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BASIC METHODS OF SPECIMEN PREPARATION IN PARASITOLOGY

June 1979

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BASIC METHODS OF SPECIMEN PREPARATION IN PARASITOLOGY

MURRAY J. KENNEDY

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PREFACE

This book is designed to give laboratory technicians and researchers a basic grounding in preparing parasites for microscopic examination. It does not pretend to be comprehensive, nor does it attempt to illustrate each technique with a specific example. The author recognized that parasitology laboratories have well-tried practical methods and that some fundamental skills can be gained only by experience.

This work was carried out and this book compiled in Bogor, Indonesia, while the author worked as a consultant for the International Development Research Centre (IDRC), at the Inland Fisheries Research Institute of the Agency for Agricultural Research and Development.

INTRODUCTION

For the past few years, various countries in the Southeast Asian region have expressed interest in expanding research on parasites, particularly fish parasites. This expansion has been hampered in part by a lack of trained personnel, inadequate faci facilities, and the non-availability of instruction manuals and other literature pertaining to the field of Parasitology. The above needs were expressed again by seven countries from Southeast Asia during the First Workshop on Tropical Fish Diseases, held in Bogor, Java, Indonesia in November, 1978. It was the consensus of opinion of the participants of the workshop that a laboratory manual outlining various techniques of parasite preparation would be of great value. It was with this in mind that the author compiled this manual.

The manual describes techniques for preparing the major groups of parasites for study. Included are methods for temporary, semi-permanent, and permanent mounting procedures. The Appendix includes formulae for preparing all of the special solutions mentioned in the text. While not exhaustive, the text does describe a variety of techniques which can be applied in most basic laboratories.

The author welcomes any comments or criticisms that may improve the manual.

Funds for publishing this manual came from IDRC. Any errors in the text are the sole responsibility of the author.

PROTOZOA

Protozoa are best studied when they are alive. However, permanently stained preparations are necessary to supplement study and to document an infection. There are two fundamentally different methods for colouring Protozoa. In the first, the Protozoa actively take up coloured particles, such as carbon particles in India ink and Carmine, into food vacuoles and become coloured. In the second, dyes infiltrate Protozoa and colour certain cellular organelles such as mitochondria (janus green B), nuclei (methylene blue), and vacuoles (neutral red). The second is of more general interest to parasitologists.

Vital staining

Normally, the organism is not killed in vital staining because low concentrations of dye are employed. There are two methods. In the first, the dye is dissolved in distilled water and a drop of the solution added to the media containing living Protozoa. Dye concentrations of 0.01% or less are usually best. In the second, the dye is dissolved in absolute ethanol. A drop of the solution is then spread on a slide and allowed to dry. A drop of media containing the Protozoa is then added to the dried film. The dye dissolves slowly in the fluid and colors the living organism. Most vital dyes are easily removed by ethanol and other organic solvents and are usually not permanent (Baker 1970). Vitally stained Protozoa are usually studied, photographed or drawn, and then discarded.

Procedures for using Neutral Red are described below and can be used for other vital dyes, although the best concentrations for each dye need to be determined by trial and error.

Neutral Red (ethanol): Place a small drop of 0.05% neutral red (dissolved in absolute ethanol) on a slide and let it dry. Add a drop of media containing the Protozoa to the dried film, apply a coverslip, and observe the specimen.

Neutral Red (aqueous): An aqueous solution of dye of 0.01% or weaker may be used. Add the solution directly to the material under observation.

Neutral Red is a basic dye that stains vacuoles and may lightly stain the nucleus.

Temporary Mounts

Temporary mounts, as the name implies, are kept for only a short period, usually a few days. The solutions used in preparing specimens are sufficiently strong to kill the organism.

Lugol's solution: Dilute the solution to 1:5 or 1:10 with distilled water and add a drop to the Protozoa under observation. The result is a general contrast stain useful for Protozoa in feces.

Acidulated methyl green: Use 1/2% methyl green in 1% acetic acid to stain nuclei a bright green against a lightly stained bluish-grey background.

Noland's solution: Mix one drop of the solution with one drop of the fluid containing Protozoa. Noland's solution contains gentian violet (= crystal violet) and stains flagella, cilia, and nuclei.

Iodine-eosin stain: Saturate one volume of 5% aqueous potassium iodide with iodine crystals and mix with an equal volume of saturated aqueous eosin (yellow aqueous eosin). Use 0.8% NaCl solution for making this solution. A general contrast stain.

Permanent Mounts (Blood smears)

Obtain blood through a heart puncture or by snipping the caudal fin of a fish. The blood sample should be taken immediately after killing the fish and before clotting occurs. There are two types of blood smears: thin films and thick films.

Thin film: Place a drop of fresh blood about one inch from the end of one of the slides (Fig. 1). Touch the end of the other slide to the leading edge of the drop, hold the slide at an angle of 20 to 45° so that the capillary action spreads the blood across the width of the angle (Fig. 2). Push the slide forward with a smooth even motion so as to drag the blood out as a uniform film over the surface of the lower slide (Fig. 3). The angle of the slides and the speed with which the smear is made will determine the thickness of the smear. A large angle and slow movement of the spreading slide both produce a thick smear. Place the slide horizontally under a dust cover and allow the film to dry. If the slide is made properly the film should form a monolayer of cells.

Fig. 1

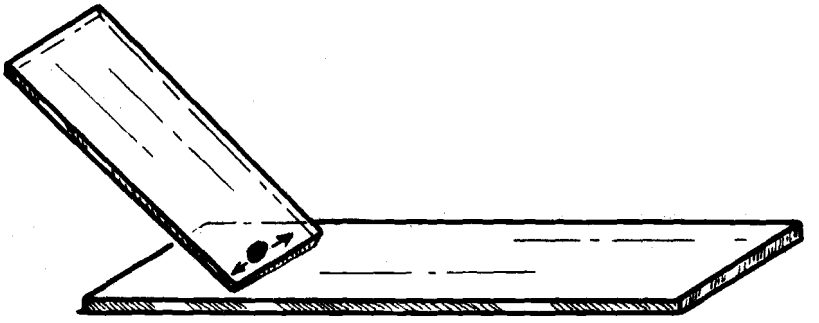


Fig. 2

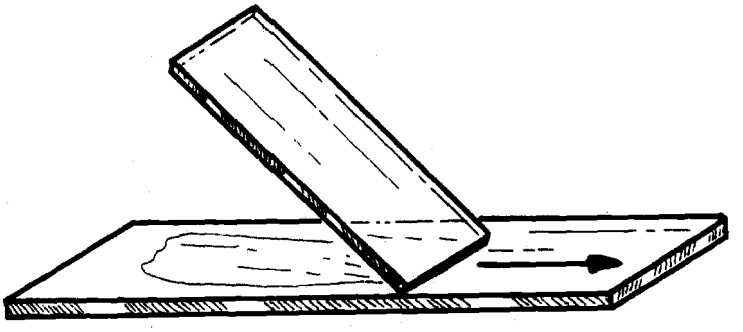


Fig. 3

FIGS. 1 to 3. PROCEDURE FOR PREPARING BLOOD SMEARS

Thick film: A thick film is best used when parasite numbers are low. Place two or three drops of blood in the center of a slide and spread it into an even layer with a needle or toothpick. Allow the film to dry. It may take one or two hours for the film to dry completely. Place the dried smear in distilled water before staining. This will haemolyze the red blood cells and render the parasites more visible.

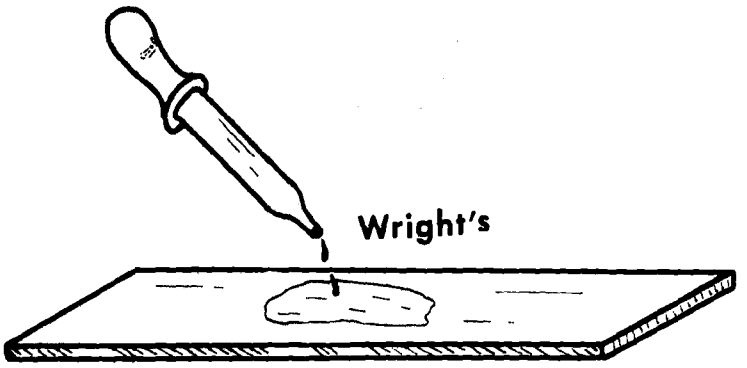
Staining Blood Smears

Wright's stain: Wright's stain contains methylene blue and eosin dissolved in methyl alcohol. To use, add 10 drops of the stain to the dried blood smear and leave for one to three minutes (Fig. 4). Tilt the slide if necessary to give an even distribution of the stain. Now add 10 drops of distilled water (or phosphate buffer pH 6.4) (Fig. 5). Allow to stand twice as long as the undiluted stain. Wash the slide with distilled water until all excess stain is removed and the thin portions of the stained film are pink (Fig. 6). Air dry the smear under a cover to prevent dust from settling on the slide. If a coverslip is to be added, put one drop of oxylol on the smear, add a drop of Canada balsam and apply a coverglass. This procedure is not applicable to thick films unless they have been haemolyzed with distilled water before staining.

Note: The most common fault in blood smears is that the erythrocytes are clumped together. This comes from either having too thick a smear or using a dirty slide. Even new slides should be cleaned before use.

Red blood cells stain yellowish red; leucocytes a dark blue nucleus and pale pink or blue cytoplasm.

Giemsa's stain: Giemsa (1902) specified Bernthsen's methylene azure, methylene blue, and eosin as constituents of his widely used stain for blood and blood protozoa. The azure stains the lymphocyte cytoplasm light blue. This blue colour is better represented in Giemsa stained than in Wright stained preparations (Baker 1970). Some researchers feel that Giemsa stained blood gives greater purity of colour and sharpness of definition, with deeper staining of chromatin. As a stain for malaria plasmodium, Giemsa's stain is preferred over Wright's stain. However, a good Wright stain can be an acceptable substitute. A thick blood smear which has been haemolyzed in distilled water before staining is preferred.



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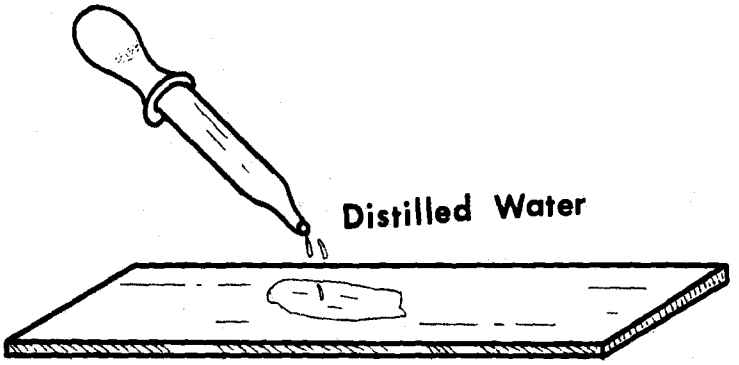

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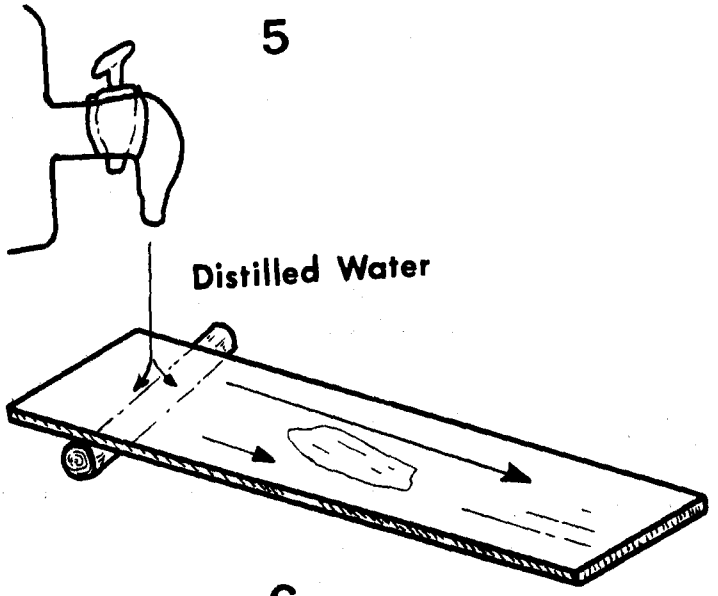

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FIGS. 4, 5, 6. PROCEDURE FOR STAINING WITH WRIGHT'S STAIN

Place the dried thin blood film in absolute methyl alcohol for about 10 minutes (Fig. 7A). Remove and dry the slide. Add the dried slide to the stain for 20 to 60 minutes (Fig. 7B). Use a dilute solution of stain (1 drop of stain to 1 ml of distilled water). Wash the slide in distilled water to remove excess stain, then allow the smear to dry (Fig. 7C). Add Canada balsam and a coverslip if desired (see Wright stain).

If the pH of the solution is correct (pH 6.0 to 6.5) erythrocytes will stain red, lymphocytes will have a blue cytoplasm and red-purple nuclei.

Permanent Mounts (General)

Iron hematoxylin: Iron hematoxylin is often used as a stain for Protozoa in feces. The following is an outline of the technique as given by Spencer and Monroe, 1961. The technique can be modified for general use by spreading a thin layer of Mayer's albumen on a slide before adding the Protozoa. The albumen should adhere the Protozoa to the slide.

Procedure: (after Spencer and Munroe 1961)

Emulsify feces in saline. Take a small drop of the suspension and make a thin smear. Place the slide in Schaudinn's fixative before the smear dried. Let stand for one hour or longer then transfer to a 70% iodine-alcohol solution for 5 minutes, then wash the slide gently in tap water for an additional 10 minutes. Transfer to a "working" solution of iron hematoxylin and stain for 4 or 5 minutes. Wash again in tap water for 10 minutes. Transfer to 70%, 95% and then absolute ethanol for 5 minutes in each solution. Transfer to xylene for 10 minutes. Mount in Canada balsam.

At no time should you allow the slide to dry.

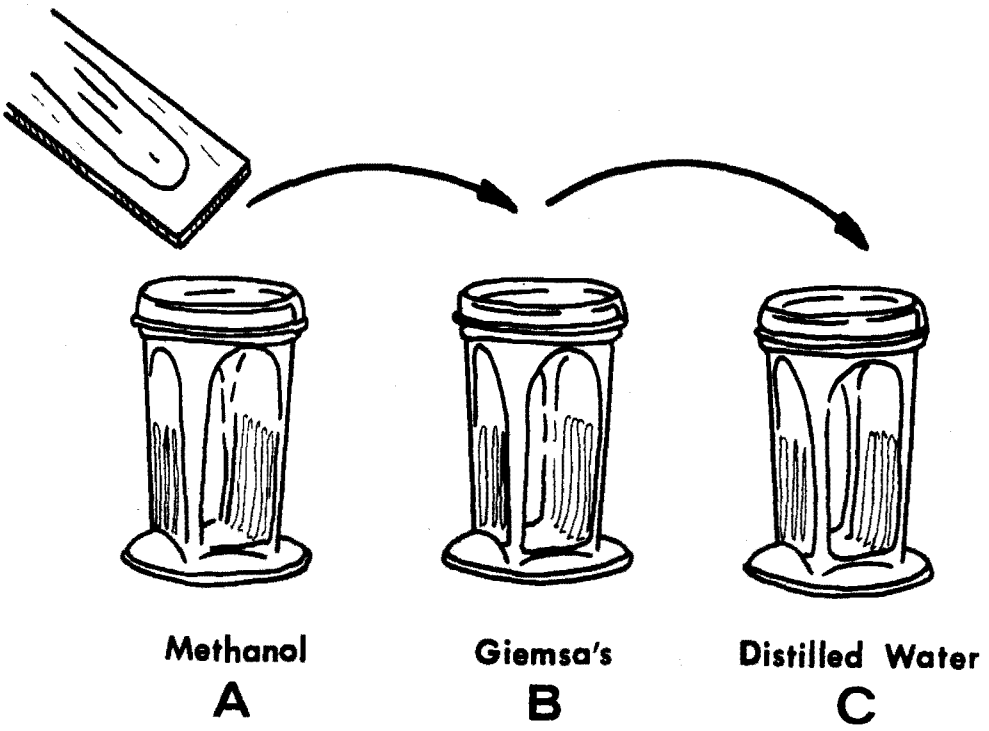


Fig. 7

PROCEDURE FOR STAINING WITH GIEMSA'S STAIN

MYXOSPORIDA
(Figs. 8 & 9)

Temporary Mounts

Lugol's solution: This solution is often employed as a test for the presence or absence of iodinophilous vacuoles within the sporoplasms of spores of the family Myxobolidae (eg. Henneguya, Myxobolus, Thelohanellus, etc. Kudo 1966). See Lugol's solution (Protozoa) for procedure.

India ink: The presence or absence of a mucous coat around some spores can be determined by studying fresh spores mounted in India ink. Add one drop of ink to an equal volume of spore suspension. Apply a coverglass.

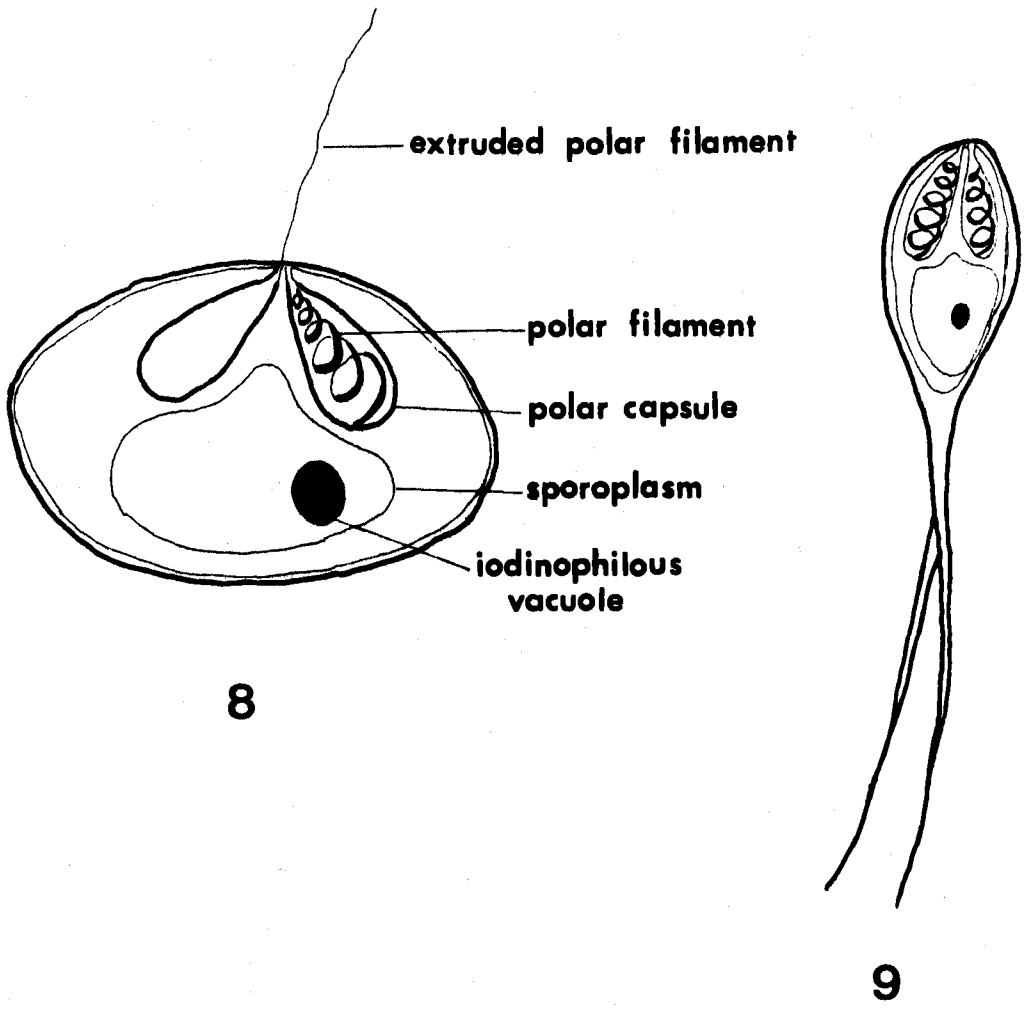
Fuchsin: Place a drop of fresh spore suspension on a slide. Form a ring of petroleum jelly or Vaseline around the drop and add one drop of 1% (W/V) aqueous basic Fuchsin. Place a coverslip on the petroleum jelly and apply light pressure to form a complete seal.

For differentiating spores and flagella:

Noland's solution: As for Fuchsin above.

Permanent Mounts

Giemsa stain: Make a smear using fresh spores and allow to air dry. Fix the spores in 10% buffered formalin and stain in Giemsa's stain for about 25 minutes. Remove from the stain, wash in distilled water until excess stain is removed then allow the slide to dry (after Cone and Anderson 1977) or by adding a 1% KOH solution and allowing the slide to dry. If polar filaments do not extrude increase the concentration of KOH (NaOH can also be used). Longer staining may be required to demonstrate the extruded filaments.



FIGS. 8 and 9. GENERAL MORPHOLOGY OF MYXOSPORIDA

HELMINTHS

Fixatives (General)

Fixation is primarily the modification of tissues in such a way that they retain their form as nearly as possible when the tissue is subjected to subsequent treatments such as dehydration, clearing, and mounting, which tend to distort tissues (Baker 1970). Fixatives also make the tissues more easily stained.

The length of time a specimen should be left in a fixative will depend, in part, on the size of the specimen and the rate of penetration of the fixative. As a general rule, it is convenient to leave the specimen in the fixative overnight (18-24 hours). Fixatives can be used at room temperature or heated to give the added advantage of minimizing contraction and culing of the specimen. Specimens fixed in some solutions (eg. 70% ethanol) may be transferred directly to a stain, while other fixatives (eg. formalin) should be washed out of tissues before staining.

Common Fixatives for Helminths

Ethanol: A 70% solution of ethanol gives good results when used as a fixative but absolute ethanol causes tissue shrinkage and hardening (Baker 1970). While ethanol has less effect on the dyeing of proteins than most fixatives, chromatin is rendered strongly colourable by basic dyes. However, ethanol alone does not stabilize chromatin and so is a poor fixative for chromosomes (Baker 1970).

Formaldehyde: Formalin is a colourless solution containing 37% by weight of formaldehyde gas in water, usually with 10-15% methanol added to prevent formation of paraformaldehyde $(CH_2O)_n.H_2O$. This commercial formalin is the same strength as that known as formalin 40% which contains 40 g of formaldehyde gas in 100 ml of the solution and is equivalent to 37% by weight (Nelson 1974).

In this manual the name formalin is restricted to the commercial product at 40% and the concentration of diluted fluids is expressed in terms of their formaldehyde content. For example, a 10% solution of formalin gives a 4% formaldehyde concentration.

Baker (1970) states that formaldehyde preserves cell structure better than any other primary fixative except osmium tetroxide. It hardens tissues faster than ethanol and preserves the external form better. Specimens fixed in formaldehyde are more readily stained by basic dyes such as Heidenhain's hematoxylin, a weakly acidic dye, is commonly used and gives satisfactory results. Borax carmine, a stronger acid dye, does not give as good results. As a general rule no special washing out of the fixative is needed before staining. However, some authors recommend transferring specimens to either water or ethanol after fixation in formaldehyde. If specimens are to be stained with hematoxylin then water is the best choice, but if carmine is to be used then wash in 70% ethanol before staining.

Bouin's fixative: This is a compound fixative containing picric acid, formaldehyde, and acetic acid. The picric acid leaves tissues soft, and helps prevent the hardening effect of ethanol (in Alcoholic Bouin's). Picric acid can be readily washed out by ethanol. Any yellow colour which remains in tissues may be removed by an aqueous solution of lithium carbonate. Cytoplasm becomes more readily stained by acidic dyes after treatment with picric acid. Therefore, Grenacher's Borax Carmine is a good choice of dye. Very little affinity for basic dye is retained (Baker 1970).

The acetic acid in Bouin's helps prevent shrinkage during fixation and prevent excessive hardening (Baker 1970). It is also an excellent fixative for muclei and chromosomes (Gabb and Latchem 1968). Formaldehyde fixes cytoplasm and makes it basiphilic. Picric acid compensates by making the cytoplasm strongly acidophil.

Alcoholic Bouin's (= Duboscq-Brasil) is a good fixative for arthropods because it penetrates the cuticle rapidly (Gabb and Latchem 1968).

A.F.A. (Alcohol:Formalin:Acetic acid): This is another commonly used compound fixative for helminths. Tissues fixed in this solution are usually stained in Semichon's acetic carmine. See above discussion for effects of the various components.

Staining Helminths

The most frequently employed formulae for staining with hematoxylin are Harris' hematoxylin and Delafield's hematoxylin, which are alum hematoxylins, and Heidenhain's hematoxylin, an iron hematoxylin. Grenacher's alcoholic-borax-carmine and Semichon's acetic acid carmine are two frequently used carmine stains. The procedures for three of the above stains are described below.

Grenacher's alcoholic-borax-carmine: Fix worms in Bouin's fixative for 18-24 hours then wash in several changes of 70% ethanol, or until most of the yellowish colour has been removed. Transfer the specimens to the stain. Overnight staining with slow destaining has been recommended for most procedures (Fernando et al. 1972). However, shorter periods will give good results. Destain in acid alcohol (see Appendix) until reproductive organs are rosy-pink and most of the stain has been removed from other tissues. Dehydrate through progressive changes of 85%, 95% and absolute ethanol. The time required for each step will depend, among other factors, on the size of the specimen and permeability of the tegument. Clear (see section on clearing agents) and mount in Canada balsam.

Semichon's acetic carmine: Fix specimen in A.F.A. or 70% ethanol. Then, transfer the specimen directly to the stain if ethanol was used, or if A.F.A. was the fixative of choice, wash in 70% ethanol before transferring to Semichon's. Destain, dehydrate, clear and mount as above.

Harris' hematoxylin: Worms fixed in formalin are often subsequently stained with Harris' hematoxylin. Wash the formalin out with several changes of distilled water. Stain, then wash excess stain from tissues by passing through distilled water. Dehydrate through 35% then 50% ethanol, then destain in 70% acid alcohol. Transfer to alkaline alcohol, dehydrate, clear and mount.

The techniques described in this section are useful for Cestodes, Digenea, and Acanthocephala (Figs. 10 and 11).

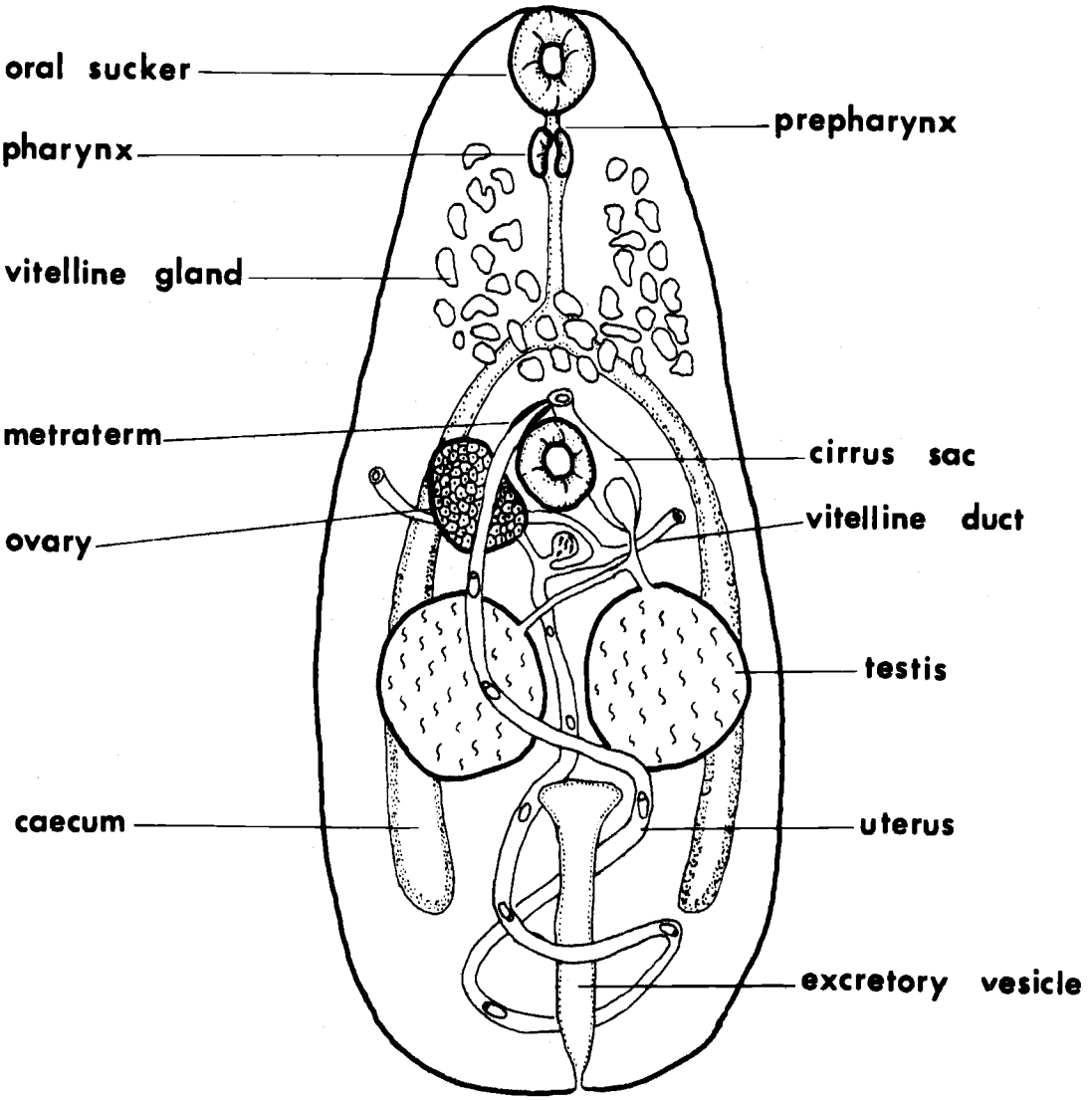


Fig. 10

GENERAL MORPHOLOGY OF TREMATODA

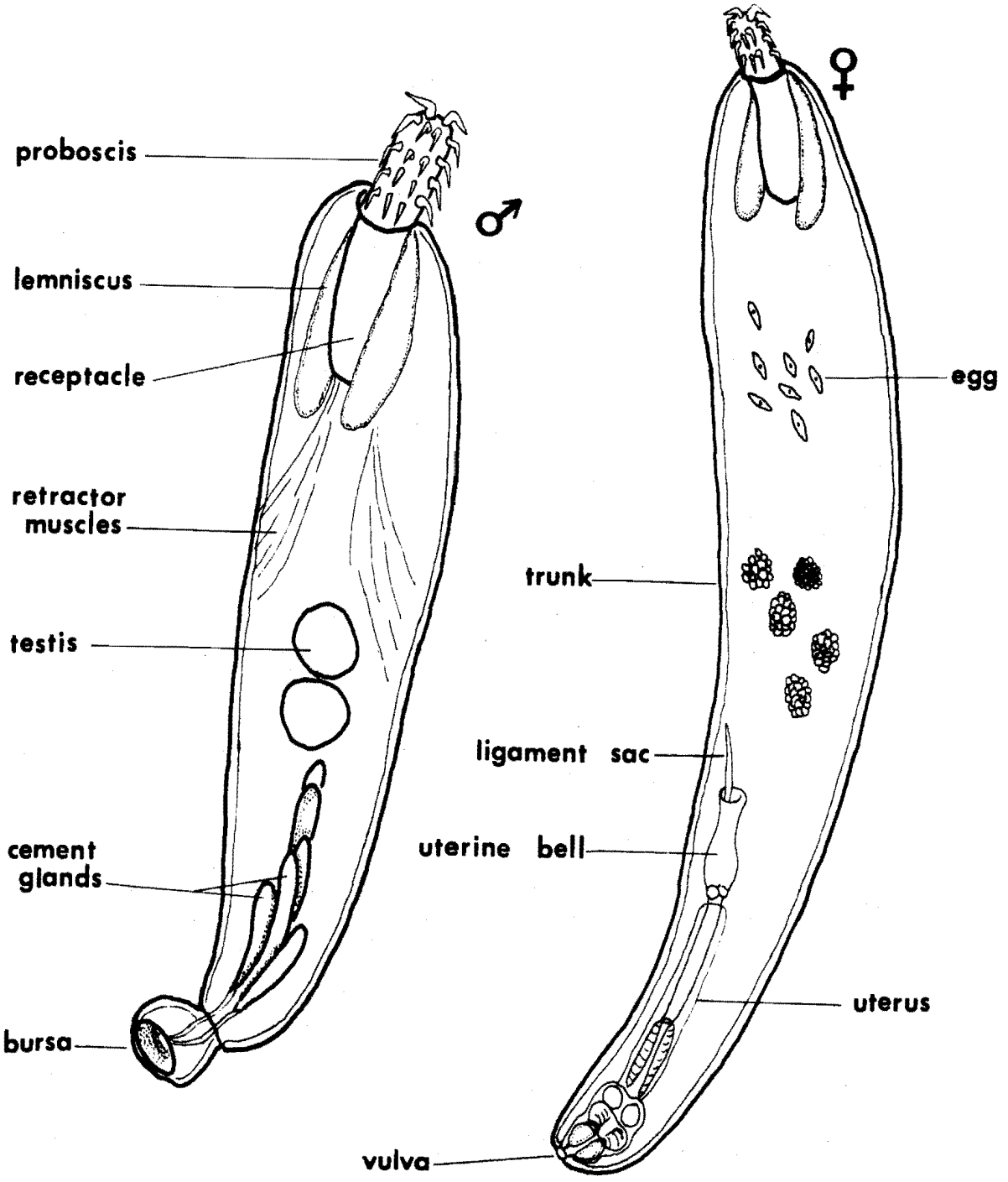


Fig. 11

GENERAL MORPHOLOGY OF ACANTHOCEPHALA

Monogenea (Fig. 12)

The techniques described in the previous section can be used to stain monogenea. However, good results are difficult to obtain.

Seamster (1938) studied five fixatives and five stains in order to determine those combinations giving the best tissue differentiation. He observed that 10% formalin-Heidenhain's hematoxylin combination produced the best results and Bouin's-Harris' hematoxylin the poorest. Seamster noted that the haptoral and copulatory complex were usually obscured by the stained surrounding tissues and concluded that unstained preparations made the best study mounts.

Mizelle (1938) came to the same conclusion. He preferred to mount worms unstained in Canada balsam for permanent mounts or in equal parts of glycerine and alcohol for temporary mounts (Mizelle 1936, 1937).

Permanent mounts can be made in the following manner: Transfer live specimens to a slide coated with Mayer's albumen. Place the slide in a finger bowl on an inverted stender dish. Add alcohol to the container to a level just below the top of the stender dish. Cover the bowl and incubate at 55°C for 1 to 5 minutes. This will coagulate the albumen and attach the specimens to the slide. After attachment fix in Gilson's fluid, remove mercuric salts by dipping slides in iodine, stain in borax-carmine and mount in Canada balsam.

A.V. Gussev (1968) recommended mounting Monogenea in a mixture of equal volumes of glucerine and a saturated ammonium picrate solution. Slides of worms mounted in this way can be sealed with a heated mixture of colophony and wax.

Ergens (1969) cautioned against the use of ammonium picrate-glycerine for permanent slides. He found that the ammonium picrate evaporates and crystalizes if slides are not sealed carefully. As well, specimens tend to turn brown after 1 or 2 years and lose their transparency. He recommended that specimens treated with ammonium picrate-glycerine be remounted in Canada balsam after no longer than 6 months if permanent mounts are desired.

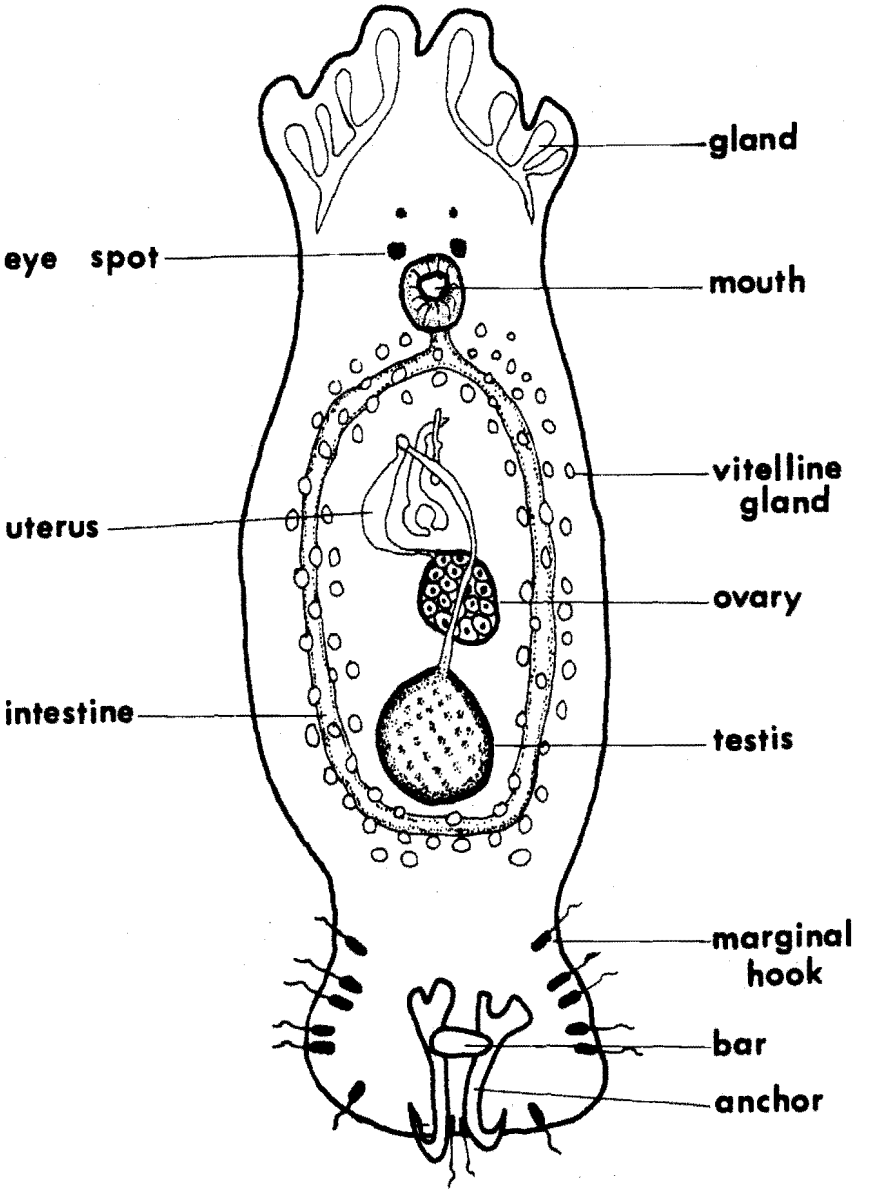


Fig. 12

GENERAL MORPHOLOGY OF MONOGENEA

Crane (1972) used the following technique to study Monogenea from the fish of california. Worms were fixed in 5% formalin, washed in distilled water, dehydrated, cleared in toluene for 6 to 8 hours, and mounted in permount.

More recently, Kritsky et al. (1978) developed a technique for staining the superficial and deep bars of the opisthaptor of Gyrodactylus spp. For temporary mounts worms are fixed in 3% formalin then transferred from the fixative to a drop of distilled water on a glass slide. The water is drawn off with filter paper and replaced with one drop of Gomori's trichrome solution. Never allow the worm to become dry. Stain for 1 to 15 minutes or until the body of the worm is a deep blue. Place a drop of Gray and Wess' mounting medium directly on the specimen and apply a coverslip. Allow 24 hours for the mountant to harden and the specimen to clear. The stain will fade after several weeks.

If a permenant mount is desired place stained specimens in 95% ethanol, then absolute ethanol. Clear in xylene and mount in Canada balsam.

Haptor bars stain orange to red, soft tissues blue.

Special Techniques

It is sometimes desirable to demonstrate the head organs, pharyngeal glandular masses, and the connecting ducts. This can be accomplished using Hortega's silver carbonate and the method of Arcadi (1948).

Place the specimens in concentrated ammonium hydroxide for 3 to 4 minutes. This will remove some of the formalin. Wash thoroughly in two changes of distilled water. Agitate the dishes while in the wash. Impregnate tissues with the ammoniacal silver solution for 50 seconds. Wash, with agitation, in two changes of distilled water. Reduce the specimens in 1% formalin for 30 seconds. Formalin reduces the ionic silver to a metallic state. Wash with agitation in two more changes of distilled water. Fix for a few seconds in 1% $Na_2S_2O_3$. Wash briefly in distilled water. Mount in the glycerine-gelatin medium of Van Ostern (1923) and adapted for gill parasites by Mizelle and Seamster (1939). The argentophilic areas are evident by their darkened appearance.

Nematoda

Nematodes will be treated separately in the next section.

Clearing

This step makes the specimen transparent and miscible with a resinous mountant such as Canada balsam. Several chemicals can be used as clearing agents. Three are described below.

Xylol (Dimethylbenzene): Will displace ethanol rapidly. Subject worms to it for only a short period because it causes tissue hardening.

Toluol (Methylbenzene): Does not cause shrinkage or tissue hardening as excessively as xylol.

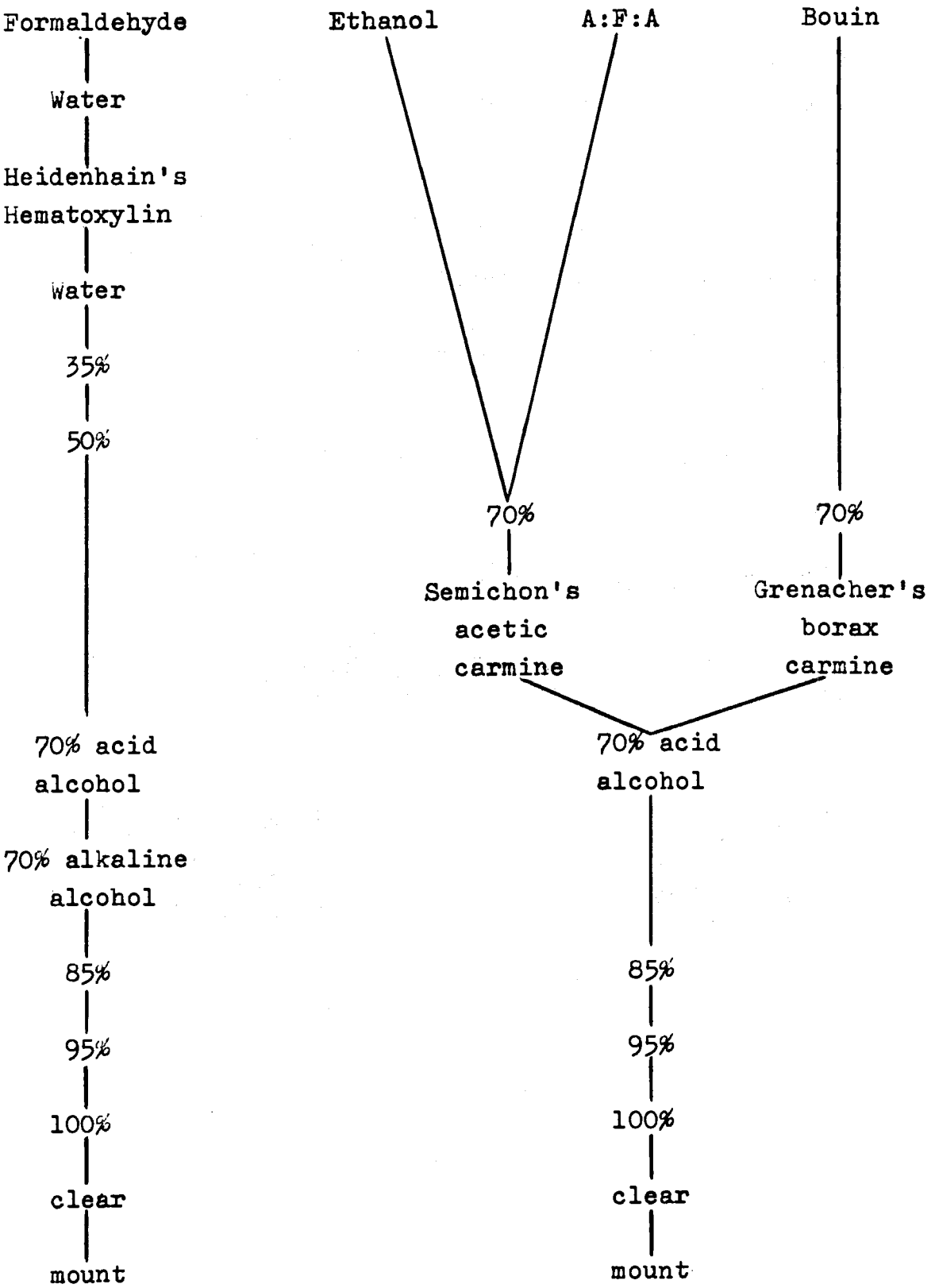
Methyl Benzoate: Will often tolerate transfering from 95% ethanol but absolute ethanol is advisable.

Benzene, methyl salicylate (wintergreen), beechwood creosote and clove oil are also used as clearing agents.

Mounting

Whole mounts do not develop maximum transparency until several months after preparation because of the time required for the mountant to diffuse through the specimen and give greater clarity (Meyer and Olsen 1975). Canada balsam and permount are two commonly used mountants. Both can be dissolved in either xylene or benzene.

Summary of Staining Procedures



% refers to ethanol concentrations.

MOUNTING HELMINTHS

Monogenea: Should be mounted with the ventral side up, and the "head" end pointing toward you (Fig. 13).

Digenea: As for Monogenea (Fig. 14).

Cestoda: Large, whole worms cannot be mounted on one slide. Short sections should be removed from the worm and mounted. The scolex is mounted with the neck and a few immature proglottids still attached. Sections containing mature and gravid proglottids should also be mounted. Mount the scolex on the right-hand-side of the slide, with the "head" nearest you. Mature proglottids are mounted in the middle, and gravid sections on the left hand side. These sections should be oriented with the cephalid portion nearest you, and the caudal portion pointing away (Fig. 15).

Acanthocephala: Mount as for Monogenea. One or two specimens can be mounted on one slide as shown in Fig. 16.

NEMATODA

There are three basic methods for mounting nematodes: water mounts, temporary mounts, and permanent mounts.

Water Mounts: Small adult nematodes and larvae can be studied alive in temporary water mounts. Details of internal structures can be determined with this procedure. Water mounts may last for several days if the edges of the coverglass are sealed with vaseline.

Procedure: Draw on a piece of paper a square which is slightly smaller than the size of the coverglass to be used (Fig. 17). Place a slide on top of the card so that the square is roughly centered (Fig. 18) and, with a small toothpick, trace the outline of the square on the slide with vaseline (Fig. 19). Now place a small drop of water, containing the nematode, in the center of a coverslip (Fig. 20). Invert the slide so that the vaseline square is underneath, and lower this onto the coverglass (Fig. 21). The vaseline should form a rim around the slide. Press the slide down until the vaseline has formed complete contact all the way around (Fig. 22).

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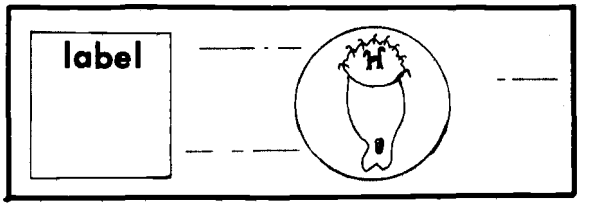

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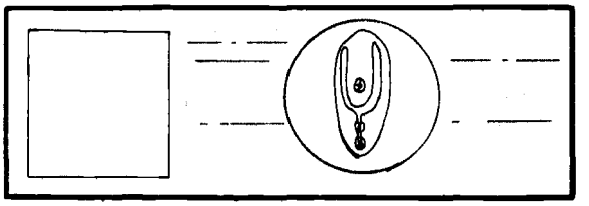

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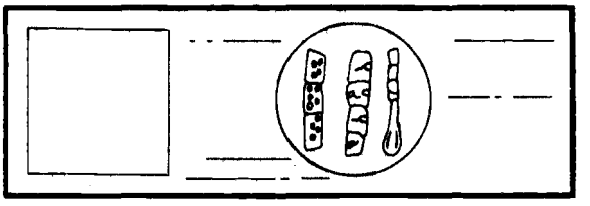

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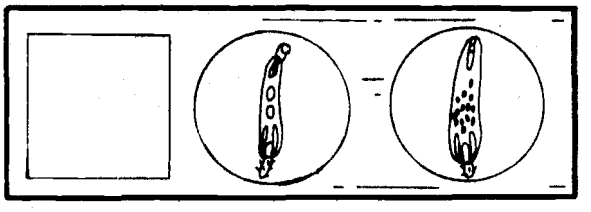
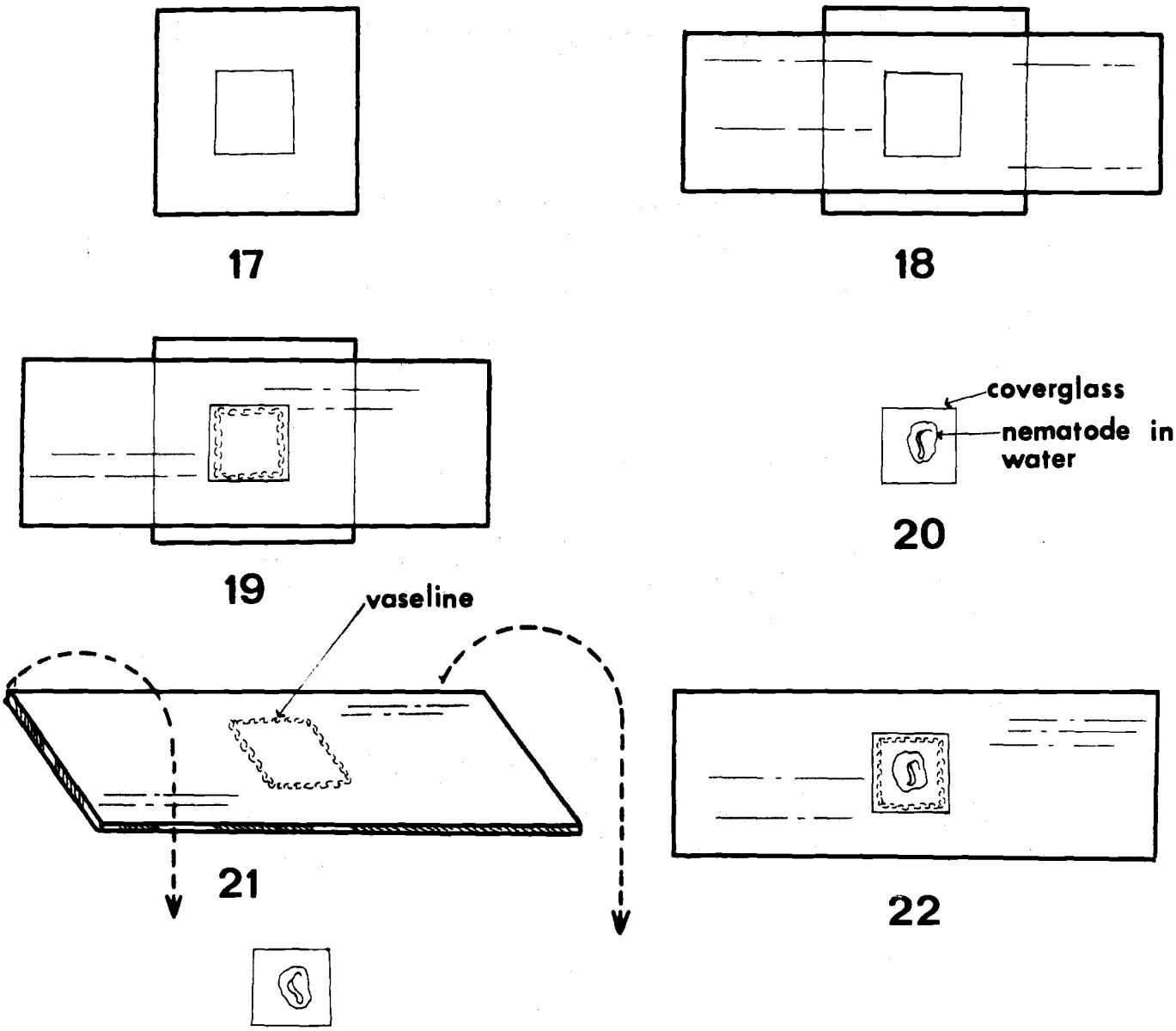


FIGS. 13 to 16. MOUNTING HELMINTHA

13. MONOGENES
14. DIGENEA
15. CESTODA
16. ACANTHOCEPHALA



FIGS. 17 to 22. PROCEDURE FOR PREPARING A WATER MOUNT

Temporary Mounts: Do not have the coverglass "glued" to the slide. This allows the specimen to be rolled under the coverglass for viewing from different positions. Worms are killed and fixed prior to examination.

Killing and Fixing

Worms should be thoroughly washed in physiological solution and cleaned or mucus and debris before fixation.

Large Nematodes: Large nematodes can be dropped into steaming acetic alcohol. This results in less shrinkage than when alcohol alone is used (Meyer and Olsen 1975). Replace this solution with 70% ethanol for storage.

Glycerine alcohol, heated to the point of steaming, may also be used as a fixative for nematodes.

Small Nematodes: Small worms may be studied alive by placing them in a drop of water under a coverglass (see Water Mounts), or mounted in pure glycerine. If glycerine is used, seal the coverglass with glyceel.

Clearing

Lactophenol: Transfer fixed worms to a slide containing a drop of lactophenol and add a coverglass. Large worms should be cleared first by placing them in a petri dish to which lactophenol has been added. When the worms clear, transfer them to a slide containing one drop of fresh lactophenol. Apply a coverglass.

Specimens left in lactophenol for a week or longer may become distorted (Meyer and Olsen 1975). If worms are to be preserved for future study, the lactophenol should be washed out through several changes of ethanol and stored in 70% ethanol.

Glycerine: Glycerine is a good mounting medium for nematodes. Transfer the specimens directly from the fixtures into a small amount of the following mixture:

ethanol, 95%	20 ml
glycerine	1 ml
water, distilled	79 ml

Place the dish containing the nematodes and the above solution on a support in a closed container containing 95% ethanol for approximately 12 hours at 35 to 40°C, or longer if the worms are large. Then remove the dish from the container and fill it with a solution of 5 ml glycerine in 95 ml of 95% ethanol. Place a lid over the dish and support it with a toothpick. Keep the container at 40°C until all of the ethanol has evaporated, leaving the worm in glycerine. Temporary or permanent mounts can be made from specimens cleared in this manner. Transfer the worms from the dish to a slide to which one drop of glycerine has been added. The amount of glycerine added should be sufficient to reach the edges of the coverslip when the coverslip has been applied.

Permanent Mounts

When permanent mounts are required drop small nematodes into 1 or 2 ml of hot 0.5% acetic acid in a well slide and then fix by transferring to a mixture of 4% formalin and 0.5% acetic acid for 24 hours (Meyer and Olsen 1975). Specimens may be stored in this fixative or processed for permanent mounts in glycerine or semi-permanent mounts in lactophenol (see Temporary Mounts). Large nematodes are fixed as per temporary mounts.

Nematodes do not require staining. Important structures show well in nematodes mounted in lactophenol or glycerine. For taxonomic purposes cleared unmounted and/or temporary mounted specimens are best.

Lactophenol or Glycerine: Place a drop of lactophenol or glycerine on a slide and place the fixed nematode in the center of the drop. Apply a coverslip. The clearing agent should not reach the edges of the coverslip.

Large nematodes may be cleared first as for temporary mounts then cleared worms transferred to the slide. Seal the edges of the coverglass with glyceel.

Canada balsam: Place nematodes previously fixed in fresh 70% ethanol. Transfer the worms slowly through 85%, 95% and then absolute ethanol. The material should be left in absolute ethanol until all water has been removed. Specimens can be left in absolute ethanol for as long as 12 hours, changing the ethanol periodically. Clear in creosote and mount in Canada balsam.

Acid fuchsin (after Stringfellow 1971): It is generally very difficult to stain whole mounts of nematodes. This is due to the relatively impermeable cuticle which impedes the movement of some dyes. The following technique has proven successful for some researchers.

Fix worms in Bouin's for 1/2 hour, then rinse excess fixative from the specimen with 70% ethanol. Now place the worms in a saturated acid fuchsin-lactophenol solution. This solution may be warmed to enhance penetration of the dye into the nematode. Rinse excess dye from the specimen with 70% ethanol. Destain in saturated picric acid-distilled water solution until no more acid fuchsin leaves the specimen. Rinse worm in 70% ethanol. Now remove picric acid and acid fuchsin from softer tissues of the nematode with saturated sodium bicarbonate-70% ethanol solution. The spicules and gubernaculum should be stained magenta (reddish-purple). Clear the specimens in 1/2 glycerine-70% ethanol (v/v) and then transfer to pure glycerine. Mount worms in glycerine and ring the coverglass with glyceel.

Methylene Blue: Nematodes can be transferred from the fixative to lactophenol or glycerine to which Methylene Blue has been added. A 0.01% or 0.0025% solution is recommended. Large nematodes should be stained in this solution first, then transferred to a slide containing a drop of fresh preparation. Seal with glyceel. Small nematodes may be mounted on the slide directly.

PARASITIC ARTHROPODS

Mites, lice, and other small arthropods can be mounted on microscope slides in Canada balsam, Gum dammar, Hoyer's solution or Berlese's fluid. Hoyer's and Berlese's are gumchloral mountants which combine fixation and mounting in one step. These solutions contain gum arabic, chloral hydrate, and glycerol, and are most useful when studying small specimens.

Parasitic copepods and isopods are frequently found attached to the skin and gills of fish. These parasites can be preserved by placing them directly in 70% ethanol. Ethanol that has been heated to the point of steaming will help prevent specimen curling.

Hoyer's Solution: Small live arthropods may be mounted directly in this solution. Specimens are fixed, cleared, and mounted in one process. The author has obtained good results with this solution on crustacea as large as Argulus spp. Specimens previously fixed in 70% ethanol can also be mounted directly in Hoyer's.

If the specimen is filled with blood, puncture it with a fine needle and place it in boiling lactic acid for a few seconds. Then transfer to a slide to which a few drops of Hoyer's has been added. Apply a coverglass.

Berlese's Fluid: Can be used in the same manner as Hoyer's. If you want to have simultaneous staining of the cuticle add a small amount of Chlorazol Black or Lignin Pink to the mounting fluid (Kabata, personal communication).

Lactic Acid: Humes and Gooding (1963) state that lactic acid is the best clearing agent for temporary mounts of whole or dissected copepods. Humes (1977) continues to use lactic acid as an aid in studying copepod morphology.

Copepods are placed live in the lactic acid or transferred to it after fixing in alcohol or formalin. Copepods may contract initially when placed in lactic acid but will regain their shape.

Permanent mounts are prepared by allowing most of the lactic acid to evaporate and then adding the preferred mountant such as Hoyer's solution or Canada balsam.

While staining is usually not desirable in lactic acid mounts, a small amount of Chlorazol Black may be added to the lactic acid. By virtue of its permanence, Chlorazol Black is superior to Lignin Pink and Cotton Blue as a copepod stain (Perkins 1956).

Chlorazol Black: Harding (1950) used a combination of KOH and Chlorazol Black to study specimens of Lernaea spp. He placed specimens in KOH (conc. not specified) to which a little dye was added. The parasites were left 24 hours in this solution.

Lernaea spp. with external growth and host tissue still attached to its surface can be cleaned off if left in 10% KOH for 2 or 3 days. The disadvantage of this technique is that eggs are destroyed, so they must be removed prior to treating with KOH.

An alternative method to the one described by Harding is to fix specimens in 70% ethanol for 24 hours, then transfer to a 1% solution of Chlorazol Black E in 70% ethanol. Stain in this solution for 10 to 20 minutes (depending on the size of the specimen and the degree of staining required), dehydrate through 85%, 95% and absolute ethanol, clear, and mount in Canada balsam.

Chitin will stain a dark blue, glycogen a pale red.

Live specimens may be placed in 10% KOH and left until they become semi-transparent. This may require several hours or days, depending on the size of the specimen. Rinse in distilled water and transfer, with a drop of water, to a slide. Using fine needles, arrange the position of the appendages, blot off most of the water but do not allow the specimen to dry. Now place 3 or 4 drops of glacial acetic acid into the specimen. The acetic acid will clear and stiffen the specimen. Remove excess acetic acid, and add Canada balsam. Apply a coverglass.

Meyer and Olsen (1975) give the following modification:

After the specimens are washed in distilled water, transfer to acidulated 70% ethanol, dehydrate, clear, and mount in Canada balsam.

Large Arthropods: Place large specimens in 10% KOH and distilled water as described above. Transfer a specimen to a slide and arrange the appendages. Add several drops of glacial acetic acid, bracket the specimen with supports. An "O" ring cut in half is good. Add a second slide to the top, tie the two slides together and place the "sandwich" in a container of acetone for 2 or 3 days. Separate the slides, add a few drops of clearing agent, drain off the clearing solution, add Canada balsam and a coverglass.

Sodium hypochlorite:(ClNaO): Johnson (1969) employed sodium hypochlorite to dissolve the soft tissues of Crustacea. (Ergasilus, Lernaea, Argulus, and Achtheres) to render taxonomic features more visible.

Specimens are fixed in formalin then placed in 5% sodium hyperchlorite (= Clorox) for 1 to 3 minutes. After this time wash in distilled water, transfer to an aqueous stain (eg. Chlorazol Black E), and wash again in distilled water. Mount in glycerin jelly.

It should be noted that some sensory setae may be lost with this method. Large specimens should be transferred to glycerin-alcohol and the alcohol allowed to evaporate slowly. This will help prevent their collapse.

RECLAIMING DRIED SPECIMENS

Preservatives may evaporate leaving stored specimens to dry. Dried specimens can often be made soft and pliable again if treated with an aqueous solution of trisodium phosphate. Van Cleave and Ross (1947) achieved good results using a 0.25 to 0.50% trisodium phosphate solution in distilled water. One or more hours may be needed to soften specimens. Warming the solution will speed softening. When worms have regained their soft texture wash in several changes of distilled water. Transfer to 35%, 50% and then 70% ethanol for storage.

Appendix

Acetic Alcohol (Meyer and Olsen, 1957)

Acetic acid, glacial	20 ml
Ethanol, 70%	60 ml

Acid Alcohol, 2%

Ethanol, 70%	98 ml
HCl, commercial	2 ml

Acid Fuchsin-lactophenol (Stringfellow, 1971)

Saturate lactophenol (see Appendix) with Acid Fuchsin.

Acidulated Methyl Green (Jahn and Jahn, 1949)

Distilled water	99 ml
Acetic acid	1 ml
Methyl green	0.5 g

A.F.A. (=F.A.A.) (Meyer and Olsen, 1975)

Ethanol, 85%	85 ml
Formalin, (40% HCHO)	10 ml
Acetic acid, glacial	5 ml

Alcoholic Bouin (=Duboscq-Brasil) (Grimstone and Skaer, 1972)

Picric acid	1 g
Acetic acid, glacial	15 ml
Formalin (40% HCHO)	60 ml
Ethanol, 80%	150 ml

Alkaline Alcohol (Meyer and Olsen, 1975)

Ethanol, 70%	500 ml
Ammonium hydroxide, conc.	0.5 ml

Berlese Fluid (Jaques, 1947)

Distilled water	20 ml
Glycerine	6 ml
Gum Arabic (=Acacia)	12 g
Chloral hydrate	20 g

Live specimens may be mounted directly in this medium. It is best to put them in water first to prevent air bubbles from forming. Alcoholic specimens should be washed thoroughly before mounting.

Bouin's Fixative (Grimstone and Skaer, 1972)

Picric acid, saturated aqueous solution	75 ml
Formalin (40% HCHO)	25 ml
Acetic acid, glacial	5 ml

D'Antoni's Iodine-Modified (Spencer and Monroe, 1961)

Potassium iodide	1.0 g
Iodine, powdered crystals	1.5 g
Distilled water	100 ml

See Lugol's solution.

Formaldehyde, 4% Buffered (=10% formalin)

Distilled water	900 ml
Formalin (40% HCHO)	100 ml
$NaH_2PO_4 \cdot H_2O$	4.0 g
$Na_2HPO_4 \cdot 2H_2O$	6.5 g

Giemsa's Stain (Lillie, 1969)

Azure A eosinate	0.5 g
Azure B eosinate	2.5 g
Methylene Blue eosinate	2.0 g
Methylene Blue Chloride	1.0 g
Glycerine	375 ml
Methyl alcohol, reagent	375 ml

To prepare the staining fluid, 0.5 g of the powder dye is dissolved in 33 ml of glycerine, then 33 ml of methyl alcohol is added and the solution allowed to stand for 24 hours before use.

Meyer and Olsen, 1975) give the following procedure for preparing Giemsa's stain:

Stock Solution:

Giemsa's powder (commercial source)	1 g
Glycerine	66 ml
Methyl alcohol, absolute	66 ml

Working Solution:

Giemsa stock solution	1 ml
Distilled water	20 ml

or

Phosphate buffer (approx. 0.1 M, pH 6.5)	20 ml

Note: If Giemsa stain is prepared from a powder, methanol which is free of acetic acid must be used (Baker, 1970). That is, the solvent must be neutral. The presence of acetic acid lowers the pH of the solution when the methanolic solution is diluted with water. Methanol of reagent grade is satiffactory.

Glycerine Alcohol, 5%

Ethanol, 70%	95 ml
Glycerine	5 ml

Glycerine jelly (Grimstone and Skaer, 1972)

Gelatin	10 g
Glycerine	70 ml
Phenol	0.25 g
Distilled water	60 ml

Soak the gelatin in water for 2 hours, then add the other constituents and heat on a water bath, stirring continuously until the mixture is smooth. Store in a refigerator. Apply melted and use a warm slide and coverslip.

For delicate specimens it may be advantageous to transfer from water via 50% glycerine before going to glycerine jelly.

Glycerine jelly (Meyer and Olsen, 1975)

Gelatin	7 g
Distilled water	40 ml
Phenol	1 g
Glycerine	50 ml

Soak the gelatin in water for 30 minutes then melt in a warm water bath and filter through several layers of cheese cloth previously moistened with hot water. Finally dissolve the phenol in the glycerine and add to the gelatin. Stir until the mixture is homogeneous.

Glycerine Alcohol, 5%

Ethanol, 70%	95 ml
Glycerine	5 ml

Grenacher's Alcoholic-Borax-Carmine (Fernando, et al, 1972)

Carmine, powder	3 g
Borax	4 g
Distilled water	100 ml
Ethanol, 70%	100 ml

Dissolve borax in water, add carmine, and boil for 30 minutes. Add an equal volume of alcohol and allow to stand until cool, then filter.

Gabb and Latchem, 1968 give the following modification:

Carmine, powder	2 g
Borax	8 g
Distilled water	200 ml
Ethanol, 70%	200 ml

Boil together the carmine, borax, and distilled water for 30 minutes. Cool and make up to 200 ml before adding 200 ml 70% ethanol.

Harris' Hematoxylin (Meyer and Olsen, 1975)

Hematoxylin crystals	2 g
Ethanol, absolute	20 ml
Distilled water	400 ml
Ammonia alum (aluminum ammonium sulphate)	40 g
Mercuric oxide	1 g

Dissolve the hematoxylin crystals in the ethanol. Heat the distilled water and add ammonium alum. When dissolved and still warm, add, while stirring, the hematoxylin solution. Boil for a few minutes then add the mercuric oxide to ripen the solution, which should now be purple. After a minute more of boiling, the flask is plunged into cold water.

Heidenhain's Iron Hematoxylin (Spencer and Monroe, 1961)

Solution 1 (Mordant)

Hematoxylin	10 g
Ethanol, absolute	1000 ml

Solution 2

Ferrous ammonium sulfate	10 g
Ferric ammonium sulfate	10 g
HCL, conc.	10 ml
Distilled water	1000 ml

Allow solution 1 to sit in the light for one week. To prepare the working solution of iron hematoxylin, mix 15 ml each of solutions 1 and 2. The working solution will last for about 7 days, and solution 2 for 6 months.

Hortega's Solution-Weak (Arcadi, 1948)

$AgNO_3$, 10%	5 ml
Na_2CO_3, 5%	20 ml
Ammonium hydroxide, conc. .... use just enough to dissolve the silver carbonate precipitate.	
Distilled water	75 ml

Hoyer's Solution

Distilled water	50 ml
Gum arabic flakes	30 g
Chloral hydrate	200 g
Glycerine	20 ml

Mix the ingredients in the order given. It may be necessary to let the solution stand at room temperature for one or two days to completely dissolve the gum arabic.

Iodine-eosine Stain (Noble and Noble, 1962)

Distilled water	100 ml
KI	5.0 g
Sodium chloride	0.8 g

Saturate the above solution with iodine crystals, above 0.2 g, and mix the resulting solution with an equal volume of saturated aqueous eosin (Yellow aq. eosine).

Lactophenol (Meyer and Olsen, 1975)

Distilled water	20 ml
Glycerine	40 ml
Lactic acid	20 ml
Phenol, melted crystals	20 ml

Keep this solution in a brown bottle.

Lugol's Solution (Meyer and Olsen, 1975)

KI	1 g
I	0.5 g
Distilled water	50 ml

Keep in a brown bottle.

Lugol's Solution (Spencer and Monroe, 1961)

KI	10 g
Iodine crystals	5 g
Distilled water	100 ml

Dissolve the KI in the distilled water. Iodine crystals are added to saturate the solution. Some undissolved iodine crystals should be present. Store in a brown bottle. The stock solution is good as long as iodine crystals are present.

Mayer's Albumen (Noble and Noble, 1962)

Egg white, fresh	50 ml
Glycerine	50 ml
Sodium salicylate	1 g

Methylene Blue Lactophenol Solution

Methylene blue, crystals	0.01 g
Lactophenol (see above)	100 ml

Noland's Solution (Jahn and Jahn, 1949)

Phenol, sat. aq. solution	80 ml
Formalin (40% HCHO)	20 ml
Glycerine	4 ml
Gentian violet	20 mgms

Salines

	Amphibian[2]	Birds/Mammals[2]	F.W. Teleosts[1]	Marine[1]
NaCl	4.24	8.0	5.50	13.5
KCl	0.148	0.2	0.14	0.60
$CaCl_2$	-----	0.2	0.12	0.25
$MgCl_2$	-----	0.05	----	0.35
$MgSO_4$	0.146	----	----	----
$NaHCO_3$	2.1	1.0	----	----
Na_2HPO_4	0.356	----	----	----
NaH_2PO_4	-----	0.04	----	----
KH_2PO_4	0.068	----	----	----
Na_2SO_4	0.092	----	----	----
Ca-gluconate	0.4	----	----	----
Glucose	4.684	1.0	----	----

Concentrations are given in grams per litre. When preparing the salines each salt should be added in the order given in the table and each should be fully dissolved before the next is added.

1 From Gabb and Latchem, 1968.

2 From Lockwood, 1961.

Schaudinn's Solution (Spencer and Monroe, 1961)

Stock solution

Mercuric chloride	11.4 g
Distilled water	200 ml
Ethyl alcohol, 95%	100 ml

Make a saturated solution of mercuric chloride by adding it to the distilled water. Add the ethanol to the saturated solution.

Working solution

Add 3 ml of glacial acetic acid to 47 ml of the stock solution. This will be good for about two weeks.

Semichon's Acetic-Carmine (Meyer and Olsen, 1975)

Acetic acid, glacial	100 ml
Distilled water	100 ml
Carmine, in excess .....about	1.5 g

Mix distilled water and acetic acid in an Erlenmeyer flask, add Carmine. Heat in boiling water bath for 15 minutes, then cool the flask in cold water and filter the contents. This stock stain should be diluted with approximately 2 parts of 70% ethanol before use.

Wright's Stain (Lillie, 1969)

Sodium bicarbonate	0.5 g
Distilled water	100 ml
Methylene blue	0.9 g
Eosine Y	0.5 g
Distilled water	500 ml

Add the sodium bicarbonate to 100 ml of distilled water and heat gently. Slowly add the Methylene Blue. Put in a boiling water bath for 30 to 45 minutes, shaking occassionally, then cool. Dissolve the Eosin Y in 500 ml of distilled water

and filter the Methylene Blue solution into it. Shake several times during the day and let stand at room temperature overnight. The following day, without shaking the flask, filter. Let the precipitate dry on paper in an oven at 37°C. This powder is dissolved in methyl alcohol (1:600) and allowed to stand for 5 to 7 days before use.

This solution can also be purchased ready made from many biological supply houses, and is probably more convenient for most laboratories.

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