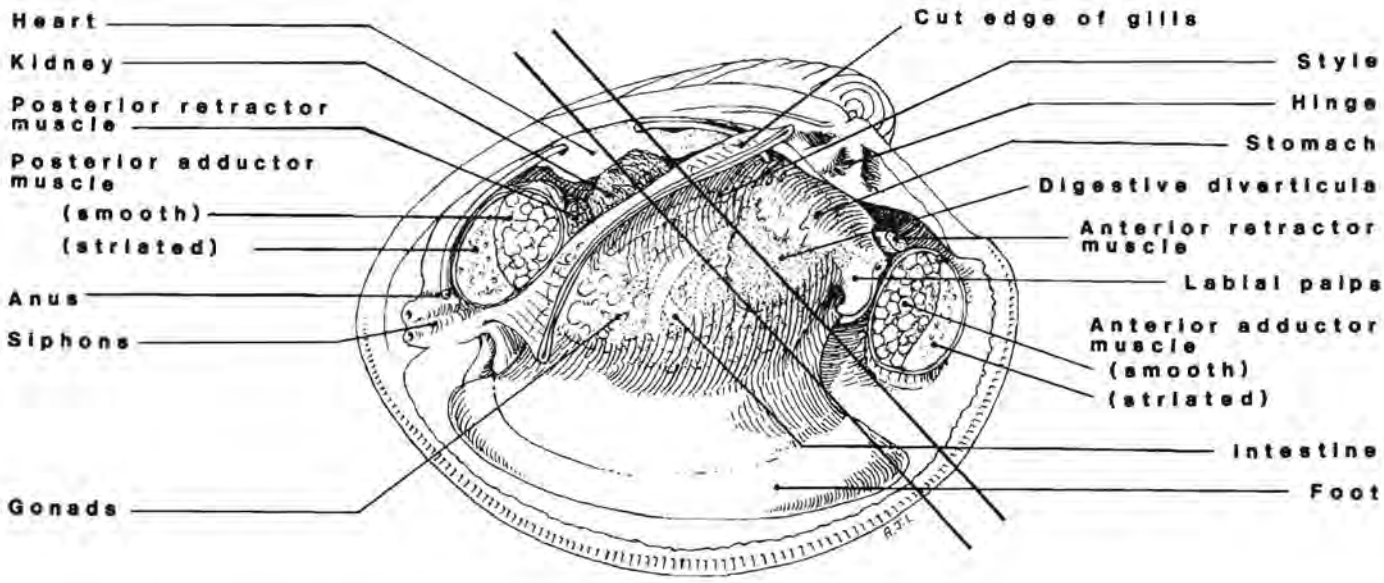


Fig. 44. Gross anatomy of hard clam. The bold parallel lines show the location where the tissue for cross-section should be taken (illustration by A.J. Lippson).

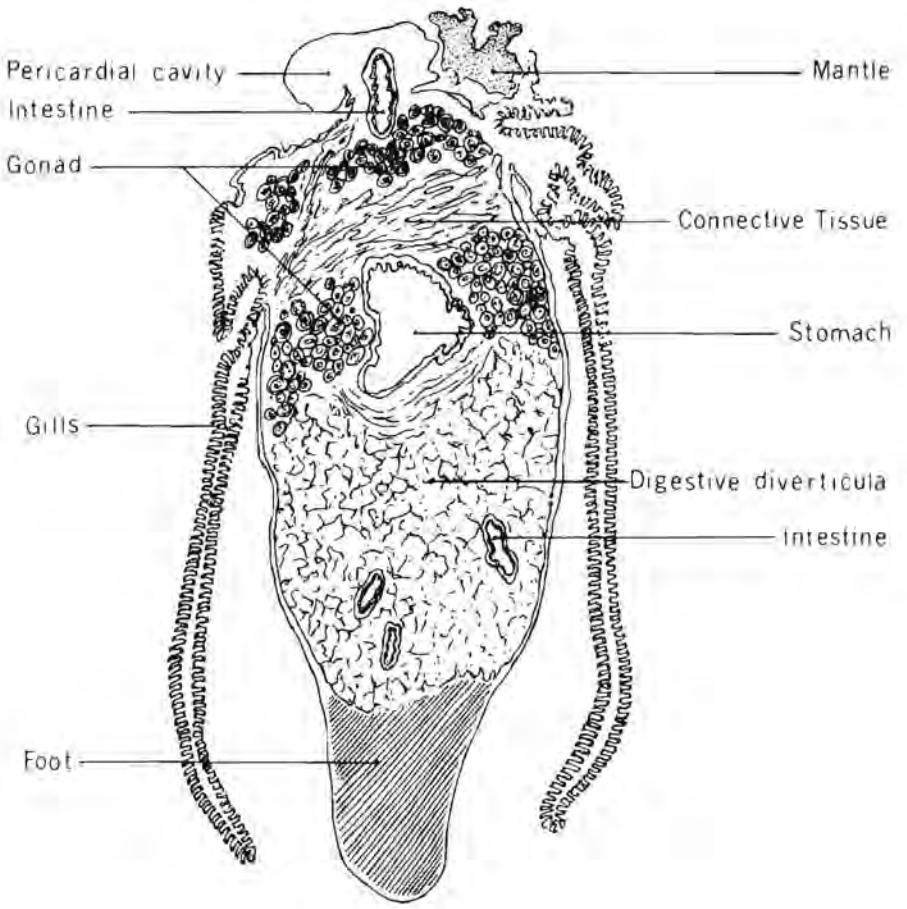
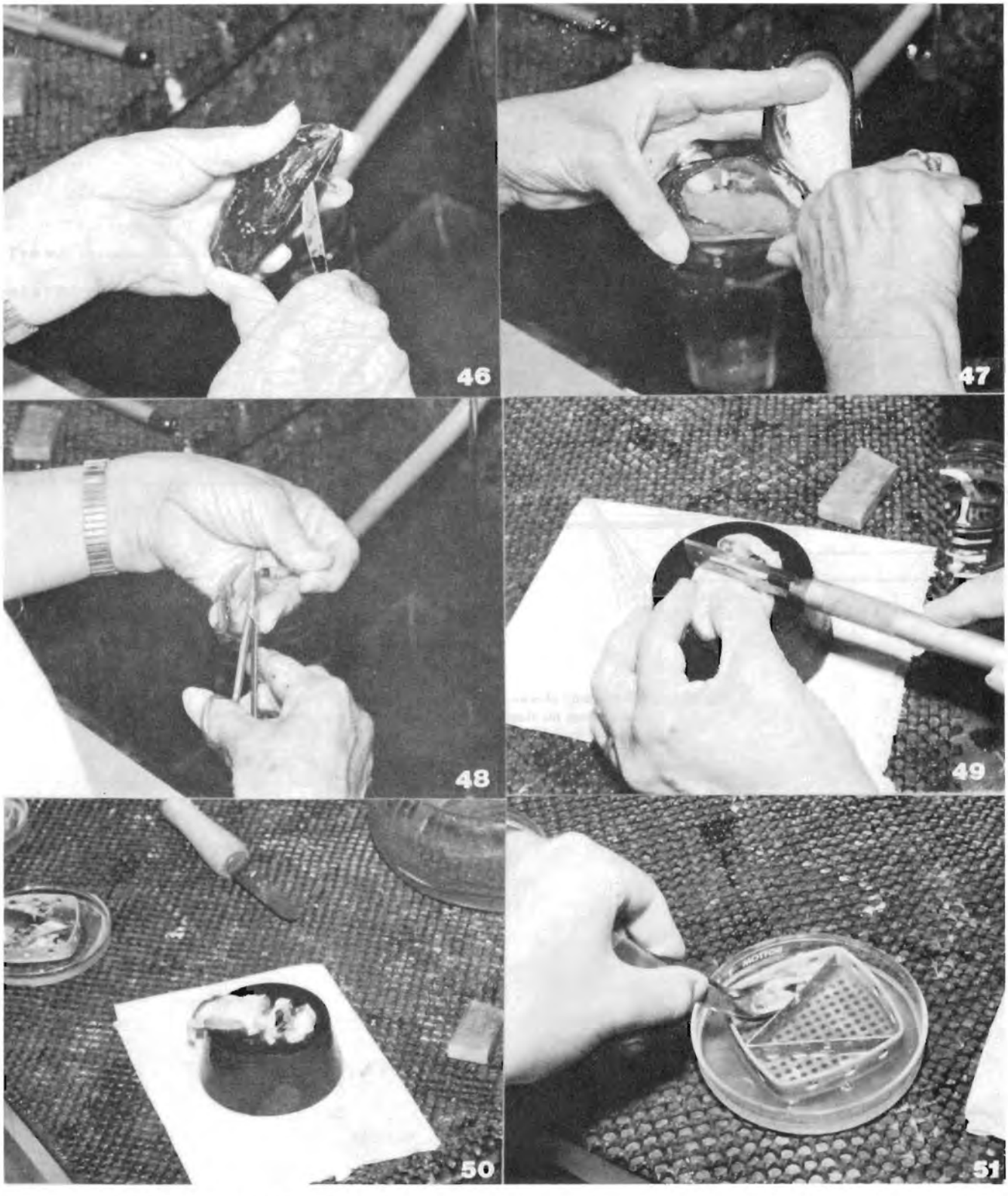


Fig. 45. Microscopic illustration of cross-section of hard clam (illustration by A.J. Lippson).



Processing of blue mussel, *Mytilus edulis*.

Fig. 46. Inserting knife.
Fig. 47. Cutting mantle along shell to expose tissue.
Fig. 48. Cutting byssus.
Fig. 49. Cutting cross-section.
Fig. 50. Orienting tissue.
Fig. 51. Placing tissue with code in autopsy tray.

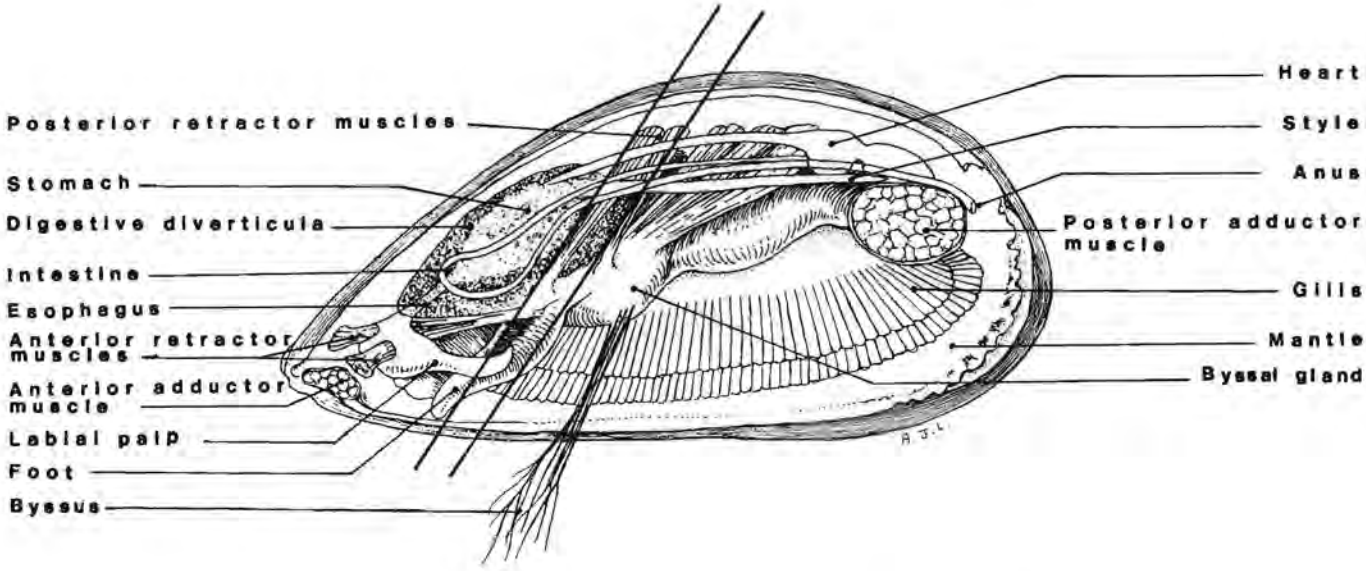


Fig. 52. Gross anatomy of mussel. The bold parallel lines show the location where the tissue for cross-section should be taken (illustration by A.J. Lippson).

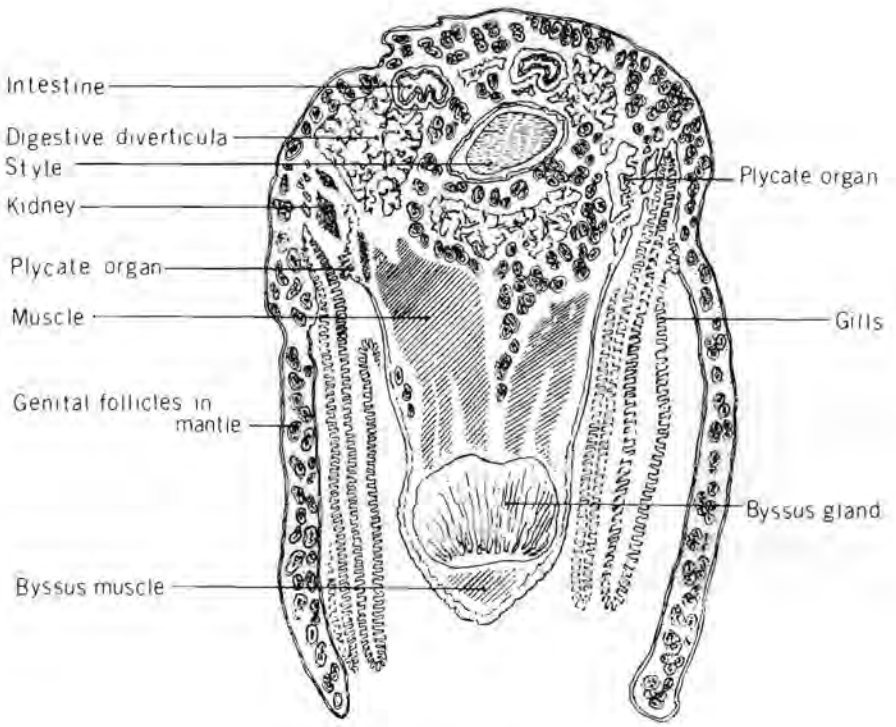
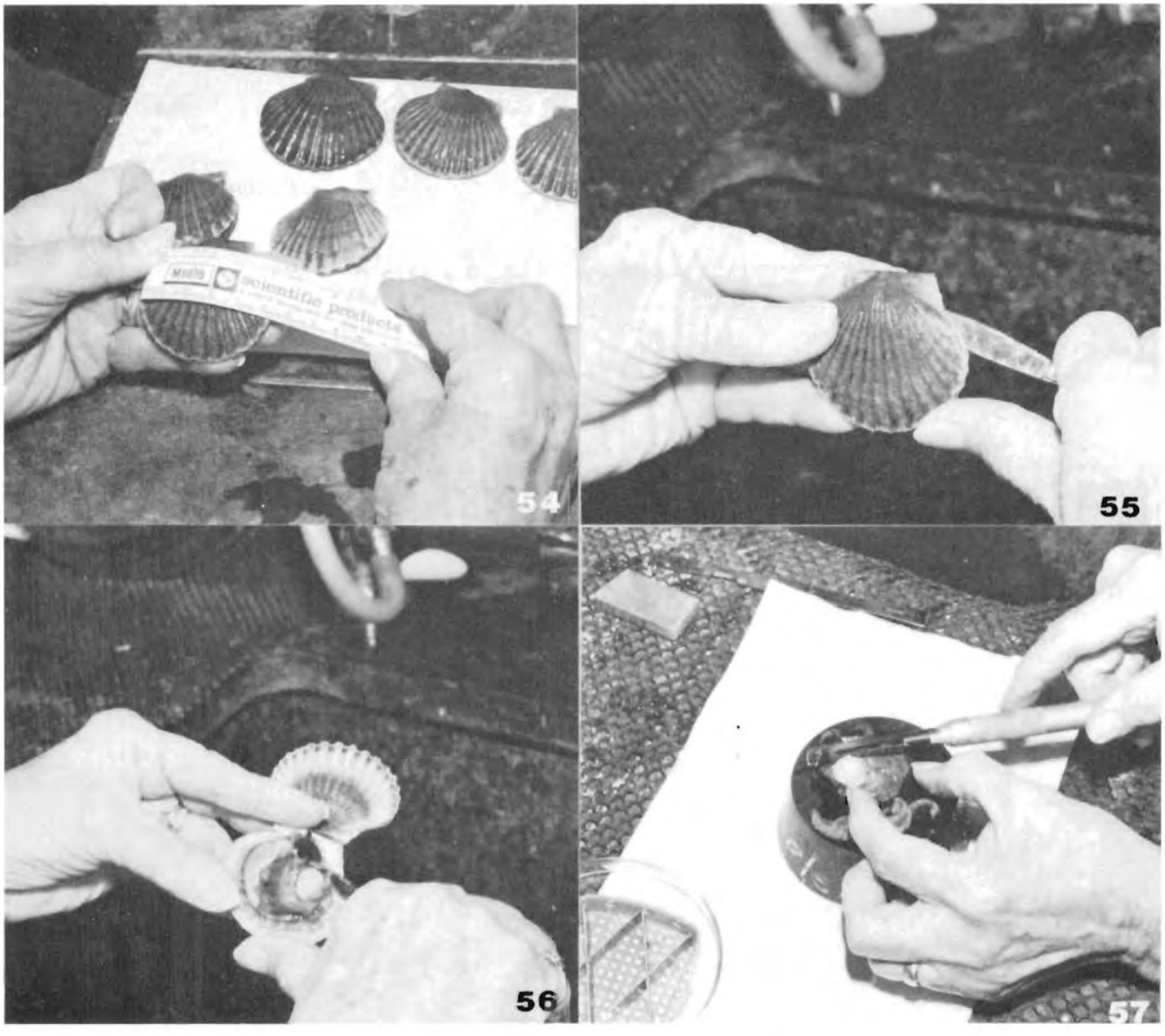


Fig. 53. Microscopic illustration of cross-section of mussel (illustration by A.J. Lippson).



Processing of sea scallop, *Argopecten irradians*.
Fig. 54. Measuring scallop.
Fig. 55. Inserting knife.
Fig. 56. Cutting muscle.
Fig. 57. Cutting cross-section.

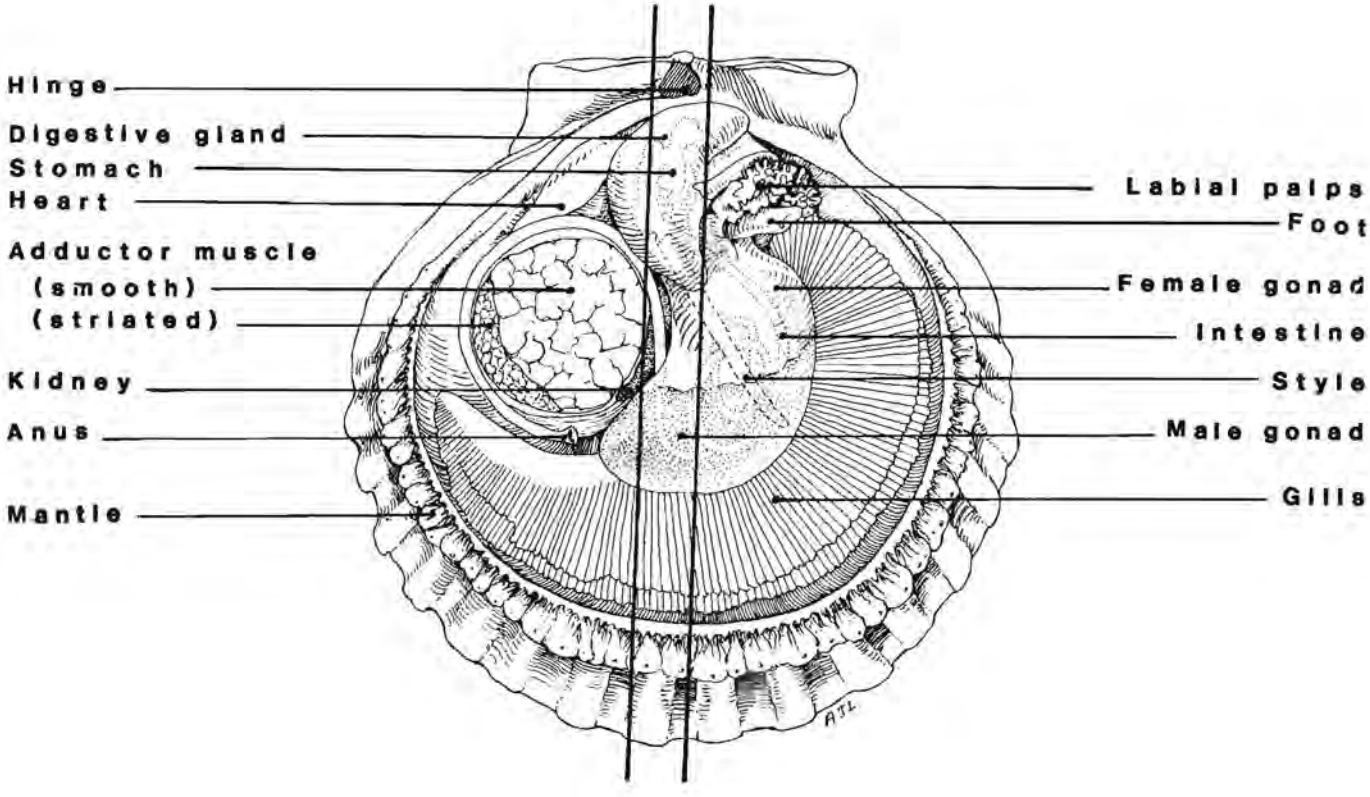


Fig. 58. Gross anatomy of scallop. The bold parallel lines show the location where the tissue for cross-section should be taken (illustration by A.J. Lippson).

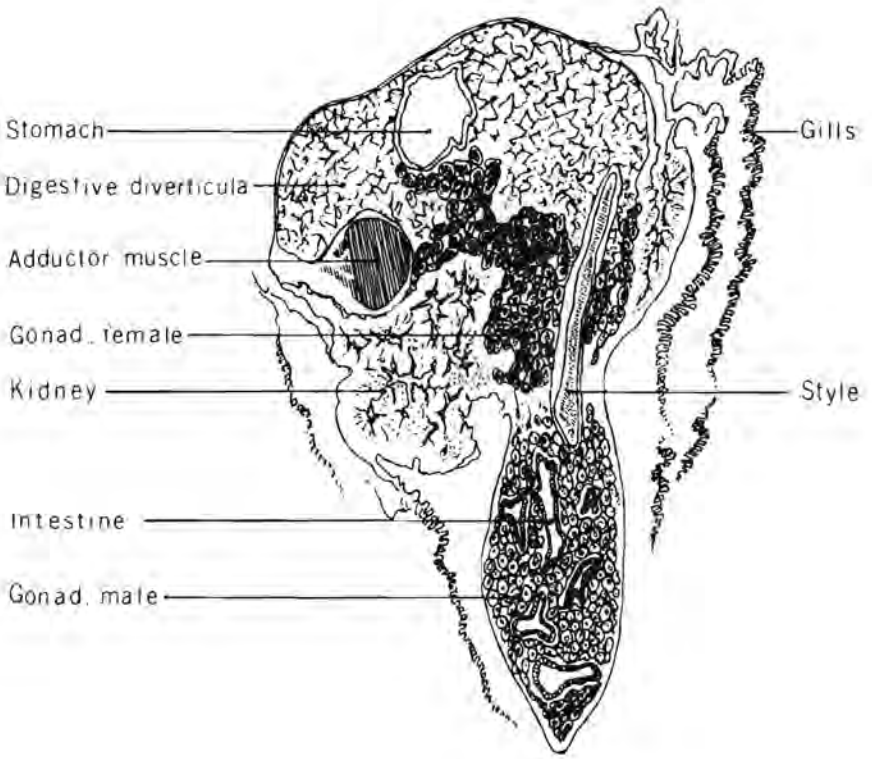


Fig. 59. Microscopic illustration of cross-section of scallop (illustration by A.J. Lippson).

CHAPTER II. FROZEN TISSUE TECHNIQUE

A. DESCRIPTION OF TECHNIQUE

Frozen tissue techniques enable a sample to be quickly surveyed microscopically for obvious parasites or lesions. Specimens which exhibit suspicious macroscopic signs such as pale digestive diverticula, watery condition, mantle recession, or other observable abnormalities may be selected for frozen sectioning and quick diagnosis.

The frozen tissue technique presented here may differ from methods used with vertebrate tissue primarily by its simplicity. Liddell (1967) outlined the following frozen sectioning method useful for most molluscan tissue:

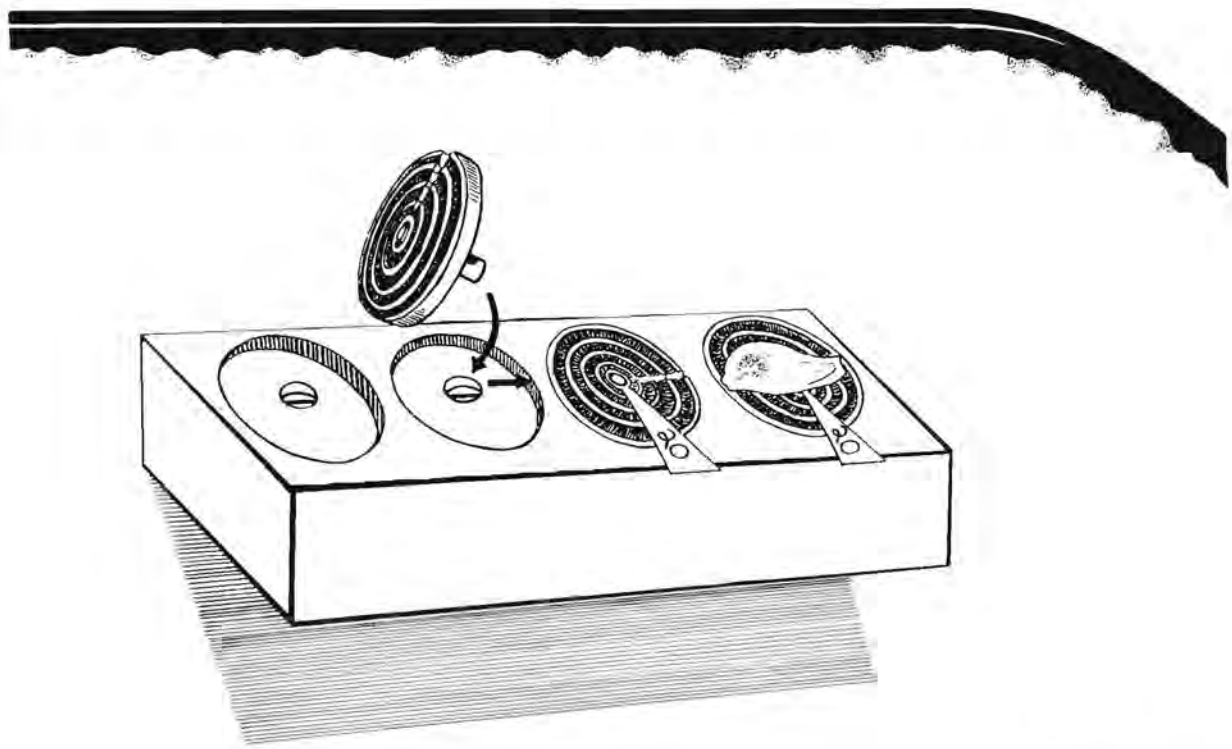
- When performing the necropsy, an extra thin cross-section is taken approximately 2 mm below the palps (see Fig. 28).
- Tissue is quick-frozen to the object holder with its appropriate code (see Figs. 60a, b, c, d).
- Object holder disc is then placed into the microtome chuck inside the cryostat.
- Tissue is gradually advanced and rough trimmed until the full surface of the cross-section is exposed.
- Knife blade and surrounding area are carefully brushed clean so that no ice chips or tissue residue will interfere with the surface of the tissue block.
- A frozen section is then cut at 6-8 μm.
- Tissue is picked up with a clean glass slide or coverglass.
- The section adheres immediately to the glass which is room temperature and the section melts into place.
- Slide is now ready for staining or frozen storage.

If immediate inspection of the tissue is desired, the section can be stained for a quick diagnosis.

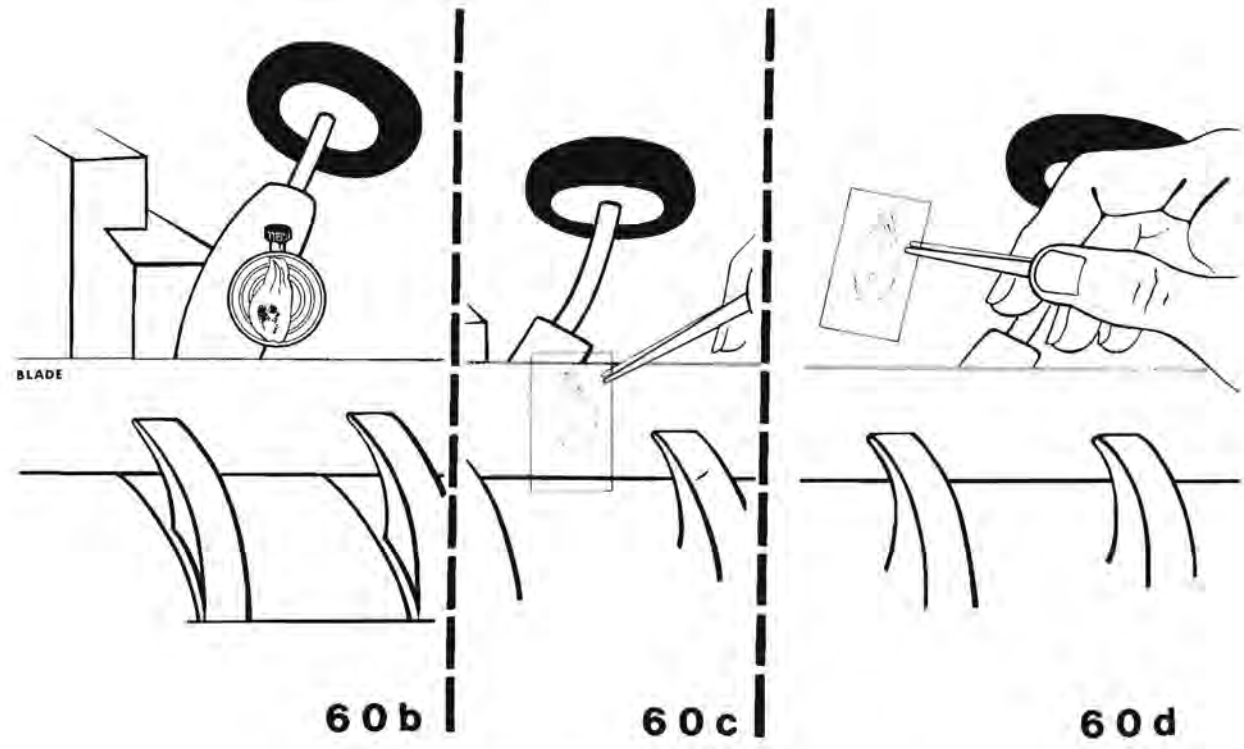
Successful cutting of frozen tissue on a cryostat or freezing microtome is accomplished by the same method that is used on a standard rotary microtome designed for sectioning paraffin blocks, the main difference being the consistency of the frozen tissue. Since molluscan tissue is soft and very moist, it should be quick-frozen while fresh. Before attempting to cut the tissue, make sure the knife and microtome have been chilled to -25°C and the knife blade is sharp and clean. Consistently good results can be obtained with fresh frozen molluscan tissue.

B. REFERENCE

Liddell, V.A. 1967. Frozen-section technique in shellfish research. J. Invertebr. Pathol. 9: 283-284.



60a



60b 60c 60d

Fig. 60. Frozen tissue technique:
a, tissue coded and frozen in object holder; b, tissue positioned; c, cut; d, picked up on coverglass (illustration by R. Tolley).

CHAPTER III. FIXATION

A. FORMULAS FOR FIXATIVES AND METHODS

The choice of a fixative depends on the nature of the tissues, the interests of the investigator, and the purpose of the study. In most cases a general purpose fixative is preferable, especially for work in the field. Ultrastructural examinations demand specifically designed fixatives. If facilities are lacking for safe disposal of toxic substances like mercury, alternative formulations should be employed. In brief, the nature of the work and the facilities at hand determine the optimum fixative for the occasion. Table 1 lists formulas for fixatives and methods.

TABLE 1. FIXATIVE FORMULAS AND METHODS.

Fixative	Fixation time	Preparation for sectioning	Remarks
BOUIN's (Coolidge and Howard, 1979) Saturated aqueous picric acid ..... 75 ml 37-40% formaldehyde .... 25 ml Glacial acetic acid ..... 5 ml	Optimum fixation time, 4-18 hr. Room temp.; pH, acid.	Optimum tissue thickness, 5-7 mm. To prepare for embedment, wash in many changes of 70% ETOH. May store larger tissue in Bouin's or 70% ETOH (if necessary).	Good fixative for protozoans or glycogen (large whole objects such as eyeballs). Not recommended for staining azure eosin or Feulgen reaction. Remove excess picric acid with many washes of 70% ETOH or 70% ETOH saturated with lithium carbonate. Good for trichrome stains and special techniques, e.g. protargol silver. *Note:* **Dry picric acid is dangerously explosive.**
DAVIDSON's (Shaw and Battle, 1957) *1 gal stock (3600 ml):* Glycerin ..... 400 ml 37-40% formaldehyde .. 800 ml 95% ETOH ..... 1200 ml Filtered ambient sea water (see III. B.) .. 1200 ml	Optimum fixation time, 24-48 hr. Refrigerate, 4°C; pH, acid.	Optimum tissue thickness, 4-5 mm. To prepare for embedment, change to 50% ETOH, 2-hr minimum; change to 70% ETOH, 2-hr minimum. May store tissue in 70% ETOH or stock Davidson's without acetic acid (if necessary).	Good standard fixative; general molluscan pathology; acceptable for most stains. Best results if stock fixative is made in order of ingredients in formula. Shake well before using stock fixative each time. *Note:* Add 1 part acetic acid to 9 parts stock Davidson's prior to fixation.
FORMALIN (10% sea water) (Lillie, 1965; Farley, pers. commun.[1]) 37-40% formaldehyde .... 10 ml Filtered ambient sea water (see III. B.) ..... 90 ml	Optimum fixation time, 24-48 hr. Room temp.; pH, neutral for best results.	Optimum tissue thickness, 4-5 mm. To prepare for embedment, wash 30 min to 4 hr, depending on size of tissue, to remove excess formalin. Store in 70% ETOH or 10% formalin.	Good general fixative, particularly good for field and cruise work because of simplicity. For seawater formalin, use filtered ambient sea water when fixing tissues in the field. *Note:* After opening a fresh bottle of commercial formalin, add a few marble chips or calcium carbonate to prevent onset of acidity.
GLUTARALDEHYDE (2%) (Feng et al., 1971) 50% glutaraldehyde ..... 4 ml Filtered ambient sea water (see III. B.) ..... 96 ml	Optimum fixation time, 2-24 hr. Temp., 4°C; pH 7.2-7.4.	Optimum tissue thickness, 1 mm. To prepare for embedment, dehydrate as routine for desired plastic embedding medium. Wash in buffered sea water, pH 7.2-7.4. Post-fix in buffered 1% osmium textroxide, pH 7.2-7.4. Store in refrigerator, 4°C.	Recommended for electron microscopy.

TABLE I. (cont.)

Fixative	Fixation time	Preparation for sectioning	Remarks
1% GLUTARALDEHYDE/4% FORMALDEHYDE (1G4F) (McDowell and Trump, 1976; Farley, pers. commun.[2])			
37-40% buffered formalin stock: Formaldehyde ..... 5 gal Na_2HPO_4 (disodium phosphate) ..... 284 gm Phenol red ..... 0.5 gm NaOH (sodium hydroxide) ..... 1.2 gm	Optimum fixation time, 24-48 hr for penetration. See original paper for specific information. Room temp.; pH 7.2-7.4	Optimum tissue thickness, 2-3 mm. Wash well in ambient sea water prior to embedment. Store tissue in fixative at room temp. until ready for embedment. Tissue will tolerate long storage in fixative.	Good fixative for tissue to be viewed for light or electron microscopy. It is an excellent protein fixative. *Note:* If not washed well, fixative will interfere with Schiff's reaction in PAS, MPAS, and Feulgen stain. May want to use the aldehyde blocker suggested in staining procedures. Store working fixative at 4°C. Stable for 3 months.
1G4F seawater fixative working solution: 37-40% buffered formalin stock ..... 120 ml 50% glutaraldehyde ..... 20 ml Tap water ..... 360 ml Filtered ambient sea water (see III. B.) ..... 500 ml *Note:* For 10% buffered formalin, dilute 120 ml of 37-40% buffered formalin stock with 88 ml of diluent (distilled or sea water, depending on tissue to be fixed).			
HELLY's (Zenker formalin) (Coolidge and Howard, 1979)			
Zenker base ..... 95 ml 37-40% formaldehyde (add just before using) ..... 5 ml	Optimum fixation time, 6-18 hr. If fixative becomes turbid, it has lost its effectiveness and should be discarded. Room temp.; pH, acid.	Optimum tissue thickness, 2-4 mm. To prepare for embedment, wash well in running water 6-24 hr, depending on thickness of tissue. Store (if necessary) in 70% ETOH. When turbid, change to fresh 70% ETOH.	See instructions for Zenker base.
ZENKER's (base) (Lillie, 1965)			
Mercuric chloride ..... 50 gm Potassium dichromate ..... 25 gm Sodium sulfate ..... 10 gm Distilled water ..... 1000 ml	Optimum fixation time, 6-24 hr. *Note:* Overfixing will dull nuclear detail. Room temp.; pH, acid.	Optimum tissue thickness, 2-4 mm. To prepare for embedment, wash well in running water 6-24 hr, depending on thickness of tissue. Store (if necessary) in 70% ETOH.	Recommended for fixation of protein; gives exceptional cytologic detail in tissue. Remove mercury crystals before staining. *Note:* Good as a mordant for metachromatic staining. Use special caution with mercury disposal. **Avoid metal containers.**
ZENKER's (acetic) (Coolidge and Howard, 1979)			
Zenker base ..... 95 ml Acetic acid (add just before using) ..... 5 ml	Optimum fixation time, 6-18 hr. Room temp,; pH, acid.	Optimum tissue thickness, 2-4 mm. To prepare for embedment, wash well in running water 6-24 hr, depending on thickness of tissue. Store in 70% ETOH.	Good nuclear fixative. Remove mercury crystals before staining. *Note:* Good as a mordant for metachromatic staining. Use special caution with mercury disposal. **Avoid metal containers.**

B. SPECIAL NOTE ON SEA WATER

When sea water is not readily available, use either of the following as substitutes where indicated:

Salt water, using "Forty Fathoms Bio-Crystals"[3]:

Approximately 40 gm/liter = 30 °/₀₀ (normal saline)
Approximately 20 gm/liter = 15 °/₀₀ (normal saline)
Approximately 14 gm/liter = 8.5 °/₀₀ (normal saline)

Note: Check the salinity of each batch of sea water with a salinometer for best results.

Salt water BSS (balanced salt solution) (Tripp et al., 1966):

Sodium chloride (NaCl)	23.50 gm/liter
Potassium chloride (KCl)	0.67 gm/liter
Calcium chloride ($CaCl_2$) (anhydrous)	1.10 gm/liter
Magnesium chloride ($MgCl_2$) (anhydrous)	2.03 gm/liter
Magnesium sulfate ($MgSO_4$) (anhydrous)	2.94 gm/liter
Sodium bicarbonate ($NaHCO_3$)	0.02 gm/liter
Potassium phosphate dibasic (K_2HPO_4) (anhydrous)	0.19 gm/liter
Glucose	0.50 gm/liter
Trehalose	0.50 gm/liter
Phenol red	0.05 gm/liter

Example: To make up 7 °/₀₀ sea water, use 7 gm of BSS in 1 liter of tap or distilled water; adjust pH to 7.2 or 7.4 with buffers (see VI. A.3.e.).

Note: This formula has been modified by deleting two sugars. Three percent of the above will make the equivalent of 30 °/₀₀ ocean sea water (Farley, pers. commun.[4]).

C. NOTES ON FIXATION

Mercury-based fixatives (Zenker and Helly's) have good penetration of tissue. They are excellent for protein fixation and preservation of nuclear detail if fixation times are observed. The mercury must be removed prior to staining. The procedure used in our laboratory to recover mercury from spent fixing fluids containing mercuric chloride was reported in *Technicon Bulletin* by D. L. Europa, Pathology Laboratories, Bellevue Medical Center, New York, N.Y. (Sanders, 1972):

- Collect fixatives containing mercury in a jug and store in a hood or well-ventilated area.
- Add 20 ml of 13% thioacetamide per liter of mercuric chloride waste. The thioacetamide can be stored in a capped glass container and is stable for approximately 1 yr.
- Mix thoroughly, cap, and allow the solution to stand in a hood for 24 hr.
- After 24 hr, filter in a hood with fluted filter paper. The residue containing mercuric salt can be stored in a glass jug indefinitely for approved disposal. The clear filtrate can be discarded.

Davidson's fixative is compatible with most of the stains used and penetrates through most tissue within 48 hr.

The 1% glutaraldehyde/4% formaldehyde (1G4F) fixative is suitable for conversion of paraffin-embedded tissue to plastic embedment for use in light and electron microscopy. Penetration is slower and less predictable with 1G4F; therefore, care should be taken to cut tissues thinner during the initial necropsy.

Ten percent seawater formalin or Bouin's is recommended for convenience in shipboard or field situations. No refrigeration is necessary and, once fixed, the tissues can be held in the fixative until returned to the laboratory. However, Bouin's is not compatible with the Feulgen-related stains.

D. PROBLEMS AND RESOLUTIONS

Table 2 includes some problems or artifacts resulting from fixation or inadequate fixation, as well as some means of resolving them.

E. NOTES

[1] Modified by C.A. Farley, National Marine Fisheries Service, Oxford, Md.; 1979.

[2] Modified by C.A. Farley, National Marine Fisheries Service, Oxford, Md.; 1965.

[3] Marine Enterprises, Timonium, Md.

[4] Modified by C.A. Farley, National Marine Fisheries Service, Oxford, Md.; 1979.

[5] Yevich, P.P. and C.A. Barszcz. Preparation of aquatic animals for histopathological examination. Mimeograph, U.S. Environmental Protection Agency, Narragansett, R.I.; 1979.

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TABLE 2. FIXATIVE PROBLEMS AND RESOLUTIONS.

Problem or artifact	Possible causes	Solution, if any
Large, irregular black deposits throughout tissue.	Mercuric chloride remaining in tissue when fixed with mercury-based solutions.	Wash tissue 6-24 hr before processing. Treat with Lugol's iodine and 2% sodium thiosulfate during hydration in staining (see VI. C.).
Small, black granular pigmentation in and around blood vessels.	Formalin pigment from stock formaldehyde becoming too acid (age will decrease pH of formalin).	Once formalin is opened, add marble chips periodically to help neutralize fixative. Wash tissue ½ hr to 4 hr, depending on size of tissue (Luna, 1968).
Tissue swells when put in water bath.	Soft tissues are more difficult to penetrate with fixing agents.	24 hr after initial fixation, immerse tissues in fresh fixative and agitate periodically. Be sure tissues are cut thin during initial necropsy (Yevich and Barszcz, unpubl.[5]).
Tissue too soft; not rigid in paraffin block.	Improper penetration.	For thorough fixation of soft tissue such as shellfish "gapers," immerse whole specimen in fixative of choice for ½ hr to 1 hr. Wash thoroughly; process as usual. This procedure should firm-up tissue before necropsy. Larger molluscan tissues should be immersed in fresh fixative after 24 hr in the initial fixative and the specimens agitated periodically during the day to ensure good penetration of fixative. When the fixative has adequately preserved the specimen, wash well and follow routine embedment process (Yevich and Barszcz, unpubl.[5]).

CHAPTER IV. EMBEDDING

A. GENERAL PROCEDURES

The embedding sequence for molluscan tissue is comparable to that used for human tissue. Once fixation is attained, the tissues must be dehydrated. They are then hand-changed from the fixative through 50% and 70% ETOH, or washed according to specific fixative requirements (see Table 1). The remaining dehydration, clearing in xylene, and infiltration of paraffin is by hand or on an automatic tissue processor such as Autotechnicon[1] or Ultratechnicon[2] (see Table 3). If the entire mollusk has been fixed (a procedure not recommended because of penetration problems), that portion which is to be used for fixation and sectioning should be cut to proper size and immersed in fresh fixative.

Paraffin (Paraplast[3]) with a melting point of 56.6°C is a most satisfactory product for year-round use and is kept ready for embedding in a paraffin dispenser. The processing basket, containing eight trays of tissues, is removed from the tissue processor and placed in a paraffin bath. A smaller paraffin bath is used for ease in handling individual trays of tissues. Commercially made aluminum molds and plastic embedding rings are employed. The rings are labeled with a code corresponding to the tissues to be embedded. In order for the paraffin blocks to be released from the molds easily, the molds are dipped before use in a release dip (95 ml of 95% ETOH and 5 ml of glycerin, Lab Tek[4]). Heated forceps are used for transferring tissues from the tray to the mold to prevent cooling of the paraffin and attachment of the forceps to the tissue. Once all the equipment is in order, embedding may begin. Slowly remove one tray from the basket and place in the small paraffin bath. With heated forceps, remove a label from one tissue in the tray. Find the corresponding embedding ring and choose a mold of appropriate size. Holding the mold by its handles with thumb and middle finger, cover the bottom of the mold (no more than one-fourth full) with heated paraffin from the dispenser. With heated forceps, turn the tissue over and gently squeeze to release any trapped air bubbles (Fig. 61). Quickly orient the tissue as desired (Fig. 62) and place the embedding ring onto the base mold with the correct identification number and hold in place with index finger. Fill the mold to the top with paraffin (Fig. 63) and place in a shallow container of ice water to cool for approximately 10 min (Fig. 64). This process should be completed as rapidly as possible to prevent the paraffin from cooling, entrapment of bubbles, and layering of the paraffin within the block. After the block has cooled, use gentle pressure to remove it from the mold, and place it on a tray to be filed and kept in the refrigerator prior to sectioning. This embedding procedure yields perfectly shaped blocks cast into plastic mounting rings, which can be quickly oriented in the microtome vise for sectioning.

The Technicon basket and trays should be placed in a bath of used xylene and soaked overnight to dissolve attached paraffin. For cleaning, rinse them in 50:50 ETOH and xylene, then in 100% ETOH, and place on paper toweling to dry. Trays will then be ready for use. *Note:* Tissues too large for aluminum molds may be embedded utilizing commercially cast lead "L's" and copper plates, and mounted on fiber blocks for sectioning.

B. EMBEDDING SCHEDULES

The schedules shown in Table 3 are recommended for specific types of tissue. The best results for dehydration, clearing, and infiltration are obtained only by allowing the tissue adequate exposure time to reagents, combined with frequent agitation of tissue.

Since molluscan tissues are soft and dense, a maximum of 5-mm thickness is recommended for complete infiltration. Fresh reagents are used for each step of embedment to prevent evaporation and carry-over of reagents. The paraffin baths should be checked regularly to prevent the possibility of overheating and breaking down of the paraffin. The temperature of the paraffin should not exceed the recommended degree range (e.g. Paraplast range, 56°-62°C).

C. PROBLEMS AND RESOLUTIONS

Table 4 describes problems that could result from incomplete dehydration, clearing, or infiltration during the embedment process.

D. NOTES

[1,2] Technicon Instrument Corp., Tarrytown, N.Y.
[3] Lancer, St. Louis, Mo.
[4] Miles Laboratories, Inc., Naperville, Ill.

E. REFERENCES

Luna, L.G., ed. 1968. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd ed. McGraw-Hill, New York.

Sheehan, D.C. and B.B. Hrapchak. 1973. Theory and Practice of Histotechnology. C.V. Mosby Co., St. Louis.

TABLE 3. EMBEDDING SCHEDULES FOR SPECIFIC TYPES OF TISSUE.

19-hr routine embedding schedule (on Autotechnicon) from 70% ETOH for molluscan tissue approximately 5-mm thick

80% ETOH	1 hr
1st 95% ETOH	2 hr
2nd 95% ETOH	2 hr
1st 100% ETOH	2 hr
2nd 100% ETOH	2 hr
3rd 100% ETOH	1 hr
1st xylene	1 hr
2nd xylene	2 hr
½ xylene/½ paraffin	2 hr
1st paraffin	2 hr
2nd paraffin	2 hr

Hold tissue in fresh paraffin bath until embedment

4-hr embedding schedule (on Ultratechnicon, under vacuum) for molluscan tissue not to exceed 5-mm thick

80% ETOH	5 min
1st 95% ETOH	10 min
2nd 95% ETOH	20 min
1st 100% ETOH	15 min
2nd 100% ETOH	20 min
3rd 100% ETOH	20 min
4th 100% ETOH	30 min
1st xylene	15 min
2nd xylene	20 min
½ xylene/½ paraffin	30 min
1st paraffin	20 min
2nd paraffin	35 min

Hold tissue in fresh paraffin bath until embedment; maximum time to hold, 3 hr.

7-hr short schedule for embedding small tissue such as larvae by hand

80% ETOH	½ hr
95% ETOH	½ hr
95% ETOH	½ hr
100% ETOH	½ hr
100% ETOH	1 hr
100% xylene	½ hr
Xylene	½ hr
Xylene	½ hr
½ xylene/½ paraffin	½ hr
Paraffin	½ hr
Paraffin	1½ hr

Note: Use vacuum oven for infiltration of paraffin.

2-hr short schedule for embedding small specimens by the Ultratechnicon or vacuum oven

80% ETOH	5 min
95% ETOH	5 min
95% ETOH	5 min
100% ETOH	10 min
100% ETOH	10 min
100% ETOH	10 min
100% ETOH	10 min
Xylene	10 min
Xylene	10 min
½ xylene/½ paraffin	10 min
Paraffin	20 min
Paraffin	15 min

Note: If ETOH is not available, isopropyl alcohol in combination with toluene (instead of xylene) may be used for processing tissues.

TABLE 4. EMBEDMENT PROBLEMS AND RESOLUTIONS.

Problem or artifact	Possible causes	Solution, if any
Tissue pulls apart while sectioning with large empty spaces; loss of tissue orientation.	Improper infiltration, dehydration, clearing, and impregnation (usually large tissues or whole mollusks).	Large tissues should be processed on a longer embedment schedule, with the aid of a vacuum system (Sheehan and Hrapchak, 1973).
Tissue too soft; the block scrapes against blade rather than cutting cleanly.	Soft tissues are not only difficult to fix, but infiltration takes longer.	Soft tissues should be cut thin and processed quickly for complete fixation. Adequate time should be allowed for impregnation of paraffin.
Crumbling or tearing of section.	Incomplete dehydration, clearing, and/or infiltration. Paraffin too hot during infiltration and/or embedding.	Consider size and tissue density when planning processing of tissue. Always check temperature of paraffin (Luna, 1968; Sheehan and Hrapchak, 1973).
Tissue smells of xylene and still too soft.	Too much carry-over of xylene; paraffin has not completely impregnated the tissue.	Immerse tissue in a clean paraffin bath and agitate, while extending the processing time (Sheehan and Hrapchak, 1973).
Round holes in tissue areas.	Air bubbles trapped under tissue while embedding in paraffin.	Once tissue is immersed in paraffin, roll it over once and orient tissue as desired; this should permit air bubbles to escape.

Embedding.
Fig. 61. Placing tissue in mold.
Fig. 62. Orienting tissue.
Fig. 63. Filling mold after adding coded ring.
Fig. 64. Blocks hardening in ice water.

CHAPTER V. SECTIONING

A. TECHNIQUES

Successful sectioning of tissue for histological examination can only be accomplished with patience and acquired skill. Sheehan and Hrapchak (1973) in *Theory and Practice of Histotechnology* pose four major requirements for producing good histological slides: (1) a skilled technologist, (2) a sharp microtome knife, (3) a proper microtome, and (4) properly prepared material. Additionally, practice, experience, and knowledge of the tissues are necessary to learn the art of microtomy.

Once the paraffin is firm, the blocks are aligned in consecutive order according to the code. They should then be refrigerated for 2 hr (5°C) before sectioning. Individual blocks should be kept cool until ready for sectioning since a warm block will not section properly. Carefully mount the ring in the microtome vise, orienting the tissue gills or identification number to the right. Also, orient the head of the microtome so that the bottom surface of the block is parallel to the knife-edge. The microtome is adjusted to cut 5- to 6-μm sections, the thickness usually employed in our studies. A knife angle of between 6° and 8° is recommended for the AO Spencer 820 microtome[1]. Using the manual advance lever, trim the block until the tissue is fully exposed. Slight angling of the block may be necessary to expose all of the tissue without cutting the block to a dangerous thinness. Lift the ribbon of paraffin sections that has been created by cutting with continual, even strokes (Fig. 65). The ribbon of sections is floated on a heated water bath (Fig. 66) adjusted to a temperature of approximately 42°C. The water bath should be changed frequently and kept clean and clear of air bubbles to avoid histological artifacts. Ribbons are preferred to individual sections since the thickness of the cut is more uniform throughout.

Pre-cleaned microscope slides with etched ends are made ready for tissue sections by placing one drop of Mayer's albumin fixative (Gray, 1954) on a slide; a second slide is placed face down on the first and the two are slid apart, evenly distributing the fixative on each slide. A thin albumin coating is desired, since if it is too thick it will adversely affect the results. The coating causes sections to adhere to the slide during staining. Before placing the tissue on the slide, and to ensure accurate identity, scratch the code number of each tissue on the reverse side of the etched end of the slide, using a diamond pen. Dip the properly prepared and coded slide, etched side up, into the water bath under the best tissue section on the ribbon (Fig. 67). Guide the section into place on the slide with a small camel's hair brush. Gently raise the slide from the water and, with the handle of the brush, draw down each side of the slide gently to cut the section from the excess paraffin of the ribbon (Fig. 68). Using the brush or a probe, orient the tissue centrally on the slide, leaving at least one-eighth-inch clearance at the end of the slide. This will prevent the coverslip from coming in contact with the groove in the storage box. Place the slides in a rack to drain excess water, then into a drying oven set at 42°C for approximately 12 hr, or until the sections are completely dry. *Note:* The temperature of the water bath and oven should not exceed 42°C for tissues embedded in Paraplast (melting point 56°- 57°C). Melting tissues on the slides can cause distortion of cells in the tissue.

The completely dry slides with affixed tissue sections are removed from the drying oven and placed in a staining holder. Grasp the holder so the handle is to the left. The slides are placed in the holder vertically, etched end up and facing away from you, front to back, in consecutive numerical order. The slides are now ready for staining.

The step-by-step preparation of slides follows.

B. PREPARATION OF TISSUE SECTIONS

1. Blocks

- Gradually chill before cutting (this cuts down on thick compressed tissue sections); extreme cold will crack the paraffin. The blocks are kept refrigerated until ready to section, and during cutting they are kept in an ice bath in intended cutting sequence.
- Block should be parallel to the knife-edge. Before cutting, the blocks can be trimmed for different slide requirements (example, serial sections) or for proper orientation of tissue.

2. Microtome

- Know your microtome; keep it properly serviced and adjusted.

(1) For the AO Spencer microtome, a knife angle of 6°-8° is recommended.
(2) When knives become too short in width (because of frequent sharpening) to fit in the microtome holder, a pencil can be used to lift the blade uniformly higher. Diamond etching pens are perfect for this purpose because they are sturdy and remain straight.
(3) Keep microtome well oiled and lubricated to assure optimum operation.
(4) Tighten set screws on knife holder and block vise before cutting.

3. Knives

- Hand honing of knives usually gives the very best results for cutting tissue sections. A combination of Ivory soap and Lava soap is recommended on a Belgian honing stone. Figure 69 illustrates the honing action.
- The AO automatic knife sharpener[2] has been very satisfactory for sharpening new or reconditioned knives. B. Coolidge and R. Howard (pers. commun.[3]) suggested the combination of an abrasive (Metadi diamond abrasive[4]) and a lubricant (Shandon microsharp lubricating fluid[5]). Best results have been attained by using only the coarse setting on the machine.

4. Sectioning

- Tissue sections are cut routinely at 5-6 μm, selecting the best section within a ribbon for uniform thickness.
- Oyster, clam, scallop, and mussel tissue ribbons are generally cut and transferred to a water bath. The bath temperature should not exceed 42°C. The sections should be picked up on

slides quickly to reduce the possibility of tissue breakdown. The water bath should be cleaned daily to eliminate albumin build-up and bacterial growth in the water. Filling the water bath with room temperature water will reduce the accumulation of air bubbles in the bottom of the bath when it has heated to 42°C.

• A warming tray set at 42°C is used when doing serial sections of small tissue or when sectioning problem tissue. Two techniques follow for special serial section studies.

Small tissue for serial sections (Fig. 70):

Shellfish larvae or seed oysters must often be embedded whole because of their size. The tissue should still be oriented for the cross-section. However, to ensure that all desired tissues are exposed for study, serial sections may be requested.

(1) Hand trim the block as close to the tissue as possible.
(2) Make sure the block is parallel to the knife after trimming to ensure better serial sections.
(3) Line up an estimate of the number of albuminized slides necessary, e.g. 10 at a time, coded consecutively.
(4) Cover the slides with a small pool of distilled water.
(5) Trim to the tissue with microtome and begin saving the ribbons.
(6) Count the number of sections which will fit under the intended coverslip size, lay sections down directly on the slide, top to bottom, and ribbons across left to right.
(7) Continue this procedure uniformly for the number of slides necessary to cut through the tissue.
(8) Carefully line up ribbon strips and drain excess water.
(9) Wipe the back of the slide dry.
(10) Place on warming tray at 42°C to allow sections to flatten evenly.
(11) Eliminate air bubbles, folds, or rolls by teasing out with gentle manipulation of two wet brushes.
(12) Place slides onto racks and put in oven at 42°C to continue drying.

Note: For problem tissue that tends to split or break apart, continue to ice block in between ribbons. Lay sections down in sequence on wax-coated paper (photographic paper liners are good for this procedure) and pick up with a wet brush to transfer to consecutive slides.

Special blocks for serial sections (Fig. 71):

To fulfill requests for study slides for instructional purposes, it may be necessary to produce a large number of slides from the same block. Since blocks may be valuable examples of a specific disease, parasite, or shellfish condition, care is taken to cut a minimum of the tissue from the block.

(1) Albuminize and label desired number of slides.
(2) Tape down black paper to the counter top. Use the highly wax-coated photographic liner paper so the sections do not stick.
(3) Carefully align the block in the chuck of the microtome.
(4) Line the slides up consecutively, but not touching, and add a small pool of water (avoid overflowing the slides). The counter surface should be flat for easier slide manipulation.
(5) Begin sectioning the chilled block; lay the ribbons on the paper in the order of cutting in a bottom-to-top sequence. Try to keep as much of a continuous cutting motion as possible so that sections will be cut in uniform thickness.
(6) Separate the sections by cutting between each with a sharp razor blade.
(7) Lift cut sections with a wet brush and float in sequence onto the coded slides.
(8) Orient tissue properly and drain water from the slides.
(9) Place slides on warming tray until sections stretch out. *Warning:* For ripe oysters, clams, or very soft tissues, reduce time on the warming tray since soft tissues will sometimes break down after long exposure to heat.
(10) Place slides onto racks and put in oven at 42°C to complete drying.

C. PROBLEMS AND RESOLUTIONS

Normally an inexperienced technician will encounter many problems when sectioning tissue. The ability to recognize the source of the difficulty and to remedy it is important. A list of commonplace problems, their causes, and possible solutions is shown in Table 5.

D. NOTES

[1,2]American Optical Corp., Buffalo, N.Y.
[3]Animal Histology Unit, National Cancer Institute, Bethesda, Md.; 1977
[4]Buehler Ltd., Evanston, Ill.
[5]Shandon Southern Instruments, Inc., Sewickley, Pa.

E. REFERENCES

Gray, P. 1954. The Microtomist's Formulary and Guide. The Blakiston Co., New York.

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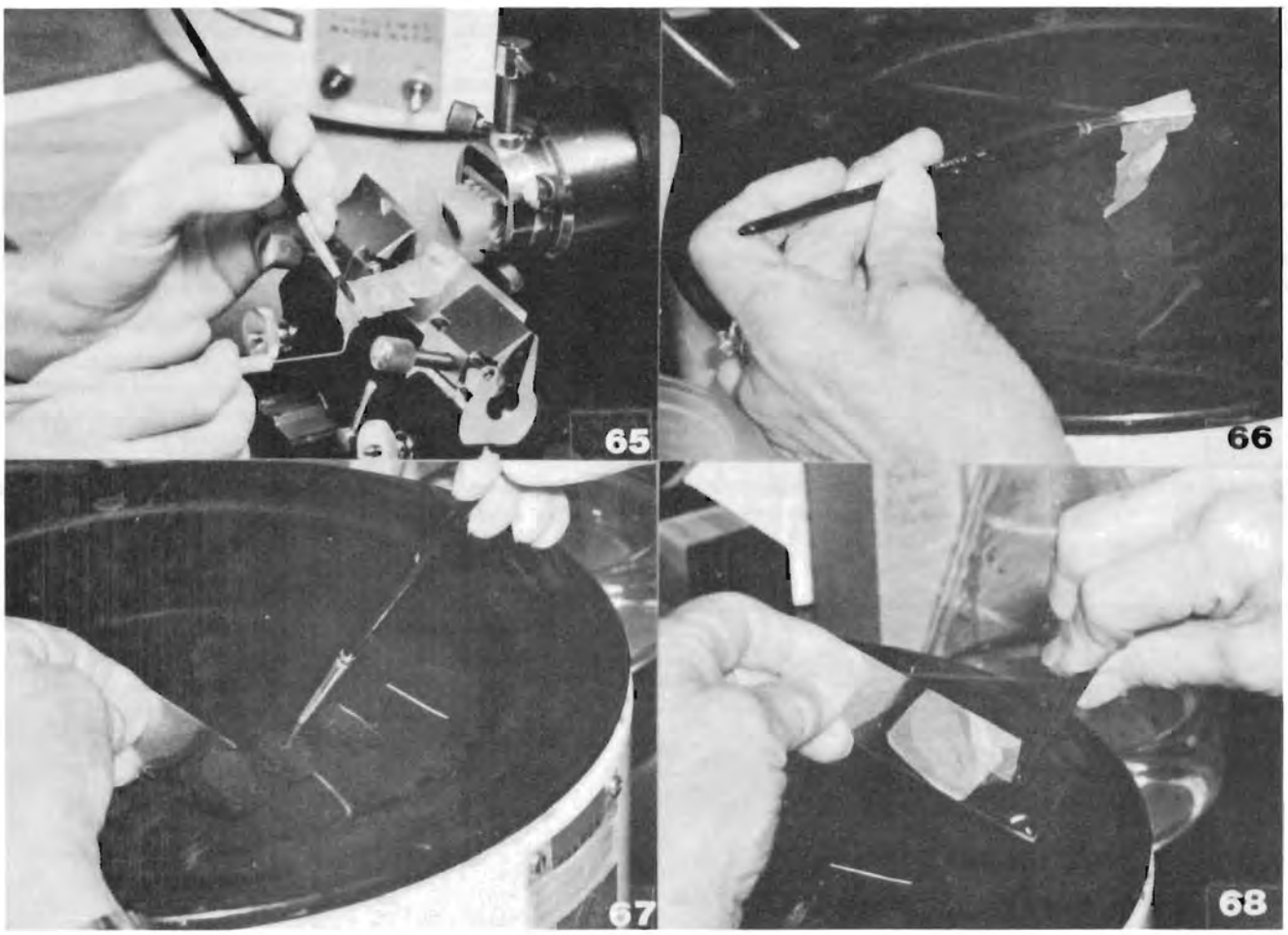
Sheehan, D.C. and B.B. Hrapchak. 1973. Theory and Practice of Histotechnology. C.V. Mosby Co., St. Louis.

TABLE 5. SECTIONING PROBLEMS AND RESOLUTIONS.

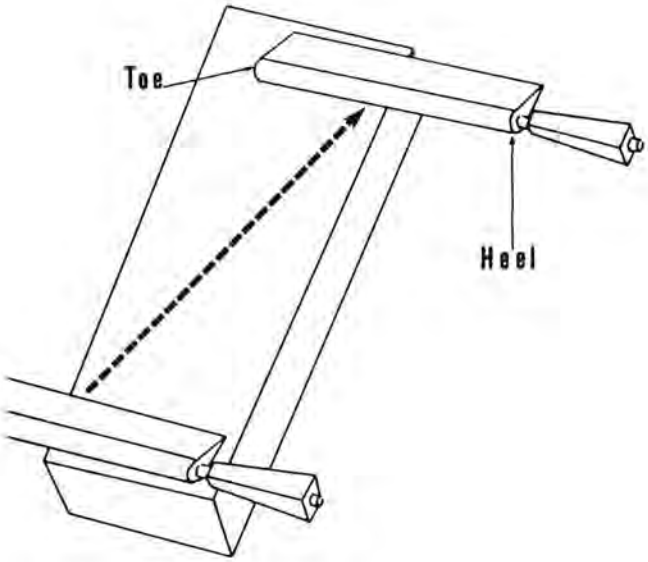
Problem or artifact	Possible causes	Solution, if any
Grooves through tissue; tissue torn and separated.	Sand, shell, miscellaneous debris left in tissue prior to fixation. Unique to species: scallop—sensory eyes; mussels—byssus; clams—crystalline style; and various bivalves—pearls. Knives need reconditioning.	Inspect tissue during preparation before cutting cross-section. Check knives under dissecting scope for damage after sectioning problem tissue. Use disposable blades for problem tissue.
Grooves through tissue, continuing from one block to another.	Nick in knife blade.	Move to new area of knife; resharpen. If not removed by sharpening, recondition knives (Luna, 1968).
Entire section splits, including clear paraffin.	Dirty knife; lint, hair, other debris, or nick in knife blade.	Clean back and front of knife carefully; move to another area on knife or resharpen knife (Luna, 1968).
Tissue rubbed or smudged, sometimes pulled apart; thickened areas indistinct.	Dirty knife, probably coated with paraffin on the back.	Clean back of knife after rough trimming of each block.
Compression (tissue section noticeably smaller than block).	Block too warm. Section too thin. Knife angle too close to vertical. Dull knife.	Sharpen knife. Strive to have tissue section comparable in size with exposed tissue in block. Keep tissue chilled before sectioning (Luna, 1968).
Shearing (horizontal parallel folds in tissue sections).	Dull knife or wrong knife angle.	Sharpen knife. Check angle of knife.
Tissue has raised round areas.	Air bubbles trapped under tissue.	Make sure water bath is bubble-free. Lay ribbon in bath gently to avoid large bubbles or folds. Bubbles can be gently teased out of tissue area by brush manipulation while tissue is floating on bath (Gray, 1954).
Moth-eaten appearance.	Rough cutting tissue.	Select tissue in middle of 6-μm ribbon. Several 6-μm sections should be taken after rough trimming of block to avoid problem (Luna, 1968).
Sections are alternately thick and thin, usually with compression of thin sections.	Set screws are loose on knife holder or the block is not tight. Block of tissue is too hard or the tilt of the knife is insufficient to clear base. This also may occur when the block is too large or too warm.	Check microtome before cutting. Adjust angle. Cool block for at least ½ hr before sectioning (Gray, 1954; Luna, 1968; Sheehan and Hrapchak, 1973).
Lifting of sections on upstroke.	Too vertical a knife angle or dull knife.	Check knife angle. Sharpen knife (Sheehan and Hrapchak, 1973).
Block lifts ribbon.	Ribbon charged by static electricity (check by testing whether or not ribbon sticks to everything else). No clearance angle. Upper edge of block has fragments of wax on it. Edge of knife (either front or back) has fragments of wax on it.	Increase room humidity. Alter knife angle to give clearance angle. Scrape under surface of block with safety razor blade. Clean knife with xylene (Gray, 1954).
Tissue with rows of round raised areas.	Shearing when section is put on water bath or warming tray.	Avoid selecting sections with shearing lines that extend into tissues.
Tissue looks distorted. Paraffin and tissue appear melted.	Water bath too hot, warming tray too hot, or slide oven too hot.	Check temperature of paraffin routinely before a tissue sample is processed; maintain warming tray and water bath temperature at 42°C.
Tissue separates quickly on water bath.	Water bath too hot, breaking down paraffin and tissue.	Maintain 42°C in water bath; transfer tissue to slides quickly.
Ribbon curved.	Edges of block not parallel; knife not uniformly sharp, causing more compression on one side of block than other.	Trim block evenly. Try another portion of knife-edge or resharpen knife. Let block cool (Gray, 1954; Sheehan and Hrapchak, 1973).

TABLE 5. SECTIONING PROBLEMS AND RESOLUTIONS. (Continued)

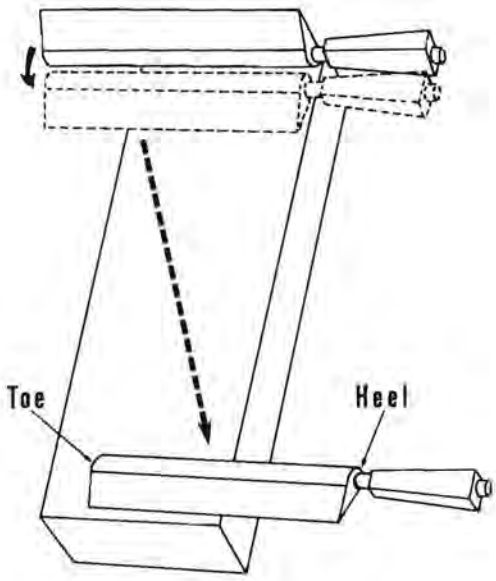
Problem or artifact	Possible causes	Solution, if any
Sections distorted.	Blunt knife. Ribbon too stretched when laid down on water bath. Tissues not properly hardened before embedding.	Use suitable knife. Handle ribbons in short lengths; lay down gently. Use more suitable fixative or fix longer. Take extra care in dehydrating, clearing, and embedding (Gray, 1954).
Sections bulge in middle.	Paraffin cool in center, warm on outside. Only sharp portion of knife is that which cuts center of block. Object may be too hard for paraffin embedment, or some clearing agent remains in object.	Let block cool evenly in ice bath. Try another portion of knife-edge or resharpen knife. Re-embed object in appropriate media or melt paraffin to remove excess clearing agent (Gray, 1954).
Tissue breaks away from paraffin or is shattered by knife.	If tissue appears chalky and shatters under knife, it is not impregnated with paraffin. If tissue shatters under knife, but is not chalky, it is too hard for paraffin sectioning. If tissue pulls away from paraffin, but does not shatter, the wrong dehydrant, clearing agent, or paraffin has been used.	Discard block and start again. If tissue is irreplaceable, try dissolving off paraffin, re-dehydrating, re-clearing, and re-embedding. Avoid xylene in clearing muscle tissue (Gray, 1954).
No ribbon forms because: paraffin crumbles; sections, though individually perfect, do not adhere; or sections roll into cylinders.	Paraffin contaminated with clearing agent. Knife angle wrong. Dull or dirty knife. Warm block.	Re-embed. (*Note*: Paraffin very readily absorbs hydrocarbon vapors) (Gray, 1954). If the section is cut very slowly, and the edge of the section held flat with a brush, ribbons may sometimes be formed. Adjust knife angle.
Sections appear wrinkled.	Blunt knife used for cutting. Water bath used for flattening too hot, so that folds in sections are fused into position.	None. Prevent by sharpening knife and cut new sections. Control temperature of water bath used for flattening. Make sure that slide is clean, so that water flows uniformly over it.
Chatter of tissue.	Occurs frequently with very ripe oyster tissues. The gonads are soft tissue and, therefore, difficult to infiltrate.	Allow block to warm to room temperature and try to ribbon. Be sure knife is sharp and tight in holder.



Sectioning.
Fig. 65. Lifting a ribbon of oyster tissue sections.
Fig. 66. Laying sections down on water bath.
Fig. 67. Directing section onto slide.
Fig. 68. Cutting excess paraffin ribbon from edge of slide.



A



B

Fig. 69. Action used in hand boning of knife blades (illustration by R. Tolley).

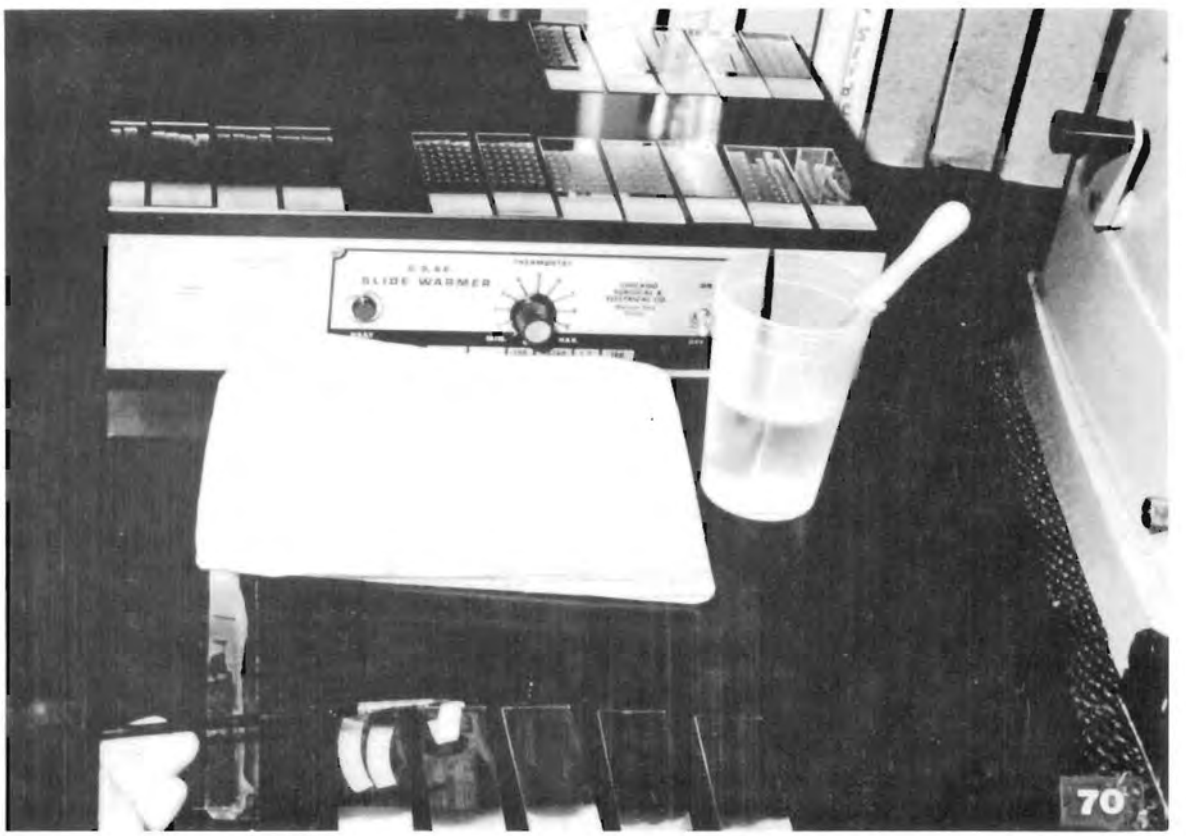


Fig. 70. Serial sections of small tissues.

Fig. 71. Serial sections of large tissues.

CHAPTER VI. STAINING

A. METHODS AND USE

The collection of staining methods described in this chapter provides routine general tissue stains, as well as a variety of special stains for the demonstration of specific tissue elements. The format for each stain description includes the staining procedure, its results, necessary reagents, additional information presented as notes, and the principal reference.

To provide for ease of use of these stain descriptions, Table 6 gives a synoptic listing of these stains, keyed to the pages on which their descriptions occur, and Table 7 provides a synoptic listing of the tissues and tissue elements to be stained for, keyed also to the appropriate pages.

TABLE 6. SYNOPTIC LISTING OF STAINS.

Name of stain	Acronym	Specific for	Approx. time	Page no.
Alcian blue	AB	Acid mucopolysaccharides	3 hr 20 min	55
Alizarin red S	ARS	Calcium	1 hr 45 min	56
Azure-eosin/Giemsa	AZE	Blood	1 hr, fresh blood	57
Azure-eosin/Giemsa	AZE	Blood; gram positive bacteria	1 hr 30 min, fixed tissue	58
Feulgen gram		Bacteria; gram positive	1 hr 50 min	60
Feulgen picromethyl blue	FPM	DNA; collagen, connective tissue	1 hr 35 min	61
Gram		Bacteria; gram positive and negative	1 hr 10 min	62
Grocott's methenamine silver	GMS	Fungi	3 hr 15 min	63
Harris' hematoxylin and eosin (progressive)	HHE_1	General tissue	1 hr 30 min	64
Harris' hematoxylin and eosin (regressive)	HHE_2	General tissue	1 hr 10 min	65
Heidenhain's iron hematoxylin and eosin	IHE	Nuclear detail	1 hr 30 min	66
Mallory's hematein		Copper metals	1 hr	67
Mallory's trichrome (modified)		Collagen, connective tissue	1 hr	68
Malt periodic acid Schiff	MPAS	Glycogen digestion and PAS positive substances	2 hr 20 min	69
Malt periodic acid Schiff-alcian blue	MPAS-AB	Neutral and acid mucopolysaccharides	5 hr	70
Oil red O	ORO	Fats	10 min, fresh frozen tissue	71
Periodic acid Schiff	PAS_1	Glycogen; fungi; neutral mucopolysaccharides	1 hr 15 min	72
Periodic acid Schiff	PAS_2	Glycogen; fungi; neutral mucopolysaccharides; good nuclear detail	1 hr 45 min	73
Perls' Prussian blue	PPB	Ferric iron	2 hr	74

TABLE 6. SYNOPTIC LISTING OF STAINS. (Continued)

Name of stain	Acronym	Specific for	Approx. time	Page no.
Sudan black B	SBB	Phospholipids	2 days	75
Toluidine blue 0	TBO	Hemocytes	30 min, fresh blood	76
Von Kóssa		Calcium	2 hr	77
Wilder's reticulum	WR	Connective tissue, reticulum	1 hr	78
Ziehl and Harris' hematoxylin	ZHH	Acid-fast spores	1 hr 45 min	79
Ziehl and methylene blue		Acid-fast bacteria	1 hr 30 min	80

TABLE 7. SYNOPTIC LISTING OF TISSUES AND TISSUE ELEMENTS TO BE STAINED FOR.

Tissues and tissue elements	Acronym	Name of stain	Approx. time	Page no.
Acid-fast:				
(a) Bacteria		Ziehl and methylene blue	1 hr 30 min	80
(b) Spores	ZHH	Ziehl and Harris' hematoxylin	1 hr 45 min	79
Bacteria:				
(a) Acid-fast (see acid-fast)				
(b) Gram positive		Feulgen gram;	1 hr 50 min	60
	AZE	azure-eosin/Giemsa	1 hr 30 min	58
(c) Gram positive and negative		Gram	1 hr 10 min	62
Blood:				
(a) Differentiation	AZE	Azure-eosin/Giemsa	1 hr, fresh blood	57
	AZE	Azure-eosin/Giemsa	1 hr 30 min, fixed tissue	58
(b) Hemocytes	TBO	Toluidine blue O	30 min, fresh blood	76
Calcium				
	ARS	Alizarin red S	1 hr 45 min	56
		Von Kóssa	2 hr	77
Connective tissue:				
(a) Collagen		Mallory's trichrome	1 hr	68
(b) Collagen	FPM	Feulgen picromethyl blue	1 hr 35 min	61
(c) Reticulum	WR	Wilder's reticulum	1 hr	78
Copper metals		Mallory's hematein	1 hr	67
DNA	FPM	Feulgen picromethyl blue	1 hr 35 min	61
Fats and phospholipids:				
(a) Fats	ORO	Oil red O	10 min, fresh frozen tissue	71
(b) Phospholipids	SBB	Sudan black B	Allow 2 days	75

TABLE 7. SYNOPTIC LISTING OF TISSUES AND TISSUE ELEMENTS TO BE STAINED FOR. (Continued)

Tissues and tissue elements	Acronym	Name of stain	Approx. time	Page no.
Fungi:				
(a) Mycelia and hyphae	GMS	Grocott's methenamine silver	3 hr 15 min	63
(b) Walls	PAS_1	Periodic acid Schiff	1 hr 15 min	72
	PAS_2	Periodic acid Schiff with Heidenhain's counterstain	1 hr 45 min	73
General tissue:				
(a) Progressive hematoxylin	HHE_1	Harris' hematoxylin and eosin	1 hr 30 min	64
(b) Regressive hematoxylin	HHE_2	Harris' hematoxylin and eosin	1 hr 10 min	65
Glycogen:				
(a) See fungi (PAS)				
(b) Glycogen digestion	MPAS	Malt periodic acid Schiff	2 hr 20 min	69
Iron	PPB	Perls' Prussian blue	2 hr	74
Mucopolysaccharides:				
(a) Acid	AB	Alcian blue	3 hr 20 min	55
(b) Neutral (see PAS, MPAS)				
(c) Neutral and acid	MPAS-AB	Malt periodic acid Schiff-alcian blue	5 hr	70
Nuclear detail	IHE	Heidenhain's iron hematoxylin and eosin	1 hr 30 min	66

1. Standard Format for Paraffin-Embedded Sections

To eliminate the repetitive cycle of solutions and times, our stain format for paraffin-embedded sections will read as follows: deparaffinize, hydrate to water, stain protocol, dehydrate, clear, and mount. Blot slides after immersion in each solution to avoid carry-over to next solution. Some stain protocols indicate a fixative of choice for best results; therefore, it is essential for the requestor to designate the fixative.

a. Deparaffinize

Immerse slides in two changes of xylene for 5 min each for sections cut from 5-6 μm thickness. Change xylene regularly to ensure complete deparaffinization.

Thicker sections will require three to four changes of xylene and longer immersion times.

b. Hydrate to Water

Carry slides through descending concentrations of ethyl alcohol to water as follows:

100% ETOH	2 changes	5 min each
95% ETOH	1 change	3 min each
70% ETOH	1 change	3 min each
50% ETOH	1 change	3 min each
30% ETOH	1 change	3 min each
Distilled water		2 min each

The 100% ETOH should be renewed frequently depending on the number of slides involved to avoid carry-over of xylene. Obvious xylene contamination in 100% ETOH will leave an insoluble scum on slides when carried into diluted alcohols. The solutions should flow smoothly off slides before descending to the next alcohol.

c. Removal of Mercury

Tissues preserved in a fixative containing mercury (Helly's, Zenker's, or Zenker acetic) must be treated with Lugol's iodine and sodium thiosulfate before staining to prevent mercury crystal artifacts.

To prepare for stain, deparaffinize slides and hydrate to water. Add Lugol's iodine solution (iodine should be approximately 10% of solution) to the first 70% ETOH and place slides in solution for 10 min. Rinse slides in 70% ETOH, change to 50% ETOH, and follow the 30% ETOH with 2% sodium thiosulfate for 5 min. Wash in running tap water 10 min, then return slides to distilled water. Continue with staining procedure (Mitchell, 1966).

Lugol's iodine:	Potassium iodide	6.0 gm
	Iodine	4.0 gm
	Distilled water	100.0 ml
2% sodium thiosulfate:	2 gm sodium thiosulfate per 100 ml distilled water	

d. Dehydrate

When the stain and counterstain are complete, the slides are taken through ascending ethyl alcohols:

95% ETOH	6 dips or 3 min
100% ETOH	1½ min
100% ETOH	3 min

e. Clear

Immerse slides in two changes of xylene for 5 min each. Change xylenes regularly to avoid alcohol carry-over.

f. Mount

Slides are ready to coverslip with the mounting medium of choice.

2. Pre-Staining Techniques

a. Aldehyde Blocking Technique

This technique precedes Schiff (Feulgen)-type stains where nonspecific aldehyde staining has occurred.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) Place slides in a saturated solution of dimedone[1] overnight
(4) Rinse thoroughly in running tap water — ½ hr
(5) Proceed with desired Schiff (Feulgen)-type stain

Reagent:

[1]Dimedone (5,5-dimethyl-1,3-cyclonexanedione) saturated solution

Dimedone	0.5 gm
Distilled water	100.0 ml

Stir or shake intermittently for at least 5 hr and filter before use.

Note: "Completeness of blockade can be tested by transferring a treated, rinsed slide directly to the Schiff's reagent, followed by the usual metabisulfite and water rinses. There should be no staining if the blockade of aldehyde groups is complete..." (Feder and O'Brien, 1968).

Reference: Feder, N. and T.P. O'Brien. Am. J. Bot. 55: 123-142; 1968.

b. Celloidin Coating for Slides

Use this technique when tissues tend to come loose from slides.

Procedure:

(1) Deparaffinize
(2) 100% ETOH — 2 changes — 5 min each
(3) Place in 0.5% celloidin[1] — 1 min
(4) Drain
(5) Place in 70% ETOH
(6) Hydrate to water
(7) Stain as usual

Reagent:

[1]1% celloidin stock solution

Parlodion	1.0 gm
100% ETOH	100.0 ml

Let stand till parlodion is dissolved.

0.5% celloidin working solution

1% celloidin stock	20.0 ml
Ethyl ether (anhydrous)	20.0 ml

(Danger. Avoid open flame or electrical discharges. Use hood. Avoid breathing vapor.)

Note: (1) For Feulgen picromethyl blue (FPM), remove celloidin after Schiff's (Feulgen), with one change of 70% ETOH and two changes of 100% ETOH, then hydrate to 70% ETOH and to water, and continue staining with picromethyl as usual. (Butanol will not remove celloidin; therefore, the celloidin must be removed in 100% ETOH before completing the stain.) (2) Do not use celloidin with alcian blue staining. The celloidin will pick up alcian blue and will mask the entire slide. (3) Use extreme caution when handling or storing celloidin. It is highly explosive.

Reference: Coolidge, B.J. and R.M. Howard. Animal Histology Procedures of the Pathological Technology Section of the National Cancer Institute. 2nd ed. Natl. Inst. Health Publ. No. 80-275, Bethesda, Md.; 1979.

c. Acid Clean Glassware

Dichromate-acid cleaning solution:

Potassium dichromate	200 gm
Distilled water	2000 ml

Dissolve with low heat.

Add slowly:

Sulfuric acid	200 ml

Extreme care must be taken in preparation of this solution and its use. Take the following precautions:

(1) **Slowly** add the acid to the aqueous solution. Place container in stone sink while pouring acid.
(2) Wear protective face mask, acid-resistant apron and gloves. Check gloves for holes each time they are used.
(3) Potassium dichromate has allergenic properties and should be handled with care.
(4) Store in acid-proof container.

Clean glassware is submerged in the cleaning solution for 2 hr or more, rinsed well in running water and several changes of distilled water. Dry and store in a dust-free cabinet.

To dispose of used acid bath, safety waste containers are recommended. **Do not pour down the drain.**

Reference: Coolidge, B.J. and R.M. Howard. Animal Histology Procedures of the Pathological Technology Section of the National Cancer Institute. 2nd ed. Natl. Inst. Health Publ. No. 80-275, Bethesda, Md.; 1979.

3. Staining Methods

a. Alcian Blue (AB) (3 hr 20 min)

Specific for acid mucopolysaccharides.

Procedure:

(1) Deparaffinize
(2) Hydrate to water and remove mercury crystals, if present (see VI. A.1.c.)
(3) Distilled water — 2 min
(4) Alcian blue[1] — 30 min
(5) Rinse briefly in running water—4-6 dips
(6) 0.5% alcoholic ammonium hydroxide[2]—2 hr
(7) Rinse briefly in running tap water—4-6 dips
(8) Counterstain in nuclear fast red[3] (Kernechtrot)—5 min
(9) Rinse briefly in distilled water (swish through in large container) — 6 dips
(10) Dehydrate
(11) Clear
(12) Mount

Results:

Acid mucopolysaccharides—brilliant blue
Nuclei—bright red or shades of blue
Background—pink to red
Other basophilic substances—various shades of blue or purple
Other cytoplasmic elements—shades of pink

Reagents:

[1]0.1% alcian blue in 0.01 M HCl (hydrochloric acid) (pH 2.2) — Helly's fixed material (Johnson, 1980)

Alcian blue 8GX	1.0 gm
1 N HCl	10.0 ml
Distilled water	990.0 ml

0.1% alcian blue in 3% acetic acid — Davidson's or formalin-fixed tissue (Johnson, 1980)

Alcian blue 8GX	0.1 gm
Distilled water	97.0 ml
Glacial acetic acid	3.0 ml

Note: Check expiration date of alcian blue stain.

[2]0.5% alcoholic ammonium hydroxide (Johnson, 1980)

Ammonium hydroxide	3.0 ml
95% ETOH	597.0 ml

[3]Nuclear fast red (Kernechtrot[a])

5% aluminum sulfate ($Al_2(SO_4)_3$):

$Al_2(SO_4)_3$	25.0 gm
Distilled water	500.0 ml

0.1% nuclear fast red:

Kernechtrot nuclear fast red	0.5 gm
5% $Al_2(SO_4)_3$	500.0 ml

Note: Refrigerate; good till loses color.

References: Pearse, A.G.E. Histochemistry, Theoretical and Applied. 2nd ed. Little, Brown and Co., Boston; 1960. (Modified by P.T. Johnson. Histology of the Blue Crab, *Callinectes sapidus*. A Model for the Decapoda. Praeger, New York; 1980.)

b. Alizarin Red S (ARS) (1 hr 45 min)

Specific for calcium (Lillie, 1965). Use a positive control test slide.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) 0.1% alizarin red S[1] — 1 hr
(4) Rinse in several dips of tap water
(5) Counterstain in 1% light green[2] — count to 15 quickly
(6) Distilled water — 6-10 dips
(7) Dehydrate
(8) Clear
(9) Mount

Results:

Calcium salts — intense reddish-orange
Background — pale green

Reagents:

[1]0.1% alizarin red S

Alizarin red S	0.1 gm
Distilled water	100.0 ml

[2]1% light green (tends to overstain) (Dahl and Dole, 1952)

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

Reference: Luna, L.G., ed. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd ed. McGraw-Hill, New York; 1968.

c. Azure-Eosin/Giemsa (AZE) (1 hr)

Specific for fresh blood smears fixed in methanol.

Procedure:

(1) Giemsa[1], pH 6.8-7.1 — 1 hr
(2) Rinse in buffered distilled water[2]
(3) Blot individual slides to prevent water spots
(4) Air-dry

Results:

Nuclei and basophilic substances — blue
Cytoplasm and acidophilic substances — pink

Reagents:

[1]Giemsa

Giemsa (azure B type) (American Scientific Cat. No. S-7712-1[b])	5.0 ml
Buffer (will vary with blood species; see McIlvaine-Lillie buffer table, VI.A.3.e.)	10.0 ml
Distilled water	185.0 ml

[2] Distilled water
Buffer to same pH of stain used

Note: (1) If coverslips are required, allow the blood smears to air-dry completely, then dip in clean xylene and mount. (2) For old fish blood smears, immerse slide in the following solution for 5-10 min:

100% ETOH	60.0 ml
Glacial acetic acid	10 drops

Rinse in distilled water and continue with Giemsa stain procedure (Drury and Wallington, 1967).

Reference: Lillie, R.D. Histopathologic Technic and Practical Histochemistry. 3rd ed. McGraw-Hill, New York; 1965.

d. Azure-Eosin/Giemsa (AZE) (1 hr 30 min)

Metachromatic stain for fixed tissues. Specific for blood studies and gram positive bacteria.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) Giemsa[1] (see table for pH, VI.A.3.e.) — 1 hr
(4) Drain 20 sec
(5) Acetone — 1½ min
(6) Acetone — 3 min
(7) 50:50 acetone/xylene — 1½ min
(8) Clear
(9) Mount

Results:

Nuclei — blue to violet
Cytoplasm — varying shades of light blue or pale pink
Muscle — bright pink
Bacteria — blue

Reagent:

[1]Giemsa

Giemsa (azure B type) (American Scientific Cat. No. S-7712-1[b])	5.0 ml
Buffer (McIlvaine-Lillie buffer table, VI.A. 3.e.)	10.0 ml
Acetone	25.0 ml
Distilled water	160.0 ml

Note: Use fresh Giemsa stain for each set of slides. The stain starts to deteriorate after 1 hr.

References: Lillie, R.D. Histopathologic Technic and Practical Histochemistry. 3rd ed. McGraw-Hill, New York; 1965. (Modified by M.W. Newman, National Marine Fisheries Service, Oxford, Md.; 1971.)

e. Fixative Guide for Giemsa stain

The following table developed by Martin Newman (pers. commun.) provides the recommended pH for buffers to be used with various fixatives. After the preferred pH has been determined, one should consult the McIlvaine-Lillie buffer table also shown below:

Fixative	pH
Formalin	4.5
Hollande	4.75
Formalin, followed by decalcifier	4.8
Zenker formalin	4.95
Davidson's	4.95
Susa	4.95
Helly's	5.1
Bouin's	5.5

McIlvaine-Lillie buffer table (Lillie, 1954)

Stock solutions:

M/10 citric acid
- Citric acid ..... 2.1 gm
- 25% methanol (25 ml methanol + 75 ml distilled water) ..... 100.0 ml

M/5 disodium phosphate (sodium phosphate dibasic)
- Disodium phosphate ..... 2.84 gm
- 25% methanol ..... 100.0 ml

Stock solutions (M/10, M/5) made in 25% methanol: pH values are for final aqueous dilution of 1:25:

pH	M/10 citric acid (ml)	M/5 disodium phosphate (ml)
2.5	20.0	0.0
2.6	19.5	0.5
2.65	19.0	1.0
2.7	18.5	1.5
2.75	18.0	2.0
2.8	17.5	2.5
2.9	17.0	3.0
3.0	16.5	3.5
3.05	16.0	4.0
3.1	15.5	4.5
3.2	15.0	5.0
3.3	14.5	5.5
3.45	14.0	6.0
3.6	13.5	6.5
3.75	13.0	7.0
3.95	12.5	7.5
4.1	12.0	8.0
4.3	11.5	8.5
4.5	11.0	9.0
4.75	10.5	9.5
4.95	10.0	10.0
5.3	9.5	10.5
5.5	9.0	11.0
5.7	8.5	11.5
6.0	8.0	12.0
6.1	7.5	12.5
6.3	7.0	13.0
6.4	6.5	13.5
6.5	6.0	14.0
6.6	5.5	14.5
6.8	5.0	15.0
6.9	4.5	15.5
7.0	4.0	16.0
7.1	3.5	16.5
7.2	3.0	17.0
7.3	2.5	17.5
7.4	2.0	18.0
7.5	1.5	18.5
7.7	1.0	19.0
8.0	0.5	19.5
8.3	0.0	20.0

Reference: M.W. Newman, National Marine Fisheries Service, Oxford, Md.; 1970.

f. Feulgen Gram (1 hr 50 min)

Specific for gram positive bacteria. Use a positive control test slide.

Procedure:

(1) Deparaffinize and hydrate to water
(2) Hydrolyze in 5 N HCl[1] (hydrochloric acid) — 30 min
(3) Distilled water — 1 min (change frequently to avoid carry-over)
(4) Distilled water — 1 min
(5) Blot well before entering Schiff's (Feulgen) stain
(6) Schiff's (Feulgen)[2] stain (refrigerated) — 15 min
(7) Distilled water — 2 changes — 1 min each
(8) Running tap water — 2 min
(9) Crystal violet[3] — 2 min
(10) Wash in running water
(11) Lugol's iodine[4] — 2 min
(12) Wash in running water
(13) Decolorize in 95% ETOH until "blue" does not bleed off slide
(14) Wash in running water
(15) 0.03% picromethyl blue[5] — 3 min
(16) Destain in 1 part 100% ETOH to 9 parts butanol — 6 dips (change for each rack)
(17) Butanol — 1½ min
(18) Butanol — 3 min
(19) Xylene
(20) Mount

Results:

Nuclei — red
Muscle — yellow
Gram positive — blue
Collagen — light blue
Background — green

Reagents:

[1] 5 N HCl (Lillie, 1965)

Distilled water	583.5 ml
Concentrated HCl sp. gr. 1.19	416.5 ml

[2] Schiff's (Feulgen) (Sanders, 1972)

Boil 200 ml of distilled water
Add 1.0 gm of basic fuchsin (stir)
Cool to 50°C (filter optional)
Add 20 ml of normal HCl (916.5 ml of distilled water and 83.5 ml of concentrated HCl sp. gr. 1.19)
Cool to 25°C (room temp.)
Add 1.0 gm of sodium metabisulfite ($Na_2S_2O_5$)
Shake vigorously
Store in a dark place; reagent takes about 2 days to turn a straw color
Add charcoal (approx. ½ gm/200 ml); shake and filter
Refrigerate before use

Note: See note for Feulgen picromethyl blue stain, VI.A.3.g.

[3] Crystal violet (Brown and Brenn, 1931)

Crystal violet	0.8 gm
Sodium bicarbonate	1.0 gm
Distilled water	100.0 ml

Note: Prepare fresh before use.

[4] Lugol's iodine (Sanders, 1972)

Iodine	1.0 gm
Potassium iodide	2.0 gm
Distilled water	100.0 ml

[5] 0.03% picromethyl blue (Farley, pers. commun.)

Methyl blue (Roboz Surgical Instrument Cat. No. 1A240[c])	0.15 gm
Saturated aqueous picric acid (Fisher Scientific Cat. No. CS-311-1[d])	500.0 ml

References: Brown, J.H. and L. Brenn. Bull. Johns Hopkins Hosp. 48: 69-73; 1931.
(Modified by C.A. Farley, National Marine Fisheries Service, Oxford, Md.; 1980.)

g. Feulgen Picromethyl Blue (FPM) (1 hr 35 min)

Specific for DNA and collagen in connective tissue.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) Distilled water — 2 min
(4) Hydrolyze in 5 N HCl[1] (hydrochloric acid) at room temp. — 30 min
(5) Distilled water — 1 min (change frequently to reduce carry-over)
(6) Distilled water — 1 min
(7) Blot well before entering refrigerated Schiff's (Feulgen)[2] stain — 15 min
(8) Distilled water — 2 changes — 1 min each
(9) Running tap water — 2 min
(10) Distilled water — 1 min
(11) 0.03% picromethyl blue[3] — 3 min
(12) Destain in 1 part 100% ETOH to 9 parts butanol — 6 dips (change for each rack)
(13) Butanol — 1½ min
(14) Butanol — 3 min
(15) Clear
(16) Mount

Results:

DNA — red
Nucleoli and muscle and other acidophilic substances — yellow
Connective tissue — blue
Cytoplasmic protein — green

Reagents:

[1] 5 N HCl (Lillie, 1965)

Distilled water	583.5 ml
Concentrated HCl sp. gr. 1.19	416.5 ml

[2] Schiff's (Feulgen) (Sanders, 1972)
Boil 200 ml of distilled water
Add 1.0 gm of basic fuchsin (stir)
Cool to 50°C (filter optional)
Add 20 ml of normal HCl (916.5 ml of distilled water and 83.5 ml of concentrated HCl sp. gr. 1.19)
Cool to 25°C (room temp.)
Add 1.0 gm of sodium metabisulfite ($Na_2S_2O_5$)
Shake vigorously
Store in a dark place; reagent takes about 2 days to turn a straw color
Add charcoal (approx. ½ gm/200 ml); shake and filter
Refrigerate before use

Note: For Schiff's (Feulgen), suggest basic fuchsin from Fisher Scientific Co. (P-389), Certified Biological Stain Color Index No. 42500. It is very important to get a good lot of basic fuchsin. Store Schiff's in refrigerator in a tightly capped bottle; always use a test slide to check stain. When the Schiff's takes on a purple tone, it will no longer stain effectively; discard. Also, when making up solution, do not double or triple batch. Make separate 200-ml portions for best results and store in separate containers.

[3] 0.03% picromethyl blue

Methyl blue (Roboz Surgical Instrument Cat. No. 1A240[c])	0.15 gm
Saturated aqueous picric acid (Fisher Scientific Cat. No. CS-311-1[d])	500.0 ml

Reference: Farley, C.A. Natl. Cancer Inst. Monogr. 31: 541-555; 1969.

h. Gram (1 hr 10 min)

Specific for gram positive and gram negative bacteria. Use positive and negative control test slides.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) Sterling's crystal violet[1] — 2 min
(4) Wash in running water — 2 min
(5) Lugol's iodine[2] — 2 min
(6) Running water — 2 min
(7) Decolorize in 95% ETOH until "blue" does not bleed off slide
(8) Running tap water — 2 min
(9) Safranin O[3] — 2 min
(10) Running tap water — 2 min
(11) Picromethyl blue[4] or picric acid[5] (optional) — 3 min
(12) Differentiate in 1 part 100% ETOH to 9 parts butanol — 6 dips (or until safranin O does not bleed off slide)
(13) Butanol — 1½ min
(14) Butanol — 3 min
(15) Clear
(16) Mount

Results:

Nuclei — red
Gram positive — blue
Gram negative — red
Background — yellow or green, depending on counterstain

Reagents:

[1]Sterling's crystal violet (Conn et al., 1962)

Crystal violet (mortared in 10 ml of 100% ETOH)	5.0 gm
Aniline oil	2.0 ml
Distilled water	88.0 ml

Note: **Aniline oil extremely hazardous. Avoid breathing vapor and contact with skin.**

[2]Lugol's iodine (Sanders, 1972)

Iodine	1.0 gm
Potassium iodide	2.0 gm
Distilled water	100.0 ml

[3]Safranin O (Conn et al., 1962)

Safranin O dissolved in 10 ml of 95% ETOH	0.25 gm
Distilled water	90.0 ml

[4]0.03% picromethyl blue (Farley, pers. commun.)

Methyl blue (Roboz Surgical Instrument Cat. No. 1A240[c])	0.15 gm
Saturated aqueous picric acid (Fisher Scientific Cat. No. CS-311-1[d])	500.0 ml

[5]Picric acid (saturated aqueous)

Distilled water	20.0 ml
Picric acid	2.0 gm

References: Brown, J.H. and L. Brenn. Bull. Johns Hopkins Hosp. 48: 69-73; 1931.
(Modified by C.A. Farley, National Marine Fisheries Service, Oxford, Md.; 1980.)

i. Grocott's Methenamine Silver (GMS) (3 hr 15 min)

Specific for fungi. Use a positive control test slide and acid-cleaned glassware.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) Oxidize in 4% chromic acid[1] — 1 hr
(4) Wash in tap water — few seconds
(5) 1% sodium bisulfite[2] — 1 min (to remove any residual chromic acid)
(6) Wash in running water — 5-10 min
(7) Rinse in distilled water — 3-4 changes
(8) Place in freshly mixed methenamine silver nitrate[3] working solution in incubator or water bath at 58°-60°C for 60 min or until section turns yellowish-brown
(9) Rinse in distilled water — 6 changes
(10) Tone in 0.1% gold chloride[4] — 2-5 min
(11) Rinse in distilled water
(12) Remove unreduced silver with 2% sodium thiosulfate (hypo)[5] — 2-5 min
(13) Wash thoroughly in tap water
(14) Counterstain in 0.2% light green[6] — 30-45 sec
(15) Dehydrate
(16) Clear
(17) Mount

Results:

Fungi — sharply delineated in black
Mucin — taupe to dark gray
Inner parts of mycelia and hyphae — old rose
Background — pale green

Reagents:

[1]4% chromic acid

Chromic acid	4.0 gm
Distilled water	100.0 ml

[2]1% sodium bisulfite

Sodium bisulfite	1.0 gm
Distilled water	100.0 ml

[3]Methenamine silver nitrate working solution

Prepare the following solutions:

(a) 5% silver nitrate

Silver nitrate	0.25 gm
Distilled water	5.0 ml

(b) 3% methenamine

Methenamine	3.0 gm
Distilled water	100.0 ml

(c) 5% borax

Borax	5.0 gm
Distilled water	100.0 ml

(d) Methenamine silver nitrate stock solution

5% silver nitrate (a)	5.0 ml
3% methenamine (b)	100.0 ml

Note: A white precipitate forms in the methenamine silver nitrate stock solution, but immediately dissolves on shaking. Clear solution remains usable for months. Store in refrigerator.

Combine for working solution as follows:

Methenamine silver nitrate stock (d)	25.0 ml
Distilled water	25.0 ml
5% borax (c)	2.0 ml

Note: Make working solution fresh.

[4]0.1% gold chloride

1% gold chloride	10.0 ml
Distilled water	90.0 ml

Note: This solution may be used repeatedly.

[5]2% sodium thiosulfate (hypo)

Sodium thiosulfate	2.0 gm
Distilled water	100.0 ml

[6]0.2% light green

Light green	0.2 gm
Distilled water	100.0 ml

Note: Acid-cleaned glassware for preparation of solutions in the above silver procedure is recommended, as well as acid-cleaned coplin jars. The silver will precipitate on the slide very easily. Keep all solutions away from metal, and be sure to coat forceps with paraffin before immersing in silver solution.

Reference: Luna, L.G., ed. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd ed. McGraw-Hill, New York; 1968.

j. Harris' Hematoxylin and Eosin (HHE_1) (Progressive Stain) (1 hr 30 min)

General tissue stain used with tissues fixed in Helly's or Zenker's.

Procedure:

(1) Deparaffinize
(2) Hydrate to water and remove mercury crystals, if necessary (VI.A.1.c.)
(3) Harris' hematoxylin[1] — 2 min
(4) Distilled water — 6 dips
(5) Running tap water — 5 min
(6) Distilled water — 6 dips
(7) 95% ETOH — 2 min
(8) Eosin bluish[2] — 20 sec-2 min (may vary with tissue and fixative used)
(9) Dehydrate
(10) Clear
(11) Mount

Results:

Basophilic substances — blue
Acidophilic substances — pink

Reagents:

[1]Harris' hematoxylin

Harris' hematoxylin (VWR Scientific Cat. No. AL33050-3[e])	200.0 ml
Glacial acetic acid	14.0 ml

[2]Eosin bluish

Eosin bluish (Fisher Scientific Cat. No. E-514[d])	1.0 gm
95% ETOH	100.0 ml

Note: Age eosin bluish 1 month before using.

Reference: Johnson, P.T. Histology of the blue crab, *Callinectes sapidus*. A model for the Decapoda. Praeger, New York; 1980.

k. Harris' Hematoxylin and Eosin (HHE_2) (Regressive Stain) (1 hr 10 min)

General tissue stain for tissues fixed in Davidson's, formalin, and Bouin's.

Procedure:

(1) Deparaffinize
(2) Hydrate to water and remove mercury, if necessary (VI.A.1.c.)
(3) Harris' hematoxylin[1] — 8 min
(4) Distilled water — 2 min (use fresh distilled water for each rack)
(5) Acid alcohol[2] — 1 dip
(6) Running tap water — 5 min (tissue should "blue" here)
(7) Distilled water — 3 min
(8) 95% ETOH — 3 min
(9) Eosin y[3] — 3 min
(10) 95% ETOH — 6 dips
(11) Dehydrate
(12) Clear
(13) Mount

Results:

Basophilic substances — blue
Acidophilic substances — pink

Reagents:

[1]Harris' hematoxylin

Harris' hematoxylin (VWR Scientific Cat. No. AL33050-3[e])	200.0 ml
Glacial acetic acid	14.0 ml

[2]Acid alcohol

70% ETOH	99.0 ml
Concentrated hydrochloric acid (HCl) sp. gr. 1.19	1.0 ml

[3]Eosin y

Eosin y	0.2 gm
Distilled water	75.0 ml
100% ETOH	25.0 ml
Just before use, add concentrated HCl	1 drop/200 ml

Note: If precipitate does not dissolve on stirring, eosin may stain too orange.

Reference: Johnson, P.T. Histology of the blue crab, *Callinectes sapidus* A model for the Decapoda. Praeger, New York; 1980.

I. Heidenhain's Iron Hematoxylin and Eosin (IHE) (1 hr 30 min)

Specific for good nuclear detail.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) Place in Lang's mordant[1] — 10 min
(4) Wash in running water — 5 min
(5) Dip in distilled water
(6) Hematein[2] or hematoxylin[3] — 10 min
(7) Dip in distilled water
(8) Destain in picric acid[4] until only nuclei remain stained — 10-20 min (check with microscope)
(9) Wash in running tap water — 5 min
(10) Dehydrate in alcohols through 95% ETOH as usual
(11) Counterstain in eosin bluish[5] — 20 sec-2 min
(12) Dehydrate
(13) Clear
(14) Mount

Results:

Nuclei — black
Muscle — pink
Background — gray

Reagents:

[1]Lang's mordant stock solution (Conn et al., 1962)

Distilled water	1000.0 ml
Glacial acetic acid	10.0 ml
Sulfuric acid	1.2 ml
Ferric ammonium sulfate	30.0 gm

Lang's mordant working solution

Lang's mordant stock	200.0 ml
Dimethyl sulfoxide (DMSO) (Mitchell, 1966)	20.0 ml

[2]Hematein stock solution (Farley, pers. commun.)

100% ETOH	200.0 ml
Hematein	20.0 gm

Hematein working solution (Mallory, 1968)

Hematein stock	10.0 ml
Distilled water	190.0 ml
DMSO	20.0 ml

[3]Hematoxylin stock solution (Lillie, 1965)

100% ETOH	100.0 ml
Hematoxylin	10.0 gm

Note: Allow hematoxylin stock solution to age 4-6 weeks before use.

Hematoxylin working solution

Distilled water	190.0 ml
Hematoxylin stock	10.0 ml
DMSO	20.0 ml

Note: Both hematein and hematoxylin are included for the above procedure for Heidenhain's iron hematoxylin. The hematoxylin is purer but takes 4-6 weeks to ripen, while hematein may be used immediately. Do not contaminate hematoxylin with mordant; stain will become ineffective. **When using DMSO, avoid contact with skin.**

[4]Picric acid (saturated aqueous)

Distilled water	20.0 ml
Picric acid	2.0 gm

[5]Eosin bluish

Eosin bluish (Fisher Scientific Cat. No. E-514[d])	1.0 gm
95% ETOH	100.0 ml

Note: Allow eosin bluish to age 1 month before use.

References: Conn, H.J., et al. Staining Procedures Used by the Biological Stain Commission. 2nd ed. The Williams & Wilkins Co., Baltimore; 1962.
(Modified by C.A. Farley, National Marine Fisheries Service, Oxford, Md.; 1979.)

m. Mallory's Hematein (1 hr)

Specific for copper and other metals. Use a positive control test slide.

Procedure:

(1) Deparaffinize
(2) Hydrate to water, but do not permit slides to remain in water
(3) Continue immediately
(4) Hematein[1] with dimethyl sulfoxide (DMSO) — 5 min
(5) Running tap water to "blue" slides — 5 min
(6) Dip in distilled water
(7) 1% safranin O[2] — 2 min
(8) Running tap water — 2 min
(9) Destain in 1 part 100% ETOH to 9 parts butanol — 1-6 dips
(10) Butanol — 2 min
(11) Butanol — 5 min
(12) Clear
(13) Mount

Results:

Background — bright red
Special contaminants, e.g. copper — deep clear blue

Reagents:

[1]0.5% hematein stock solution (Farley, pers. commun.)

Hematein	1.0 gm
Distilled water	200.0 ml

Hematein working solution

0.5% hematein stock	10 parts
DMSO	1 part

Note: Be careful not to contaminate hematein; a dark color change will indicate the stain is no longer usable. Make up immediately prior to use. It will not keep overnight after DMSO is added. **Avoid contact of DMSO with skin.**

[2]1% safranin O (Conn et al., 1962)

Safranin O	1.0 gm
Distilled water	100.0 ml

References: Lillie, R.D. Histopathologic Technic and Practical Histochemistry. 3rd ed. McGraw-Hill, New York; 1965. (Modified by C.A. Farley, National Marine Fisheries Service, Oxford, Md.; 1980.)

n. Mallory's Trichrome (Modified) (1 hr)

Specific for collagen in connective tissue. Use a positive control test slide.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) 0.5% acid fuchsin[1] — 7 min
(4) Dip twice in tap water
(5) Blot slide well
(6) Aniline blue-orange G[2] — 15 min
(7) Rinse in tap water — 2 quick dips
(8) Transfer to several changes of 95% ETOH (quickly)
(9) Dehydrate (quickly, because slides can lose too much aniline blue in this process)
(10) Clear
(11) Mount

Results:

Nuclei — red
Collagen fibers — intense blue (also reticulum, pale blue)
Elastic fibers — pale pink, pale yellow, or unstained
Muscle — red-purple to blue (depending on specimen)
Erythrocytes and myelin — yellow

Reagents:

[1]0.5% acid fuchsin

Acid fuchsin	0.5 gm
Distilled water	100.0 ml

Note: Acid fuchsin stain gives best results after aging a few days.

[2]Aniline blue-orange G (best for tissues fixed in Davidson's formalin)

Dissolve in this order:

Aniline blue	0.5 gm
Orange G	2.0 gm
Phosphomolybdic acid	1.0 gm
Distilled water	100.0 ml

Aniline blue-orange G (best for Helly's or Zenker's fixed tissue) (Johnson, 1980)

Aniline blue	0.5 gm
Orange G	2.0 gm
Phosphotungsic acid	2.0 gm
Distilled water	100.0 ml
Glacial acetic acid	2 drops

References: Pauley, G. B. J. Invertebr. Pathol. 9: 268-269; 1967. (Modified by P.T. Johnson. Histology of the Blue Crab, *Callinectes sapidus*. A Model for the Decapoda. Praeger, New York; 1980.)

o. Malt Periodic Acid Schiff (MPAS) (2 hr 20 min)

Specific for PAS positive substances other than glycogen.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) Immerse in malt[1] (diastase) — 1 hr at 40°C
(4) Wash well in running tap water — 10 min
(5) Distilled water
(6) Oxidize in 1% periodic acid[2] at room temp. — 5 min
(7) Wash in running tap water — 5 min
(8) Blot well and place in refrigerated Schiff's[3] (Feulgen) reagent — 5 min
(9) Wash well — 10 min
(10) Hematoxylin[4] of choice
(11) "Blue" in tap water — 5 min
(12) Dehydrate
(13) Clear
(14) Mount

Results:

Glycogen is digested
Other PAS positive substances — pink-magenta
Basophilic substances — blue-black

Reagents:

[1]Malt for MPAS (Lillie, 1965)

Diastase malt	0.2 gm
Buffer, pH 5.5-6.0	20.0 ml
Distilled water	180.0 ml

Buffer for malt (Lillie, 1965)

Boil distilled water	1000.0 ml
Sodium phosphate monobasic ($NaH_2PO_4.H_2O$)	1.97 gm
Sodium phosphate dibasic (Na_2HPO_4)	0.28 gm

Note: pH 5.5 to 6.0; check pH meter. Phosphate buffer same as Sorenson's. Add 1 thymol crystal when cool to prevent mold, and refrigerate.

[2]1% periodic acid (Drury and Wallington, 1967)

Periodic acid	1.0 gm
Distilled water	100.0 ml

[3]Schiff's (Feulgen) (Sanders, 1972)

Boil 200 ml of distilled water
Add 1.0 gm of basic fuchsin and stir
Cool to 50°C (filter optional)
Add 20 ml of normal hydrochloric acid (HCl) (916.5 ml of distilled water and 83.5 ml of concentrated HCl sp. gr. 1.19)
Cool to 25°C (room temp.)
Add 1.0 gm of sodium metabisulfite ($Na_2S_2O_5$)
Shake vigorously. Store in a dark place; reagent takes about 2 days to turn a straw color
Add charcoal (approx. ½ gm/200 ml); shake and filter
Refrigerate before use

Note: See note for Feulgen picromethyl blue stain, VI.A.3.g.

[4]Harris' hematoxylin

Harris' hematoxylin (VWR Scientific Cat. No. AL33050-3[e])	200.0 ml

Reference: Lillie, R.D. Histopathologic Technic and Practical Histochemistry. 3rd ed. McGraw-Hill, New York; 1965.

p. Malt Periodic Acid Schiff-Alcian Blue (MPAS-AB) (5 hr)

Specific for differentiation of acid and neutral mucopolysaccharides. Use a positive control test slide.

Procedure:

(1) Deparaffinize
(2) Hydrate to water and remove mercury, if present (see VI.A.1.c.)
(3) Immerse in malt[1] (diastase) — 1 hr at 40°C
(4) Wash well in running tap water
(5) Oxidize in 1% periodic acid[2] at room temp. — 5 min
(6) Wash in running water — 5 min
(7) Blot well and place in refrigerated Schiff's[3] (Feulgen) reagent — 5 min
(8) Wash well in running water — 10 min
(9) Harris' hematoxylin[4] — 2 min
(10) "Blue" in running tap water — 5 min
(11) Rinse in distilled water
(12) Alcian blue[5] — 30 min
(13) Rinse briefly in running tap water
(14) 0.5% alcoholic ammonium hydroxide[6] — 2 hr
(15) Rinse in running tap water
(16) Dip in distilled water
(17) Dehydrate
(18) Clear
(19) Mount

Results:

Acid mucopolysaccharides — bright intense blue
Neutral mucopolysaccharides — magenta
PAS positive tissue — magenta
Nuclei — blue

Reagents:

[1]Malt (Lillie, 1965)

Diastase malt	0.2 gm
Buffer, pH 5.5-6.0	20.0 ml
Distilled water	180.0 ml

Buffer for malt (Lillie, 1965)

Boil distilled water	1000.0 ml
Sodium phosphate monobasic ($NaH_2PO_4.H_2O$)	1.97 gm
Sodium phosphate dibasic (Na_2HPO_4)	0.28 gm

Note: pH 5.5 to 6.0; check pH meter. Phosphate buffer same as Sorenson's. Add 1 thymol crystal when cool to prevent mold, and refrigerate.

[2]1% periodic acid (Drury and Wallington, 1967)

Periodic acid	1.0 gm
Distilled water	100.0 ml

[3]Schiff's (Feulgen) (Sanders, 1972)
Boil 200 ml of distilled water
Add 1.0 gm of basic fuchsin and stir
Cool to 50°C (filter optional)
Add 20 ml of normal hydrochloric acid (HCl) (916.5 ml of distilled water and 83.5 ml of concentrated HCl sp. gr. 1.19)
Cool to 25°C (room temp.)
Add 1.0 gm of sodium metabisulfite ($Na_2S_2O_5$)
Shake vigorously
Store in a dark place; reagent takes about 2 days to turn a straw color
Add charcoal (approx. ½ gm/200 ml); shake and filter
Refrigerate before use
Note: See note for Feulgen picromethyl blue stain, VI.A.3.g.

[4]Harris' hematoxylin

Harris' hematoxylin (VWR Scientific Cat. No. AL33050-3e)	200.0 ml

[5]0.1% alcian blue in 0.01 M HCl (pH 2.2) — Helly's or Zenker's fixative (Johnson, 1980)

Alcian blue 8GX	1.0 gm
1 N HCl	10.0 ml
Distilled water	990.0 ml

0.1% alcian blue in 3% acetic acid — Davidson's or formalin-fixed tissue (Johnson, 1980)

Alcian blue 8GX	0.1 gm
Distilled water	97.0 ml
Glacial acetic acid	3.0 ml

Note: Check expiration date of alcian blue stain.

[6]0.5% alcoholic ammonium hydroxide (Johnson, 1980)

Ammonium hydroxide	3.0 ml
95% ETOH	597.0 ml

References: Lillie, R.D. Histopathologic Technic and Practical Histochemistry. 3rd ed. McGraw-Hill, New York; 1965.
Pearse, A.G.E. Histochemistry, Theoretical and Applied. 2nd ed. Little, Brown and Co., Boston; 1960.
(Modified by P.T. Johnson. Histology of the Blue Crab, *Callinectes sapidus*. A Model for the Decapoda. Praeger, New York; 1980.)

q. Oil Red O (ORO) (10 min)

Specific for fat in frozen sections.

Procedure:

(1) Frozen sections, air-dried or fixed (if fixed, wash out fixative)
(2) Rinse in dilute 49.5% isopropyl alcohol[1]
(3) Oil red O[2] — 5 min
(4) Rinse in dilute 49.5% isopropyl alcohol
(5) Rinse in distilled water
(6) Mount in glycerin jelly

Results:

Fat — bright orange-red

Reagents:

[1]49.5% isopropyl alcohol

99% isopropyl alcohol	50.0 ml
Distilled water	50.0 ml

[2]Oil red O stock solution

Supersaturated:

Oil red O	0.5 gm
99% isopropyl alcohol	125.0 ml

Oil red O working solution

Saturated:

Oil red O stock	12.0 ml
Distilled water	8.0 ml

Mix, allow solution to stand for 5 min, and filter.

Note: Use working solution until precipitate begins to form.

Reference: Thompson, S.W. Selected Histochemical and Histopathological Methods. Charles C. Thomas Publ., Springfield, Ill.; 1966.

r. Periodic Acid Schiff (PAS_1) (1 hr 15 min)

Specific for glycogen, fungi, and neutral mucopolysaccharides.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) Oxidize in 1% periodic acid[1] at room temp. — 5 min
(4) Wash in running tap water — 5 min
(5) Blot well and place in refrigerated Schiff's[2] (Feulgen) reagent — 5 min
(6) Rinse immediately in running tap water — 10 min
(7) Distilled water — 6 dips
(8) Weigert's hematoxylin[3] — 30 sec
(9) Running water — 6 dips
(10) Distilled water — 6 dips
(11) Dehydrate
(12) Clear
(13) Mount

Results:

Glycogen — magenta
PAS positive substances — red-pink
Basophilic substances — blue-black

Reagents:

[1]1% periodic acid (Drury and Wallington, 1967)

Periodic acid	1.0 gm
Distilled water	100.0 ml

[2]Schiff's (Feulgen) (Sanders, 1972)

Boil 200 ml of distilled water
Add 1.0 gm of basic fuchsin (stir)
Cool to 50°C (filter optional)
Add 20 ml of normal hydrochloric acid (HCl)
(916.5 ml of distilled water and 83.5 ml of concentrated HCl sp. gr. 1.19)
Cool to 25°C (room temp.)
Add 1.0 gm of sodium metabisulfite ($Na_2S_2O_5$)
Shake vigorously
Store in dark place; reagent takes about 2 days to turn a straw color
Add charcoal (approx. ½ gm/200 ml); shake and filter
Refrigerate before use

Note: See note for Feulgen picromethyl blue stain, VI. A.3.g.

[3]Weigert's hematoxylin for PAS_1 (Lillie, 1965)

Solution A:	Hematoxylin	1.0 gm
	95% ETOH	100.0 ml
Solution B:	Ferric chloride ($FeCl_3 \cdot 6H_2O$)	2.5 gm
	Distilled water	99.0 ml
	HCl	1.0 ml

Mix equal parts of solutions A and B immediately before use.

Note: When using Weigert's hematoxylin in this procedure, work quickly: rinse in running water to avoid overstaining and precipitation of stain on slide; acid-cleaned slides will prevent stain precipitation. This stain will not keep; make fresh each time.

Reference: Lillie, R.D. Histopathologic Technic and Practical Histochemistry. 3rd ed. McGraw-Hill, New York; 1965.

s. Periodic Acid Schiff (PAS_2) (Heidenhain's Hematoxylin Counterstain) (1 hr 45 min)

Specific for nuclear detail, fungi, glycogen, and neutral mucopolysaccharides.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) Oxidize in 1% periodic acid[1] at room temp. — 5 min
(4) Wash in running tap water — 5 min
(5) Blot well and place in refrigerated Schiff's[2] (Feulgen) reagent — 5 min
(6) Go directly to running tap water — 10 min
(7) Distilled water — 6 dips
(8) Mordant in 3% ferric ammonium sulfate[3] — 5 min
(9) Wash in running tap water — 2 min
(10) Heidenhain's hematoxylin[4] — 2-5 min, according to intensity desired, at room temp.
(11) Differentiate in picric acid[5] until only nuclei remain stained — 10-20 min (check with microscope)
(12) Wash in running tap water — 2 min
(13) Dehydrate
(14) Clear
(15) Mount

Results:

PAS positive substances — red-pink
Glycogen, collagen, reticulum, mucin, and neutral mucopolysaccharides — red-pink
Basophilic substances — blue to black

Reagents:

[1]1% periodic acid (Drury and Wallington, 1967)

Periodic acid	1.0 gm
Distilled water	100.0 ml

[2]Schiff's (Feulgen) (Sanders, 1972)

Boil 200 ml of distilled water
Add 1.0 gm of basic fuchsin (stir)
Cool to 50°C (filter optional)
Add 20 ml of normal hydrochloric acid (HCl) (916.5 ml of distilled water and 83.5 ml of concentrated HCl sp. gr. 1.19)
Cool to 25°C (room temp.)
Add 1.0 gm of sodium metabisulfite ($Na_2S_2O_5$)
Shake vigorously
Store in a dark place; reagent takes about 2 days to turn a straw color
Add charcoal (approx. ½ gm/200 ml); shake and filter
Refrigerate before use

Note: See note for Feulgen picromethyl blue stain, VI.A.3.g.

[3]3% ferric ammonium sulfate

Ferric ammonium sulfate	3.0 gm
Distilled water	100.0 ml

[4]Heidenhain's hematoxylin (Mitchell, 1966)

0.5% hematein stock (VI.A.3.m.)	10 parts
Dimethyl sulfoxide (DMSO)	1 part

Note: Do not contaminate hematoxylin with mordant; stain will become ineffective. **Avoid contact of DMSO with skin.**

[5]Picric acid (saturated aqueous)

Distilled water	20.0 ml
Picric acid	2.0 gm

References: Lillie, R.D. Histopathologic Technic and Practical Histochemistry. 3rd ed. McGraw-Hill, New York; 1965.
Conn, H.J., et al. Staining Procedures Used by the Biological Stain Commission. 2nd ed. The Williams & Wilkins Co., Baltimore, Md.; 1962.

t. Perls' Prussian Blue (2 hr)

Specific for ferric iron. Use a positive control test slide and acid-cleaned glassware.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) Expose sections to a fresh mixture of equal parts of 2% potassium[1] or sodium ferrocyanide[2] and 2% hydrochloric acid (HCl)[3] — 30-60 min (if longer period is used, change solution after first 30 min)
(4) Wash in distilled water
(5) Counterstain nuclei with 1% neutral red[4] — 3 min
(6) Wash in running tap water
(7) Dehydrate
(8) Clear
(9) Mount in Canada balsam (a neutral mounting medium)

Results:

Ferric iron — Prussian blue to green
Nuclei — red
Background — pink

Reagents:

[1]2% potassium ferrocyanide
Potassium ferrocyanide ..... 2.0 gm
Distilled water ..... 100.0 ml

[2]2% sodium ferrocyanide
Sodium ferrocyanide ..... 2.0 gm
Distilled water ..... 100.0 ml

[3]2% HCl
Concentrated HCl sp. gr. 1.19 ..... 2.0 ml
Distilled water ..... 98.0 ml

[4]1% neutral red
Neutral red ..... 1.0 gm
Distilled water ..... 100.0 ml

Note: Keep solutions away from metal to avoid contamination. Acid fixatives interfere with iron. Versenate decalcification interferes with results (Drury and Wallington, 1967).

Reference: Drury, R.A.B. and S.A. Wallington. Carleton's Histological Technique. 4th ed. Oxford Univ. Press; 1967.

u. Sudan Black B (SBB) (allow 2 days)

Specific for phospholipids. Use duplicate slides.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) Post-chrome in 3% potassium dichromate[1] — 1-2 hr (1 hr 15 min for Helly's fixative)
(4) Wash in running water — 10 min
(5) Dehydrate to 70% ETOH and stain in 0.3% SBB[2] at room temp. — 16 hr (time varies for specific tissue)
(6) Rinse in 70% ETOH and wash—5-10 min (one set of test slides goes into acetone for 7½ hr and is washed in running water for 5-10 min, counterstained, and mounted for comparison response of results)
(7) Counterstain in 1% neutral red[3] — 1 min
(8) Wash in running water — 5-10 min
(9) Dip in distilled water
(10) Mount in glycerin jelly

Results:

Phospholipids — black-blue, black
Background — red
Bound lipids and remaining neutral lipids — black, blue-gray, or brownish
Other tissue components — pink to red or unstained
Following the acetone extraction, lipid components are unstained

Reagents:

[1]3% potassium dichromate

Potassium dichromate	3.0 gm
Distilled water	100.0 ml

[2]0.3% SBB

SBB	0.3 gm
70% ethyl alcohol	100.0 ml

[3]1% neutral red

Neutral red	1.0 gm
Distilled water	100.0 ml

References: Pearse, A.G.E. Histochemistry, Theoretical and Applied. 2nd ed. Little, Brown and Co., Boston; 1960. (Modified by P.T. Johnson. Histology of the Blue Crab, *Callinectes sapidus*. A Model for the Decapoda. Praeger, New York; 1980.)

v. Toluidine Blue O (TBO) (fresh blood, 30 min)

Specific for hemocytes.

Procedure:

(1) Prepare slide with fresh blood
(2) Fix in 1% glutaraldehyde/4% formaldehyde (1G4F) fixative (made with ambient sea water) — 2 min
(3) Wash in running tap water — 1 min
(4) Distilled water — 6 dips
(5) 0.5% toluidine blue O[1] — 5 min
(6) Running tap water — 6 dips
(7) Destain in 1 part 100% ETOH to 9 parts butanol — 6 dips
(8) Butanol — 2 min
(9) Butanol — 4 min
(10) Clear
(11) Mount

Results:

Nuclei — blue
Granules — purple or blue
RBCs — green

Reagent:

[1]0.5% toluidine blue O

Toluidine blue O	0.5 gm
Borax	0.5 gm
Distilled water	100.0 ml

Heat gently to dissolve the stain, then filter solution.

Note: Stable for several months.

References: Trump, B.F., et al. J. Ultrastruct. 5: 343-348; 1961. (Modified by C.A. Farley, National Marine Fisheries Service, Oxford, Md.; 1980.)

w. Von Kóssa (2 hr)

Specific for calcium. Use a positive control test slide and acid-cleaned glassware.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) Place slides in 5% silver nitrate[1] — 30-60 min, exposed to direct sunlight
(4) Rinse in distilled water — 2-3 changes
(5) 5% sodium thiosulfate[2] — 2-3 min
(6) Wash well in distilled water (2 dips each in 2 changes)
(7) Counterstain in nuclear fast red[3] (Kernechtrot) — 5 min
(8) Wash well in distilled water — 6 dips
(9) Dehydrate
(10) Clear
(11) Mount

Results:

Calcium salts — black
Nuclei — red
Cytoplasm — pink to rose

Reagents:

[1]5% silver nitrate

Silver nitrate	5.0 gm
Distilled water	100.0 ml

[2]5% sodium thiosulfate

Sodium thiosulfate	5.0 gm
Distilled water	100.0 ml

[3]Nuclear fast red (Kernechtrot[a])

5% aluminum sulfate ($Al_2(SO_4)_3$):

$Al_2(SO_4)_3$	25.0 gm
Distilled water	500.0 ml

0.1% nuclear fast red:

Kernechtrot nuclear fast red	0.5 gm
5% $Al_2(SO_4)_3$	500.0 ml

Note: Refrigerate; good till loses color.

Note: For best results, stain on a sunny day. Hi-intensity lamps do not work nearly as well as natural sunlight.

Reference: Sanders, B.J. Animal Histology Procedures of the Pathological Technology Section of the National Cancer Institute. Government Printing Office, Washington, D.C.; 1972.

x. Wilder's Reticulum (WR) (1 hr)

Specific for reticulum (single slide manipulation). Use acid-cleaned glassware.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) Oxidize in 10% phosphomolybdic acid[1] — 1 min
(4) Rinse well in running water
(5) Sensitize in 1% uranium nitrate[2] — 1 min
(6) Rinse in distilled water — 10-20 sec
(7) Ammoniacal silver[3] —1 min (change solution frequently)
(8) Dip quickly in 95% ETOH and go straight to reducing solution[4] — 1 min (change solution frequently)
(9) Rinse well in distilled water
(10) Tone in gold chloride[5] (until section is lavender) — 1 min
(11) Rinse well in distilled water
(12) 5% sodium thiosulfate[6] — 1 min
(13) Wash well in tap water
(14) Distilled water — quick dip
(15) Counterstain as desired (usually use nuclear fast red)
(16) Nuclear fast red[7] (Kernechtrot) — 1-4 min (depending on age of stain)
(17) Dip in distilled water — 6 dips
(18) Dehydrate
(19) Clear
(20) Mount

Results:

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

Reagents:

[1]10% phosphomolybdic acid

Phosphomolybdic acid	10.0 gm
Distilled water	100.0 ml

[2]1% uranium nitrate

Uranium nitrate	1.0 gm
Distilled water	100.0 ml

[3]Ammoniacal silver

10.2% silver nitrate:

Silver nitrate	10.2 gm
Distilled water	100.0 ml
or	
Silver nitrate	1.02 gm
Distilled water	10.0 ml

3.1% sodium hydroxide:

Sodium hydroxide	3.1 gm
Distilled water	100.0 ml
or	
Sodium hydroxide	0.31 gm
Distilled water	10.0 ml

To 5 ml of 10.2% silver nitrate add 28% ammonium hydroxide (concentrated ammonium hydroxide) until precipitate which forms is almost dissolved. Add 5 ml of 3.1% sodium hydroxide and barely dissolve the resulting precipitate with a few drops of ammonium hydroxide. Make solution up to 50 ml with distilled water. Use at once.

[4]Reducing solution

Distilled water	50.0 ml
40% neutral formalin	0.5 ml
1% uranium nitrate	1.5 ml

Note: Make fresh and just before use add calcium carbonate in excess, or add one marble chip.

[5]1% gold chloride stock solution

Gold chloride	15 grains/glass vial
Distilled water	100.0 ml

0.2% gold chloride working solution

1% gold chloride stock	10.0 ml
Distilled water	40.0 ml

[6]5% sodium thiosulfate

Sodium thiosulfate	5.0 gm
Distilled water	100.0 ml

[7]Nuclear fast red (Kernechtrot[a])

5% aluminum sulfate ($Al_2(SO_4)_3$):

$Al_2(SO_4)_3$	25.0 gm
Distilled water	500.0 ml

0.1% nuclear fast red:

Kernechtrot nuclear fast red	0.5 gm
5% $Al_2(SO_4)_3$	500.0 ml

Note: Refrigerate; good till loses color.

Note: Use acid-cleaned slides and glassware for preparation of reagents and for staining. Silver will precipitate on the slide very easily, and frequent changes of the solutions are recommended. Keep metal away from all solutions, and be sure to coat forceps with paraffin before immersing in silver solution.

Reference: Luna, L.G., ed. 1968. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd ed. McGraw-Hill, New York; 1968.

y. Ziehl and Harris' Hematoxylin (ZHH) (1 hr 45 min)

Specific for acid-fast substances (e.g. detection of acid-fast organisms, haplosporidan spores). Use a positive control test slide.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) Ziehl's fuchsin[1] — 15 min
(4) Distilled water — 6 dips
(5) Destain in 0.1 N sulfuric acid[2] — 10-15 min (time varies with specific tissue)
(6) Running tap water — 10 min
(7) Harris' hematoxylin[3] — 2 min
(8) Distilled water — 6 dips
(9) Lithium carbonate saturated solution[4] — 2 min (not necessary if running tap water is slightly alkaline)
(10) Running tap water — 5 min
(11) Dehydrate
(12) Clear
(13) Mount

Results:

Basophilic substances — blue
Acid-fast substances — bright red, including mature haplosporidan sporoplasms, lipofuscins, and acid-fast bacteria

Reagents:

[1]Ziehl's fuchsin (Gray, 1954)

Basic fuchsin (magenta) (Fisher Scientific Cat. No. P-389[d])	1.0 gm
Phenol (liquefied)	5.0 ml
90% ETOH	10.0 ml
Distilled water	100.0 ml

Grind the fuchsin with phenol in a mortar. When dissolved, add the alcohol in 10 successive lots while grinding. Then wash stain from mortar with ten 10-ml washings of distilled water. Save and filter accumulated washings.

[2]0.1 N sulfuric acid (H_2SO_4)

Distilled water	997.2 ml
H_2SO_4 sp. gr. 1.84	2.8 ml

[3]Harris' hematoxylin

Harris' hematoxylin (VWR Scientific Cat. No. AL33050-3e[e])	200.0 ml
Glacial acetic acid	14.0 ml

[4]Lithium carbonate saturated solution

Lithium carbonate	1.0 gm
Distilled water	78.0 ml

Reference: Farley, C.A. J. Invertebr. Pathol. 7: 144-147; 1965.

z. Ziehl and Methylene Blue (1 hr 30 min)

Specific for acid-fast bacteria (AFB). Use a positive control test slide.

Procedure:

(1) Deparaffinize
(2) Hydrate to water
(3) Ziehl's fuchsin[1] — 15 min
(4) Distilled water — several dips — (approx. time) 1 min
(5) Destain in 0.1 N sulfuric acid[2] — 10-15 min (time varies with specific tissue)
(6) Running tap water — 10 min
(7) Distilled water — 3 dips
(8) Methylene blue[3] — dip quickly (stains quickly, depending on tissue)
(9) Running tap water — 5 min
(10) Dehydrate
(11) Clear
(12) Mount

Results:

Background — pale blue
AFB — brilliant red

Reagents:

[1]Ziehl's fuchsin (Gray, 1954)

Basic fuchsin (magenta) (Fisher Scientific Cat. No. P-389[d])	1.0 gm
Phenol (liquefied)	5.0 ml
90% ETOH	10.0 ml
Distilled water	100.0 ml

[2]0.1 N sulfuric acid (H_2SO_4)

Distilled water	997.2 ml
H_2SO_4 sp. gr. 1.84	2.8 ml

[3]Methylene blue stock solution (Luna, 1968)

Methylene blue	1.4 gm
95% ETOH	100.0 ml

Methylene blue working solution (Luna, 1968)

Methylene blue stock	10.0 ml
Tap water	90.0 ml

References: Gray P. The Microtomist's Formulary and Guide. The Blakiston Co., New York; 1954.
Luna, L.G., ed. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd ed. McGraw-Hill, New York; 1968.

B. PROBLEMS AND RESOLUTIONS

Table 8 includes some problems or artifacts resulting from staining, as well as some means of resolving them.

C. NOTES

[a]Roboz Surgical Instrument Co., Inc., 810 18th Street, N.W., Washington, D.C.

[b]American Scientific Products, 8855 McGaw Road, Columbia, Md.

[c]Roboz Surgical Instrument, Co., Inc., 810 18th Street, N.W., Washington, D.C.

[d]Fisher Scientific Company, 711 Forbes Avenue, Pittsburgh, Pa.

[e]VWR Scientific, Inc., 6601 Amberton Drive, Baltimore, Md.

D. REFERENCES

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TABLE 8. STAINING PROBLEMS AND RESOLUTIONS.

Problem or artifact	Possible causes	Solution, if any
Tissues have greasy, oily appearance.	Tissues are not completely dehydrated; water is not miscible with xylene; therefore, the tissues will not accept the xylene. Large amounts of water cause whitish scum on tissues.	Take tissues back to 100% ETOH (or dehydrant used in staining protocol) and dehydrate; use new xylenes to clear before trying to coverslip.
Tissues appear dull and whitish after using nuclear fast red.	Excess nuclear fast red remains in tissue.	Rinse slides well in distilled water before dehydration.
Iron reaction does not work on control.	Solutions contaminated.	Avoid metal racks or forceps. Use acid-cleaned glassware.
Silver precipitate all over slides.	Dirty slides or contaminated solutions.	Use acid-cleaned slides and glassware (critical) and change solutions frequently; avoid metal contamination.
Reactions faded on selected special stains.	Slides left in water for extended times.	Occasionally check pH of distilled and tap water, particularly before special stains.
Sections will not take stain, or stain irregularly.	Paraffin not completely removed before staining. Section not uniform in thickness. Tissue "old" (long storage in alcohol or, worse, in fixative). Fixative not suitable for staining technique. Fixative not fully removed.	Return sections through proper sequence of reagents to xylene. Leave until paraffin removed. Restain. Change xylene frequently. Return sections through proper sequence of reagents to water. Wash overnight and restain. Store tissues as embedded paraffin blocks—never store tissues in liquids for extended time. Mordant sections in recommended fixative.
Alcian blue not distinct.	Check expiration date of stain.	Check pH of stain and use test slide before main staining run.
Schiff's (Feulgen) reagent nonspecific on tissues fixed in 1% glutaraldehyde/4% formaldehyde (1G4F).	If fixed with 1G4F, fixative was inadequately washed out before processing tissue.	Wash tissues well to remove excess glutaraldehyde before processing. May try aldehyde blocking agent.
Schiff's (Feulgen) reagent faded or weak reaction.	Schiff's has become inactive or taken on too much moisture.	Make new batch; color is often indicative of the stain's potency. Best results are with a peach-straw color. *Note:* After reagent becomes clear, a slight purple tint will show stain to be ineffective. Always use test slide.
Hematein in Heidenhain's iron hematoxylin faded, not clear.	Contamination with the mordant.	Take care not to expose hematein directly to mordant. If using solution more than once, color change from brown to black-brown will indicate contamination.
Yellow, water-spotted appearance of stain on slide after using picromethyl blue.	Too much butanol carry-over in xylene.	Change xylenes frequently if staining a large number of slides.
Loss of picric acid in picromethyl blue.	Too much ethyl alcohol used in dehydration.	Use 1:9 ratio of 100% ETOH to butanol; tissues can be destained with acid alcohol and restained with picromethyl blue if color not green enough.
Tissue falls off slide.	(1) Moisture left under tissue; (2) tissue did not dry flat; (3) swollen tissue, does not hold together well; (4) tissues difficult to cut (hard, brittle), do not hold together well; (5) effect of stain too harsh on tissue, use alternative; (6) alkaline reagents dissolve albumin adhesive, sections start to work loose in course of staining or dehydrating; (7) using heat to speed reaction time is usually hard on tissue; or (8) slide dirty or greasy.	Dry slides adequately before staining. Spread tissue folds if they cannot be avoided, before drying tissue. Coat suspected loose tissue slides with celloidin after second 100% ETOH (except with alcian blue). For hard or brittle tissues, use extended staining time, e.g. overnight in solution instead of 1 hr at 60°C. Be sure slides are clean before albuminizing.

CHAPTER VII. COVERSLIPPING

A. TOOLS AND TECHNIQUES

Individual techniques of coverslipping tissue may vary from technician to technician. Exactly how to hold the slide or coverslip will vary with one's dexterity and comfort. However, the basic procedure is simple.

Materials needed are: (1) fume hood, (2) good light, (3) coverslip, (4) probe (straight), (5) paper towels, (6) Kimwipes[1], and (7) mounting medium.

Once the slides have been stained, dehydrated, and placed in xylene, they are ready to be coverslipped for permanence. This work should be done under a fume hood.

Remove slides singly in numerical order. Be sure the etched, coded side of the slide is up. With a lint-free tissue (Kimwipes), remove excess xylene from the front and back of the slide (Fig. 72), and place one drop of mounting fluid on the tissue on the slide (Fig. 73). Choose the proper size coverslip and gently lower the coverslip across the slide gradually (Fig. 74) so that the mounting fluid flows evenly from bottom to top. (Do not drop coverslip onto slide because this will usually trap air bubbles under the coverslip.)

Once the coverslip is attached, blot the edges of the slide on a paper towel (Fig. 75) and examine for occasional air bubbles. If present, gently lead the bubble to the edge, out from under the coverslip, using a probe. This procedure should be done quickly, precisely, and with care to prevent damage to the tissue. Place the slide on a flat tray (Fig. 76) along with an identifying label with the code, stain, and date of coverslipping. The slides are then placed in a drying oven set at approximately 42°C for at least 2 weeks to allow the mounting medium to harden.

After the mounting medium hardens, the slide can be cleaned, labeled, and filed for examination (Fig. 77). Using a single-edged razor blade, gently but firmly remove all excess mounting medium from around the coverslip. Be careful not to nick the coverslip with the razor blade. Brush off the loosened mounting medium, dip the slide in xylene, and gently wipe clean. When all the slides have been cleaned, make sure they are in the correct series and numerical order. Using a Koh-I-Noor rapidograph pen[2] containing India ink, write all pertinent codes on the etched end of the slide, including staining procedure used and date fixed.

Warning: Coverslip and clean all slides under a fume hood.

B. PROBLEMS AND RESOLUTIONS

Table 9 includes some problems or artifacts resulting from coverslipping, as well as some means of resolving them.

C. NOTES

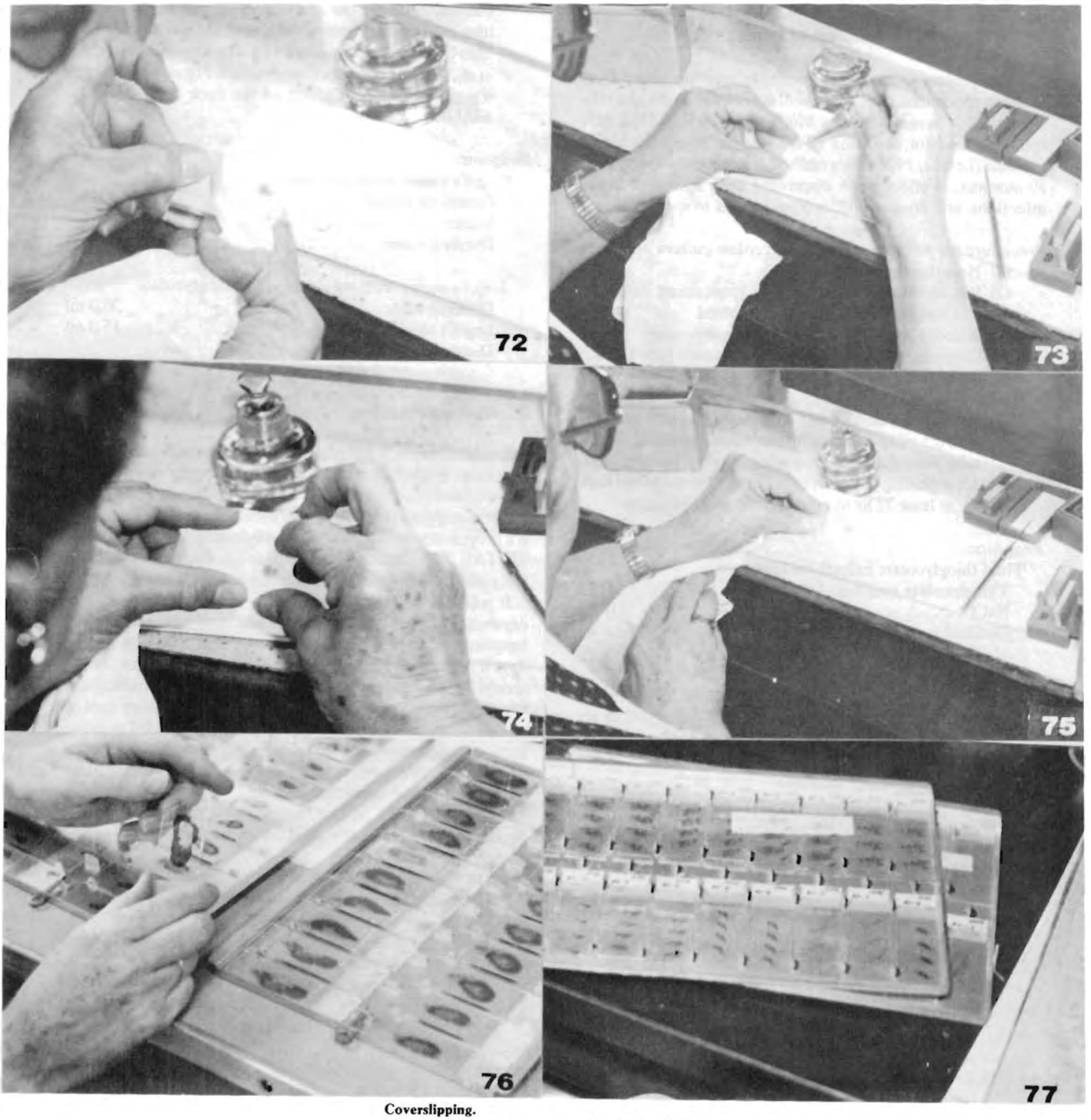
[1]Kimberly-Clark Corp., Neenah, Wisc.
[2]Koh-I-Noor Rapidograph, Inc., Bloomsbury, N.J.

D. REFERENCE

Luna, L.G., ed. 1968. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd ed. McGraw-Hill, New York.

TABLE 9. COVERSLIPPING

Problem or artifact	Possible causes	Solution, if any
Mounting medium will not flow across tissue.	Slide has dried out. Mounting medium too thick: part of the solvent may have evaporated.	Redip slide into xylene and start process again. Use new mounting medium or carefully thin down medium with solvent.
Coverslip tends to slip out of place.	Slide too wet with xylene. Mounting medium too thin. Too much mounting medium.	Wipe excess xylene from around tissue and from back of slide before adding mounting medium. Blot edge of slide once coverslip is in place. Mounting medium should have the right consistency. Use fresh mounting medium.
Air bubbles will not release from under slide.	Grit or loose debris under slide. Old coverglasses may be warped or dirty.	This often occurs with very sandy tissues. Remove coverslip by soaking in xylene and start process again. If trouble spot can be identified, remove it. However, if it lies within the tissue, try mounting with thicker mounting medium. Re-coverslip with a new coverglass.
Black, singed-looking, or parched tissue.	Tissue dried out before or during coverslipping or large air pockets formed from warped coverslips.	Make sure tissue is covered with xylene before coverslipping; tissue should never be allowed to dry out after deparaffinizing (Luna, 1968).
Sections appear opaque or have highly refractive lines outlining cells and tissues.	Clearing agent evaporated before mounting medium added. Sections insufficiently cleared or clearing agent not miscible with mounting medium.	None. Soak off cover. Clear properly. Check quality and nature of clearing agents and mounting medium (Luna, 1968).
Cloudy slide.	Too much mounting medium or too thick.	Mounting medium should be free-flowing; only use amount necessary and blot edge of slide after coverslipping to remove excess mounting medium.
Black air rings in tissue.	Air trapped during coverslipping.	Press coverslip down gently and use probe to guide bubbles to outer edges of coverslip (Luna, 1968).
Tissues dislodged out of place, layered, or unrecognizable.	Too much pressure on probe to remove air bubbles	If there is a large air bubble or many air bubbles, do not force out. Re-coverslip instead to avoid damaging tissue.



Coverslipping.

Fig. 72. Wiping excess xylene from back of slide.
Fig. 73. Dropping mounting medium over tissue section.
Fig. 74. Lowering coverglass over tissue.
Fig. 75. Draining excess mounting medium from slide.
Fig. 76. Placing a stained slide on a flat tray to dry.
Fig. 77. Slides cleaned and labeled, ready to file.

APPENDIX

A. SPECIAL TECHNIQUES

1. Culture of *Perkinsus marinus*

Thioglycolate cultures are routinely used on many fresh shellfish specimens. The thioglycolate culture technique described below was devised by Mackin and Ray in 1966. It is routinely used for detection of the marine fungus, *Perkinsus marinus* (Levine, 1978). This method is apparently specific for *P. marinus*, is effective in discerning both light and heavy infections, and does not differ from species to species.

Procedure for preparing fluid thioglycolate culture medium[1]*:*

(1) Heat distilled water to a boil
(2) Stir in thioglycolate medium (containing dextrose) and NaCl (salt) till both are dissolved
(3) Remove from heat and add chloromycetin[2]
(4) Dispense 9.5 ml of the medium into each tube (should fill 50 tubes)
(5) Autoclave 15 min at 15-17 lb pressure
(6) Store in the dark at room temperature until needed
(7) Add 0.5 ml of mycostatin[3] suspension into each 9.5-ml tube immediately before using. *Do not shake tubes.*
(8) After placing tissues in culture tube, return to the dark for at least 72 hr to culture before examination

Reagents:

[1]Fluid thioglycolate culture medium

Thioglycolate medium	14.6 gm
NaCl	10.0 gm
Distilled water	485.0 ml

[2]Chloromycetin (chloramphenicol, Sigma Cat. No. C-0376[a])

Chloromycetin	0.25 gm
Distilled water	10.0 ml

[3]Mycostatin suspension (nystatin, Sigma Cat. No. N 3503[b])

Mycostatin	500,000 usp units
Sterile distilled water	125.0 ml

Shake well before using. Refrigerate unused portion for further use.

Procedure for preparing slides for examination:

(1) Excise rectal tissue from mollusk during initial necropsy (Fig. 78)
(2) Remove tissue from tube with a thin glass rod with hooked end (Fig. 79) and place on the correspondingly coded slide with a drop of Lugol's iodine working solution[4] (Fig. 80)
(3) Tease tissue apart with probes (Fig. 81) and add another drop of Lugol's (Fig. 82) iodine working solution
(4) Place a coverslip on the slide (Fig. 83)
(5) Place finished slide on a tray
(6) Wait 15 min, then examine the slide

Note: Clean instruments after handling of each specimen to avoid contamination

Results:

Examine with the use of a dissecting microscope. A positive infection of "dermo" will show symmetrically round blue-black to solid black spheres. See Ray, 1966 and Kern et al., 1973 for additional information. Figure 84 illustrates a positive fungus infection of the duck clam, *Macoma balthica.*

Reagents:

[4]Lugol's iodine stock solution

Potassium iodide	6.0 gm
Iodine	4.0 gm
Distilled water	100.0 ml

Lugol's iodine working solution for thioglycolate

Distilled water	30.0 ml
Lugol's stock	15.0 ml

Dispense from 50-ml dropper bottle

2. Decalcification

Decalcification is not often utilized with molluscan tissues. However, in special circumstances, for example when processing unshucked larvae of bivalves, we have found the versenate method of decalcification to give excellent results. (Versenate is a proprietary term for disodium ethylenediaminetetraacetate (EDTA)). This chelating method of decalcification works gradually and does not damage the internal animal tissues.

It will take different time periods for decalcifying larvae, depending on species and size. The best results are obtained by frequently changing the versenate solution over a period of days if necessary. Pearls found in oysters, mussels, and clams should be removed prior to embedment unless the investigator specifically requires their presence. Also, the sensory eyes of scallops, located along the mantle, should be avoided in tissue sections unless necessary for specific diagnosis. If either pearls or scallop eyes are required by the investigator, they can be decalcified by the versenate method.

Procedure for versenate method of decalcification (Birge and Imhoff, 1952):

(1) Fix tissue in fixative of choice
(2) Wash well in running water (the time will depend on fixative used and size of tissue)
(3) Place tissue in a saturated solution of versenate[1] until decalcification is complete; check daily
(4) Wash well in running water – ½-1 hr (depending on size of tissue)
(5) Place in 70% ETOH until ready to process

Reagents:

[1]Versenate solution

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

Note: For the most efficient use of versenate as a decalcifier, change tissues to a fresh solution every 2-3 days. We have had great success using versenate on bones found in fish tissue.

"... specimens may be left in the versenate solution for as long as 14 days without noticeable effect on the staining qualities of the cells" (Birge and Imhoff, 1952).

Warning: Versenate may remove iron and other metals in the process of decalcification, according to *Carleton's Histological Technique* (Drury and Wallington, 1967).

3. Transfer of Tissue from Broken Slide (Coolidge and Howard, 1979)

When it is not desirable to mount a broken slide on another slide because of excessive thickness (when high resolution is needed on the microscope) or if a slide has been broken in too many pieces, use the following method:

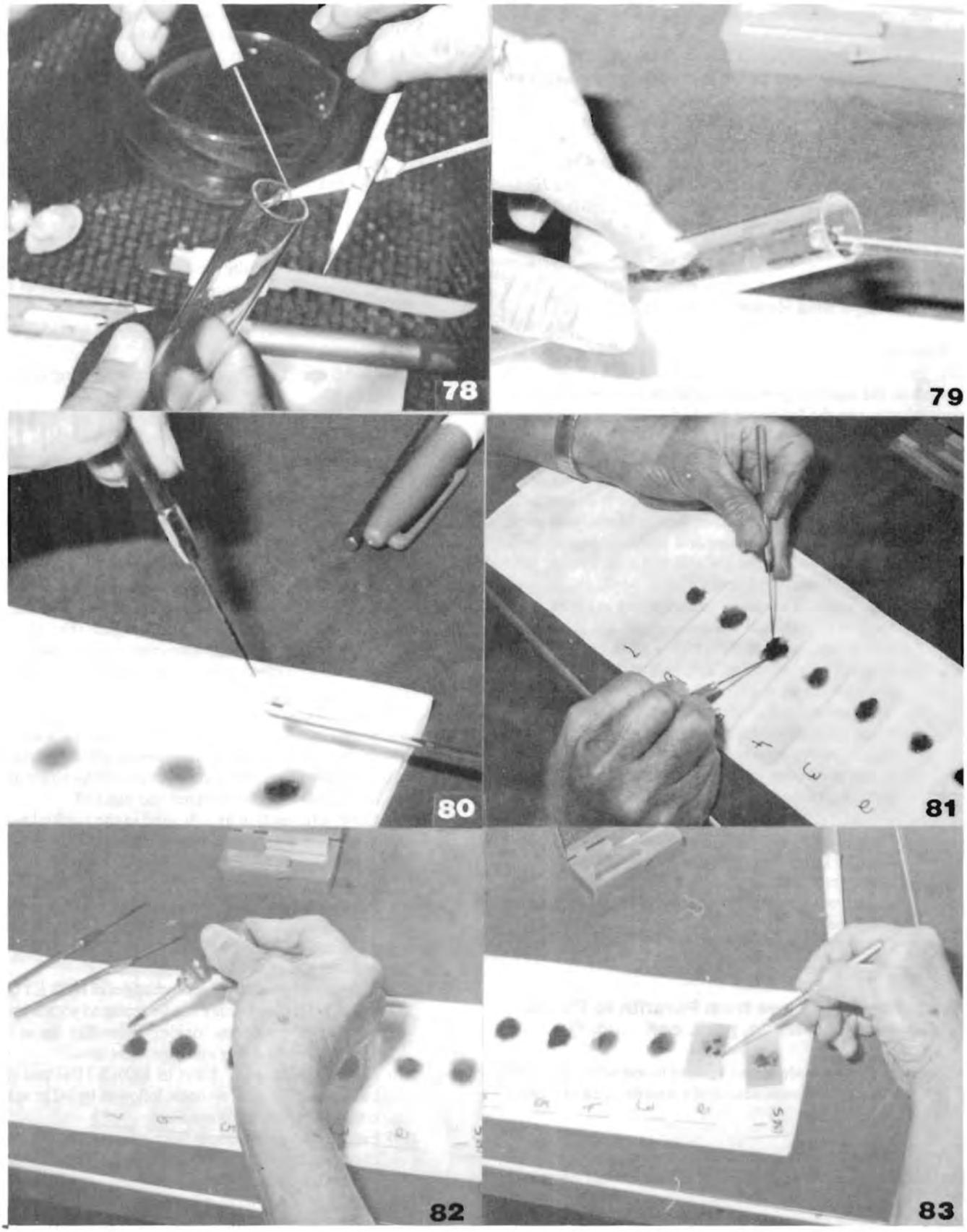
(1) In a shallow pan, assemble all piêces of broken slide on another slide.
(2) Slowly pour xylene in pan until slide is covered.
(3) Soak until all pieces of coverslip can be easily removed. Lift slide from container and place on paper towel. While broken pieces are still wet with xylene, pour Diatex[c] (plastic) on entire slide.
(4) Dry overnight at room temperature or in a 60°C oven for 2 hr (plastic will be hard).
(5) Cut around edge of slide with a razor blade.
(6) Soak in tap water (about 2 hr) until plastic can be easily peeled from slide.
(7) Trim around tissue with scissors.
(8) Place plastic-coated section on clean albuminized slide. Cut a paper towel the size of the slide, dampen it, and place it on top of the plastic film. Place a clean slide on top. An object should be placed on top of slide to hold it in place. Leave at room temperature or place in 37°C oven overnight (towel will be dry).
(9) Remove top slide. Put slide with film and paper towel into xylene. The towel will soak loose, and the slide should remain in xylene until the plastic is completely dissolved.
(10) Coverslip.

4. Transfer of Tissue from Paraffin to Plastic (Howard and Wade, pers. commun. [d])

The following procedure was devised to use when it is desirable to examine the ultrastructure of a specific area of interest on a histological slide.

A modification of the tissue transfer process from *Animal Histology Procedures* of the Pathological Technology Section of the National Cancer Institute (Coolidge and Howard, 1979) is used to re-embed a cut and stained section of tissue from paraffin to resin without loss of orientation:

(1) Begin by cutting a practice block at 30-50 μm of the same type of tissue as your specimen at room temperature. The goal here is to cut evenly through the tissue without breaking apart the section.
(2) Align the block of interest in the chuck of the microtome. Cut two 6-μm sections before changing the cutting gauge to desired thickness (30-50 μm). The thicker the tissue can be cut without damage, the greater the prospect of finding the desired areas.
(3) Moisten the block and cut through the whole face of the block with an even motion. A thick section will generally come off the microtome knife in a thick roll.
(4) Gently and carefully raise the tissue roll with the aid of brushes to an albuminized slide moistened with a small pool of distilled water.
(5) Drain excess water and place slide on warming tray at 42°C.
(6) Unroll tissue slowly, carefully manipulating brushes (Fig. 85).
(7) Continue to unroll section (Fig. 86); gently flatten section and place slide in oven at 42°C.
(8) Rechill block which is still in the chuck with a piece of ice and cut another 6-μm slide section.
(9) Once the slides are thoroughly dry, deparaffinize and stain the 6-μm sections in Harris' hematoxylin and eosin.
(10) Check to see if desired area is present.
(11) Proceed with thick section. Deparaffinize and stain with hematoxylin (1 min), eosin (30 sec), and mount with Diatex synthetic liquid coverglass (Fig. 87).
(12) Dry overnight at room temperature or in slide oven at 42°C until plastic is hard; check under microscope.
(13) Cover slide with paper towel and soak with tap water (about 2 hr) until plastic is pliable and can be gently peeled away from slide (spatula may be used for thicker sections to keep from damaging tissue section).
(14) Compare plastic section with 6-μm slides where area of interest has been located and marked.
(15) Cut plastic section as indicated in the marked 6-μm sections; use a specific shape (e.g. triangle or truncate pyramid) (Fig. 88) to ensure proper orientation of tissue later.
(16) Place tissue in a porcelain spot plate or small shallow glass dish; add several changes of xylene until tissue is free from all Diatex; pipette new xylene on and off, trying not to disorient tissue.
(17) Remove xylene with several changes of 100% ETOH. The ETOH may or may not be replaced with several changes of propylene oxide, depending upon the choice of embedding medium to be used.
(18) Leave section in a 1:1 mix of 100% ETOH and embedding medium for an hour, followed by 1-2 hr in the complete embedding medium.
(19) Embed section of tissue in fresh embedding medium; check again for orientation and place mold carefully in 70°C oven for 48 hr.
(20) Before cutting thin sections from the cured blocks, cut and stain a 1-μm section for positive identification of the tissue desired.



Procedure for determining *Perkinsus marinus* in thioglycolate cultured rectal tissue of *Macoma balthica*.

Fig. 78. Putting freshly cut rectal tissue in thioglycolate.
Fig. 79. Removing cultured tissue with glass hook.
Fig. 80. Placing tissue into drop of Lugol's iodine.
Fig. 81. Teasing tissue apart for better penetration of iodine.
Fig. 82. Placing another drop of iodine on tissue.
Fig. 83. Tissues covered and ready for inspection.

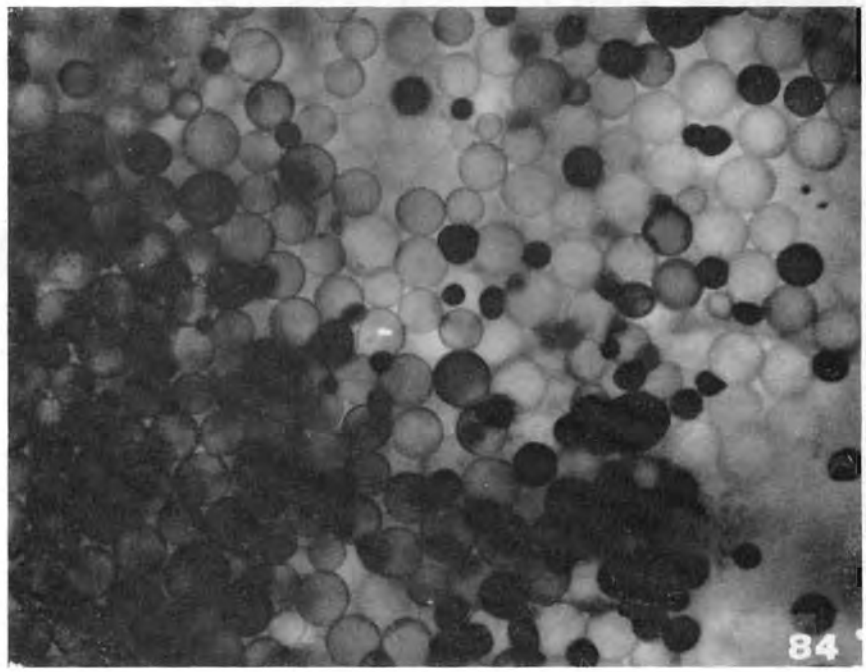
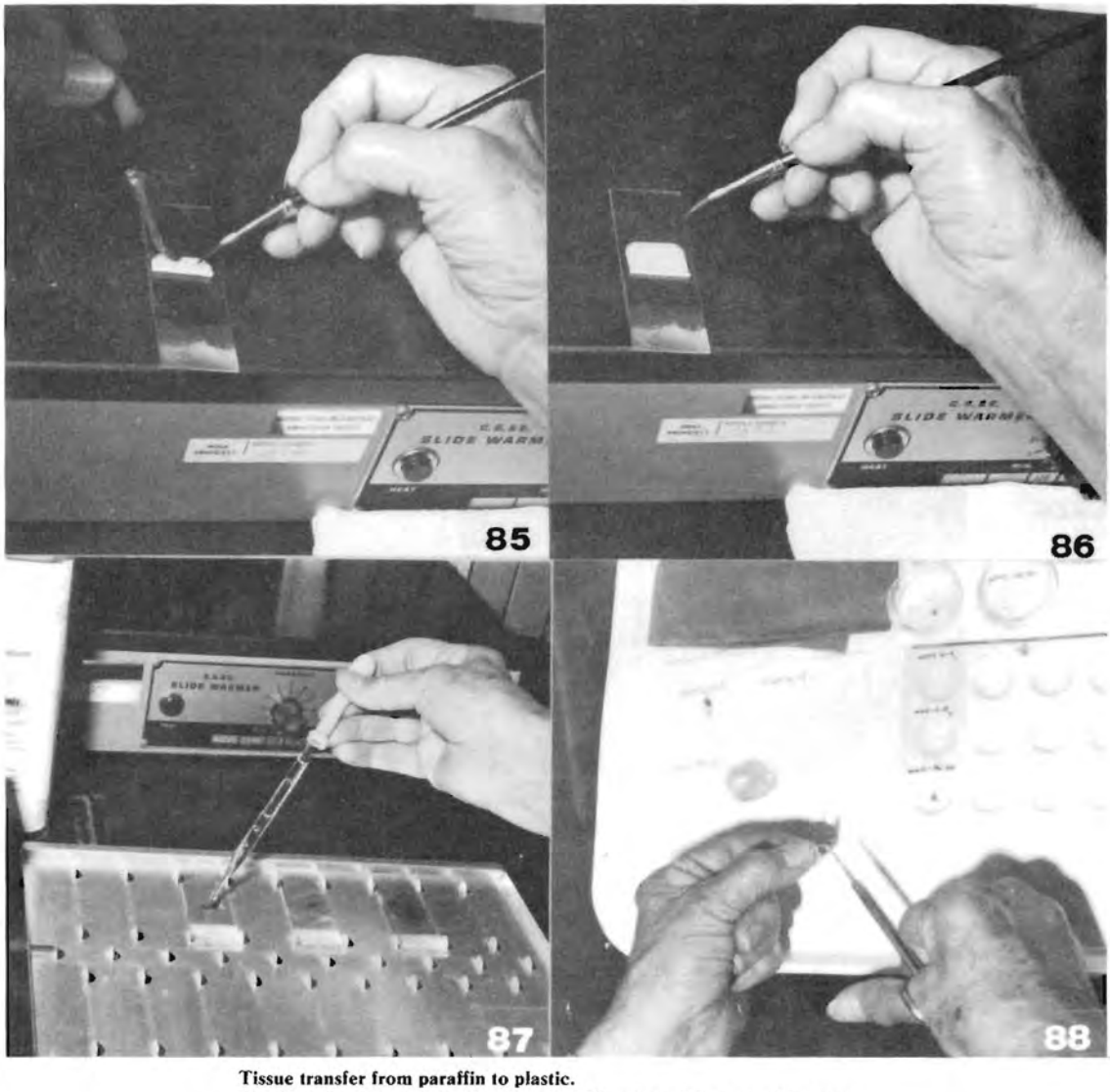


Fig. 84. Positive fungus infection of duck clam, *Macoma balthica*.



Tissue transfer from paraffin to plastic.

Fig. 85. Placing thick section (approx. 50 μm) on slide and rolling open by careful manipulation of brushes.

Fig. 86. Section rolling open.

Fig. 87. Covering stained section with Diatex liquid coverglass.

Fig. 88. Cutting plastic section in a specific shape (e.g. truncate pyramid) to ensure proper orientation of tissue during embedment.

5. Record Keeping

The two data forms shown on the next two pages cover a wide range of information. Each form follows the sample through a specific step from arrival and code designation through the final histological examination. A summary of all information is statistically presented on the *Molluscan Pathology Report.* All of the compiled data are filed in a permanent information bank (data forms courtesy of C.A. Farley).

6. Filing and Slide Storage

All samples are kept in permanent files. Once the sample is coded, either in the field or after arrival, it is recorded with its historical data. Each investigator is responsible for keeping his/her own records, but we compile all the information given us. It is recorded in our master files and entered into a current sample index. In the index, the code number, specimen or identification type, date of arrival, fixative, and embedment, date of sectioning and staining, identity of requestor, and source are recorded. A work ledger is also kept in which we record daily work activities performed by the laboratory. Maintaining up-to-date records is imperative.

Once the permanent slides have been completed and issued to the requestor, the tissue blocks are filed consecutively in trays and stored. A record book identifies the location of all blocks in storage. They can be readily retrieved by code numbers. The block storage area is analogous to a library. Study projects with consecutive code numbers are filed together. Samples from similar geographic areas are also consolidated when possible. A record guide specifies block location by aisle, row, and cabinet. If study blocks have been removed, this is so indicated, and their current location is recorded. This system enables us to keep an orderly collection of blocks which is of value for retrieval and future retrospective study.

B. NOTES

[a,b]Sigma Chemical Company, P.O. Box 14508, St. Louis, Mo.
[c]American Scientific Products, 8855 McGaw Road, Columbia, Md.
[d]D.H. Howard and J.T. Wade, National Marine Fisheries Service, Oxford, Md.; 1976.

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FIELD DATA ON OYSTER SAMPLES

USNMFS, Oxford, Maryland
Comparative Invertebrate Pathology

Date sample collected_____

Date sample received_____

Source: State_____ No. oysters_____Code designation_____

Estuary_____ Bar_____Depth water_____ft.

Bottom water: temp._____°C salinity_____o/oo

Nature of bottom: hard, medium, soft, sandy, mud, shell,

other_____

Additional comments on field collection and condition of oysters:

GROSS EXAMINATION OF INDIVIDUAL OYSTERS in Laboratory (over), by_____

Date_____ Time_____ oysters examined and fixed;

Fixative_____.

MOLLUSCAN PATHOLOGY REPORT

Date sample received _____ Purpose _____ Date sent _____

Requested by _____ Phone _____

Send report to _____

_____

Environmental data:

Temperature _____ °C Salinity _____ o/oo pH _____ Oxygen _____ ppm Other _____

Species _____ Stock _____

Origin _____

History _____

_____

Location sample came from _____

Number in sample _____ Date collected _____ Date fixed _____ Code _____

Fixative _____ Other histologic data _____

GROSS PATHOLOGY

Condition			Mantle recession	Pale digestive gland	Cliona	Polydora	Other
Fat	Medium	Watery					
__%	____%	____%	_____%	_____%	____%	____%	____%

MICROSCOPIC PATHOLOGY

Pathologic lesions				Parasites			
Category	Type of lesion	Prevalence	Degree (0-9)	Group	Species	Prevalence	Degree (0-9)
Inflammation	_____	_____%	_____	Viruses	_____	_____%	_____
_____	_____	_____%	_____	_____	_____	_____%	_____
_____	_____	_____%	_____	Chlamydia	_____	_____%	_____
_____	_____	_____%	_____	_____	_____	_____%	_____
_____	_____	_____%	_____	Bacteria	_____	_____%	_____
_____	_____	_____%	_____	_____	_____	_____%	_____
_____	_____	_____%	_____	Fungi	_____	_____%	_____
_____	_____	_____%	_____	_____	_____	_____%	_____
_____	_____	_____%	_____	Protozoa	_____	_____%	_____
_____	_____	_____%	_____	_____	_____	_____%	_____
_____	_____	_____%	_____	_____	_____	_____%	_____
_____	_____	_____%	_____	Metazoa	_____	_____%	_____
_____	_____	_____%	_____	_____	_____	_____%	_____

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