

The Collection and Preservation of Animal Parasites (Technical bulletin)

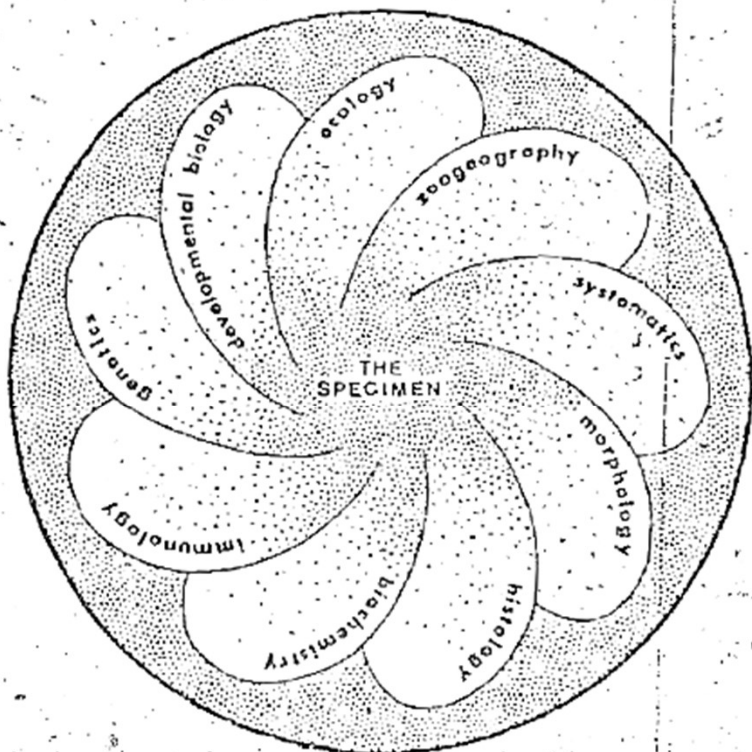
- M. H. Pritchard & G. O. Kruse, 1982. The collection and preservation of animal parasites. Technical Bulletin No. 1. The Harold W. Manter Laboratory, University of Nebraska Press, 141 pp.

Pritchard, Mary Hansen

Note: This is not the actual book cover

INTRODUCTION

Animal parasites may be found anywhere in or on the body of vertebrates and many invertebrates. The collector should examine not only the gut of an animal, but the whole body. The more thorough the autopsy of the host, the more reliable the data obtained for that host. Whether the time required for such thoroughness is commensurate with the objectives of the collection must be determined by the investigator. In general, it is better to collect more information than required than too little.



The purposes of collecting and preserving animal parasites are identification for diagnosis and treatment of disease, morphological studies, systematic studies, zoogeographical studies, and additions to systematic collections--of parasites and/or hosts. Improperly handled and preserved specimens will not permit accurate identification or correct description. It is a waste of time to collect and process such specimens.

This guide presents some basic formulae and techniques in a single, easy-to-use book. Special techniques for the various groups of animal parasites are included. Many of these methods are available in diverse publications (texts, manuals, and journals), but it would be difficult, time-consuming, and expensive for the student or non-specialist to assemble such a reference library. It is, in fact, impossible to acknowledge all original sources for the commonly used formulae and techniques contained herein. No personal names are cited except as standard references to solutions (e.g., Wright's stain).

The host is an essential item in collecting data for the parasite; the parasites are equally important biological information for anyone studying the host. Thus this guide will be a useful reference to collectors of potential hosts who may encounter parasites, as well as to specialists of one parasitic group wishing to collect other parasites, graduate students, undergraduate students, veterinary and medical laboratories, and public health laboratories.

Although at least one satisfactory method for collecting and preserving each type of animal parasite has been presented, others are available. The back of each page is blank so that users may make annotations or additions to

their personal copies. A decade ago we might not have included methods for preparing specimens for electron microscopy; we cannot now anticipate technological advances of the future or information which may limit the use of certain chemicals.

Revisions of this guide are anticipated. Readers who wish to share new or revised techniques which they know produce superior specimens may send them, together with permission to reprint them, to:

The Harold W. Manter Laboratory
Division of Parasitology
University of Nebraska State Museum
W-529 Nebraska Hall
University of Nebraska
Lincoln, Nebraska 68588-0514
U.S.A.

Collecting is not always easy. "Feast or famine" may apply to hosts or parasites. When hosts or parasites are plentiful, the parasitologist may work far into the night; furthermore, he/she may work the same long hours and have nothing to show for it if the hosts are not infected. If one attempted to list rules for a collector, they might look like this:

1. The collector must be aware of and abide by regulations relating to the collections of hosts and must secure the required legal permits. Also, it is common courtesy to obtain permission when entering private property.
2. The collector must be ready to work hard and long.
3. Nothing must be allowed to interfere with the collecting.
4. The collector must respect the law for prevention of cruelty to animals.
5. Specimens, once collected, must be attended to immediately (much hard-earned material is often ruined because laboratory procedures are postponed until the next day).
6. The collector must possess some mechanical skill and use ingenuity in meeting specific problems.

Caution: The collector must be careful about microbial and parasitic infections which are transmissible from animals to man.

CHECKLIST OF MATERIALS NEEDED TO COLLECT PARASITES
(A suggested collecting kit)

The field equipment listed below includes all the necessary tools and solutions one ordinarily needs for collecting and preserving parasites in the field.

Equipment:

- ☒ Dissecting microscope or strong hand lens
- ☐ Dissecting scissors (two sizes, one pointed, one blunt or enterotome)
- ☐ Forceps (two sizes, fine-pointed tips)
- ☐ Dissecting needles (insect pins may be fastened into glass rods)
- ☐ Petri dishes (several, may be plastic)
- ☐ Beakers (two sizes, one 500ml or larger, slender and tall)
- ☐ Alcohol lamp and matches
- ☐ Glass slides
- ☐ Cover glasses
- ☐ Pipettes
- ☐ Brushes (small)
- ☐ Vials (several sizes) and jars with lids
- ☐ Paper towels
- ☐ Ruler (in cm)
- ☐ Diamond-point pencil
- ☐ Pen with permanent ink (some India inks are not permanent) or pencil
- ☐ Paper (white bond or rag for labels in vials; black for background under collecting dish)
- ☐ Bags (plastic or paper)
- ☐ Collecting book

Solutions:

- ☐ 70% ethyl alcohol (EtOH)
- ☐ Formalin
- ☐ AFA (alcohol-formalin-acetic acid)
- ☐ Glacial acetic acid
- ☐ Glycerol
- ☐ Physiological saline or tap water

HOSTS

The determination of the host species is essential in making parasite collections. If the collector is not familiar with the host taxonomy, the host should be preserved for exact determination by specialists. The reference number (see Collection data, p. 16) must accompany the host specimen.

The animal to be dissected should be freshly killed. Both internal enzymes and external agents such as bacteria contribute to the rapid breakdown of cells. Parasites survive longest in cold-blooded hosts and in the cyst stages, but many parasites deteriorate quickly amid dead and dying cells; therefore, examine only one host at a time.

Some parasites (e.g., arthropods) leave the host very soon after its death, and some migrate within the host. Regardless of how the hosts are handled, the parasites will recognize and react to any change in their environment.

If numerous hosts are suddenly available and it is impossible for the collector to autopsy them immediately, the hosts can be treated by one of the following methods:

- A. Maintaining hosts alive. Small animals may be kept alive in aquaria or cages for days or even weeks. The length of time will vary according to host species, the ability to provide hosts with an adequate diet, the cage or aquarium conditions, and the virulence of parasite infection.

Two important things should be remembered:

1. Some parasites, especially intestinal helminths, will be eliminated from the host during the early days, or even hours, of captivity.
2. Other parasites, especially ectoparasites, will multiply and/or transfer from one host individual, or even species, to another.

If it is impossible to keep the hosts alive, then we suggest the following methods which will aid in preserving the parasites and keeping them in the proper location on/in the host.

- B. Cooling. The hosts should be kept as cool as possible until they can be autopsied. Cooling slows the lysosome reactions which continue even in the dead animal. Refrigerating or icing is effective for short periods of time, perhaps as long as 24 hours. If time and space permit, it is preferable to separate the organs into containers of saline and then refrigerate them.

- C. Heat treatment. Fresh organs can be placed in a beaker to which boiling water is added. Let organs heat thoroughly, then transfer to fixative. Most helminths will relax completely before the fixative reaches them. Upon recovery later, they will be found in excellent condition for subsequent processing.
- D. Chemical preservation. Many collectors routinely preserve specimens in 10% formalin. This method also preserves the parasites in situ and permits the collection of parasites from hosts preserved for long periods of time, as from museum collections. An abdominal incision or additional formalin injected by syringe into the coelomic cavity and GI tract can fix the parasites even more quickly. Under these conditions parasites will contract. It is very difficult to straighten them properly when they are collected at a later time. A method for straightening and slight flattening is described on p. 80.

Note: The proboscis of an acanthocephalan, which is of taxonomic importance, will often not be extended during chemical preservation.

Note: The volume of fixative should always be at least nine times the volume of the tissue to be fixed. Warm fixative will penetrate tissue more rapidly than cold fixative. If the liquid discolors, it should be changed. To store tissue for longer periods of time, always use fresh fixative after 48 hours (5% formalin is sufficient).

- E. Freezing. It is not advisable to freeze the host in toto, but the faster the freezing, the better the result. Under conditions of relatively slow freezing, the parasite begins to autolyse before it is frozen. It undergoes further maceration during the thawing process. Small parasites recovered from frozen hosts should be dropped immediately into fixative.

The above methods are in declining order of preference. Again, the best preparations are obtained when living parasites are collected and treated appropriately.

COLLECTING PARASITES FROM HOST

Freshly collected, living parasites always make more satisfactory study specimens than those that have undergone even slight maceration.

Thorough examination in the proper sequence is important to prevent losing some parasites while looking for others. The preferred sequence is: (1) blood, (2) gills (if present) and skin, (3) internal organs and (4) musculature.

Note: It is important, when possible, to record the precise site of the infection.

Collecting data. Keep an autopsy record for each host examined (see samples, pp. 16 and 17). Make a record of the number and kinds of parasites found in each organ; record also negative findings! When parasites are removed, they should be treated appropriately and placed in a suitable container together with the host number assigned on the autopsy record.

Labels for immersion in fixatives, alcohols, and other liquids should be written with pencil or waterproof ink (India ink is not recommended, as only certain kinds are reliable) on white bond (rag) paper, such as good writing paper (not pad, colored, or lined notebook paper, which may break down and stain the specimens). Place the label into the container with the preserved specimens.

Note: If label has been written with ink, wash it first under running water before placing with the specimen.

A. Smears.

Clean glass slides are essential for satisfactory blood smears. Even pre-cleaned slides just unpacked should be cleaned thoroughly with a detergent soap and rinsed in distilled water or washed in a 1:1 mixture of 95% EtOH and 100% acetone, and then dried carefully with a soft, lint-free cloth.

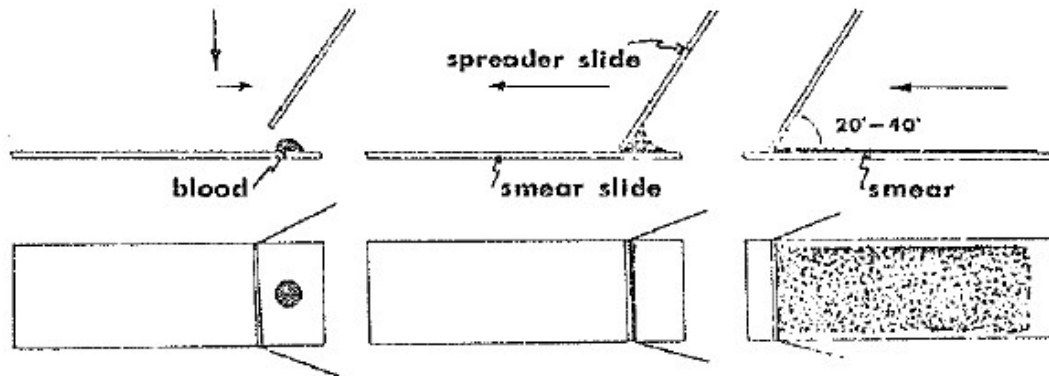
1. blood smears. Blood should be taken from an animal immediately after death, before clotting occurs. If too much time has elapsed between the death of the host and the making of the blood smear, too many erythrocytes settle, and one will obtain primarily serum.

Note: It is important to stain the smears within a few days of taking the sample, especially when collecting under humid conditions.

- a. Thin smears: Make 2-4 thin blood films for staining later with Giemsa or Wright's stain. If possible, take blood from different areas of the host. The blood may be drawn with a syringe from a vein or the heart or taken from an open source.

Caution must be taken if the blood is drawn from an open source, such as the body cavity, where there may be contamination by the contents of the gut or other organs and tissues.

To make a blood smear:



Place a drop of blood near one end of a clean ("smear") slide. Touch the end of another ("spreading") slide to the center of the first one and draw it toward the drop of blood. Upon contact, the blood will flow along the edge of the spreading slide. The two slides should be held at an angle of 20 to 40° to each other. Push the spreading slide over the length of the smear slide with an even, smooth motion, dragging the blood behind it. The thickness of the smear may be controlled by altering the angle between the slides (e.g., increasing the angle thins the smear).

Air-dry blood smears. They may be stored until stained. Mark slides with the collecting (host) number with a diamond pencil.

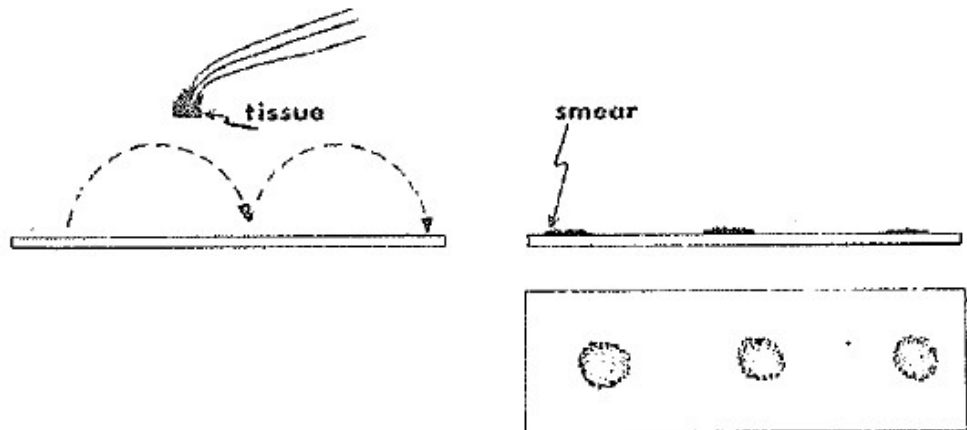
- b. Thick smears: Two or three drops of blood are spread on a clean slide over an area of about 4cm² using a needle, toothpick, or corner of a second glass slide with a circular stirring motion. Allow to clot and dry thoroughly. They may be stored until stained later.

Note: Thick films should not be fixed (i.e., treated with alcohol or killing solution) at this time.

To stain smears, soak in distilled water for 20 minutes or until color disappears. If one end of the slide is elevated (use a glass rod), the ruptured red blood cells will wash away. Leucocytes and parasites are left more visible. The film is then dried again and stained. Thick films concentrate parasites, but they are more difficult to interpret than thin films. Mark slides with collecting number.

Note: If thick smears have been stored for long periods of time, add 1 to 3 drops of glacial acetic acid to the distilled water to enhance hemolysis.

2. Tissue smears. Protozoan and mesozoan parasites in tissues may be exposed by cutting through a fresh organ. A tissue smear is simply made by pressing the cut surface of the organ against a slide several times:



Air-dry tissue smears; they may be stored and stained later. Mark slides with collecting number.

3. Staining blood and tissue smears. Most histologists prefer to use Giemsa (p. 98) or Wright's stain (p. 110) for blood and tissue smears. Giemsa stain is the best for most blood smears, but Wright's is an easy alternative.
4. Skin parasites. The skin should be carefully inspected. Ectoparasites, fleas in particular, leave the host upon its death.
1. Terrestrial hosts. It is advisable to transport the host in a tightly closed plastic or paper bag. Some workers add a piece of cotton soaked with ether or chloroform to the bag with mammalian or avian hosts. Wash out the bag and inspect the washings with a dissecting microscope.

A simple field method for obtaining ectoparasites involves shaking the host over a white piece of paper. The dark parasites are conspicuous when they fall onto the white background.

Furry or feathery animals may be scrubbed with a small, fine-bristled brush (such as an old tooth brush) which is periodically dipped into soapy water. Collect run-off into a container and inspect with a dissecting microscope.

Ectoparasites are obtained from large, living animals by directly picking them off with fine forceps, taking a deep scraping with a scalpel

dipped into glycerol, or cutting the hair or feathers from the infected area. Inspect under a microscope.

Skin, scabs, or lesions may be scraped with a scalpel and the scrapings smeared on a glass slide for examination. It helps to put a drop of water or glycerol on the scrapings and add a cover glass.

Note: Handle arthropods carefully, since rough treatment may damage delicate legs and setae. Fine-tipped brushes moistened in alcohol are excellent tools for picking up the parasites.

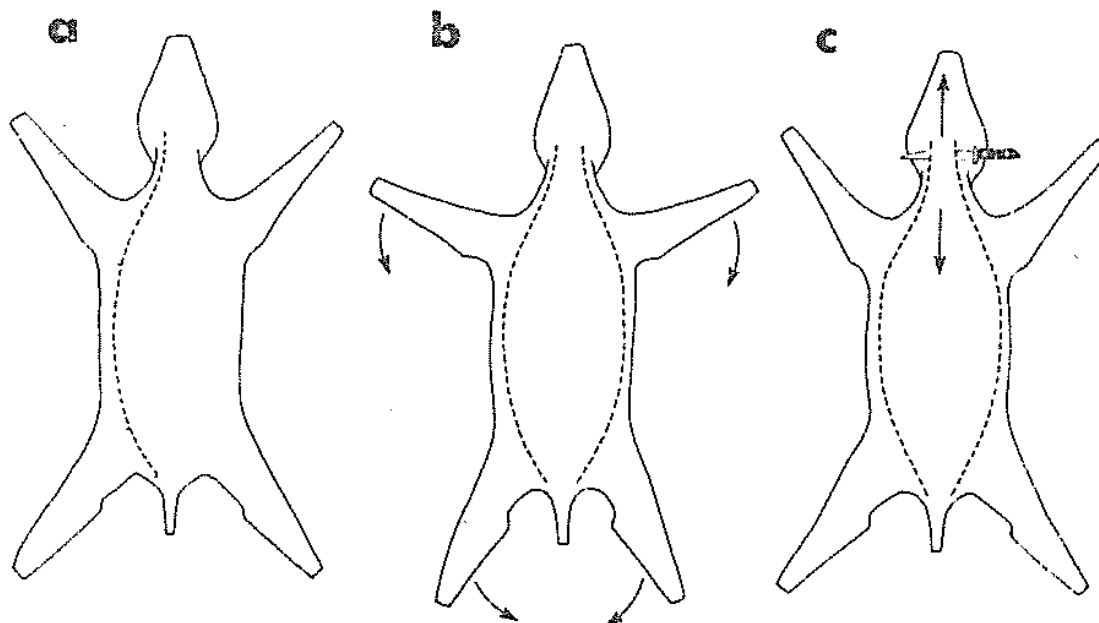
2. Aquatic hosts. Most aquatic hosts harbor parasites ranging from protozoans (below and p. 32) to monogeneans (p. 37), leeches (p. 57), copepods and isopods (p. 59), and glochidia (p. 62).
3. Cysts on (in) the skin of aquatic hosts usually contain protozoans or larval trematodes. To recover specimens, the cyst may be teased apart or:
 - a. Helminth cyst. Excyst the larvae by the chemical method described for larval trematodes (p. 44) or under Artificial digestion technique (p. 78).
 - b. Protozoan cyst. Make a tissue squash by pressing a cover glass or second slide onto the cyst to break it. Let the preparation dry, and stain with Giemsa (see p. 98) like tissue smears.
 - c. Otherwise, excise the tissue with the cyst in situ and fix in 10% formalin for embedding and sectioning (see p. 88) or in 1% gluteraldehyde for TEM studies (see p. 86).
- C. Mouth and nasal parasites. The oral cavity can more easily be exposed by cutting through the corners of the mouth with heavy scissors or bone cutters. Some parasites inhabit the buccal cavity, but be aware that others may migrate to that area upon the death of the host. When some marine fishes are brought up to the surface in a catch, the stomach is everted, pushing the contents into the buccal cavity.

The nasal cavity can be flushed by holding the head pointed downward over a small dish and running a fine stream of water from a large-bulbed pipette through it.

If present, excise gills and examine for parasites (see Monogeneans, p. 37; Copepods and Isopods, p. 59).

- D. Internal parasites. In smaller animals, cut through the body wall of the host along the ventral side and extend the incision backward through the pubic symphysis, around the anus and around the urogenital opening if

present. Anteriorly, extend the cut to a point between the mandibles, cutting through the ribs and sternum if necessary.



In larger animals (mammals), it is easier to make the incision more laterally (see figure above). Lay the animal on its side, lift up the legs, and cut the skin medially, connecting the cuts between front and hind legs. Extend to pharynx. Turn the animal on the other side and do the same. Lift up skin in pharyngeal region and pull posteriorly, cutting the sternum and diaphragm.

Expose the viscera fully. Look for parasites that may be free in the body cavity, in the mesenteries or in the peritoneal cavity. Examine the surface of organs for cysts.

Remove viscera and place organs in individual containers with saline. Separating the organs leaves no question as to the original site of infection. Containers should be kept cool or shaded when working in the field. If a game animal is shot in the field, its internal organs should be stored in saline as soon after death as feasible (or choose other preservation techniques). Such jars of entrails must be kept cold if the parasites are to survive a day or more.

Organs should be torn open or cut apart with sharp scissors. This will allow exposure of parasites and will cause the least damage to specimens present. Frequently, attached parasites will free themselves if organs are allowed to stand in water for as long as an hour.

Separate stomach, cut it longitudinally, and shake the contents into saline solution (p. 130) or water (for marine animals use 1/3 sea water and 2/3 fresh water). Scrape inner lining of stomach deeply with scalpel. Proceed with the other parts of the digestive tract in similar fashion.

The intestine should be freed from the mesentery, straightened out, and opened with blunt scissors (preferably an enterotome). If the intestine is very long, it may be cut into convenient sections (if necessary, measure and keep in order to record location of parasites). Definitely separate the diverticular ceca when present.

A good way to empty and scrape the intestine is to grasp one end of the intestine, which is in a large container, with a pair of forceps and to scrape off the inner lining with a scalpel as it is drawn from the container. Large intestinal parasites will be seen immediately.

Gut content may be thoroughly examined by mixing it with saline. One easy way is to place the contents into a tall, slender, screw-cap container and shake vigorously for 15 seconds. Let the jar stand for 3 to 10 minutes to allow sedimentation of parasites. This process also helps to remove mucus, fecal matter, and bits of tissue which frequently obscure small parasites.

Decant the turbid fluid. Several washings may be necessary if much mucus is present. The sediment should be diluted for examination if it is so dense as to cover any parasites that might be present. Examine sediment under dissecting microscope over a black background.

If they are available, a series of graduated sieves will speed the examination of the sediment. Parasites are easily recognized by their movements, size, shape, and color.

Dense organs such as liver should be torn apart and minced in saline. Examine suspension as above.

Remove parasites from the host to clean saline, and wash them. Use a soft brush to gently remove all adhering mucus and debris that cannot be removed by shaking.

Note: Fixative will congeal mucus and make its removal more difficult.

Some authors recommend pouring boiling water over organs isolated in small beakers and allowing to stand. Minimum times for heat to penetrate intestines and give satisfactory results are 10 seconds for small amphibians, 30 seconds for small birds and fish, and up to 2 minutes for larger animals. A number of preparations can be handled at one time in this way. After this treatment, organs are placed into separate containers of AFA for fixation and storage until examination. Worms fixed by this method remain in their microhabitats, but detached from the host tissue. When the heat-treated and fixed organs are teased apart in dishes of EtOH (30 to 50%), well-relaxed and fixed worms are released.

Note: When making microscopic examinations aboard a ship, reduce the amount of liquid to an absolute minimum. Transfer the organs to separate dishes, pipette a few drops of saline solution or water over the surface, and cover with wet paper towels until time for examination. Cut organs and larger blood vessels longitudinally, add some drops of saline to the contents of the lumen, and examine with a dissecting microscope. Living helminths are quickly located by their movements and transferred to slides for fixation. Sediments from scrapings may be examined in small quantities with no or only a little additional liquid or fixed for later examination.

- E. Internally encysted parasites. Follow instructions in section B, 3 above (p. 9).

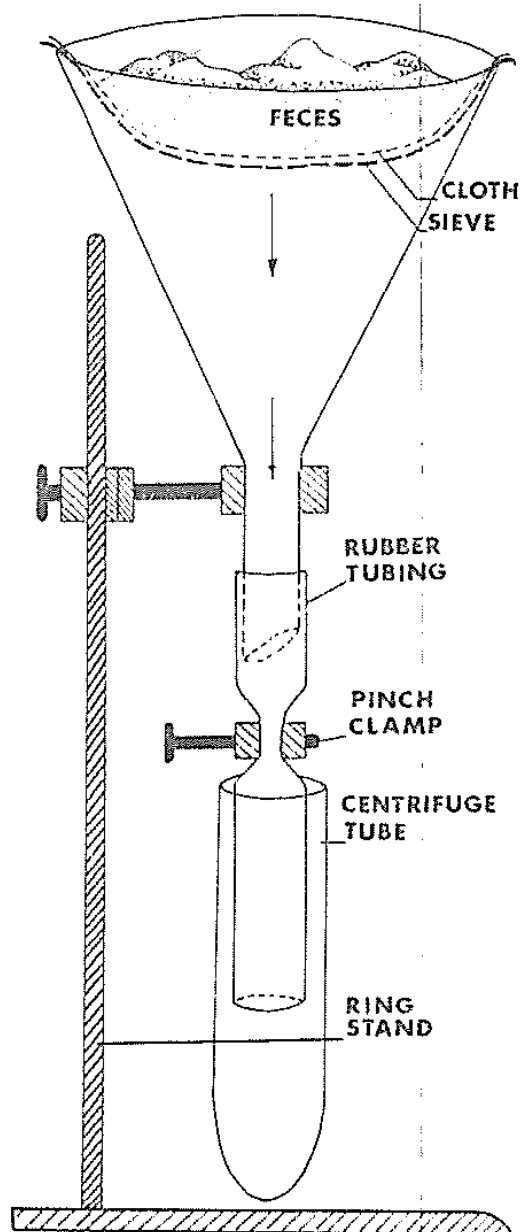
Note: After gut of small vertebrate has been scraped, view it villi down with dissecting microscope. Stroke surface with scalpel. Small cysts and larvae in wall can be seen as scalpel passes over them.

- F. Tumors. Excise the abnormality which may occur in any tissue and fix in 10% formalin for light microscopy or in 3% gluteraldehyde for TEM. If the tumor is large, make a crosscut penetrating about halfway to permit better penetration of fixative.

- G. Keeping parasites alive. It is often desirable to keep parasites alive. Von Brand (1952. Chemical Physiology of Endoparasitic Animals. Academic Press Inc., New York) and Taylor and Baker (1968. The Cultivation of Parasites In Vitro. Blackwell Scientific Publications, Oxford) have published some methods and survival times for in vitro studies.

h. Separation and concentration of living nematode larvae from soil or feces.

1. Assemble apparatus (Baermann) as shown in figure below. A funnel 15 to 25cm in diameter is usually satisfactory.
2. Pour lukewarm water (about 45°C) into the funnel until the water surface touches the bottom of the wire screen basket.
3. Place two layers of gauze on the wire screen basket to prevent filtering particles of soil debris from falling into centrifuge tube.
4. Place fecal or soil sample (well broken up) on gauze and cover with a piece of filter paper.
5. Let stand for 1 hour. The larvae will migrate to the warmer water and settle at the bottom of the funnel. A piece of ice placed on top of the filter paper hastens the migration of the larvae. The sample may be allowed to stand overnight.
6. Collect the larvae by opening the rubber tube clamp.
7. Centrifuge for 5 min. and remove supernatant fluid with pipette.
8. Examine sediment on a glass slide under a microscope with reduced light.



PRESERVING PARASITE SPECIMENS

In order to preserve animal tissue, one needs to fix it, that is, to alter the tissue constituents in a way which renders them no longer susceptible to autolysis or decay through microbial action.

The parasitologist balances quality, time, and conditions (in field camp, at sea, etc.) in choosing methods of killing and fixing. Often it is a question of when the time will be spent on the specimens.

- a. Relaxing, killing, and fixing. Improper killing and fixation of helminth parasites frequently render them practically useless for identification purposes. Worms have a tendency to contract, curl up, or otherwise become distorted prior to death.

If at all possible, one wants to kill the parasites in the most relaxed form and also to fix them at the same time. The secret lies in proper relaxation and fixation so that they are preserved in as life-like a posture as possible.

Heating and freezing are physical ways to relax specimens, but chemical methods are sometimes preferred for certain helminth groups.

Several methods of killing parasites may be used. A good killing and fixing agent penetrates rapidly and stops metabolic activities. Most agents penetrate faster when warm.

Fixation should make as permanent and as true a picture of the organism as possible. It is perhaps the most critical step in the preparation of helminths for study and identification. Fixation calls for experience and judgment on the part of the worker because decisions are made on an individual basis.

Remember: The fixative used will affect the staining and differentiation of the stain in the final preparation of the specimen. In general, fixatives which contain acids will allow for better staining later. We have found in our work that AFA (p. 119) is a good all-around fixative, useful for all helminth preparations. The properties of the solutions contained in AFA are well balanced to compensate for shrinkage or expansion of the tissue. For T.E.M. studies, see p. 86.

Specimens may sometimes be stored in fixative, but it is advisable to wash them in 70% EtOH after 4 to 48 hours fixation, and then to store them in fresh 70% EtOH.

More details about fixing agents are given under

(cont. p. 16)

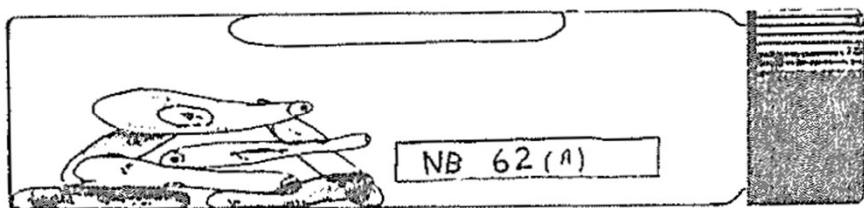
specific headings of parasites (p. 31 ff.). Formulae for fixatives are given on p. 117 ff.

Note: Should no fixative or preservative be available, most parasites can be killed and fixed in any local brew, such as schnapps, vodka, gin, rum, or whiskey. Even methylated spirit may be used to preserve a suddenly available specimen. Transfer parasites into 70% EtOH as soon as the latter is available after carefully pouring out the spirit.

- B. Collection data. No specimen has much scientific value if the collection data do not accompany it. An infallible method is to put the information on a label in the vial together with the specimen.

Labels for immersion in fixatives, alcohols, and other liquids should be written with soft pencil or waterproof ink (not India ink) on white bond (rag) paper.

Note: If the label is written with ink, the excess ink should be rinsed off before immersion in the liquid.



If this method is not feasible, an identifying number should be kept in the vial with the specimen and full records entered into a collecting book or card file.

Collecting records, either card file or collecting notebook with autopsy records (examples given below),

No. <i>NB-62</i>	Host: <i>CASTOR CANADENSIS</i>
Common name: <i>BEAVER</i> Sex: ♂ Size: <i>78cm</i>	
Locality: <i>BEAVER LAKE, PIONEERS PARK, LINCOLN, NEBR.</i> Date: <i>16-V-76</i>	
Coll: <i>G. KRUSE</i>	
Parasite: <i>STICHORCHIS SUBTRIQUETRUS (RUD. 184)</i>	
Type: <i>PIGENES (135+)</i>	
Location: <i>SMALL INTESTINE</i>	
Number: <i>135+</i>	Condition: <i>ALIVE</i>
Killed or preserved in: <i>AFA → 70% ETOH</i>	
Identified by: <i>G. O. W. KRUSE (22-IX-76)</i>	
Remarks: <i>81 SLIDES DEPOSITED IN HAROLD W. MANTER LAB,</i> <i>Coll. No. 21308</i>	

should include the identification of the host, the locality from which the host was collected (town, township, state, river, lake, or geographical coordinates and depth of trawl when collecting at sea), the number of parasites (record also negative findings!), their location within the host, and the date. If the collection is made for another party, the collector should be identified in the data.

AUTOPSY RECORD				
HOST:		HOST No _____		
Scientific name: _____	Date collected _____		Collector _____	
Common name: _____	Exact locality _____		_____	
Size, age, etc: _____	_____		_____	
Sex _____		Autopsy by: _____		
Health: _____		Date of autopsy _____		
Method of collection: _____		_____		
Remarks _____				
name of parasite	location in host	number found	parasite number	remarks

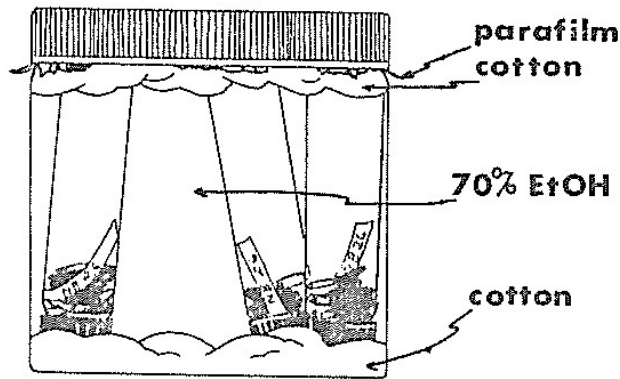
Packing specimens for transport. The safe transport and shipment of glass vials is sometimes a problem. Plastic ammunition boxes available at sporting goods stores are made in at least two sizes; one is ideal for half-dram vials, and the other accommodates three-dram vials. Each vial fits snugly in a plastic cell and needs no further packing as long as the boxes are handled with moderate care.

Another method is to place many small vials upright in a larger jar filled with the same preservative as used in the vials. Each vial has a plastic cap or plug (do not use cotton, because specimens may become entangled in fibers and lost), and layers of cotton should be placed in the bottom of the jar and on top of the tubes to hold them firmly in place.

Specimens are less likely to be damaged by shaking during transport if the vials are completely filled with fluid. As a precaution against evaporation, especially in hot climates, wax paper or Parafilm (American Can Company) may be stretched over the mouth of the vial before the cap is screwed in place. Parafilm is equally effective in sealing the large jar if the second method of packing is used.

Plastic electrical tape should be wrapped around each lid and upper part of jar to prevent the lid from unscrewing during transport.

- D. Storage of specimens in liquid. Many museums store "liquid specimens" in vials which are placed upside down into a larger container filled with the same preservative. Thus, if the preservative evaporates, the fluid in the vials will be the last to go.



The use of Parafilm beneath the caps of vials or jars and regular monitoring are the best means of keeping the specimens from drying out. Another way to avoid evaporation is to dip the capped end of vials into melted paraffin wax.

Caution: Extreme variation in temperatures may cause the paraffin to form "blisters" due to the expansion of air inside the vials.

An added safety measure is the use of 5% glycerol in 70% EtOH for long term preservation. Before specimens are moved through the alcohol series to be stained, they should be washed several times with fresh 70% EtOH to free them of any glycerol.

After washing, transfer the specimens to glacial acetic acid for 15 minutes. The acid removes some cytoplasmic elements, making the parasites more receptive to stain. Wash specimens again for 1 hour. Transfer then through the alcohol series depending on the stain used.

Staining procedure for Mayer's hematoxylin.
The time the specimen spends in each solution depends on its size and thickness.

Storage (70% EtOH)		
70% EtOH	15 to 30 min.	
50% EtOH	15 to 30 min.	
35% EtOH	15 to 30 min.	
Distilled water	15 to 30 min.	
Stain (diluted to 1/5 strength) ..	30 min.	
After specimens are stained (over-stained), transfer them to tap water for differentiation		
	5 min.	
Distilled water	5 min.	
35% EtOH	15 to 30 min.	
50% EtOH	15 to 30 min.	
70% EtOH	15 to 30 min.	
Destain (see below)		
Wash in 70% EtOH	15 min.	
85% EtOH	15 to 30 min.	
95% EtOH	15 to 30 min.	
100% EtOH	15 to 30 min.	
100% EtOH	15 to 30 min.	
Xylene (clearing see below)	10 to 15 min.	
Mounting specimen (see below)		

3. Destain if necessary in acid alcohol (p. 119) while observing specimens with a dissecting microscope. A compound microscope may be used for very small specimens. The cortical layer should be free of stain, but enough stain should remain to color the internal organs. It is often better to destain slightly more than the exact shade desired because there is a tendency for specimens to darken slightly during the clearing process. If the acid alcohol becomes quite pink before the specimen is the desired color, it should be changed.

The destaining process will often turn the specimen red. Stop destaining by washing well in 70% EtOH. The blue color of the hematoxylin stains can be restored by transferring the parasites to a dish with alkaline alcohol (p. 120) for a few minutes.

4. Dehydration is the process of replacing the water with an anhydrous solution, such as absolute alcohol. The usual steps of 35, 50, 70, 85, 95%, and absolute alcohol are generally used in dehydration, allowing about 30 minutes (or more if necessary) in each grade. (Replacing the alcohol

with water, the reverse of dehydration, is known as hydration.)

Note: Keep dishes covered to prevent contamination by dust, evaporation of solutions, and absorption of moisture from the atmosphere.

5. Clearing. After all traces of water have been removed from the specimen, it is next transferred to a clearing agent of choice.

The clearing agent renders the parasite transparent and is miscible with the mounting medium. Despite advantages of different clearing agents (see discussion on p. 121), xylene is the most widely used in laboratories, and its use is therefore described here.

Transfer specimens with a fine brush from absolute (100%) EtOH to xylene. Leave in clearing solution only until specimen is transparent (not more than 1 hour). Delicate objects may be transferred first to a mixture of 1/2 absolute alcohol and 1/2 xylene.

Note: Always use fresh xylene which has not collected moisture. Test old xylene with litmus paper before use. If it is acid, shake it with a little powdered CaCO_3 and filter.

- B. Mounting. Specimens are mounted in a solution of some resin which hardens as the solvent evaporates. There are a number of synthetic mounting media, but they should not be used for archival material. Canada balsam remains a proven resin which responds very little to changing environmental factors (humidity, temperature, etc.). It is neutral (pH 7) when two or three sodium bicarbonate tablets or marble chips are kept in the container. A drop or two of cedarwood oil in the container also improves the quality of the mount.

1. Equipment to have on hand when preparing to mount specimens:

Small hair brush to move specimens from clearing agent onto slide (cut out extra bristles to make brush of 4-10 hairs depending upon size of specimens to be moved)

Glass slides (pre-cleaned)

Cover glasses (no. 1), various sizes

(smaller cover glasses can be made by cutting regular sized ones with a diamond pencil)

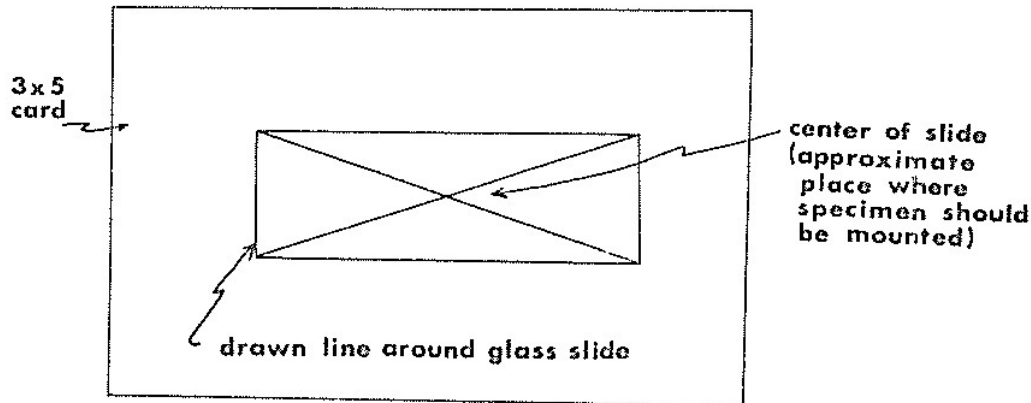
Forceps with angled tip

Container with mounting medium thinned enough to spread easily and glass rod for applying medium

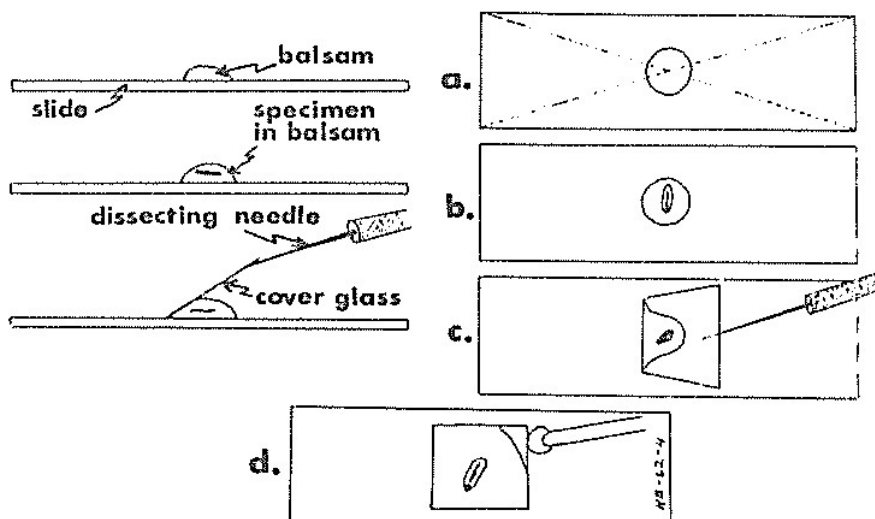
Diamond pencil to mark slides

Microscope

Prepare a white 3 x 5 card by drawing around a slide and joining opposite corners. The lines will intersect at the center of the slide, and that point can be used as a guide for centering specimens on the slide.



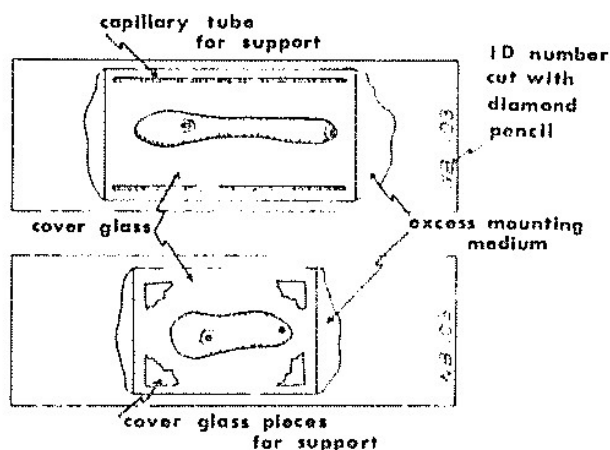
2. To mount specimens. Care must be exercised to place the specimen properly on the slide, to select a cover glass of proper size, to add the proper amount of mountant, to prevent the cover glass from tilting to one side, and to prevent the inclusion of air bubbles in the mount.
 - a. Place a drop of balsam at the center of the slide.
 - b. Lift the cleared specimen from the clearing agent with a fine brush and position it in the balsam (i.e., cover specimen with balsam) anterior end down (it will then appear right side up when viewed with a compound microscope). Add more mounting medium if necessary. Do not expose the specimen to the air.



- c. If necessary, inspect with microscope to assure a ventral mount (i.e., ventral side up).

Large or thick specimens may need small pieces of cover glass, slide, nylon string, or glass rods (like capillary tubes which have been drawn out over a flame and sorted by thickness) on either side to balance the cover glass.

Some workers recommend sheet plastic which can be cut with scissors in different thicknesses. Also pieces of cardboard which, though opaque, do work and also have the advantage of coming in different thicknesses. Use plenty of balsam so that there is an excess. When the preparation dries, this will be partially withdrawn.



- d. Add cover glass. Hold cover glass with forceps, touch mounting medium at one side, and carefully lower it over specimen. It often helps the spreading of the medium to dip the cover glass into clearing agent or xylene before applying it to the preparation. If needed, additional medium can be added from the side.

One should try to use the smallest cover glass that will adequately cover the specimen. Not only does this result in a more attractive mount, but it also means a saving of mountant, which has become quite expensive.

- (1) Air in specimens. If dull black spots are found in the freshly mounted specimen, the clearing solution has partly evaporated out of the parasite before the cover glass was in place. Return such individuals to the clearing agent, dissolve all mounting medium to allow the air to leave the specimens, and then remount.

Frequently, the thinner medium (clearing agent) is not replaced by the thicker (mounting) medium, and a vacuum results. The remedy which suggests itself is the use of thinner balsam. This works well with smaller specimens, but thick

material does not mount well in thin balsam. For these specimens we suggest clearing in creosote.

Air in specimens may also result from acetic acid in fixative or acid alcohol in differentiation reacting with calcareous corpuscles releasing CO_2 . This happens frequently in metacercariae, marine digenes and cestodes.

In case both methods suggested above fail, place the material to be mounted in very thin balsam in a dish covered with lens paper. Place the container in a warm place (45 to 50°C) and allow gradual evaporation to thicken the balsam. Specimens may then be mounted in balsam of the same density.

(2) Water in specimens. If the specimens appear dull, almost milky, water is present. Remove all mounting medium in clearing agent, and return parasite to absolute EtOH, preferably a fresh solution. Leave for one hour or more before clearing again and remounting.

- e. Add identifying number or letters to slide with diamond pencil.
- f. Harden preparation at room temperature, in a drying oven, or on a slide warmer not warmer than 50°C. Keep slides in level position until hardened to avoid a shift of specimen or cover glass.

A paper-clip spring (see p. 43) is useful whenever there is need to hold a cover glass in place as the medium hardens. Little weights (like bullets, not the cartridges) are useful to hold down the cover glass until the balsam is dry enough for the slide to be handled.

- g. When dry, remove excess medium with a razor blade.

If air bubbles become trapped in the medium of a freshly prepared slide, they will move to the margin when the slide is placed in a drying oven.

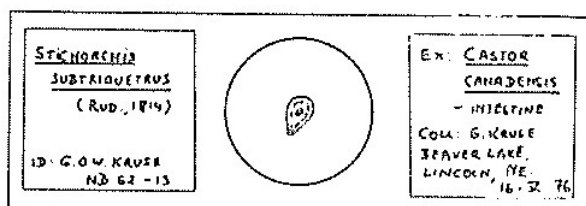
If the medium shrinks or was insufficient to fill the space under the cover glass, additional medium can be added from the side; it will spread by capillary action.

Note: Whole mounts do not develop their maximum transparency until several months after preparation because of the time required for the mountant to diffuse evenly through the specimen and to harden to an

increased refractive index with greater clarity.

- C. Labeling. One or two slide labels may be used as space permits. The information on them should be printed in India ink. If two labels are used, the left one contains parasite and locality data; the right one contains host data.

Caution: Always place the label on the same side of the slide as the cover glass.



- D. Care of preparations. Store preparations in a level horizontal position in slide boxes (to prevent drifting of the specimen) away from light and heat.
- E. Packing and mailing of specimens. If specimens are to be sent to other laboratories or to museums for deposit, it is essential that they be well packed. Good packaging protects not only the specimen in transit but also the personnel handling it in case of breakage.

A safe packaging procedure which complies with the Universal Postal Union Rules is as follows:

1. Specimens in liquid.

- a. Enclose specimens in a bottle or tube which can be well sealed (Parafilm or wax).
- b. Place glass container in suitable material for insulation and absorption.
- c. Pack in cardboard container and wrap for shipment. (If several tubes are packed together, they must be individually wrapped in soft paper or cloth to provide adequate cushioning between them.)
- d. Leakage frequently occurs, particularly when outside pressure decreases during air transport. Therefore, it must be ascertained that air- and fluid-tight closures are used.

2. Mounted specimens.

- a. Place slides with mounted specimens in slide mailer, slide holder, or slide box (depending on the number of slides sent). Wrap each slide individually to prevent it from shaking

(vibrating) within the box holder and to contain slide pieces should breakage occur.

d. Follow steps b and c above for packaging.

Note: Make sure that mounting medium has hardened enough before mailing slides.

- F. Deposition of type and voucher specimens. It is desirable that as much as possible of the following information should accompany the specimens deposited in any collection.

Harold W. Manter Laboratory		Collection No.
Division of Parasitology		
University of Nebraska State Museum		Storage No.
Parasite		
Host		
Body location		
Locality		
Collected by	Date	
Identified by	Date	
No. slides	Type designation	
Remarks		

1. Holotype specimens should be deposited immediately in a national depository. For the United States, this is the U.S. National Museum Helminthological Collection, which is housed at the Beltsville Agricultural Research Center. Pack vials and/or slides carefully and send to:

Animal Parasitology Institute
B.A.R.C. East, Building 1180
Beltsville, Maryland 20705
U.S.A.

2. Paratype and voucher specimens cited in primary literature should be deposited in a public collection either immediately or at some future time by prior arrangement. If sufficient material is available, specimens should be placed in established collections on different continents. Specimens should remain where they will be loaned, used, and cared for properly.

Our museum welcomes paratype and voucher specimens for deposit or entire collections which are in jeopardy. Send specimens or write to:

The Harold W. Manter Laboratory
Division of Parasitology
W-529 Nebraska Hall
University of Nebraska State Museum
Lincoln, Nebraska 68588-0514
U.S.A.

MICROSCOPIC EXAMINATION

The microscope is an essential tool for parasitologists. It is not only needed to find some parasites, but it is also used during the histological preparation of specimens and, finally, for morphological studies.

A. Microscope (compound)

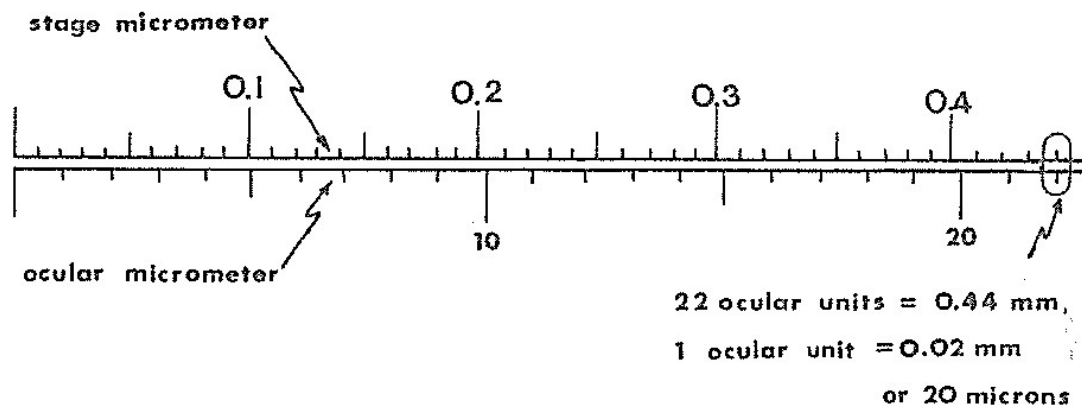
A good, clean, properly aligned microscope should be available in the laboratory. The instrument should be equipped with at least three objectives: low power--10X (16mm); high dry--44X (4mm); and oil immersion--97X (1.8mm); or another similar series of magnifications.

B. Calibration of ocular micrometer

To measure an organism accurately, a calibrated ocular micrometer is required. The units on the micrometer are arbitrary; the value of the divisions varies from lens to lens and must therefore be calculated for each combination of lenses. This is done by comparing the ocular micrometer units with a scale of known dimensions.

1. Place the stage micrometer (slide with calibrated scale of 0.1mm and 0.01mm divisions) on the stage and focus on some part of the scale.
2. Replace regular ocular with the one that contains the ocular micrometer.
3. Look through the scope and adjust the stage micrometer until the 0-line on the ocular micrometer is exactly superimposed upon the 0-line of the stage micrometer (see figure below).

Example of a Calibration



4. Without moving the stage micrometer, find a second set of lines which are also superimposed (the longer the distance between superimposed lines, the better).
5. Each of the large divisions of the stage micrometer equals 0.1mm. Determine the total distance (in mm) between the two points of superimposition on the

stage micrometer, and then determine the number of small units necessary to cover the same distance on the ocular micrometer.

Example: 22 ocular units = 44 stage units (=0.44mm)
1 ocular unit = .44mm/22 = 0.02mm or 20µm

Repeat for each objective.

Note: The calibration refers to a specific ocular micrometer and a specific objective (usually, this means a specific microscope). Many hours of calculations will be saved if the ocular micrometer is assigned to the microscope and kept with it.

Note: The preparation of a permanent chart for each objective showing values of 1 through 100 ocular units, also 200, 300, 400, and 500 units, permits a quick conversion from ocular units to micrometers.

6. Specimens, organs, and structures are measured by aligning the ocular micrometer along the axis of the object, counting the divisions in the ocular micrometer, and multiplying by the calculated factor for the objective (20 in the above example) or referring to the chart.

10x		1 unit = 20µm	
1. 20	26. 520	51. 1020	76. 1520
2. 40	27. 540	52. 1040	77. 1540
3. 60	28. 560	53. 1060	78. 1560
4. 80	29. 580	54. 1080	79. 1580
5. 100	30. 600	55. 1100	80. 1600

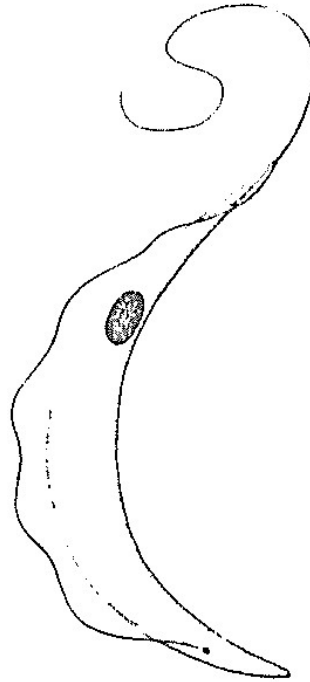
7. If the specimen or organ is longer (wider) than the ocular micrometer, choose a distinctive marker within the range of the ocular micrometer, record the units to that marker, move the specimen so that the marker is at the 0-line, and complete measurement. Repeat if necessary, following the median line of the specimen or organ if possible.
8. Often a sketch with a camera lucida projection helps to verify such measurements.

Note: Always project and draw the stage micrometer and label its value at the same time the specimen is drawn.

PROTOZOANS AND MESOZOANS

With a few exceptions parasitic protozoans are microscopic. They may be intra- or extracellular and may be found as trophozoites or cysts in the digestive system, the circulatory system, the muscles, or the skin of the host. Some protozoans are found on the body surface.

Mesozoans are microscopic, ciliated animals which parasitize the excretory or reproductive systems of marine invertebrates. The body is composed of two layers of cells. They are rarely encountered. To collect them, make tissue smears (p. 8) of the infected organs and treat like protozoans.



A. Intestinal protozoan forms. Fresh fecal samples should be examined within 24 hours (48 hours if tightly covered and refrigerated). Containers such as baby food jars will maintain moisture and prevent dehydration of the sample. Parafilm (p. 17) stretched across the top of the container aids against dehydration. Be sure host information accompanies the fecal sample.

1. Fecal samples and smears.

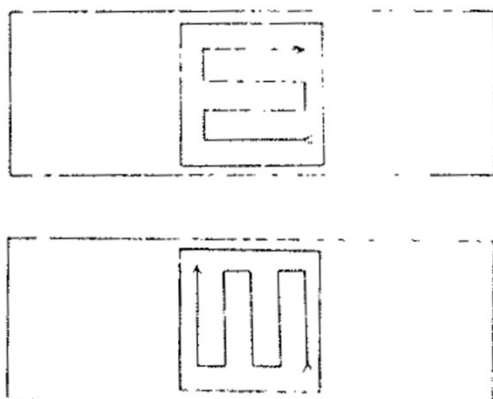
(Preparation of concentrations from fecal samples is described on p. 66.)

a. Direct wet smear technique. The first step in the microscopic examination of a fecal sample is the preparation of a direct smear (temporary mount).

(1) Unstained preparation. Smear a small portion of feces (bloody or mucous portion if present) on a clean microscope slide and mix it with a few drops of saline. Add a cover glass and examine with a compound microscope. The smear should be thin enough to read print through it, but should not be so thin that the material floats under the cover glass.

Note: Trophozoites are characteristic of loose stools; cysts of formed stools.

Examine preparation using low power and very low light intensity to find mobile amebas, flagellates, and ciliates. Specimens are very small, and the slide should be scanned carefully.



Begin at one corner, and read across to the opposite side of the cover glass; overlap the field, and again read across to the opposite side of the cover glass; repeat, going back and forth until all the material under the cover glass has been examined. To reduce a tendency to become dizzy, some people prefer to read the preparation vertically rather than horizontally.

- (2) Iodine stained. Place a drop of fecal material on a slide, add a drop of iodine-eosin (see p. 100), and mix; add cover glass. Everything will stain at once, and the cysts will be bright and colorless against a pink background. The cysts gradually become tinged with yellow, and the nuclei become dark brown. Examine preparation as above.

Warning: Iodine also stains starch, both within protozoan cysts and in the surrounding material. Large yeast cells may also have delayed staining.

- b. PVA-fixed specimens. An advantage of Burrow's polyvinyl alcohol (PVA), fixative is that organisms in fluid samples can be stained. The solution serves as an adhesive as well as a preservative.

- (1) Transfer part of fecal sample to a vial or bottle. Add no less than 2 parts and no more than 3 parts of PVA-fixative solution (see p. 128). It helps to make marks on the container before adding the specimen and fixative. *become stained*
- (2) Stir thoroughly with an applicator stick or stirring rod.
- (3) Replace stopper or cap and shake vigorously.
- (4) With an applicator stick, smear some of the material from this vial onto a microscope slide, avoiding smears that are too thick or

- too thin (i.e., be able to read print through smear).
- (5) Allow to dry for 18 to 24 hours.
 - (6) Follow steps 3 through 6 of procedure for Permanent mounts below.

- c. Formalin or PAF-fixed specimens. See p. 72 for procedure.
- d. KCB-fixed specimens. See p. 104 for procedure.
- e. MIF-fixed specimens. See p. 68 for procedure.
- f. Permanent mounts. Excellent results for permanent slides can be produced by using Schaudinn's fixative (p. 131) and an iron alum hematoxylin (p. 102) stain. Many workers feel that there is enough albuminous material in feces to insure adhesion of organisms to the slide. Using Mayer's albumen affixative (p. 126) will minimize the possibility of losing specimens during fixation and staining.

Procedure:

- (1) Make a thin smear of fecal sample on a clean glass slide.

Note: Never let the preparation dry until it is finished!

- (2) Have Schaudinn's fixative in a Coplin (staining) jar or petri dish; immerse slide for 20 minutes at room temperature or 2 minutes at 60°C;
- (3) Remove mercuric chloride by rinsing the slide in 70% EtOH and then placing it in iodine alcohol (p. 125) for 5 minutes.

Note: If iodine color fades, replace with fresh iodized alcohol.

- (4) Return slide to 70% EtOH. The preparation may be stored for a time in 70% EtOH.
- (5) Stain in iron alum hematoxylin (p. 102) or Wheatley's trichrome (p. 114).
- (6) Write with a diamond pencil the host number onto the slide.

Note: Smears can be made on cover glasses. Carry out all procedures in Columbia jars; they hold 10ml of solution.

B. Blood smears (see pp. 6-8).

C. Muscle, internal organs, and skin samples (see tissue smears, p. 8; cysts in and on skin, p. 9). If parasites cannot be collected by smear techniques, they should be teased from the host; or the host tissue

should be fixed with parasites in situ and sections made (see p. 86 or p. 88).

Note: Prepared blood smears may be left uncovered indefinitely. To study the parasites with oil-immersion lenses, the immersion oil is put directly onto the smear. To remove most of the oil, the slide is laid upside down onto a paper towel or tissue soaked in xylene. Do not rub dry! Some oil will remain on the specimen, but this causes no harm if slides are stored in slide boxes protected from dust.

For better protection, permanent slides may be made for study collections by adding a thin layer of Canada balsam and a cover glass.

See also: Fixation for EM, p. 86
Embedding and sectioning for
light microscopy, p. 88.

NOTES:

MONOGENEANS

Monoogeneans are found inside the mouth, on the gills and skin, in the gill chambers and cloaca of marine and fresh-water fishes, and in the urinary bladder of amphibians and, very rarely, of reptiles.

When possible, record the precise site of infection.

Note: Large monoogeneans are treated the same as digeneans; follow procedures for that group (p. 41).

A. Collecting, relaxing, killing, and fixing small monoogeneans.

1. Place small fish (or amphibian) or gills (gill arches should be separated from one another) and/or fins in a container of 1:4,000 formalin.

Or: Freeze the gills or entire fish for 6-24 hours to kill the worms in a relaxed position and to loosen the mucus which frequently clings to the worms. Place the gills or fish in a vial or jar of tap water for thawing, then shake the container vigorously for one minute.

Or: Isolated worms, body washings, gills and/or entire fish (or amphibian) are placed in a container 1/3 full of saline, tap or sea water, and shaken vigorously for several minutes. Then add (a) an equal volume of the same liquid with chloretone (1g in 250ml of water) or (b) a few drops of fixative (to make a 1:10,000 solution), and again shake vigorously for several minutes.

2. Following any procedure above, pour the liquid into a tall vessel, let stand for a few minutes to allow worms to settle, and then remove extraneous particles by decanting the supernatant fluid. Examine the sediment by placing small amounts in a petri dish and scanning under a dissecting microscope. Remove specimens with a pipette and drop into AFA.

B. Storing. These worms are so small that they may be transferred from the fixative to 70% EtOH in an hour or two. Include collection number.



- C. Mounting and clearing small monogeneans. Small monogeneans are usually mounted unstained in glycerol jelly (p. 124), which also clears them.

Equipment needed:

Water bath on a hot plate at 45°C
Glycerol jelly with applicator stick
Glass slides
Cover glass (round) of two different sizes
(e.g., 18mm and 22mm)
Razor blade
Jar with Canada balsam (for double slide technique)

1. Place vial of glycerol jelly in warm water bath until the jelly is liquefied.

Note: Excessive heat will destroy the jelly by changing it to an irreversible colloid which will not harden.

2. Put one drop of liquid jelly on glass slide, position specimen, and add a round cover glass.
3. Let preparation cool and harden. Remove excess jelly with razor blade.
4. Warming the slide on a hot plate permits removal of cover glass and/or rotation of the specimen, but small monogeneans are so fragile that this procedure is not recommended.

Note: When mounting in glycerol or other aqueous media, transfer specimens from water (or 70% EtOH), not from absolute alcohol!

Note: Glycerol media have a very high osmotic pressure which may cause collapse and shrinkage even in fixed specimens. Transfer delicate objects via intermediate dilutions of glycerol (as alcohol evaporates, add glycerol to bring back to original volume, allowing several hours or days in each dilution)!

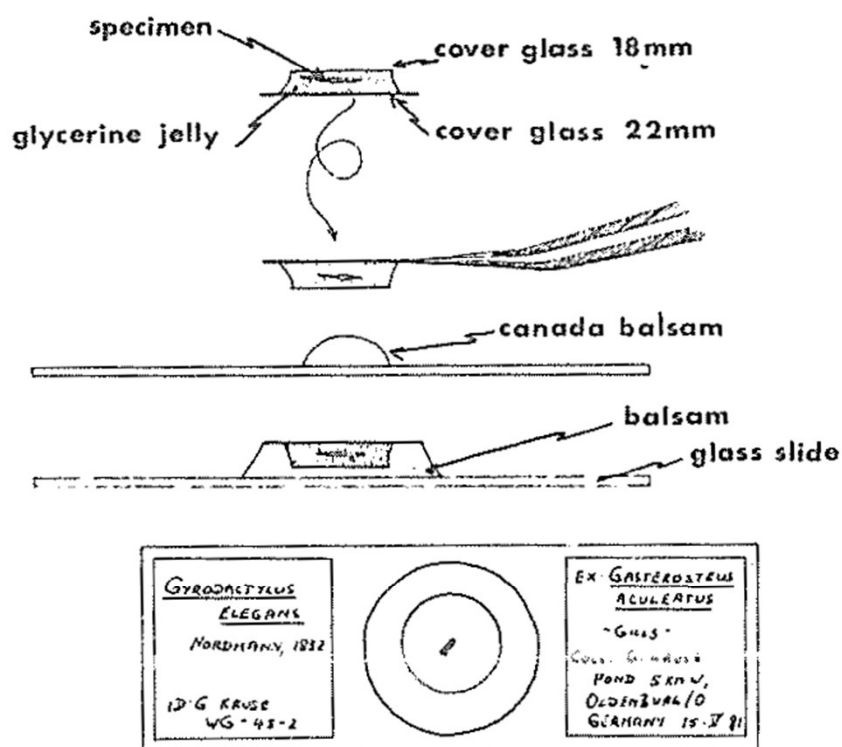
Note: If mounts in glycerol jelly are not stored in a cool dark place, they may become fluid and run off the slide!

5. Permanent mounts of the preparations using the above technique are simply made by ringing the cover glass with a double layer of clear fingernail polish. Another method is:

Double cover glass mount.

- a. Place a larger cover glass (22mm) on the edge of a warming plate.

- b. Add a drop of the melted jelly in the center of the cover glass and orient the specimen within the jelly (ventral side down because the specimen will later be viewed through the large cover glass).
- c. Slowly lower the second, smaller cover glass (18mm) onto the preparation.



- d. Let the assembly cool and the jelly set at room temperature overnight.
- e. Scrape off excess jelly with a razor blade.
- f. Put a drop of balsam in the center of a glass slide.
- g. Turn the double cover glass assembly over so that the larger glass is on top, and then lower it onto the drop of balsam. The balsam will spread under the small cover glass and extend to the edge of the larger glass, forming an effective and permanent seal (see figure above).

Note: Such preparations must be dried at room temperature for several weeks. Putting them into a drying oven would melt the jelly.

See also: Fixation for EM, p. 86
Embedding and sectioning for
light microscopy, p. 88.

DIGENEANS AND ASPIDOGASTREANS

Developmental stages of digeneans (sporocysts, rediae, and cercariae) are found in molluscs. Metacercariae are common in tissues of many invertebrates and vertebrates. Infected tissue may be fed to chicks or ducklings, and after a period of 1 to 3 weeks the adult trematodes can sometimes be recovered from the intestine of these "foreign" hosts.

Adult digeneans are most commonly found in the intestine of vertebrates, although many organs may be infected.

Aspidogastreans are parasites of fish, turtles, molluscs, and crustaceans. Various organs of the hosts are infected. They should be treated the same as the digeneans.



- A. Relaxing, killing, and fixing adults. Other things being equal, the quality of the fixation will determine the quality of the final preparation. Trematodes in general are very delicate. They have to be fixed soon after they are found in order to prevent maceration. For best results, fix them while they are still alive.

Frequently trematodes are so packed with eggs that internal structures are obscured. The worms can usually be made to discharge some of the eggs by putting them into distilled water for a few minutes. If they do not discharge some eggs in 15 minutes, then add glacial acetic acid drop by drop.

Large trematodes can be relaxed in ice water or cooled in the refrigerator, or they can be anesthetized with menthol (sprinkle a few crystals in dish holding worms in saline) for 1/2 hour. The most widely used fixative for this group is AFA (see p. 119).

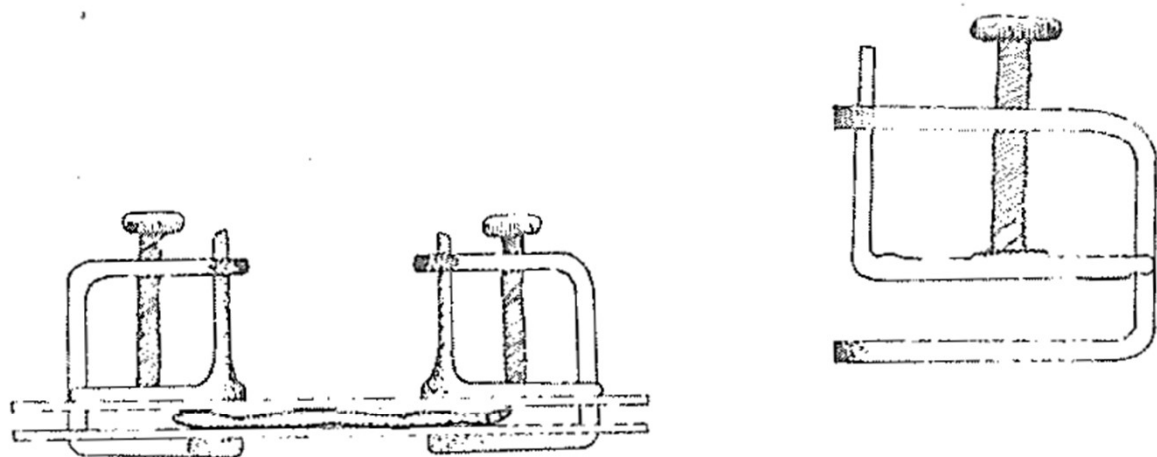
Procedure for flattening worms while fixing.

1. Using a brush or pipette, transfer a specimen to a slide with a few drops of saline.
2. Position specimen dorsoventrally.
3. Add a cover glass over the fluke.
4. Draw off excess saline with a paper towel (absorbent material) on one side while adding fixative on the opposite.
5. Apply enough pressure on the cover glass so that the specimen is straightened to approximately its average degree of extension. Use an easily controlled tool such as a flexible dissecting needle. Observe with a hand lens or dissecting

microscope while applying pressure. Do not crush!
Continue pressure until the specimen becomes opaque.

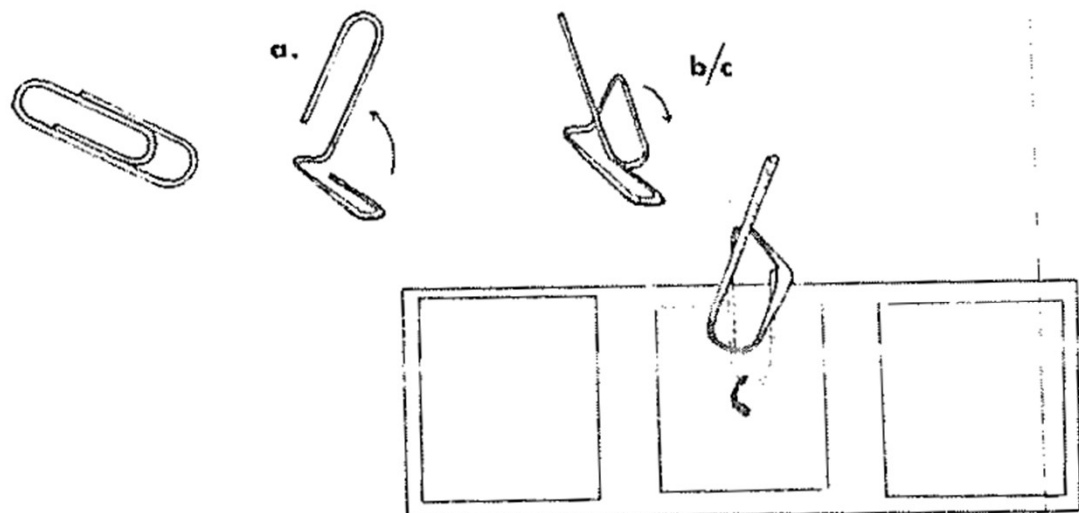
Note: Many workers have given up "flattening" specimens because the internal structures become distorted and can not readily be used in comparing measurements. It should be pointed out here that when we use the term "flattening," we do not imply making a "pancake image" of the trematode, but rather straightening it to its "normal" shape. Small worms rarely need flattening, and all other specimens should be fixed under slight pressure.

Since worms vary much in size and muscularity, the amount of pressure varies proportionally. Often the weight of half of a cover glass will flatten small specimens (less than 3mm) as the liquid is withdrawn. On the other hand, some worms are so muscular that one cannot flatten them with pressure applied to a cover glass. In such cases, one can use another glass slide. In extreme cases, for example the hirudinellids, a worm is put between two glass slides, and two adjustable clamps (see figure below) are applied to assure equalized pressure.



The entire preparation is then placed in a beaker of fixative for 24 hours. Since most of the body surface is covered by glass and penetration occurs only on the free surfaces, the worm should stay in the fixative another 24 hours after the clamps and glass slides have been removed to assure total penetration.

Another simple technique to flatten specimens under a cover-glass is to use a spring made of a paper clip. Bend the clip as shown in the diagram below:



- a. Pull the two arms of a paper clip apart to an angle of approximately 60 degrees.
- b. Bend the longer arm near the middle so that the rounded ends of the two arms meet.
- c. Adjust the amount of pressure exerted.
- d. The wire tip extending upward may be used as a handle.

Caution: If compressed specimens are not immersed in fixative, take care that they do not dry out on the slide!

After flattening, remove specimens to a vial of AFA for further fixation. Replace fixative with 70% EtOH after 24 to 48 hours.

Some workers prefer to kill and fix specimens without pressure in a hot (not boiling) liquid. The helminths are transferred from the organ parts to stender dishes of fresh saline and killed by adding hot water. A few seconds later the worms are transferred to a vial of cold fixative. This method is reported to be equally suitable for trematodes and cestodes. A variation of this method is to heat-treat entire organs by pouring boiling water over them as they are separated in small beakers; then the organs are placed into fixative for fixation and relatively short-term storage until examined.

The quickest and simplest of all methods would be to kill and fix the parasites in situ by preserving the slit organs in cold 10% formalin. (The volume of the fixative should be at least 9 times the volume of the tissue.)

Note: Fixation of parasites in situ usually results in tangled, contracted, and mucus-covered worms that make poor specimens later. This method should not be used unless you want to demonstrate what the parasite looks like in situ, if sectioned material is to be made from it anyway, or if other, more desirable methods, cannot be used.

The parasitologist balances quality, time, and conditions (in a field camp, at sea, etc.) in choosing methods of killing and fixing. Often it is a question of when and where the time will be spent on the specimens.

- B. Killing and fixing larval trematodes and fragile adults. Pipette worms into a vial of hot water or AFA (about 50° C). Swirl until worms relax and die. Fix in cold AFA and store in 70% EtOH.
- C. Excysting living larval trematodes. Trematode cysts can be teased apart with dissecting needles, or the larvae (metacercariae) or progenetic adults may be excysted chemically. Often it is enough to drop the cysts in 0.6% NaCl solution and allow to stand at room temperature until the larvae excyst.

Another method for excysting is:

1. Drop cysts into digestive solution for 30 minutes:
HCl (0.3%) 10 parts
pepsin (0.5%) 4 parts
2. Wash in saline (according to type of host).
3. Transfer to solution of 0.5% trypsin in 0.85% NaCl(K₂HPO₄), pH 7.8, for 10 minutes.
4. Wash in saline.
5. Add 1:20,000 streptomycin to saline.

Excysted worms may be killed and fixed or may be used for in vivo studies (see vital staining, p. 112).

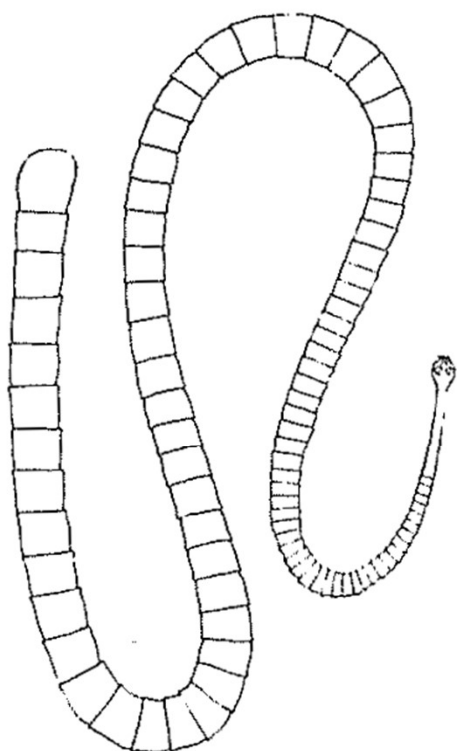
- D. Storing. Store in 70% EtOH with collecting number.
- E. Staining. Mayer's hematoxylin and Mayer's carmalum are good stains for trematodes. Follow general procedure described p. 19. Another beautiful stain, using Malzacher's technique (p. 106), differentiates the various organs sharply.
- F. Mounting. Follow general procedure described p. 23.

See also: Fixation for EM, p. 86.
Embedding and sectioning for
light microscopy, p. 88.

CESTODES

Adult tapeworms are common in the intestine of most vertebrates. Proceroid larvae are normally found in the hemocoel of crustaceans. Plerocercoid larvae (spargana) are solid larvae found commonly in fish and snake muscle, coelom, liver, etc. Cysticeroid larvae are usually encysted in the hemocoel of crustaceans, insects, millipedes, oligochaetes, and other invertebrates, but less commonly in vertebrates. Cysticercus larvae (bladder worms) occur in various organs of reptiles and mammals, but seldom birds.

Caution: Be careful in working with cestode larvae since several species can infect directly.



Cestodes are the hardest of the platyhelminths. Collecting and relaxing may be done somewhat more leisurely, but gentle treatment is necessary. The neck region and strobilae may break.

- A. Collecting and relaxing. Care must be taken to recover the scolex of the tapeworm. Often scraping deeply with a blunt scalpel will free the mucosa in which the scolex is embedded. In other cases, excise a small portion of intestinal wall in which the scolex is embedded, and chill along with the worm in saline. This may induce the worm to loosen its hold, or the worm may do so spontaneously if left for a time at room temperature. Very carefully tease the host tissue from the point of insertion and allow the scolex to float free.

Cestodes tend to contract and twist unless they are relaxed before fixative is added. Chilling the worms, either in saline in the refrigerator or in ice water for several hours, relaxes them with the least handling. Another method is to place them into 5 to 10% EtOH at room temperature.

Medium-sized to large cestodes may be swirled with a blunt instrument (like glass rod) in a dish of warm water or diluted fixative. The worm will lengthen noticeably as it relaxes. Cut the larger tapeworms into workable-size sections before swirling them in the warmed fixative.

- B. Killing and fixing. Common fixatives are AFA or Bouin's fluid. After the tapeworms are relaxed:
1. Small cestodes may be handled like digenetic trematodes, taking care to keep scolex and strobila together (attached).

2. Medium-sized cestodes (up to 30cm). Beginning with the large end of the worm, draw it onto a glass plate along with some fluid. Cover with glass slides, and pipette fixative along the edges of the slides. The purpose is to obtain flattened specimens, so take care not to "float" the slides. Allow to stand 30-45 minutes, but do not permit the specimens to dry. Remove slides, cut strobilae into 3-4cm lengths, and transfer to a dish of fixative. Place each specimen in a separate dish.
3. Large specimens may be wound around a bottle or beaker. Be sure that no parts overlap! Pour hot fixative quickly over entire specimen. Unwind, cut into 3-4cm lengths, and transfer to a dish of fixative for several hours or overnight.

Note: The vapor from heated AFA (evaporating alcohol) is highly flammable.

Medium-sized and large cestodes may also be killed and uncoiled by dropping them into hot water (about 75°C). Fix afterwards in Bouin's fluid or AFA.

Alternatively, spread them on long glass plates which are covered with water-moistened filterpaper (paper towels). Carefully straighten the worms with a soft brush and cover with a second glass plate. Pour fixative onto paper extending over the margins of the glass plates. Fix afterwards in AFA.

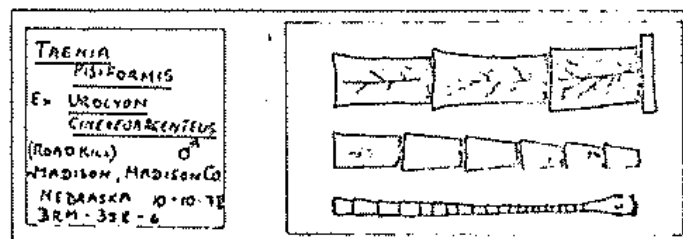
4. Larval stages. Proceed as for larval or small digenetic trematodes (p. 44).
5. Artificial evagination of cysts. The viable cysts are carefully removed from the host and placed in saline solution. Each cysticercus should be liberated from its cyst wall, if necessary (see p. 78), and placed in a warm (37-38°C) bile-salts solution (p. 120). Invaginated specimens, upon being put into the salt solution, generally show movement within 20 seconds. Then proceed as for small digeneans (p. 44).

When collecting conditions do not favor prompt recovery and treatment of tapeworms, the entire gut and its contents may be preserved in 10% formalin. This method often produces twisted or coiled specimens, which should be treated as described on p. 80.

Another method of fixing cestodes in situ is described under Heat treatment p. 5.

Storing. Transfer to vials or bottles of 70% EtOH, keeping sections as straight as possible. Remember to include a specimen number!

- D. Staining. Stain as described in general procedures, p. 19. Often carmine stains (like Mayer's carmalum, p. 108, or Semichon's aceto-carmin, p. 110) are used for cestodes. Another staining method is Malzacher's technique (p. 106) which stains tapeworms well by differentiating the various organs sharply.
- E. Mounting. Apply moderately viscous balsam to the right 2/3 of a glass slide in parallel rows (see figure below). Position the lengths of the worm along the rows of balsam. Dip the cover glass in xylene or clearing agent, touch the corner to a paper towel to remove excess fluid, and carefully affix the cover glass by beginning at one end and slowly lowering it over the balsam and specimens. There should be enough balsam on the slide to fill the spaces between the pieces of worm. If specimens are particularly thick, narrow pieces of slide may be placed between the specimens to support the cover glass. Additional balsam may be added from the edge.



Some compression may be required when mounting worms until the balsam is dry enough to hold the cover glass in place. Spring clamps (see p. 43) may be used, but take care that mountant does not harden around clamps.

- F. Demonstration of cestode hooks. Berlese's medium (see p. 120) is an ideal mountant to demonstrate rostellar hooks. This solution clears the surrounding tissue without altering the size of the hooks as may happen with glycerol.

Mount scolex in Berlese's medium and ring preparation (see p. 76) or make a double cover glass mount (see p. 38).

See also: Fixation for EM, p. 86.
Embedding and sectioning for
light microscopy, p. 88.

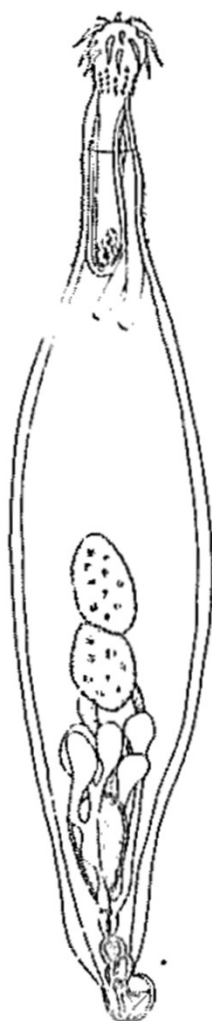
ACANTHOCEPHALANS

Adult acanthocephalans are normally found only in the intestine of vertebrate hosts. The thorny proboscis is embedded in the gut wall.

Some of the major taxonomic characters of thorny-headed worms are on the proboscis; therefore, the proboscis should be fully extended.

Note: Cystacanths should be treated as adults. Other larval stages may be prepared as Digenea, but are not excysted.

- A. Collecting. Use great care in removing acanthocephalans from the host so that the hooks of the proboscis are preserved intact and in place. The safest procedure to collect the adult worms is to cut out a small square of the intestinal wall containing the embedded proboscis, then tease away the host tissue. Remove as much host tissue as possible from the hooks as the worm is being freed from the host's intestine. Do not puncture the living worm. Tease tissue apart with dissecting needles around the attached proboscis.
- B. Killing worm and extending proboscis. Place worm in cold or room-temperature distilled or tap water and leave it there as long as it lives. This may require up to 48 hours. Larger worms should be refrigerated. Monitor with dissecting microscope. The water will move into the worm by osmosis, and the increased internal pressure will evert the proboscis.
- C. Fixing. Leave the parasite in fixative (AFA) about 48 hours before it is transferred to EtOH for storage. The body wall should then be punctured 3-4 times with an insect pin near the midbody and at the base of the proboscis to allow exchange of fluids. Leave in fixative about 48 hours.
- D. Storing. Store in 70% EtOH. Include collecting data!
- E. Staining.
1. Small specimens should be stained with Mayer's carmalum or Mayer's hematoxylin (general procedure, p. 19).
 2. Large specimens (such as Macracanthorhynchus) which cannot be mounted on glass slides are not stained. They are cleared and stored in glycerol.



- F. Cleairing. Additional caution should be taken when going from absolute alcohol to xylene because fragile specimens may collapse. This can be avoided by placing clearing fluid under absolute EtOH.

First, pour in enough clearing agent to half fill the dish. Then enough absolute alcohol is carefully added with a pipette to fill the dish. Add the specimen to be cleared. It will first float at the interface of the two fluids; the exchange of the liquids occurs gradually. The object sinks down into the lower layer.

When the specimen has sunk, the alcohol is pipetted off and the clearing agent replaced with fresh solution before mounting.

- G. Mounting. Follow general procedure, p. 23.

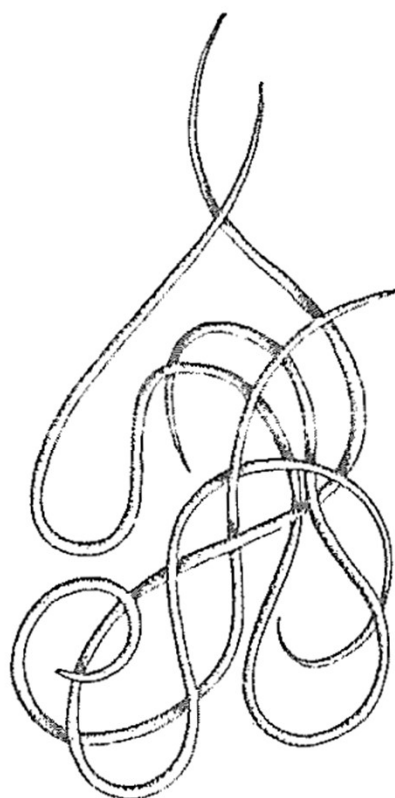
See also: Fixation for EM, p. 86.
Embedding and sectioning for
light microscopy, p. 88.

NOTES:

NEMATODES

The phylum Nematoda is the largest and most diverse of the helminth phyla. Parasitic nematodes may be found free or encysted in any tissue or organ system of any host. The lack of circular muscles gives the worms a characteristic whipping movement.

When dropped into formalin (or other fixatives that cause shrinkage), the cuticle tends to make the worms coil.



- A. Collecting. At the time of collection, it is often difficult to determine whether specimens are adults or juveniles, but techniques are the same for both. Nematodes are often free in the lumen of organ systems and are easily removed with pipette or forceps. Those in tissues can be teased out or fixed in situ and studied as serial sections.

For collection of soil stages, see description, p. 13.

Microfilariae in the blood are collected in thick blood smears and treated like the blood protozoans (see p. 7).

- B. Relaxing. Most nematodes are relatively extended when found and will remain that way. Those that are loosely coiled may be extended by swirling them for a few minutes in cold glacial acetic acid. Small nematodes can be relaxed in saline or water on a glass slide by flash heating them over a flame. Larger specimens can be dropped into steaming (not boiling) 0.5% acetic acid and left until they become uncoiled.
- C. Killing and fixing. To obtain good study specimens, nematodes should be killed as straight as possible. Use near boiling 70% EtOH (no open flame!) or Looss's fluid; However, hot alcohol is rarely available during field work. A simple alternative method is to use cold acetic acid to kill and fix nematodes; it also clears quickly and may be used as a preservative while in the field. Transfer specimens to 70% EtOH after a few minutes. Nematodes may also be killed and fixed in any of the fixatives used for other helminths.

Note: The nematode cuticle is comparatively impermeable. If nematodes are to be used for

histological studies, cut off the anterior and posterior parts while the worm is immersed in the fixing fluid.

D. Clearing.

1. Place specimen in a mixture of half 70% EtOH and half glycerol. Gradually add more glycerol to unstoppered vials or bottles as the EtOH evaporates. The specimens will slowly clear in this solution as the percentage of the glycerol increases. If evaporation proceeds too fast, add more EtOH to keep the worms from collapsing.
2. Specimens may be cleared quickly by placing them in phenol alcohol [absolute alcohol (1 part) and melted phenol crystals (4 parts)]. This solution is useful for clearing specimens quickly; it works equally well from either water or alcohol mixtures. It is especially useful for observation of cuticularized structures such as spicules. The solution will crystallize at low temperatures, but this can be prevented by the addition of glycerol to the solution (5% of volume). The addition of glycerol also permits temporary mounts to be kept for study over several days if additional solution is added periodically at the edge of the cover glass. The specimen should be washed in 70% alcohol to remove phenol prior to storage.

Note: There are three important disadvantages to using phenol alcohol for clearing nematodes:

- (a) The solution darkens if exposed to light. Therefore, it must be stored in a brown bottle, and the specimens in temporary mounts must be kept in darkened boxes when not being studied.
 - (b) Because the clearing process damages some delicate tissue, such as the intestinal cells of some nematodes and causes the cuticle to swell, nematodes that have been cleared in phenol alcohol are not good specimens for sectioning and staining.
 - (c) Phenol alcohol is unpleasant to work with. It will burn exposed skin if not washed off immediately.
3. PVA-lactophenol is a quick clearing agent. The nematodes can be dropped from water, saline, or alcohol directly into this agent, where they clear. The clearing can be speeded up if the preparation is placed on a slide warmer for an hour or so.

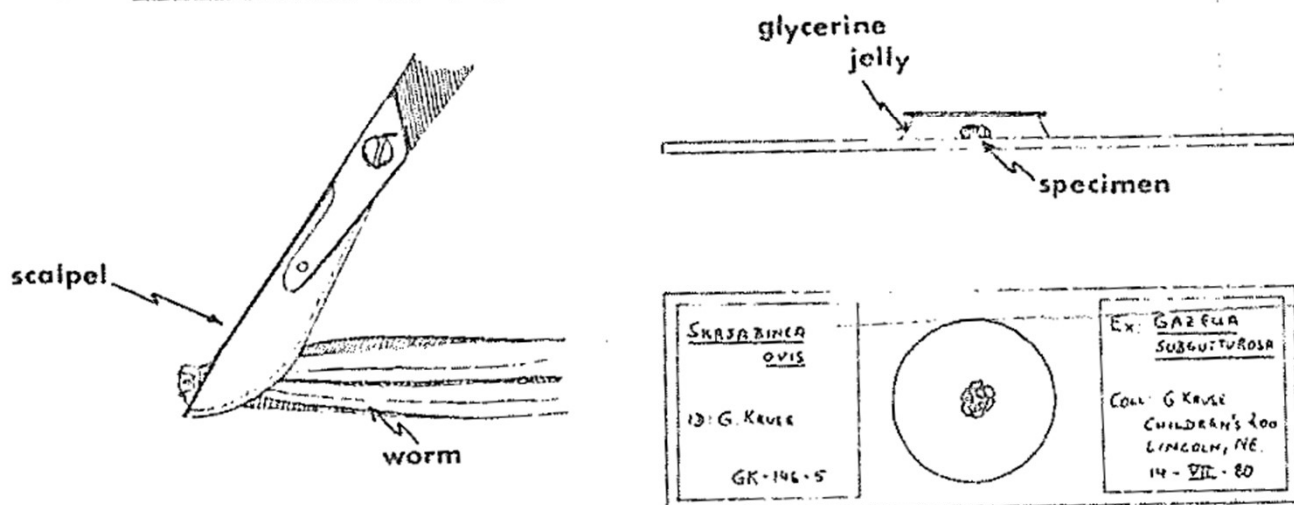
E. Storing. Specimens may be stored in a mixture of a little EtOH and glycerol after being cleared. The alcohol will prevent fungal or bacterial growth which may destroy the specimen. Include collection number!

F. Staining. Generally, nematodes are not stained, but there are a few methods to stain certain structures.

1. Some small nematodes may be stained by placing the living worm in a drop or two of egg albumin on a slide to which one of the vital stains (e.g., neutral red) has been added.
2. The addition of Giemsa stain to the PVA-lactophenol method will give color to nematode structures and improve optical properties of the preparation. The amount of stain added should be determined by personal experimentation.
3. Spicule and gubernaculum stain. After fixation in Bouin's (or overnight in picric acid/distilled water if fixative is unknown):
 - a. Place nematodes in saturated acid fuchsin-lactophenol solution and heat on a hot plate.
 - b. Rinse with 70% EtOH.
 - c. Destain specimens in saturated picric acid-distilled water solution until no more acid fuchsin leaves the specimen.
 - d. Rinse in 70% EtOH.
 - e. Remove picric acid stain with saturated sodium bicarbonate-70% EtOH solution.
 - f. Clear as described above and mount as described below.

G. Mounting. Nematodes are usually not mounted permanently. Smaller specimens may be mounted in glycerol jelly (see pp. 38 and 124). Larger specimens are kept in glycerol and are removed to a slide with some glycerol when studied. They must remain free to be turned in various positions.

H. Preparation of "en face" view. For study of cephalic



structures, which are important in the taxonomic study of nematodes, one has to cut off the anterior end and mount it under a cover glass, orienting it so that the mouth opening is upwards.

Cut the anterior end with a very fine and sharp razor blade not farther back than one body width. Treat this piece by normal procedure for nematodes and mount it in glycerol jelly (see pp. 38 and 124).

See also: Fixation for EM, p. 86.
Embedding and sectioning for
light microscopy, p. 88.

NOTES:

LEECHES

Small leeches can be treated like digenetic trematodes. Large leeches must be relaxed before they are killed!

A. Relaxing. Leeches are very muscular and must be completely relaxed before attempting to kill and fix them. A recommended method is:

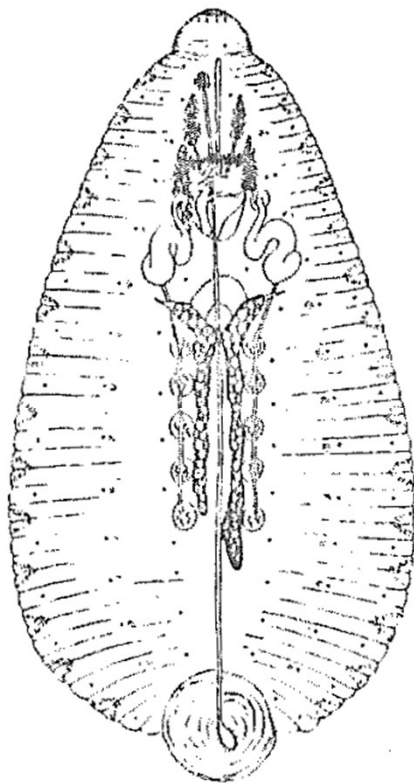
1. Place leech or leeches in small dish of saline or water.
2. Float menthol crystals on surface of liquid.
3. Allow to stand until the leeches are completely limp and do not respond to stimulus.
4. Allow to stand another hour longer than you think necessary! Leeches are notorious for rolling up into neat little balls after fixative has been added to "completely relaxed specimens."
5. Remove liquid and menthol crystals.

B. Killing and fixing.

1. Add fixative as for digenetic trematodes, p. 41.
2. Flatten, if necessary, as for digenetic trematodes, pp. 41-43.

C. Storing. Store in 70% EtOH, include collecting number.

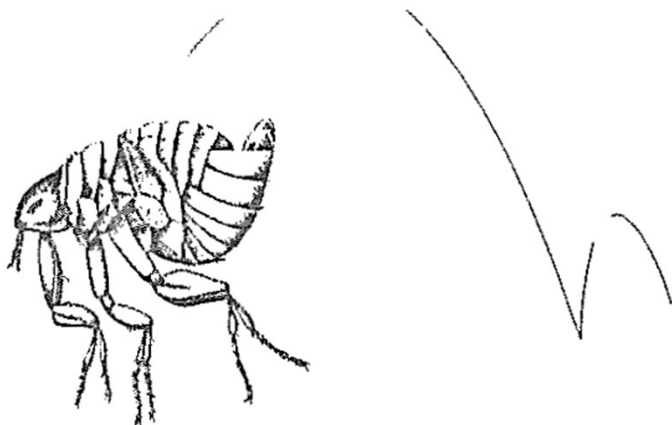
D. Staining and mounting. Follow general procedures, p. 19.



See also: Fixation for EM, p. 86.
Embedding and sectioning for
light microscopy, p. 88.

ARTHROPODS

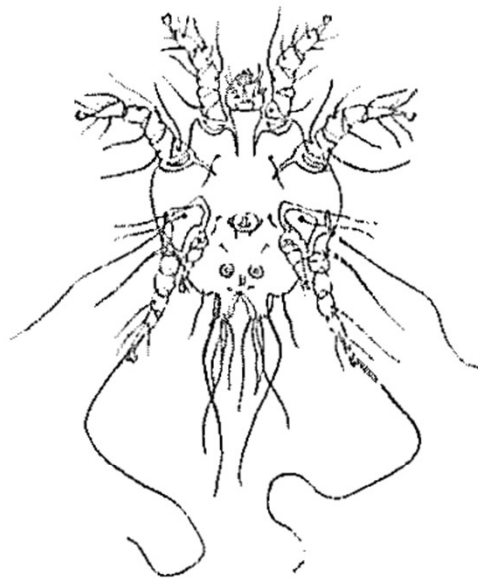
The phylum Arthropoda includes several groups of parasites, most of which are external. They come in many sizes, and some may be lost because they are not visible to the naked eye. A few, such as the pentastomes in the respiratory tracts of vertebrates, are internal. Since most identifying structures are external, these parasites should be handled carefully.



A. Collecting.

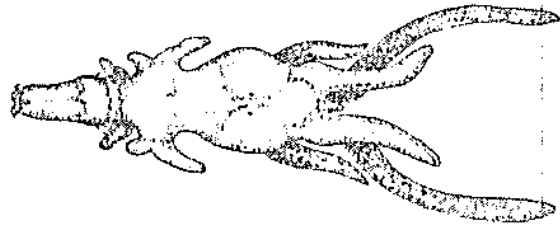
1. Fleas and lice. The simplest method is to suspend a freshly killed animal over a funnel large enough to concentrate whatever drops from the body. In time, depending on ambient temperature and humidity, the fleas and lice will leave the host as the body temperature drops. If this method is not convenient, place the freshly killed host in a plastic bag (see p. 8).

2. Mites. Scrub fur or feathers or scrape skin (see p. 8). Feather mites do not leave the host and may be carefully combed from the feathers of stuffed and mounted birds or from study skins many years after their preparation. Burrowing mites are removed by skin digestion (see p. 78).

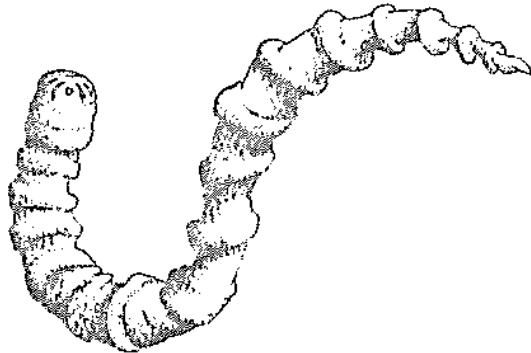


3. Ticks. Ticks are usually embedded in the skin. If pulled from a living or freshly killed host, some or all of the diagnostic mouth parts may remain in the skin. A drop of EtOH or other irritant chemical (such as oil, glycerol, or diluted fixative) placed directly on the tick will cause it to release its hold. If the host is dead, use fine-pointed forceps to grasp the skin beside the point of penetration and free the tick, or remove the portion of skin to which the tick is attached and fix.

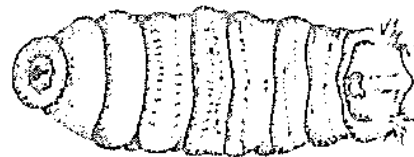
4. Copepods and isopods are frequently found attached to the skin and gills of fishes. If easily detached, drop individuals into a small vial of 70% EtOH. If not easily detached, cut out a small piece of tissue containing the parasite and carefully remove the tissue from the parasite before preserving. It is also possible to preserve the parasite with the tissue. The host tissue is more easily removed this way.



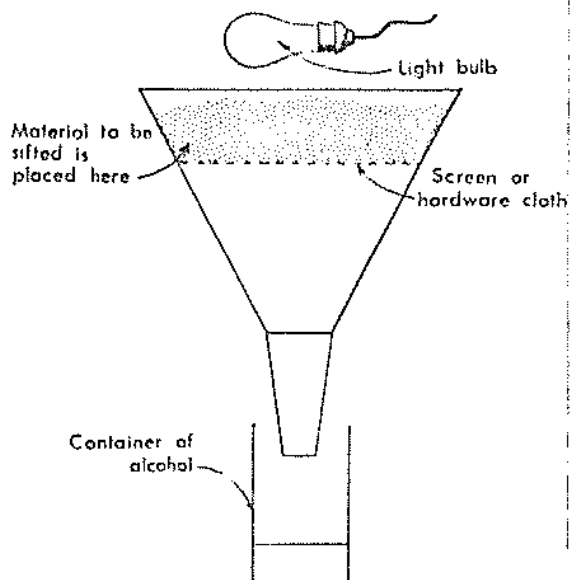
5. Pentastomes. The hooks in the mouth region are embedded in the tissue of the respiratory system. Remove from point of attachment as for ticks (see above).



6. Parasitic insect larvae. Bot fly larvae may be found embedded in the skin or in the tissues surrounding the esophagus. Their mouth hooks and/or posterior spiracles are of taxonomic importance.



7. Bedding and bird nests. A funnel (perlese) may be used to collect fleas, lice, and mites from bedding of mammal burrows and bird nests. Place material in the funnel. Put an electric light above it, and collect the arthropods from the small end of funnel where they have been driven by the heat.



- B. Killing. If the arthropods are not dead when collected, place them directly in 70% alcohol (unless they are holometabolous larvae which must be killed in boiling water or Peterson's solution, see p. 127).
- C. Clearing. Specimens (except pentastomes and mites) are transferred directly from 70% alcohol to 10% KOH and left until internal tissue is dissolved and the exoskeleton becomes relatively transparent (from 12 to 48 hours depending on size and color of specimens). Larger specimens should be punctured with an insect pin. Warm (not boiling) KOH is faster and will clear specimens in 10 to 15 minutes.

Note: Pentastomes are not cleared.

Note: Mites are cleared like monogeneans (p. 38).

- D. Storing. Place specimens in vials or bottles of 70% EtOH. Adding 5% glycerol to the alcohol will help to prevent brittle appendages and serve as a safeguard against complete drying if the alcohol evaporates. If the preservative becomes discolored during storage, discard the solution and refill with fresh fluid. Always add collecting information or number to the container holding the specimens.

- E. Mounting. Arthropods are mounted unstained.

1. Hoyer's medium. Fleas, lice, mites, and ticks may be mounted directly from distilled water or 70% EtOH into Hoyer's medium (see p. 125). If mites are filled with blood, puncture them and place them in boiling lactic acid solution for a few seconds. Wash thoroughly.

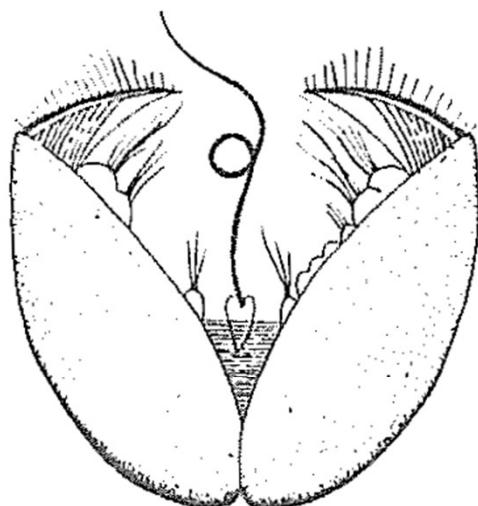
Whole pentastomes usually are not mounted permanently. The hooks should be removed on one side and permanently mounted in Hoyer's medium. Small males less than 1cm long may be mounted permanently in this medium if the reproductive system is to be studied.

2. Glycerol jelly. Mites are mounted like monogeneans (see pp. 38 and 124).
3. Canada balsam. An alternative method for mounting is to dehydrate through the alcohol series to 100% (the time for each step will depend on the size of the organisms; a satisfactory time for small arthropods is 15 minutes each step), xylene (two changes, 10 minutes each), mount in Canada balsam, and add cover glass.

See also: Fixation for EM, p. 86.
Embedding and sectioning for
light microscopy, p. 88.

MOLLUSCS

Parasitism is rare among adult molluscs, but the larvae of freshwater bivalves, family Unionidae, are generally parasitic. The larvae, known as glochidia, develop within the adult clam, pass out of the parent mollusc, and become attached to the gills or body surface of the fish or amphibian host. Within a few hours after the larvae become attached, they are surrounded by host tissue.



Note: Molluscs serve as intermediate hosts for digeneans and may contain larval stages of these flatworms (see p. 41).

- A. Collecting. Excise tissue surrounding the larva and tease cyst apart, using a pair of fine-pointed needles, or fix in toto.
- B. Killing and fixing. Common fixatives are Bouin's (contains picric acid which will soften the shell of the developing clam for sectioning) or 10% formalin.
- C. Storing. Transfer to a vial (include specimen number!) and keep in fixative or 70% EtOH. When transferring from Bouin's to EtOH, change the alcohol periodically until it becomes clear and shows no trace of yellow (picric acid).
- D. Staining. Many workers prefer not to stain glochidia and treat them like monogeneans (see pp. 38). If the specimens are to be stained, follow the general procedure (see p. 19). Often carmine stains are used for this group.
- E. Mounting. Unstained material may be mounted like monogeneans in glycerol jelly (see p. 38). Stained material is dehydrated and mounted in moderately viscous balsam (see p. 23).

See also: Fixation for EM, p. 86.
Embedding and sectioning for
light microscopy, p. 88.

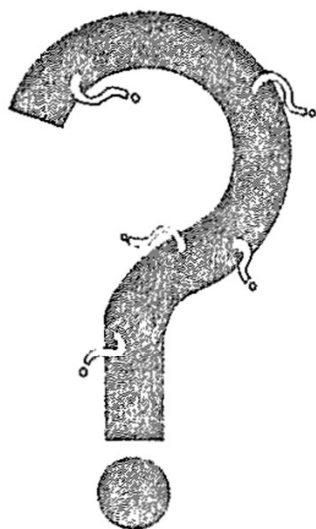
MISCELLANEOUS PHYLA

As stated in the introduction, parasites may be found in or on the body of vertebrates and invertebrates, and they may, themselves, come from practically any phylum. It would be too repetitious to list all the minor groups showing some form of adaptation to parasitism. In most cases, special methods are not required for collection and preservation.

In general, the organism may be killed and fixed in buffered 10% formalin (9 times the volume of the organism) and stored in either the same fluid (cheaper!) or 70% alcohol (preferably EtOH).

To make study preparations (depending on the size of the organism), they may be stained by using hematoxylin or carmine stains and mounted as whole mounts in Canada balsam (see general procedure, p. 19). If sectioning is preferred, follow procedures p. 88 (for light microscopy) or p. 86 (for electron microscopy). Larger specimens are left untreated in storing agent.

NOTES:



I. CONCENTRATION TECHNIQUES FOR FECAL EXAMINATION

In medical and veterinary parasitology, it is often necessary to diagnose infections without seeing the mature parasites. If they live in the gastrointestinal tract, they may produce large numbers of eggs, larvae, or cysts that leave the body of the host with the feces.

- A. Collection of fecal sample. Fresh fecal samples should be examined within 24 hours (48 hours if tightly covered and refrigerated). Containers such as baby food jars will maintain moisture and prevent dehydration of the sample. Parafilm (p. 17) stretched across the top of the container aids against dehydration.

Note: Fecal samples which have been deep frozen are unsatisfactory for flotation techniques.

- B. Concentration of parasites from feces may be accomplished in a number of ways, of which we are listing the more common ones.

Apparatus and materials needed:

Microscope (with low power, 100X; and high power, 400X)
Centrifuge (preferably electric type)
Glass slides (clean)
Cover glasses (18mm²)
Centrifuge tubes (15ml capacity)
Beakers (100ml)
Wire gauze (30 mesh/inch) or cloth gauze
Glass rod (or wooden applicator sticks)
Wooden tongue depressor
Forceps (fine pointed)
Flotation solution (depending on concentration technique used)

1. Centrifugal flotation technique. This method depends on mixing the fecal sample with a liquid, the specific gravity of which is greater than that of the worm eggs, yet less than the specific gravity of most of the fecal debris. Thus the worm eggs rise to the top of the flotation fluid by gravity.

Note: Not all helminth eggs are brought to the surface. Eggs with opercula and heavy eggs remain at the bottom of the container. For these, the sedimentation technique (described below) should be used.

The most commonly recommended flotation fluids include saturated aqueous solutions of NaCl, ZnSO₄, sucrose, or 33% ZnCl₂. Since the price of sugar has gone up, it is advisable to use a salt solution (see zinc sulfate solution, p. 132).

The method listed below will allow workers in a laboratory to establish relative worm loads over a period of time. It is advisable to keep records of the number of eggs counted and the number of worms recovered post mortem.

Note: Different helminths deposit varying numbers of eggs.

- a. Weigh out 10g of feces.
 - b. Mix thoroughly with 300ml of tap water.
 - c. Pour part of the solution through a fine screen into a beaker. Keeping the solution in motion, pour 15ml of the strained solution into a conical centrifuge tube.
 - d. Centrifuge for 3 minutes at 1000 rpm (or higher), then pour off the supernatant fluid. This washing may be repeated until the supernatant fluid is clear (3 washings).
 - e. Fill the centrifuge tube with saturated salt solution. Using a glass rod, mix the sediment with the liquid. Add more salt solution to the centrifuge tube until a meniscus forms at the top.
 - f. Place a square cover slip (22mm²) on top of the meniscus. This must be done with care so that air bubbles are not trapped.
 - g. Centrifuge for 3-5 minutes at 1000 rpm. The parasite eggs will be forced up and adhere to the underside of the cover glass.
 - h. Keeping the cover glass level, remove it with a quick upward movement. This is important, the object being to remove all of the parasite eggs along with a drop of solution. (Eggs may also be removed with a wire loop, but do not use loop as a dipper by going below the surface; simply touch the surface.
 - i. Place the cover glass on a glass slide, and count the number of parasite eggs under the microscope. The number of eggs under the cover glass represents the number of worm eggs in 0.5g of feces and can easily be converted to an estimated e.p.g. (eggs/gram).
2. Merthiolate-iodine-formalin (MIF) preservation and concentration. Perhaps the most desirable of all concentration techniques is this one. It has become a standard in most medical technology laboratories for fresh fecal material, especially when protozoan parasites are sought. It is particularly recommended when only one procedure can be performed, because it preserves trophozoites, cysts, eggs, and larvae. Using this technique for preservation will allow later concentration with excellent results. A high percentage of positive cases tested is detected when this procedure is followed.

Note: Satisfactory permanent mounts can be made from MIF-preserved material using a trichrome stain (see p. 114).

Stock MF solution:

Distilled water	50ml
Formalin (commercial)	5ml
Tincture of merthiolate (1:1000)	40ml
(Note: Use tincture of merthiolate, No. 99, 1:1000, Eli Lilly and Co.)	
Glycerol	1ml

- a. Mix well.
- b. Store in brown bottle

Lugol's solution:

See page 126.

Procedure for concentration:

- a. Add solutions in the following ratio:
0.6ml Lugol's to 9.4ml MF stock
immediately before use to make 10ml of
MIF preservative.
- b. Mix thoroughly about 1 part of feces
with 3 parts of MIF.
- c. Pour suspension through two layers of
wet gauze into a 15ml conical centrifuge
tube.
- d. Top off with more MIF and centrifuge at
1000 to 1500rpm for 1 minute.
- e. Carefully pour off supernatant fluid.
Break up the sediment and resuspend it
in 10ml of fresh MIF solution. Let
stand for 5 minutes to allow complete
fixation.
- f. Add 2 to 3ml of ether. Stopper the tube
with rubber stopper and shake vigorously
for one minute. (Should the ether
remain separated after shaking, add some
drops of water and shake again.)
- g. Centrifuge the tube at 1500 rpm for one
minute. Four layers will form: An
ether layer on top, a plug of fecal
solids, an MIF layer (clear), and the
sediment containing the parasites.
- h. Loosen the plug of solids with an
applicator stick by ringing it, and pour
off all liquid except the sediment.
- i. Mix the sediment with fluid remaining in
tube. More MIF may be added if needed.

Note: The concentrated parasites may be
stored in a vial with MIF solution for
later studies.

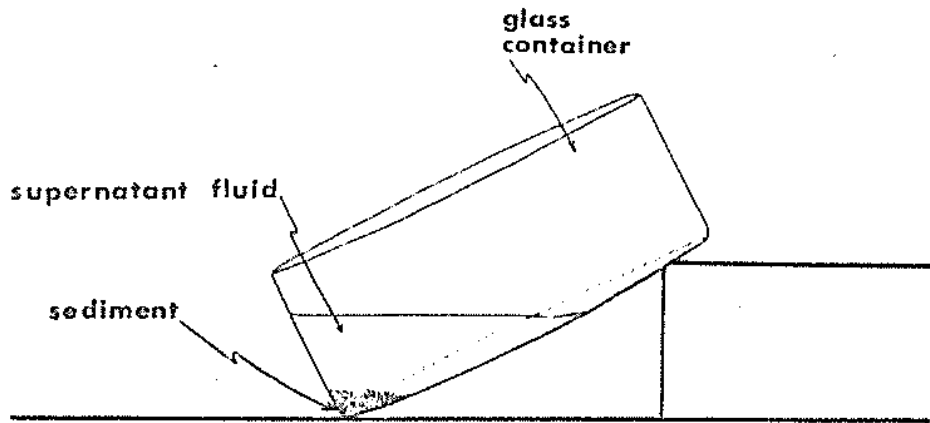
- j. Transfer a drop of the suspension to a

- glass slide, add a cover glass, and examine.
- k. To make permanent slides, transfer a drop of suspension to glass slide prepared with Mayer's albumen affixative (see p. 126), let dry for 3 to 5 minutes, and stain with Wheatley's trichrome (see p. 114).
3. Formalin-ether or PAF sedimentation technique. This method is similar to MIF concentration. It permits concentration of most helminth eggs, including those of schistosomes and acanthocephalans. In comparison with the flotation techniques (above), this one always scores higher in recovering helminth eggs.
- Transfer about 3g of feces to a test tube (15ml) and nearly fill with saline solution. Thoroughly emulsify.
 - Strain through two layers of wet gauze (or cheese cloth).
 - Centrifuge for 2 minutes at about 1800 rpm.
 - Wash twice in saline solution, centrifuging as above and pouring off supernatant fluid each time.
 - Mix sediment with 10ml of formalin or PAF (p. 127) and allow 5 minutes for fixation.
 - Add 3ml ether, invert, and shake vigorously.
 - Centrifuge for 2 minutes at about 1800 rpm.
 - Decant ether, detritus, and formalin.
 - Use a wire loop or pipette to transfer sufficient sediment to a slide. Add a cover glass and examine under a microscope. If insufficient fluid drains back from the tube wall, preventing use of a wire loop or pipette, add a few drops of saline and mix with sediment.
4. Concentrating eggs for SEM work or life-cycle studies. The concentration techniques described above alter the shape and surface morphology of the helminth eggs, mainly because of different osmotic pressures, crystal formation from the solutions used, or chemical reactions due to ingredients. It is therefore important to abstain from these procedures when the eggs must retain their original micro-morphology for SEM studies or viability for life-cycle experiments where infective eggs must be collected without being able to collect the adult worm.

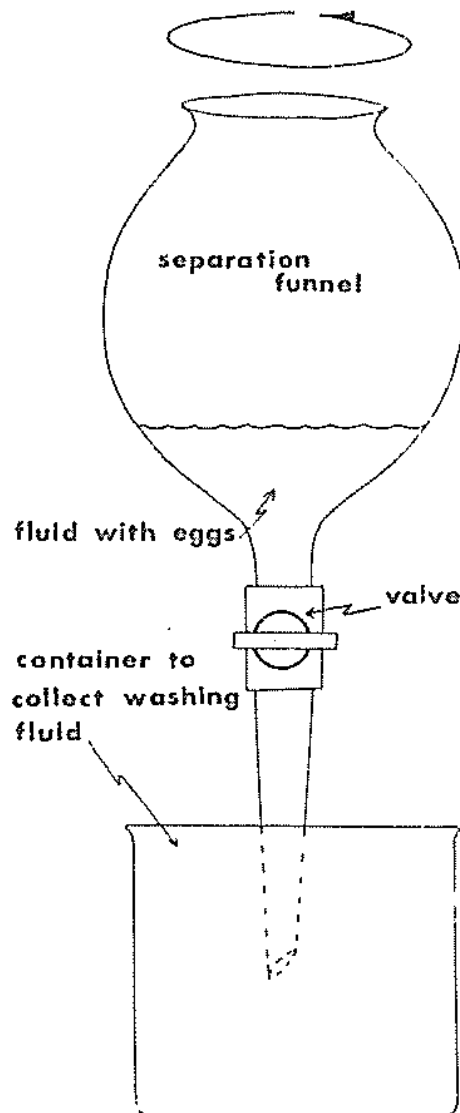
The method described below is rapid, does not require expensive reagents to perform, and maintains embryos in living condition.

Procedure:

- a. Collect fecal material in water.
- b. Mix well.
- c. Let run through a set of sieves or wet gauze and collect fluid into a white container.



- d. Tilt container with fluid and let stand for 10 minutes.
- e. Decant supernatant fluid and add a little fresh water.
- f. Pour the sediment into a dry separation funnel and with a carefully circular motion swirl the sediment. At this time, the eggs will adhere to the side of the glass.
- g. Open valve and drain excess fluid.
- h. Wash eggs off glass wall with tap water and collect.

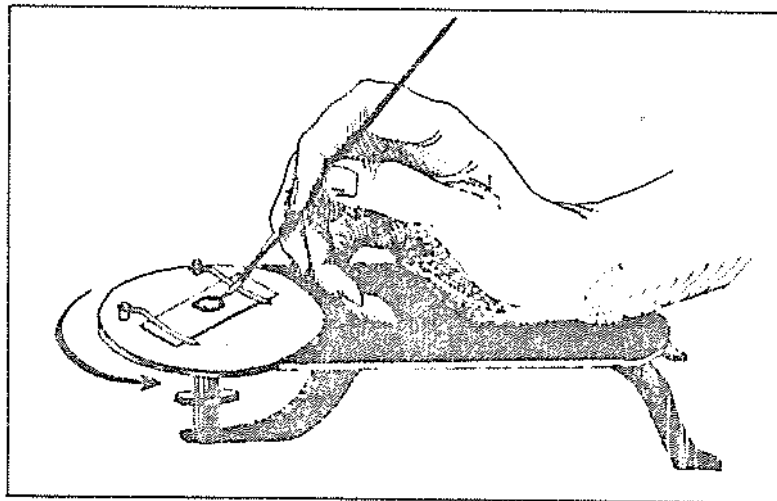


To collect the majority of the eggs, steps f-h may be repeated with the washing fluid.

5. Study collections of worm eggs may be made using the above techniques. After the eggs have been concentrated, they can be pipetted off and transferred to a vial containing 70% EtOH (or formalin, which will shrink the eggs somewhat). Nematode eggs of certain groups (ascarids) can be concentrated and stored in formalin for future experiments requiring viable eggs.

Permanent mounts may be prepared using a variation of the double cover glass method described for monogeneans (p. 38). Place a small drop of concentrated formalin-preserved eggs at the center of a large cover glass; cover with glycerol jelly and top with a smaller cover glass, making sure that the liquid does not extend beyond the small cover glass; invert preparation and mount directly in fairly viscous balsam.

Liquid cell mounts can easily be made from fixed material. Place a slide on a turntable and spin a ring with fingernail polish or asphaltum cement (diameter a little less than a large round cover glass). Let slide dry and have ready for later use.



At the time the preparation is to be made, place a fresh layer of polish or cement on the dried layer. Immediately add a drop of the fixed fecal material in the center of the circle and slowly lower a cover glass so that the liquid spreads (letting the air escape) and fills the whole cell. Gently press on the edges of the cover glass to insure contact with the soft polish or cement.

Set slide aside overnight at room temperature. Seal the edges of the cover glass with a final layer of fingernail polish or asphaltum cement.

II. ARTIFICIAL DIGESTION TECHNIQUE

This method is useful for the recovery of flesh- or skin-dwelling metacercariae and larval nematodes, especially if the parasites are few or when large numbers are needed. Some parasites tolerate this procedure very well; others die rather quickly.

The usual method is to grind the tissue containing the larvae in a kitchen food chopper or to macerate for a few seconds in saline in a blender. Add approximately 1g of host tissue to 20ml of digest fluid and place in a jar along with a few glass beads to aid agitation. Digestion is accelerated if the jar is placed in a water bath at 35-39°C. Best results occur if this can be coupled with a water bath shaker, but shaking the jar by hand every few minutes is satisfactory.

Most host tissue will be well digested in 1 hour or less. Such preparations should stand undisturbed for 5 to 10 minutes before the supernatant fluid is decanted. The bottom material containing the cysts and worms should be rinsed 2 or 3 times in saline. After the final decantation, the bottom material can then be examined in a petri dish under a dissecting microscope.

The recommended composition of digestive fluid is:

Saline (Ringer's)	199ml
HCl (concentrated)	1ml
Pepsin	1g

For optimum results, this fluid should be mixed fresh each time. It is of critical importance that the pepsin be reasonably fresh.

Note: The above fluid and technique can be used to recover Trichinella larvae with 2 modifications: Use Locke's solution instead of Ringer's, and double the quantity of HCl.

III. TREATMENT OF WHOLE MOUNTS THAT HAVE TURNED BLACK

- A. Remove cover glass by placing slide in xylene and leave until mounting medium is dissolved.
- B. Soak specimen for 3-5 days in pure beechwood creosote.
- C. Hydrate to 70% EtOH.
- D. Transfer for the same time (3-5 days) to 5% acid EtOH (70%). If necessary, restain.
- E. Dehydrate, clear, and mount in balsam.

IV. AIR IN WHOLE MOUNTS

When worms are transferred from xylene to balsam, they are leaving one medium for another which has a higher density. The thinner medium, xylene, will migrate from the specimen to the balsam. If this dilution occurs too rapidly, the xylene in the specimen is not replaced by the balsam, and opaque spots result. The remedy which suggests itself is the use of thinner balsam. This works well with thin specimens, but thick material does not mount well in thin balsam. For these specimens, proceed more slowly from clearing agent to balsam by passing them through two changes of thin balsam (1:1 xylene-balsam and then 1:6 xylene-balsam).

Other clearing agents are often used successfully. Oils do not allow air bubbles to form because they are removed more slowly than xylene. Beechwood creosote and methyl benzoate are examples of such clearing agents.

V. RECLAIMING DRIED SPECIMENS

Dried-out specimens which had been stored in EtOH are often discarded. Material of distinct taxonomic value is often included in the losses. Various methods suggested to reclaim such specimens have shown definite limits to their usefulness.

The chemical with the greatest value for treating dried, hardened, or somewhat shriveled specimens seems to be trisodium phosphate (Na_3PO_4) in aqueous solution. Treatment with this chemical does not destroy the internal tissue. It can be used at low concentration; 0.25-0.5% trisodium phosphate in distilled water is effective. To speed up the process, place the dried specimen in the solution in an oven at 35°C.

Potassium hydroxide and lactic acid are reagents with some usefulness for dried arthropods and nematodes.

VI. STRAIGHTENING PARASITE SPECIMENS FIXED IN SITU

Although it is important to straighten platyhelminths for study purposes, unflattened specimens may become available because:

- A. The parasites were fixed in situ with the host tissue (p. 5), e.g., museum collections, or
- B. The collector did not take the time to straighten them during collection.

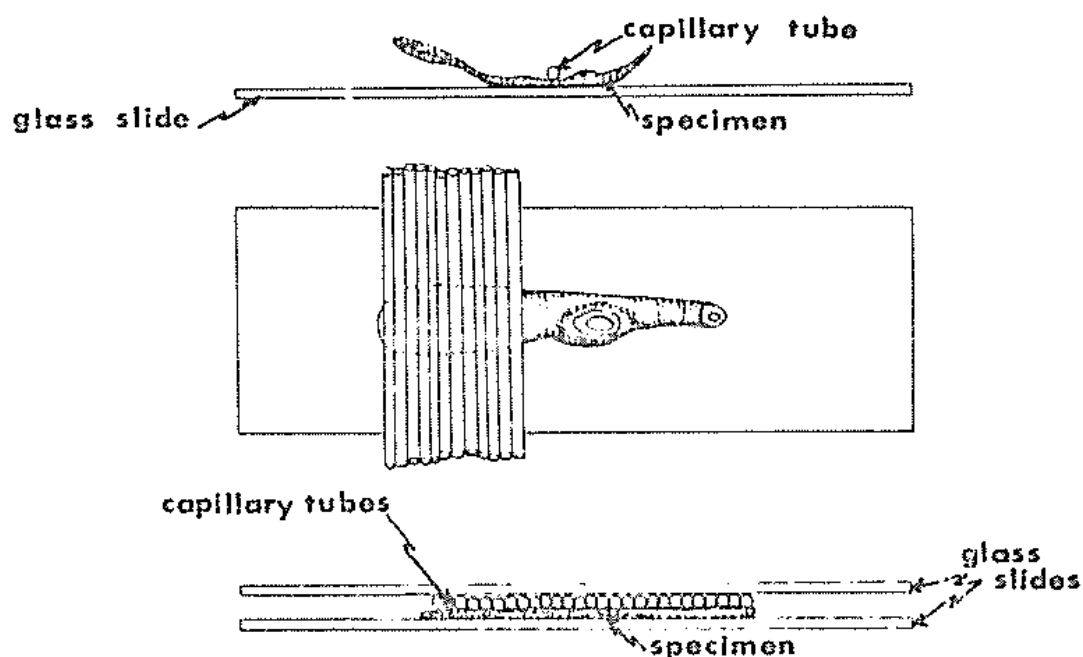
Can these worms be used?

(cont. p. 82)

Platyhelminths collected this way may be very brittle and may break without special treatment. To soften these worms, transfer them to 70% EtOH and glycerol (1:1) and let stand for 48 hours to one week depending on the size of the specimens. The glycerol is removed by several changes of 70% EtOH or when the parasites are moved down the alcohol chain.

Distorted specimens should be stained by standard procedures, and no attempt should be made to uncoil or straighten them until they have been destained and transferred to fresh 70% EtOH.

A single specimen is placed on a glass slide and a capillary tube positioned on top of it where it touches the slide. This process is continued as the worm is uncoiled, resulting in a specimen that is completely covered with capillary tubes.



A second glass slide is then placed on top of the capillary tubes (add clamps if needed) and the entire preparation transferred to a large petri dish. Dehydrate specimen in alcohol series. (For trematodes it is often enough to place another glass slide on top of the worm and not use a series of capillary tubes.)

The time for dehydration should be at least doubled at each step. After a suitable period in 95% EtOH (about one day or two), the top slide and capillary tubes can be removed and the specimen transferred to absolute alcohol.

VII. REMOUNTING TECHNIQUES

A. Replacing broken cover glass

1. Place damaged slide in freezer for 30 to 60 minutes or longer.
2. Immediately upon removal from freezer, flick off cover glass fragments with needle or scalpel.
3. Brush away any small particles of mounting medium.
 - a. If hairline cracks appear in mounting medium, place a drop of xylene on the surface of the mounting medium.
 - b. If specimen sticks to cover glass, follow instructions below under section C.
4. Place a drop of thinned Canada balsam on resin containing specimen.
5. Add new cover glass and set it with appropriate pressure.
6. Place in drying oven or air dry for several days until the balsam is firm enough so that the slide may be handled.

Note: This method is equally effective for whole mounts and sections.

Salvaging specimens in crystallized mounting medium. Certain synthetic resins may crystallize after 10 or more years and will endanger mounted specimens or interfere with viewing them. The action is progressive and will continue until all the medium is affected.

1. If crystallized resin does not engulf specimen, follow procedure for Replacing broken cover glass (steps 1-3). After removing the cover glass, scrape away the crystallized resin with a scalpel, place a drop of xylene on the surface of the old mounting medium, add Canada balsam and a new cover glass.

Note: This method is equally effective for whole mounts and sections.

2. If crystallized resin engulfs the specimen, follow procedure for Replacing broken cover glass (steps 1-3). Then place entire slide in petri dish of xylene until all mounting medium is dissolved. Use enough xylene so that the specimen is always covered. Continue by mounting the specimen on a new slide (see p. 24).

C. Specimens from broken slides

Follow instructions under A (1 and 2); place the piece of slide vertically in a staining dish with xylene, and leave until mounting medium is dissolved. Transfer specimen to a new slide and mount (see p. 24).

Illustrations by M. Marcason.

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The H. W. Mantel Laboratory, Lincoln, Nebraska

The H. W. Mantel Laboratory
3600 Mantel
Lincoln, Nebraska

VIII. FIXATION PROCEDURES FOR ELECTRON MICROSCOPY

The use of electron microscopes as tools in parasitological research has sharpened our concepts of cell structures, host-parasite interfaces, and fine surface structures. This type of work has both supported and altered previous information obtained with the light microscope. Today, this part of our research is quite common, and for that reason we include some standard preparation techniques used in electron microscopy.

A. TEM (transmission)

Two fixatives are generally used: 3% glutaraldehyde and 1 or 2% osmium tetroxide. Both are made up in the same 0.1M phosphate buffer at pH 7.3.

Buffer:

Solution A:

0.2M solution of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (27.6g/l)

Solution B:

0.2M solution of Na_2HPO_4 (28.4g/l)
(53.65g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$)

To make buffer at pH 7.3:

23ml of solution A

77ml of solution B

dilute to 200ml

results in 0.1M solution

Fixation and embedding schedule:

1. Fix 1-4 hours in 3% glutaraldehyde at room temperature.

Note: If the parasites or tissue to be fixed are thicker than 1mm, they need to be cut into smaller pieces for TEM work. Place specimen on a plate of wax in a pool of fixative and cut up with a new tempered steel razor blade, which has been cleaned with acetone.

2. Wash one hour in 5 changes of buffer with 10% sucrose added (room temperature).
3. Post-fix in 1 or 2% osmium tetroxide for 1-2 hours at room temperature.
4. Rinse briefly in distilled water twice.
5. Move specimens up through alcohol chain: 15%, 30%, 45%, 60%, 75% (process can be stopped here), 95%, 4 times in 100% EtOH at 10-minute intervals.
6. Propylene oxide (2 changes, 5 minutes each). Propylene oxide/epon mixture (1:1), overnight in uncapped vial.
7. Fresh epon mixture, under vacuum for 1-2 hours or longer if tissue is difficult to infiltrate.
8. Place tissue in capsule and place capsule at

60°C to polymerize (about 48 hours).

For further information, refer to a TEM manual (e.g., Daves, C. J. 1971. Biological Techniques in Electron Microscopy. Barnes and Noble, Inc., New York).

B. SEM (scanning)

The same fixation process as for TEM may be used for SEM, but it is now widely accepted that parasites may also be fixed in the same solutions as used for light microscopy. The specimens to be studied should not be flattened in any way, but it is very helpful to relax them.

For further information on SEM-techniques, refer to an SEM manual (e.g., Hayat, M. A. 1978. Introduction to Biological Electron Microscopy. University Park Press, Baltimore).

IX. EMBEDDING AND SECTIONING FOR LIGHT MICROSCOPY

The specimen (or tissue) which is processed for embedding and sectioning is handled similarly to those specimens intended for whole mounts. The most common fixative used is buffered 10% formalin, but parasites fixed in AFA and other fixatives can be used. Processing by the paraffin technique is accomplished most rapidly and gives the best results when thin sections of soft tissue are desired. Since paraffin is not water-soluble, the specimen is dehydrated and then cleared in solutions miscible with paraffin before impregnation.

Step by step infiltration by the required reagents produces well-processed specimens. The end result will be sections closely resembling the living state of the specimen in which every cell type is recognizable.

- A. Dehydration. Remove water from specimen (tissue) with EtOH series as described on page 22.
- B. Clearing. Reagents (e.g., xylene) must be miscible with both the dehydrant and the paraffin.
- C. Impregnation is the complete substitution of the clearing agent by paraffin. Use two or three changes of paraffin baths. Most laboratories use paraffin with a melting point of 56-58°C, but the choice of paraffin (see p. 127) depends on the hardness of the tissue and the thickness of the sections to be cut.

Note: Alongside the usual histological methods, the use of plastic media has lately become more popular. Certain microtomes allow cutting sections of 1-3µm

thickness satisfactorily. Follow steps 5 through 8, starting with 75% EtOH, p. 86.

- D. Embedding is the orientation of tissue in melted paraffin which, when solidified, provides a firm medium for keeping the tissue intact when sections are cut.

Each laboratory has a preferred method for embedding. One of the easiest methods, now commonly used, is the TISSUE TECH embedding system.

Processing time:

70% EtOH	holding point
80% EtOH (2 changes)	1 hour each
95% EtOH (2 changes)	1 hour each
100% EtOH (3 changes)	1 hour each
100% EtOH/xylene (50:50)	1 hour
Xylene	1 hour
Xylene/paraffin (50:50)	1 hour
Paraffin (3 changes)	1 hour each

Vacuum the last change. Embed and cool quickly (plunge into cold water) and cut sections at about 5-10µm thickness.

Note: Add identification number to the embedded tissue.

Note: Blocks of tissue embedded in paraffin may be stored indefinitely.

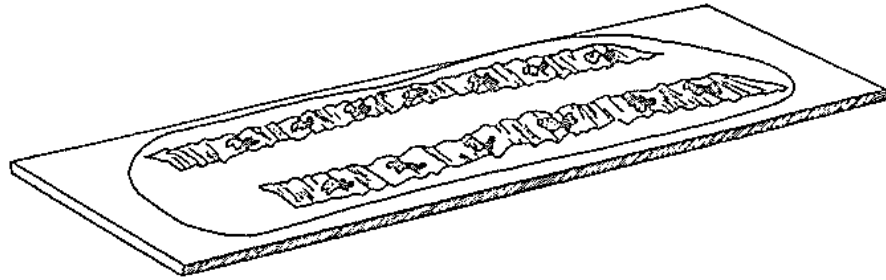
- F. Mounting sections. The cut sections are mounted on glass slides and are covered later (after staining) with a cover glass. The tissue sections need to be attached to the glass slide to provide support while staining or to permit the mounting of several sections in sequence.

The customary means of fixing sections to slides is attachment with egg albumen (p. 126) and water. With one alcohol-cleaned finger, smear a thin film of albumen fixative on the slide, and with a second finger, wipe off excess albumen.

After sections have been cut, they can be floated in a water bath (temperature 5°C below paraffin melting point) and picked up onto the albumenized slide by dipping it under the floating section and removing it from the bath. Hold the section in place with a needle while draining off excess water. Dry the slide on a warming table or in an oven below melting temperature of paraffin.

(cont. p. 92)

Note: In systematic studies parasitologists often need serial sections of the specimen. For this purpose the paraffin block containing the specimen must be trimmed so that its borders are parallel and close to the specimen. Sectioning then produces a straight ribbon which is cut in appropriate lengths (2/3 of slide length), floated in parallel rows on a thin film of distilled water on a slide and placed on a hot plate only until the ribbon expands (but does not melt!).



Draw off the water and air-dry the slide.

- G. Routine Mayer's hematoxylin and eosin staining procedure. This staining method is commonly used in laboratories because it is simple and gives consistent results even when a person is learning the technique. The solutions are in a series of containers that can hold several slides at one time, such as Coplin jars or Tissue Tech II. The slides may be left in hematoxylin for hours without overstaining.

- | | |
|--|-------------------|
| 1. Xylene (deparaffinize) | 2 min. |
| 2. Absolute EtOH | 2 min. |
| 3. 95% EtOH | 2 min. |
| 4. Mayer's hematoxylin | 15 min. |
| 5. Wash in running tap water | 20 min. |
| 6. Counterstain with eosin | 15 sec. to 2 min. |
| (depending on the depth of counterstain desired) | |
| 7. 95% EtOH | 2 min. |
| 8. Absolute alcohol | 2 min. |
| 9. Absolute alcohol | 2 min. |
| 10. Xylene | 2 min. |
| 11. Xylene | 2 min. |
| 12. Mount in Canada balsam. | |

For further information on embedding, sectioning, and staining for light microscopy, refer to a technique manual (e.g., Humason, G.L. 1967. Animal Tissue Techniques. W.H. Freeman and Co, San Francisco).

STAINS AND STAINING METHODS

The following entries are in alphabetical order. Formulae for all solutions mentioned as ingredients are included here or under reagents and solutions (p. 117). Most stains may be obtained commercially, but that often does not assure a knowledge of the ingredients.

Aqueous hematoxylin

Hematoxylin	1g
Distilled water	100ml

Dissolve powder with gentle heat, stirring constantly. Cool.

This solution should ripen at least 6 months. Add 3 drops of saturated aqueous lithium carbonate before use.

Delafield's hematoxylin

Alum solution:

$\text{AlNH}_4(\text{SO}_4)_2$	about 10g
Distilled water	100ml

1. Add alum to hot distilled water, stirring continuously until no more crystals will dissolve.
2. Let solution cool. Excess alum will crystallize.
3. Pour off solution and store in glass-stoppered bottle. Will keep indefinitely.

Stock stain:

Hematoxylin crystals	1g
EtOH (95%)	6ml
Alum solution	100ml
Methyl alcohol (acetone-free)	25ml
Glycerol	25ml

1. Dissolve hematoxylin in EtOH and slowly add alum solution.
2. Cover container loosely with cotton or gauze, and place it so that it is exposed to sunlight for two weeks until stain turns dark.
3. Mix solution with glycerol and methyl alcohol.
4. Filter before use.

Staining procedure:

1. Dilute stock stain (1 part) with distilled water (9 parts) or until one can "read print" through it.
2. Transfer specimens directly from water to staining solution.
3. Allow to stain overnight.
4. Follow general procedures for staining (p. 19).
(See testing for quality under Hematoxylin solutions, p. 100.)

Ehrlich's hematoxylin stain

Hematoxylin (powdered)	2g
EtOH (95%)	100ml
Glycerol	100ml
Glacial acetic acid	10ml
Distilled water	100ml
Potassium alum	3g

1. Dissolve the hematoxylin in the alcohol.
2. Add the glycerol and the acetic acid.
3. Dissolve the alum in warm distilled water.
4. Slowly pour that solution into the hematoxylin solution while stirring.

This is a stock solution which should ripen for three to four weeks. For use, dilute one part stock solution with three to four parts of 35% EtOH.

(See testing for quality under Hematoxylin solutions, p. 100.)

∞ Eosin

Eosin is used as one type of counterstain for hematoxylin.

Eosin stock:

Eosin Y (water-soluble)	1g
Distilled water	20ml
Dissolve and add:	
EtOH (95%)	80ml

Working solution:

Eosin stock	1 part
EtOH (80%)	3 parts
Just before use, add 0.5ml of glacial acetic acid to each 100ml of working solution.	

Giemsa stain

There are probably as many different methods of Giemsa staining as parasitologists who use the stain. The following has been found to be a rapid method with excellent results.

Stock:

Glycerol	40ml
Methyl alcohol	65ml
Giemsa powder	1g

1. Mix well for 1-2 days.
2. Filter.
3. Dilute stock 3:97 with buffer.

Buffer:

Na_2HPO_4	6g
KH_2PO_4	5g
Distilled water	1000ml

- Dilute buffer (1:10) before diluting stock with it.
- Mix only what is immediately needed.
- Keep buffer refrigerated.
- Mixed stain should be a dark blue.

Staining method:

1. Air-dry smears.
2. Fix in absolute methanol (1 minute); shake off liquid, but keep wet.
3. Place smears in stain for 10 minutes or place some stain on the slide.
4. Gently rinse in small stream of tap water.
5. Air-dry and observe (add a thin layer of immersion oil).
6. May add cover glass for long-term storage.

Hematoxylin solutions (testing for quality)

Hematoxylin stains will keep for months. To test them, add several drops of solution to tap (not distilled) water. If it turns bluish purple immediately, it is still satisfactory. If it changes slowly or stays reddish or brownish, it has weakened or broken down and should be discarded.

Iodine-eosin stain

Potassium iodide 5g
Distilled water 100ml
(Sometimes an 0.8% NaCl solution is preferable to distilled water to make up this stain.)

Iodine (crystals) as needed

Eosin (saturated aqueous) 100ml

1. Dissolve potassium iodide in water.
2. Saturate solution with iodine crystals.
3. Mix with saturated aqueous eosin.

Iron alum (ferric alum)

Ferric alum 4g
Distilled water 100ml

Keep in refrigerator to prevent precipitation on sides of bottle.

Note: Use only violet crystals of ferric alum, discard brownish crystals.

Iron alum hematoxylin (Johnson's)

For use with thin films of PVA- or Schaudinn-fixed fecal material after excess mercuric chloride has been removed (see p. 35).

The directions given below presuppose that:

- The staining solutions are made of good quality ingredients and the hematoxylin has been properly ripened.
- The smear is not allowed to dry at any time.
- Solutions are filtered before use (last 24 hours).
- The iron alum is not more than 1 month old.

Solutions used:

Iron alum	100ml
Aqueous hematoxylin	100ml

Pour each of the above solutions into separate staining dishes and have at least one other dish on hand.

Staining procedure:

1. Bring smears from 70% EtOH to distilled water, leaving slides in each solution for 2 minutes.
2. Place in iron alum solution for 30 minutes at room temperature or 5 minutes at 60°C.
3. Rinse in running tap water for 1 or 2 minutes.
4. Stain in aqueous hematoxylin for 30 minutes at room temperature or 5 minutes at 60°C.
5. Rinse briefly in distilled water.
6. Differentiate in 1% iron alum solution. Remove the slide from the staining dish as soon as the intense black color of the smear turns dark grey. Check under the microscope and continue destaining if necessary.
7. Wash in running tap water for 20-30 minutes to remove every trace of the iron alum.
8. Dehydrate by passing through 70%, 85%, 95% and absolute alcohol for 2 to 5 minutes each.
9. Clear in 2 changes of xylene (5 minutes each).
10. Mount with cover slip in Canada balsam.

- absolute alcohol for 2 to 5 minutes each.
9. Clear in 2 changes of xylene (5 minutes each).
 10. Mount with cover slip in Canada balsam.

Kohn's chlorazol black (KCB) fixative-stain

A simple procedure for intestinal protozoans in feces in which fixative and stain are combined into a single solution.

EtoH (90%)	170ml
Methyl alcohol	160ml
Glacial acetic acid	20ml
Liquid phenol	20ml
Phosphotungstic acid (1%)	12ml
Distilled water	618ml
Chlorazol black	5g

1. Mix liquids.
2. Weigh out dye, grind in a mortar, and add small amounts of the solution. Grind until a smooth paste is obtained.
3. Add more solution and continue to grind.
4. Let settle, pour off supernatant fluid into separate container.
5. Add more solution and repeat grinding, settling and pouring until all dye is in solution.

Staining procedure:

1. With a wooden applicator stick, smear some fresh feces or gut contents onto a clean slide. The area covered should be slightly less than 3/4" square (about 1cm²). Avoid smears that are too thick or too thin.
2. Do not let smear dry!!! After making smear, plunge slide directly into a staining dish with the chlorazol black fixative-stain. Stain for 1-2 hours in undiluted solution.

Note: Staining time varies slightly from batch to batch of stain, but it is easily determined by a trial run.

3. Transfer to 95% alcohol for about 2-5 minutes.
4. Dehydrate in absolute alcohol for 5 minutes.
5. Transfer to xylene (2 changes) for 5 minutes each.
6. Mount in balsam with cover glass.

Malzacher's staining technique

Whole mounts of cestodes and trematodes stain particularly well with this method. The technique is still little known. It uses Borax-carminc stain and Astra blue stain to differentiate the various organs sharply.

Astra blue stain:

Astra blue	1g
Tartaric acid	3g
Distilled water	100ml

Mix ingredients well. Stain can be used immediately.

X X

Borax-carminc stain:

Carminc	3g
Borax	4g

Mix ingredients and boil for about 30 minutes or until carminc is dissolved. Cool and add:

EtOH	100ml
------------	-------

Allow to stand 1-2 days and filter.

Staining procedure:

1. Transfer specimens from 70% EtOH into borax-carminc for 15 minutes.
2. Transfer then to distilled water until the parenchyma looks pale.
3. Transfer to astra blue stain for a few (3-5) minutes.
4. Wash in several changes of distilled water until the rinse is clear.
5. Dehydrate (start with 70% EtOH), clear, and mount.
6. Dehydrate (start with 70% EtOH), clear, and mount.

Mayer's acid carmine

Distilled water	15ml
HCl (concentr.)	15 drops
Carmine (alum lake)	4g
EtOH (85%)	95ml

1. Boil carmine in acid and water until dissolved.
2. Cool.
3. Add alcohol.
4. Neutralize (one drop of concentrated ammonium hydroxide is required to neutralize 20ml of stock).
5. Before using, one part of stock is added to four parts of 70% EtOH.

Staining procedure:

1. Put specimens from 70% EtOH into the stain.
2. After organisms are stained (overstained), destain in acid alcohol.
3. Dehydrate, clear, and mount.

Mayer's carmalum stain

Distilled water	10ml
Potassium alum	6g
Carmine (alum lake)	5g
Acetic acid	25ml

1. Dissolve dye and alum in boiling water and simmer for one hour.
2. Cool.
3. Add acetic acid.
4. Allow to stand 10 days.
5. Filter and add a few thymol crystals (to prevent mold growth).
6. Dilute with distilled water as desired just before use.

Staining procedure:

Same as for Mayer's hematoxylin (p. 22).

Staining procedure:

Same as for Mayer's hematoxylin (p. 22).

Van Cleave's combination hematoxylin stain

Delafield's hematoxylin stain	1ml
Ehrlich's hematoxylin stain	1ml
Distilled water	100ml
Potassium alum	6g

Dissolve alum in water before adding to stain.

Trematodes: stain 30 minutes.

Acanthocephala: stain overnight.

Destain in acid alcohol if necessary.

Vital staining

Vital (often called intra vitam) staining is advantageous for studying many living parasites because of the selective staining of structures and the simplicity of its use.

Depending on the purpose, one may use basic or acidic vital stains. The basic vital stains are less toxic. Some examples of basic stains are: methylene blue (now a controlled substance in the U.S.), neutral red, and janus green. Examples of acidic stains are trypan blue and carmine. The acidic vital stains are more easily fixed in the organism.

With the finger tip, spread a very small drop of the aqueous stain on a slide and let dry. Add a drop of water containing the living specimens, cover, and examine immediately or add a drop of stain to solution containing the parasites.

Example of a vital stain preparation:

Methylene blue solution (basic)

Methylene blue powder (medicinal)	3.5g
Distilled H ₂ O (or saline)	100ml

1. Add stain to water and mix well.
2. Let solution stand for few hours, shaking at intervals.
3. Filter or decant the solution into a screw-capped or glass-stoppered bottle.

Wheatley's trichrome stain

Chromotrope	0.60g
Light green	0.15g
Fast green	0.15g
Phosphotungstic acid	0.70g
Glacial acetic acid	1.00ml
Distilled water	100.00ml

1. Put the dry stains into a clean flask.
2. Add the glacial acetic acid, shake to mix, and allow the mixture to stand ("ripen") for 30 minutes.
3. Add the distilled water. Shake to mix thoroughly.

Note: The stain is stable and is used without diluting. Good stain is deep purple, almost black.

Staining procedure:

Place slides containing dried fecal smear or tissue sections in:

1. 95% EtOH for about 5-10 minutes.
2. 70% EtOH for 5 minutes.
3. Trichrome stain 10 minutes.
4. Dip briefly into distilled water.
5. 90% acid EtOH for 5 seconds.
6. Dip into 100% EtOH.
7. 100% EtOH (2 changes) for 5 minutes each.
8. Xylene (2 changes) for 5 minutes each.
9. Mount in balsam with cover glass.

Wright's stain

Wright's stain is relatively inexpensive and one of the easiest to use for blood smears. The fixing solution, methyl alcohol, is combined with the stain. The stain is commercially available in liquid form (ready for use) and also as a powder (which must be dissolved in methyl alcohol before use).

Preparing stain from powder:

Wright's stain powder is ground up with methyl alcohol (0.1g to 60ml). The alcohol must be labeled neutral and acetone-free.

Grind thoroughly in a clean glass mortar. Gradually add the alcohol while grinding the powder into solution. Pour off the solution containing dissolved dye into storage container; add more alcohol to the mortar and grind again.

Repeat this process until all dye is dissolved in the premeasured methyl alcohol. Keep stain in a tight-stoppered bottle.

Staining procedure:

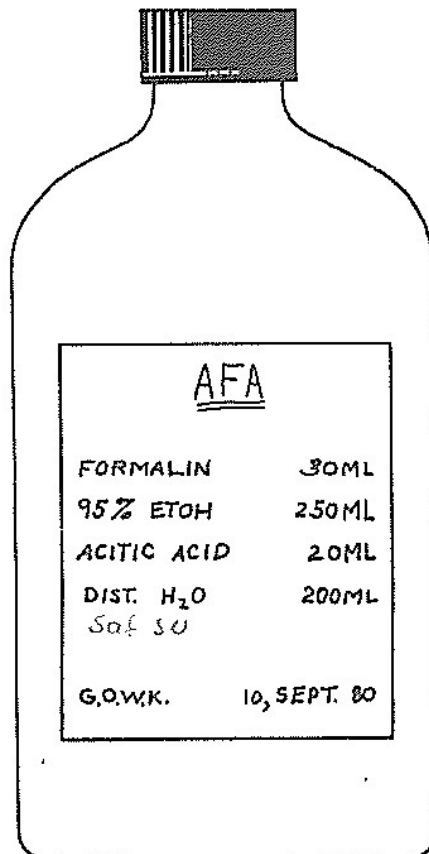
1. Flood the dried smear with about 20 drops of the stain and let it stand for 2 minutes.
2. Add distilled water drop by drop until the surface of the mixture appears to have a green scum. Usually this scum will appear when the amount of water equals the volume of stain.
3. Let stand 1 to 5 minutes and wash in distilled water. Do not wash longer than is required to remove excess stain.
4. Allow to dry thoroughly.

The solutions included under this section are needed while collecting in the field and during the preparation of specimens in the laboratory. They are probably found in most laboratory stocks and should always be available for sudden opportunities to recover parasites and to prepare them.

All chemicals listed should, if not otherwise stated, be reagent grade.

Percentage solutions. It should be indicated in some way whether the percentage is determined by weight or volume: Either it should be written out in grams (g) and milliliters (ml or cc) or it should be expressed as w/v (weight in grams in 100ml volume of solution) or v/v (volume in ml in 100ml total volume of solution).

Although it is erroneous, a long-established habit of technicians is maintained. Percentage solution of liquids is diluted as though the reagent solution is a 100% concentration (e.g., 1% solution of acetic acid is 1ml of glacial acetic acid in 99ml of distilled water).



Note: ALL CONTAINERS MUST BE LABELED

Include:

1. Name of mixture, solution, or reagent.
2. Proportion of ingredients.
3. Date of preparation
4. Name or initials of preparer.

Acetic acid

See Glacial acetic acid. ✕

Acid alcohol

EtOH (70%)	99 parts
Concentrated HCl	1 part

(If this solution is not strong enough to destain parasites, then a drop of HCl may be added to the jar containing specimens to be destained.)

AFA (alcohol-formalin-acetic acid)

(also known as FAA, Carl's, or Kahle's solution)

This solution is an excellent one for general work since it acts as a good killing and fixing agent (marginal for histological purposes) and has the additional advantage that the specimens may be allowed to remain in it for some time without injury.

Formalin (commercial)	6 parts
EtOH (95%)	50 parts
Glacial acetic acid	4 parts
Distilled water	40 parts

Some workers add the acetic acid just before use of this fixative.

Alcohols

Any alcohol which can be diluted with water can be used in any of the alcohol series listed in this manual. Most of the so-called radiator alcohols are not miscible with water and thus not suitable.

Fully denatured alcohol is also unsatisfactory for biological work. There are various formulae for treating EtOH to make it entirely unfit for internal consumption. Most of the fully denatured alcohols have some substance added which causes the solution to turn milky when water is added to it.

Alcohol can be obtained as 100% (absolute) or 95%. It is diluted with distilled water to get the concentration needed. It is advisable to use 95% alcohol because it is less expensive.

The formula for dilution is:

95% alcohol as many parts as per cent desired;
add distilled water to make a total of 95 parts.
E.g.: To prepare 70% alcohol by diluting 95%,
70 parts of 95% alcohol + 25 parts of
distilled H₂O = 95 parts of 70% alcohol.

Alkaline alcohol

Add a few drops of 1% sodium bicarbonate or ammonium hydroxide to 100ml of 70% alcohol.

Beechwood creosote

See Clearing agents.

Benzene

See Clearing agents.

Berlese's medium (mountant)

Gum arabic (flakes)	8g
Distilled water	10ml
Chloral hydrate	74g
(in the U.S., a controlled substance)	
Glacial acetic acid	3ml

1. Dissolve gum arabic in water.
2. Add other ingredients and mix well (it will take several days to dissolve).
3. Filter solution (this will take several days).

Bile-salts solution

Bile salts	1 grain (=65mg)
(bile salts are available from the druggist as 1 grain, chocolate-covered, bile-salts tablets.)	
Saline	30ml

Grind a bile-salts tablet finely in a mortar and add saline. Filter after a few minutes to remove excess chocolate.

Bouin's fluid (fixative)

Picric acid (saturated aqueous solution)	75ml
Formalin (commercial)	25ml
Glacial acetic acid	5ml

Buffer (phosphate) solution

This solution can be used with various other solutions to make them neutral (e.g., formalin).

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	3.31g
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	33.77g
(or Na_2HPO_4	17.88g)

Dissolve in 1 liter of distilled water.

Canada balsam

Canada balsam is a natural resin which is used as a mounting medium (mountant). It is composed of terpenes, carboxylic acid, and esters.

The ideal mounting medium for stained preparations should have the same refractive index as the mounted object. This is about $N = 1.54$ for fixed and cleared cell constituents. The refractive index of Canada balsam approaches this value.

Balsam dries slowly and will become acid, fading the stains and turning them yellow. This handicap can be overcome by adding two or three tablets of sodium bicarbonate to the container. A few drops of cedarwood oil will reduce the golden color and keep mounted specimens from yellowing.

We have observed in our museum that after many years (60) the specimens mounted in balsam are in much better condition than those mounted in the available synthetic resins.

Cedarwood oil

See Clearing agents.

Chloretone (acetone chloroform)

Useful in weak (1%) aqueous solutions for narcotizing many small aquatic animals. Squirt small quantities with a pipette every 5 minutes onto the surface of the water containing specimens.

Clearing agents

Hydrocarbons (e.g., benzene, toluene, and xylene) are reagents commonly used for the step between dehydration and mounting called clearing. During this step, the reagents remove (or clear) opacity from dehydrated tissues, making them transparent.

Caution: Discard clearing agents that have absorbed moisture. See that bottles are clean and dry before adding new clearing agent.

Note: Old stocks may have gathered a film of water at the bottom!

Note: The efficiency of anhydrous CuSO_4 in keeping clearing agents dry is uncertain.

Benzene presents few hardening problems like xylene, but it has a very low boiling point and evaporates quickly. Dangerous and flammable!

Cedarwood oil is another well known clearing agent which does not harden specimens.

With a long, thin dropping pipette run a few ml of cedarwood oil carefully to the bottom of a small specimen tube containing the specimen in absolute alcohol. Specimens float at the interface; gradually they clear and sink. Pipette off the alcohol. Change cedarwood oil after a few hours.

Overnight in cedarwood oil is usually required to completely replace the alcohol in the tissues. This method is expensive since it involves the use of a costly oil.

Creosote (beechwood) can be used in the same way as cedarwood oil. It is preferred by many workers because it does not evaporate during mounting.

Warning: Creosote should not come into contact with the skin, for it burns severely. Should accidents occur, wash affected parts immediately with liberal application of 95% EtOH followed by soap and water.

Methyl benzoate is a good clearing agent if whole mounts are made. In addition, if absolute alcohol must be avoided (some moisture remaining in specimen), methyl benzoate may be used. It is not completely soluble in paraffin, and before embedding, one must transfer the specimen to benzene, xylene, or cedarwood oil.

Toluene is safe for specimens; it does not harden as excessively as xylene, and it has a higher boiling point than benzene.

Xylene has the greatest tendency to harden specimens.

Digestive fluid (artificial)

This fluid is used to expose and collect parasites which are embedded in tissue (e.g., Trichinella in muscle).

Pepsin (fresh powder)	5g
HCl (commercial)	7ml
Distilled water (warm)	1000ml

For fish tissue, increase the pepsin to 7g and reduce HCl to 4ml.

Ether

Commercial grade. Very commonly used as killing agent, but must be handled with extreme caution as the fumes are highly explosive. No open flame or smoking can be permitted in the room, and care should be taken that no fumes are inhaled.

Ethyl alcohol (EtOH or ethanol)

This is the alcohol commonly used in most laboratories, often purchased in denatured form. Ethyl alcohol hardens the specimens and may cause shrinkage. (For dilution, see Alcohols.)

Formalin

This is the fixative most strongly recommended for all-round routine use. It is best in buffered form (10% buffered formalin is 10 parts formalin and 90 parts buffer). Use water instead of buffer if plain (normal) formalin is appropriate.

The name of this solution often causes confusion. Commercial formalin is a saturated aqueous solution of formaldehyde gas and contains about 40% formaldehyde by weight. This solution is known as commercial or 100% formalin and is diluted accordingly to specified concentrations.

Glacial acetic acid

Concentrated form of acetic acid. It will crystallize at room temperature, hence the name. This acid will cause cells to swell and is often used to "straighten" nematodes or is combined with fixatives which have shrinking effects (e.g., formalin to make AFA). It leaves tissue soft and prevents hardening.

Glutaraldehyde

This is a commonly used fixative for TEM since it does not block various enzyme functions. Hence, histochemical localization of enzymes can be performed on sectioned material.

Glycerol alcohol

EtOH (70%)	90 parts
Glycerol	10 parts
(or equal parts of 70% EtOH and glycerol)	
Mix and store in a well-stoppered bottle.	

Glycerol jelly

This mounting medium has an advantage over other media because specimens mounted in it need not be stained or dehydrated. Structures can be observed better by reducing the amount of light coming through the microscope.

Granulated gelatin	8g
Distilled water	52ml
Glycerol	50ml
Phenol (crystalline)	0.1g

1. Soak the gelatin in the water for an

hour or longer, and then dissolve it by heating in a water bath at a temperature of 65 to 75°C, but not higher.

2. Add the glycerol and phenol.
3. Stir the mixture, and again heat in the water bath for 30 minutes.
4. A wide-mouthed bottle with a tight-fitting glass stopper or screw cap is most convenient for storing the medium. Store in refrigerator.
5. A short time before the medium is to be used, immerse the container in a water bath maintained at about 50°C.

Hoyer's medium

Gum arabic (flakes)	30g
Chloral hydrate	200g
(In the U.S., this is a controlled substance and the user must obtain a license.)	
Glycerol	20ml
Distilled water	50ml

1. Mix ingredients at room temperature (begin with water, then add others in order).
2. Place a drop of the medium on a slide, put the specimen in the drop, and cover with a cover glass.
3. The preparation may be heated gently. Heating expands the specimen, hastens clearing, and sets the mounting fluid.

Iodine alcohol

Saturated solution of iodine in 70% EtOH.

Isopropyl alcohol

Isopropanol is an excellent substitute for ethyl alcohol. Isopropyl alcohol causes less shrinkage and hardening than ethyl alcohol, and it is free of Internal Revenue Service restrictions. One disadvantage must be remembered: It cannot be used for staining solutions since dyes are not soluble in it.

KAAD solution

See Peterson's solution.

Kahle's solution

See AFA.

Kohn's chlorazol black fixative-stain (KCB)
See p. 104.

Locke's solution
Saline for warm-blooded vertebrates (see Saline solutions).

Looss's fluid
Used as killing and fixing solution for nematodes; it works better when hot.

EtOH (70%)	9 parts
Glycerol	1 part

Lugol's solution
This solution is often used as temporary stain for protozoans. There are various formulae to which this name has been applied.

Weigert's variation:

Iodine	1g
Potassium iodide	2g
Distilled water	100ml

First dissolve the potassium iodide in the water; then the iodine will go readily into solution. Store in a brown bottle away from light.

Mayer's egg albumen
Egg white (fresh) 50ml
Glycerol 50ml

1. Whisk the egg and transfer to a cylinder. Remove any suspended material that floats to the top.
2. Add glycerol and mix well.
3. Filter through coarse filter paper or cheese cloth (gauze, several thicknesses).
4. Add a crystal of thymol as preservative.

Note: Commercial egg albumen can be purchased ready for use and can be substituted for the fresh egg white above.

Methyl alcohol (methanol)
This alcohol should be purchased as neutral and acetone-free.

Methyl benzoate
See Clearing agents.

Osmium tetroxide (osmic acid)

This fixative is known to provide superior preservation of cytoplasmic detail. The fumes are harmful to eyes, respiratory tract, and skin; work should be carried out under a vent. Solution should be kept in glass-stoppered bottles and in the refrigerator.

PAF fixative (Zamboni's)

PAF is a mixture of picric acid and formaldehyde. It is very stable and not sensitive to light.

Paraformaldehyde	20g
Picric acid (double filtered, saturated aqueous)	150ml
1. Heat to 60°C to change paraform-	
aldehyde into formaldehyde.	
2. Add drops of a 2.52% aqueous NaOH	
solution to alkalize until solution	
clears.	
3. Filter and allow to cool.	
4. Make up to 1000ml with buffer.	

Note: Burrow's PAF is phenol-alcohol-formaldehyde and is not the same as Zamboni's.

Paraffin

The melting point of paraffin varies. Soft paraffin melts in either the 50-52°C or 53-55°C ranges; hard paraffin, in the 56-58°C or 60-68°C ranges.

The choice of paraffin (melting point) depends upon the thickness at which the tissue is to be sectioned (soft paraffin for thick sections), or upon the type of tissue (hard paraffin for hard tissue). For sections of 5-10µm, use a paraffin in the 56-58° grade.

Peterson's solution (KAAD)

Kerosene (white)	1 part
EtOH (95%)	9 parts
Glacial acetic acid	2 parts
Dioxane	1 part

If this mixture is used on soft-bodied organisms, the amount of kerosene should be reduced. The dioxane is added only to make the kerosene miscible and may be omitted. Soft arthropods killed in this mixture should be transferred to alcohol after 4-6 hours, hard-bodied ones after 12-36 hours.

PVA fixative (Burrow's polyvinyl alcohol)

This solution furnishes a means of preserving protozoans (trophozoites and cysts) for subsequent examination and is particularly useful for shipping specimens to other laboratories. Fresh specimens or other materials suspected of containing protozoans are mixed with PVA. Immediately, or months later, permanently stained films can be prepared from this preserved material.

PVA-fixative solution is made from two components, (A) modified Schaudinn's fixative and (B) PVA mixture, which are prepared separately and later mixed. (See formulae below.)

A. Modified Schaudinn's fixative:

Mercuric chloride crystals	4.5g
EtOH (95%)	31.0ml
Glacial acetic acid	5.0ml

1. Dissolve the mercuric chloride in the alcohol in a stoppered flask (50 or 125ml size) by swirling at intervals.
2. Add the acetic acid, stopper, and mix by swirling.
3. Set aside until needed.

B. PVA mixture:

Glycerol	1.5ml
PVA powder*	5.0g
Distilled water	62.5ml

* Not all PVA powders are suitable for PVA fixative solution. When ordering PVA powder, specify pre-tested powder for use in PVA fixative (e.g., Elvanol 71-30 from E. I. Du Pont de Nemours & Co.).

1. In a small beaker add the glycerol to the PVA powder and mix thoroughly with a glass rod until all particles appear coated with the glycerol. Scrape the mixture into a 125ml flask.
2. Add the distilled water, stopper, and leave at room temperature for 3 hours to overnight. Swirl mixture occasionally to mix.

C. Preparation of PVA-fixative solution:

1. Heat a waterbath (or large beaker of water) to 70° or 75°C. Adjust heat to maintain this temperature range.

2. Place the loosely stoppered flask containing the PVA-mixture in the bath for about 10 minutes, swirling frequently.
3. When the PVA powder appears to be mostly dissolved, pour in the Schaudinn's fixative solution, restopper, and swirl to mix.
4. Continue to swirl mixture in the bath for 2 to 3 minutes to dissolve the remainder of the PVA, to allow bubbles to escape, and to clear the solution.
5. Remove the flask from the water bath and let cool. Store the PVA-fixative solution in a screw-cap or glass-stoppered bottle.

PVA lactophenol

Clearing agent for nematodes.

Polyvinyl fixative (see p. 128) as needed
 Lactic acid (reagent grade) 44ml
 Phenol crystals (loose, clear) 44ml
 Distilled water 100ml

1. Slowly sift dry PVA into 100ml of distilled water to form a consistency of thin library paste.
2. Sift through fine silk (or double layer of nylon stocking); agitate the beaker frequently.
3. Add phenol to lactic acid. Allow crystals to dissolve.
4. Pour the mixture into the PVA solution and let stand overnight.
5. Keep in tightly stoppered bottle.

Ringer's solution

A saline for cold-blooded vertebrates. See Saline solutions.

Saline solutions

Salines are physiological salt solutions which vary in composition depending upon the type of vertebrate involved.

Cold-blooded vertebrates:

(Ringer's solution)

Sodium chloride 8.0g
 Sodium bicarbonate 0.2g
 Potassium chloride 0.2g
 Calcium chloride (anhydrous) 0.2g
 Dextrose 1.0g
 Distilled water 1000ml

Or simply make a 0.7% sodium chloride solution for amphibians and reptiles and a 0.6% solution for fish.

40
 1
 1
 1
 5
 5 4

Warm-blooded vertebrates:

(Locke's solution)

Sodium chloride	9.0g
Sodium bicarbonate	0.2g
Potassium chloride	0.4g
Calcium chloride	0.3g
Dextrose	2.0g
Distilled water	1000ml

or simply make a 0.85% sodium chloride solution.

Schaudinn's fixative

Mercuric chloride (saturated, aqueous)	100ml
EtOH (95%)	50ml
Glacial acetic acid	
(add just before use)	7ml

Sea water (artificial)

chlorinity=19.0‰

salinity=34.33‰

NaCl	23.991g
KCl	0.742g
CaCl ₂	1.135g
(or CaCl ₂ •6H ₂ O	2.240g)
MgCl ₂	5.102g
(or MgCl ₂ •6H ₂ O	10.893g)
Na ₂ SO ₄	4.012g
(or Na ₂ SO ₄ •10H ₂ O	9.100g)
NaHCO ₃	0.197g
NaBr	0.085g
(or NaBr•2H ₂ O	0.115g)
SrCl ₂	0.011g
(or SrCl ₂ •6H ₂ O	0.018g)
H ₃ BO ₃	0.027g
Dissolve in distilled water and make up to	
1 liter.	

Thymol

This chemical is a good antiseptic, stopping bacterial or fungal growth.

Toluene

See Clearing agents.

Xylene (xylol)

See Clearing agents.

Zamboni's fixative

See PAF.

Zinc sulfate solution

$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	336g
Distilled water	1000ml

This solution has a specific gravity of 1.180.

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