DOCUMENT RESUME

ED 200 430 SE 034 564

TITLE	Medical Service Clinical Laboratory Procedures--Parasitology.
INSTITUTION	Department of the Air Force, Washington, D.C.; Department of the Army, Washington, D.C.
REPORT NO	AFM-160-48; TM-8-227-2
PUB DATE	1 Aug 74
NOTE	200p.; Contains colored pictures and small print which may not reproduce well.
AVAILABLE FROM	Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402 (Stock No. 008-070-00351-1; $7.00).
EDRS PRICE	MF01/PC08 Plus Postage.
DESCRIPTORS	*Allied Health Occupations; *Armed Forces; Biology; Government Publications; Health Occupations; Higher Education; Instructional Materials; *Laboratory Manuals; *Laboratory Procedures; Medical Education; *Medical Technologists; *Resource Materials; Science Education
IDENTIFIERS	*Parasitology

ABSTRACT

This manual presents techniques for the collection and examination of specimens in the diagnosis of parasitic disease and in field surveys conducted to determine the extent of parasitic infections in human and animal populations. It discusses areas in which parasites are most likely to be found and the relationships of parasites, vectors, and environment. Methods of preserving, staining, and mounting specimens are included. Definitive characteristics useful in differentiation and identification of parasites are given. (Author/CS)

DEPARTMENTS OF THE AIR FORCE
AND THE ARMY
Washington DC 20330

AF MANUAL 160-48/TM8-227-2

1 August 1974

Medical Service

CLINICAL LABORATORY PROCEDURES—PARASITOLOGY

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Supersedes AFM 160-48, 1 October 1960 and TM8-227-2, 24 August 1961. (For summary of revised, deleted, or added material, see signature page.)
OPR: SGPSE (Prepared by SHCS/MST)
DISTRIBUTION: (See signature page)

For sale by the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402

Stock No. 008-070-00351-1/Catalog No. D 301.7:160-48/3

PART ONE
PARASITES OF THE GASTROINTESTINAL AND GENITOURINARY TRACTS

Chapter 1
ROUTINE SPECIMEN PROCESSING

1-1. Introduction:

a. Accurate clinical diagnosis of parasitic disease is difficult, making laboratory confirmation very desirable. Demonstrating the diagnostic stage or stages of the parasite by directly examining specimens is the most reliable method of establishing the diagnosis of most parasitic infections. Recently, indirect (serologic) methods have become available for a few infections in which the organism is not readily demonstrated, including trichinosis, echinococcosis, extraintestinal amebiasis, chronic schistosomiasis, toxoplasmosis, trypanosomiasis, and leishmaniasis.

b. This manual is concerned with direct laboratory procedures used in recovery and identification of human parasites and related species. Portions of the procedures can be cookbooked. The difficult part, microscopic examination, cannot. Successful microscopy varies directly with the degree of intensive training and experience of the examiner. A laboratory's success in recovering and identifying parasites invariably depends on the following factors:

(1) Having adequate personnel who are trained to properly examine specimens and make accurate identification.

(2) Availability of adequate laboratory facilities, especially a good microscope with dark-field and phase contrast if possible.

(3) The collection of satisfactory specimens.

c. The primary purpose of this manual is to present a ready reference for the laboratory technician who is required to perform parasitologic examinations. Although many good procedures are not included, it does contain representative procedures that are reliable and that will cover most circumstances.

1-2. Determining the Type of Procedure Required:

a. Many procedures have been devised to facilitate the laboratory confirmation of intestinal parasites. Each laboratory has to establish a regimen based upon local conditions and needs. In general, the more types of examinations included in the routine, the greater the number of infections discovered. However, from a practical standpoint, the laboratory has to decide at what point the increase in positive findings warrants the time and expense of performing the additional procedures. It is best to select certain procedures for routine use and to reserve others for special purposes.

b. To render satisfactory service, a laboratory must examine permanently stained smears and material from a concentration procedure. Concentration procedures greatly increase the chance of finding organisms in light infections. The examination of stained slides increases the chances of locating organisms that are missed on wet preparations, and it enables you to study organisms in greater detail.

1-3. Regimens for Hospital and Clinic Laboratories. Establish a routine that insures the delivery of the specimens to the laboratory as soon as possible after collection. The following regimen is provided as a guide.

a. **Normally Passed Fecal Specimens:**

(1) Upon receipt of the specimen, prepare one or two smears and place them in Schaudinn's fixative or, if preferred, emulsify a portion of the feces in PVA fixative. Smears prepared in such a manner will be available for permanent staining if they are needed. They can be left in Schaudinn's fixative for a day or two without harm.

(2) Examine the fresh specimen macroscopically. If the specimen is soft, loose, or watery, begin the microscopic examination as soon as possible. Formed specimens may be left for a few hours or even overnight without deteriora-

tion, but for best results, they should also be examined as soon as possible.

(3) Prepare stained and unstained direct wet mounts. If mucus is present, include some of the material in the preparations. Examine the unstained saline preparation first. Protozoan cysts that are found in the unstained smear can frequently be identified in the temporarily stained smear. If trophozoites are present, or if there is any question as to the identity of any cysts found, stain and examine permanent smears. Estimate the numbers of eggs of each helminth species on the wet smear.

(4) Perform a concentration procedure on all stool specimens except where only trophozoites are being looked for. In cases of suspected helminthiasis, concentrations should be performed on all specimens. The MIFC procedure is one of the most effective for routine laboratory use.

(5) If organisms suggestive of *Entamoeba histolytica* or any questionable objects are seen in the wet mounts, stain and examine one of the fixed smears. Permanently stained smears should also be examined if the specimen contains abnormal elements (blood, mucus, or other material) or is of soft, loose, or watery consistency. The trichome and chlorazol black stains are especially useful for hospital and clinic laboratories. When chlorazol black is used, the smears are prepared and placed directly in the staining solution while wet because it contains both the fixative and the stain. Ideally, permanent stains should be made routinely.

(6) Cultures for protozoa need not be employed as a routine part of the fecal examination. However, when trophozoites are found that cannot be identified, the specimen should be cultured. Cultures are also helpful in persistent cases of suspected amebiasis when no organisms can be found. When organisms are cultured, mix the culture sediment with PVA fixative, and stain for identification.

b. **Followup After Treatment.** The disappearance of symptoms in a patient with a parasitic infection is not sufficient evidence of a "cure." After the patient has been treated, specimens have to be examined to determine the effectiveness of therapy.

(1) Patients treated for protozoan infections frequently stop shedding organisms for several weeks even though the infection has not been eradicated. Therefore, the first post-treatment specimen need not be collected until 4 weeks after therapy unless symptoms reappear earlier. Additional examinations after 3 and 6 months are recommended to detect later relapses.

(2) In most helminth infections, post-treatment examinations can be made 1 to 2 weeks after therapy. However, for the post-treatment followup of pinworm infections three consecutive daily preparations should be examined 7 to 10 days after therapy. Reinfection is common and, if specimens become positive after being negative for a month, the patient has probably reacquired the parasite.

(3) Tapeworm infections require considerable post-treatment follow-up. After treatment, the patient is purged to evacuate the worm, which is usually passed intact. Examine the material that is passed to make certain that the worm's head or scolex has been passed because a cure is not obtained as long as the scolex persists. Additional followup examinations should be made at 3-month and 6-month intervals to make certain that a worm has not been regenerated by a missed scolex.

c. **Post-cathartic Specimens.** Concentrate the first specimen from a series of post-cathartic specimens. Examine temporary wet mounts of all specimens from the series. Prepare permanently stained smears from any specimen containing protozoa or questionable structures. Prepare a permanently stained smear from at least one of the soft specimens even if no organisms are found in the direct wet mounts or the concentration. Examine any flecks of mucus either as wet mounts or stained slides. If the case is sufficiently suspicious for the patient to be purged, one or more of the specimens should be cultured. After a patient has been purged, several days must pass before the protozoan organisms can redevelop to a demonstrable level so there is no point in collecting any additional stools for at least a week.

d. **Anal Swabs, For Pinworms.** Handle the specimens according to the type of collection preparation used (cellulose tape slide or vaseline-paraffin swab). Either is satisfactory. It is recommended that specimens for pinworm

examinations be collected on 3 consecutive mornings.

e. **Urine.** Urine requires no special methods of examination to reveal any protozoa or helminths present. Simply centrifuge the specimen and search the sediment microscopically.

f. **Sputum.** Make a direct wet smear of any blood-tinged material. Look carefully for trophozoites if extraintestinal amebiasis is suspected. Fix a smear with PVA or Schaudinn's fixative for permanent staining when suspicious objects are observed on the smear. Concentrate helminth ova in sputum specimens by mixing the material with 3 percent sodium hydroxide and centrifuging.

g. **Vaginal and Urethral Material:**

(1) Vaginal and urethral material is frequently examined for *Trichomonas vaginalis* on a STAT basis in hospitals and clinics. The specimen should be collected with cotton swabs and placed in saline. Have it delivered to the laboratory as soon as possible after collection. Immediately after the specimen arrives, prepare a wet smear and examine for motile trichomonads.

(2) It is sometimes more convenient to culture vaginal and urethral material for trichomonads than it is to examine direct mounts. A culture is also considered to be a more sensitive procedure in light infections. Inoculate the media as soon as possible after the specimen is collected.

(3) Dried vaginal and urethral smears are generally unacceptable for detecting trichomonads due to severe distortion of the parasites on drying. However, if flagellated protozoans can be seen, report them, because they are most likely *T. vaginalis*. Viewing dried slides may be aided by staining with stains such as Leishman's, Giemsa's, Wright's or Iron-hematoxylin.

(4) When it is impossible to examine a direct wet preparation or to culture the specimen within 30 to 45 minutes after collection, fix a wet smear as soon as possible in Schaudinn's fixative or preserve the material in PVA fixative. Then stain the specimens when time permits.

h. **Duodenal Drainage.** Examine direct smears of the duodenal specimen, and if there is sufficient material available, centrifuge it to concentrate the elements. For protozoans, preserve specimens that cannot be examined immediately in PVA-fixative. Use formalin to preserve helminths.

i. **Sigmoidoscopic Material.** Set up a microscope in a room next to the room in which the sigmoidoscopy is performed. Prepare direct saline smears immediately after the specimen is collected. If organisms or suspected organisms are found, add a drop of Quensel's stain or buffered methylene blue solution to stain the internal structures. When sigmoidoscopy is performed away from laboratory facilties, have the material placed in a very small quantity of saline and delivered at once to the laboratory. The specimen should be kept warm during transit. If too much saline has been added, gently centrifuge (about 1,000 rpm for 1 minute) and make wet mounts from the sediment. In the event organisms are observed, add two or three drops of PVA fixative to the saline mount, spread into a smear, and stain with a permanent stain. When the specimen cannot be sent to the laboratory immediately, have it put into PVA fixative for staining at a later time.

j. **Aspirated Material.** Examine material aspirated from abscesses or other lesions as soon as possible after collection. Such specimens are usually submitted for the diagnosis of extraintestinal amebiasis. Only the trophozoite stage can be expected to be present. Examine the specimens by the routine procedures used for trophozoites in feces. Begin with wet saline mounts, and if trophozoites are found, add temporary stains such as Quensel's or buffered methylene blue to confirm the findings. Make permanent stains by the same methods used for fecal preparations. If the aspirated material is watery, mix some of it with PVA fixative before preparing smears. If the material is coagulated or very viscous, treat it with streptodornase before making the smears. When possible, culture the material for protozoa.

k. **Biopsied Material.** Biopsied material can be examined by several methods: sectioning and staining, compression, teasing, and digestion. The methods used depend upon the type of tissue and the organisms suspected. (See section on tissue parasites.)

1-4. Regimens for Referral Laboratories:

a. **Fecal Specimens:**

(1) Central diagnostic or referral laboratories have to depend upon specimens delivered by carrier or by mail. Therefore, provide the contributors using your services with shipping kits.

(2) Furnish the contributors two vials (one containing 5% or 10% formalin and the second PVA fixative). Include illustrated direction sheets (Figure 1-1) with the kits to insure that the proper collecting and shipping procedures are followed. Label the PVA fixative containers with the appropriate expiration date (2 months is a practical time limit).

(3) When the specimen arrives at the laboratory, examine a direct wet mount of the formalin-preserved material. Then prepare a formalin-ether concentrate on the feces preserved in formalin, and make permanently stained films from the portion preserved in PVA fixative. Examine the wet mounts for cysts, eggs, and larvae, and search the stained films for trophozoites and cysts.

b. **Other Specimens.** The most common type of specimen sent to referral laboratories is feces, but occasionally anal swabs, vaginal exudates, aspirates, and other materials need to be sent. Most of the problems with such specimens arise during the collection and handling before examination. Detailed instructions for collection and examination of such specimens are given in other sections. However, make sure that *formalin* is used for urine or sputum that is to be examined for helminth eggs and that *PVA-fixative* is used for urine, sputum, vaginal material, or urethral exudate to be examined for protozoa.

1-5. Instructions for Field Surveys:

a. Surveys to determine the prevalence of intestinal parasites in general require the use of procedures that will recover all stages of parasites. However, keep the routine limited enough to be practical. The best choices of specimens include formalin-preserved and PVA-preserved specimens.

b. Preserve the specimens in the field as they are collected, and when possible send them to a central laboratory to be examined. Existing conditions may require that more of the work be done in the field. In such a case, you can do formalin-ether concentrations on the spot, and the portion of the specimens preserved in PVA fixative can be examined later in the central laboratory. On other occasions you can concentrate the specimen locally and ship the sediments in formalin to the laboratory for examination. Also, the PVA-fixed specimens could be smeared, dried, and shipped to the laboratory for later staining and examination.

c. When laboratory support is limited, restrict the number of stained PVA films examined. Prepare and examine them only in cases where suspicious objects are found in the concentrations or the specimens are very soft or contain blood or mucus.

d. Surveys conducted to determine only the prevalence of intestinal helminth infections present fewer problems than surveys for parasites in general. Specimens need not be prepared for permanent staining. In surveys where the specimens are to be sent to a central laboratory for examination, the best all-around preservative is probably formalin. At the laboratory the formalin-ether concentration procedure can be performed on the formalized specimen. In surveys where the specimens are to be examined locally a good alternative procedure to the formalin-ether concentration is the cellophane-covered thick smear. The only elaborate equipment necessary for the procedure is a microscope, and the only supplies needed are the treated cellophane strips and glass slides. The procedure takes little time, is very effective for helminth eggs and larvae, and is no more difficult to perform than a direct wet smear.

e. Surveys are a prime test of ingenuity and patience. Always keep the purpose of the survey in mind; however, it is often necessary to make compromises when operating under field conditions. Be flexible and try to keep the routine simple. Do not ask the population that is to be surveyed to follow long, detailed instructions. It will not work!

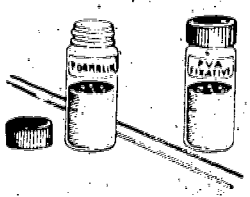
1-6. Reporting the Results of Parasitologic Examinations:

a. Report all parasites by their proper scientific names. All helminths are considered pathogenic or potentially so, but certain of the intestinal protozoa are commensals or, at least,

nonpathogens. The presence of commensals indicates that the patient has ingested material contaminated with feces. Therefore, it stands to reason that the individual is quite likely to also be infected with a pathogen, and additional specimens should be examined.

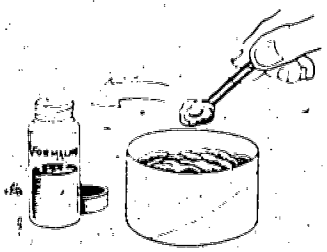
b. When protozoa, especially amebae, cannot be specifically identified, do not give a "maybe" result. A report of "organisms resembling *Entamoeba histolytica*" might be interpreted as a final identification of the organism and could lead to the needless treatment of the patient. If the organism cannot be specifically identified, report as "unidentified amebae found," "unidentified flagellates found," or "unidentified eggs or larvae found." Additional specimens can then be examined before the patient is treated. Do not report examinations as "negative." Report "no parasites found" if none are found. If additional laboratory procedures had been used or if some other person had examined the specimens, organisms might possibly have been found.

NOTE BOTH SOFT AND *FORMED* SPECIMENS SHOULD BE SUBMITTED BY THIS METHOD
SPECIMENS MUST BE *FRESH* WHEN PLACED IN VIALS

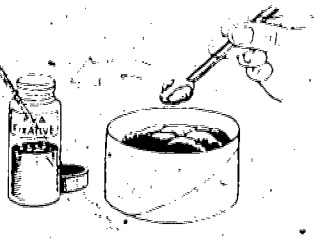


1 THE KIT CONSISTS OF TWO GLASS VIALS (ONE WITH 5% FORMALIN AND ONE WITH PVA FIXATIVE) AND TWO APPLICATOR STICKS

2 THE STOOL SHOULD BE PASSED INTO A DRY CONTAINER URINE SHOULD NOT BE PASSED INTO THE SAME CONTAINER

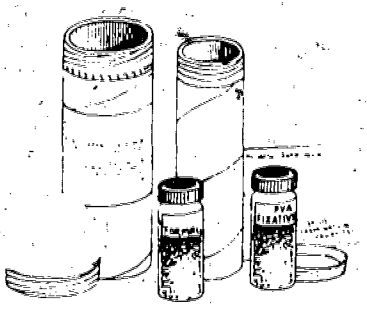


3 USING APPLICATOR STICKS, PLACE A QUANTITY OF THE STOOL (ABOUT THE DIAMETER OF A QUARTER) INTO THE VIAL CONTAINING FORMALIN. SCREW CAP ON TIGHTLY



4 PLACE A SIMILAR QUANTITY INTO THE VIAL CONTAINING THE PVA-FIXATIVE

5 THOROUGHLY BREAK UP SPECIMEN IN THE PVA FIXATIVE, USING APPLICATOR STICKS. SCREW-CAP ON TIGHTLY AND SHAKE VIGOROUSLY



6 PACK THE TWO VIALS SO AS TO PROTECT AGAINST BREAKAGE, ENCLOSE APPROPRIATE IDENTIFICATION, AND MAIL OR DELIVER TO YOUR PUBLIC HEALTH LABORATORY

Figure 1-1. Stool Specimen Handling Directions. (From Melvin and Brooke, 1969)

Chapter 2
COLLECTION

2-1. Factors to Consider:

a. The diagnostic stages of intestinal parasites are eggs and larvae of helminths and protozoan trophozoites and cysts. Specimens obtained for diagnosis should be collected and handled so that any of the above forms will reach the laboratory in an identifiable condition. Reliable diagnoses of parasitic diseases are dependent upon satisfactory specimens; therefore, inadequate, old, or poorly preserved materials make microscopic examinations as well as macroscopic examinations of little value and can lead to erroneous conclusions.

b. It is impractical to attempt to find, or to rule out, parasitic infections of all possible types. Usually, clinical observations and a history of possible exposure to infection suggest particular parasites. Such factors lead to the choice of particular specimens to be collected for examination. Intestinal parasites are most commonly diagnosed in the laboratory when feces are examined; but other specimens, such as sputum, urine, aspirates, tissue scrapings, and biopsy material, are sometimes the best choice.

2-2. General Rules for Collecting Fecal Specimens. The collection of fecal specimens is usually a nuisance to the patient and an unpleasant task for corpsmen and others in attendance. Although unpleasant, fecal examinations are necessary to diagnose many parasitic infections of the intestine, liver, lungs, and blood. Therefore, it is important to make the collection of fecal material as convenient and as simple as possible. It is up to the laboratory to provide adequate instructions and proper containers for collecting the specimens to insure that satisfactory material will be received for examination.

a. **Containers.** Disposable, leak-proof containers are recommended for collecting fecal specimens. These containers should have a capacity of about half a pint, wide mouths, and tightly fitting lids. Various plastic and waxed-paper containers have been used.

b. **Labels.** Identification labels should be placed on the side of each carton. Minimum requirements for the labels include spaces for the patient's name, date of collection, and hour of passage. Time of passage is important because trophozoites soon die outside the body. Therefore, the time of passage must be noted to prevent old specimens from being erroneously reported as negative.

c. **Instructions.** Printed instructions should be a basic part of a collection kit provided by the laboratory. Instruct the patient to defecate directly into the carton or on a clean sheet of paper and transfer a portion of the specimen to the carton. In the hospital or clinic, instruct the individual responsible for the collection of a specimen just as carefully as you instruct the patients because he probably has little or no knowledge of the correct procedures. It is permissible to collect feces from a bed pan, but care must be taken to insure that it is not contaminated with urine. Urine distorts certain types of eggs and cysts and causes many trophozoites to rupture. Water will also destroy trophozoites and may allow some helminth eggs (schistosomes) to hatch and be missed in the examination. Therefore, you should take care to prevent specimens from being collected from toilet bowls. Instruct patients not to defecate on soil because free-living larvae, plant nematodes, and other contaminants can cause erroneous reports to be sent out. In very cold climates, caution patients to protect fecal specimens they collect themselves from low temperature because freezing and thawing destroys protozoan cysts and trophozoites.

2-3. Types of Fecal Specimens:

a. **Normally Passed Feces:**

(1) In general, normally passed stools are formed, mushy, or both. Such stools can be expected to contain helminth eggs and larvae and protozoan cysts, but not usually protozoan trophozoites. When received, it is generally not necessary to immediately examine the specimen since the eggs, larvae, and cysts will remain practically unchanged for several hours. They

can be examined at the convenience of the laboratory sometime during the day. If examination must be postponed to the following day, the stool should be kept in the refrigerator to retard degenerative changes.

(2) Usually, the only time that it is necessary to immediately examine a fresh stool is when identification of *Entamoeba histolytica* has to be made on the trophozoite stage, e.g., in cases of dysentery when the patient is not expected to pass stools containing cysts. A fresh stool in this case may contain trophozoites in active motility, and they will not survive for more than a few hours. Of course, stools *must* be examined while fresh if an attempt is made to recover and identify the trophozoite stages of protozoans and any non-cyst forming protozoans.

(3) Certain drugs and compounds make a stool specimen unsatisfactory for examination. Antidiarrheal compounds, antibiotics, antacids, mineral oil, bismuth, and barium should not be administered before feces are collected for parasitologic examination. Feces should not be collected for parasite examinations for 7 to 10 days after barium or bismuth have been given because crystals and particles of these compounds will interfere with examination. Antibiotics often cause a temporary decrease or absence of organisms in the stools, making reliable recovery impossible for 2 to 3 weeks. Mineral oil appears in the feces as tiny refractile globules that interfere with examination.

b. **Purged Specimens:**

(1) Intestinal protozoa, including cyst-formers and some helminths, especially *Strongyloides* larvae, are more likely to be found in mushy rather than in formed stools. Consequently, purgation increases the possibility of finding some organisms. Keeping this in mind, the laboratory may suggest that a cathartic be administered when a patient passes only firm formed stools and yet there is some special indication for a most thorough search for such organisms.

(2) In administering a cathartic for these purposes, it is important to remember that the object is to produce a mushy and not a watery stool. Saline cathartics, such as sodium sulfate or buffered phospho-soda should be recommended; also, cascara sagrada in moderate dosage is one of the best. Castor oil, mineral oil, bismuth, or magnesia compounds are unsatisfactory because they obscure the organisms.

2-4. Collecting Multiple Fecal Specimens:

a. The passage of certain parasites from the host is definitely intermittent; therefore, the possibility of finding organisms is increased greatly by examining two or more specimens. In general, nematodes such as *Ascaris lumbricoides*, hookworm, and *Trichuris trichiura* shed eggs more or less constantly and can be detected daily in feces. Protozoa are passed irregularly, and the eggs of certain helminths, particularly schistosomes and *Diphyllobothrium latum*, are produced irregularly. The proglottids of *Taenia* species are also passed at intervals.

b. Due to the intermittent passage of certain parasites and the limitations of diagnostic procedures, only one-third to one-half of the species present are detected in a single fecal specimen. Three normally passed stool specimens, spaced at 2- to 3-day intervals, should be examined before other procedures such as catharsis or sigmoidoscopy are considered. Although specimens obtained by purgation frequently yield more trophozoites, they probably have little advantage over a series of normally passed specimens. Furthermore, normally passed specimens are more likely to contain cysts which can be identified with greater reliability than trophozoites.

2-5. Specimens Other Than Feces. Some of the intestinal parasites pass diagnostic stages in material other than feces. Occasionally, intestinal parasites and other related species are located in the liver, lungs, intestinal mucosa, genitourinary tract, or other areas. It may then be necessary to examine specimens other than feces to properly diagnose such infections.

a. **Sputum.** Sputum specimens should be collected in suspected cases of paragonimiasis. *Paragonimus westermani* eggs are frequently coughed up in sputum and then swallowed. Obviously, they will be found in fecal specimens as well as sputum. The specimen of choice is any sputum with brown or rusty colored flecks seen scattered in streaks of blood. Pulmonary amebiasis and echinococcosis can also be detected by

examining sputum specimens on occasions. Trophozoites of *Entamoeba histolytica* must be differentiated from those of *Entamoeba gingivalis*, which are normally found in the mouth. To help avoid such confusion, instruct the patient to rinse his mouth with dilute (3%) hydrogen peroxide. It is important to have the patient make an effort to cough deep within his lungs and bring up sputum from the lower respiratory passages. A clean, dry, wide-mouthed, screwtop specimen jar is suitable for collecting such specimens.

b. **Sigmoidoscopic Material:**

(1) In suspected cases of amebiasis when stool specimens are negative, material can be obtained from the intestine by sigmoidoscopy. A cleansing enema should not be given prior to the procedure because it will remove any organisms from the areas which are viewed through the sigmoidoscope.

(2) Cotton swabs should not be used to obtain the specimens. A serologic pipet (1-ml) with a rubber bulb should be used to aspirate material from visible lesions of the mucosa. Dry ulcers that are not covered by readily removable exudate, and some moist lesions as well, have to be gently curetted or scraped.

c. **Duodenal or Biliary Aspirates.** In a small percentage of *Strongyloides* infections, larvae can be found in duodenal aspirates more readily than in feces. On rare occasions the same is true for *Giardia* infections. In cases where such a diagnosis is very important, but cannot be established by fecal examinations, duodenal drainage material can be helpful. Also, there can be uncertainty as to whether large trematode eggs in stools are those of the liver fluke, *Fasciola*, or of the intestinal fluke, *Fasciolopsis*. The presence of eggs in aspirates of pure, or nearly pure, bile indicates *Fasciola*.

d. **Urine.** Urine specimens are used in the diagnosis of *Trichomonas vaginalis* and *Schistosoma haematobium* infections.

(1) The best urine specimen for finding eggs of *S. haematobium* is one collected at about noon and not an early morning specimen. The eggs are most frequently present in the last few drops of the specimen rather than in the first portion voided. They are also more likely to be found in urine containing blood or pus. Because schistosome eggs are not passed regularly, repeated daily examinations should be made in suspected cases which are negative on the first and second examinations.

(2) Fresh urine should be examined for *T. vaginalis*. In this case, the first portion of a first morning specimen is best. Old specimens are not satisfactory because the flagellates quickly become immotile and die in voided urine. Also, the urine can become contaminated with a species of *Bodo*, a nonparasitic flagellate, which can be confused with *T. vaginalis*.

e. **Vaginal and Urethral Exudates.** Vaginal swabs or scrapings and urethral swabs in females and urethral exudates or prostatic secretions in males are commonly collected for diagnosis of *T. vaginalis*. In female patients, a sterile, nonlubricated, dry speculum should be used, and the patients should not douche for 3 to 4 days before the specimen is collected. Care should be taken to prevent the specimen from being contaminated with glove powder. Examine the specimen immediately after collection regardless of source. If that is not possible, it should be placed in a tube containing a small amount of saline and sent directly to the laboratory for examination. If the examination will be delayed any further, a portion of the specimen should be inoculated directly into the proper culture medium and the remainder should be smeared on slides and fixed for staining.

f. **Perianal Material.** Anal swabs are the best means of detecting *Enterobius vermicularis* infections because the female worms ordinarily deposit their eggs on the perianal folds rather than within the intestinal tract. Two types of swabs, the cellulose-tape slide and the vaseline-paraffin swab, are the most commonly used for routine purposes. When using either method, collect the specimens a few hours after the patient has gone to bed or the first thing in the morning before a bowel movement or bath. For practical purposes, three consecutive daily collections should be routinely employed. They will normally reveal 90 percent of the positive cases. Anal swabs by either of the methods can also be used for collecting *Taenia* eggs, especially in infections where proglottids migrate out of the anus. Occasionally, any of the helminth eggs that occur in feces and resist drying are recovered by these methods.

Chapter 3
SPECIMEN STORAGE, PRESERVATION, AND SHIPMENT

3-1. Storage of Specimens:

a. From the time a specimen is collected it is necessary to expedite its examination. Until the examination is completed, the specimen should be protected from physical damage as well as general deterioration. Time, contamination, and temperature are the most important factors influencing the condition of specimens to be examined.

b. Protozoan trophozoites do not multiply or encyst outside the body. They only degenerate and die in time. Therefore, unpreserved specimens should be examined as soon as possible. Specimens should be collected in close proximity to the laboratory so they can be examined shortly after passage. The specimen should be protected from extreme changes in temperature before it is examined. Specimens from suspected protozoan infections should be examined while warm. If the examination is delayed, the specimen should not be kept warm artificially because that will hasten the degeneration of the organisms. Helminth eggs and larvae are more resistant to environmental changes than are protozoan cysts and trophozoites, but in some cases they continue to develop and may even hatch. All of these things complicate diagnosis; therefore, if a fresh specimen cannot be examined immediately, put it in the refrigerator or, if necessary, keep it at room temperature for the next few hours. Even when refrigerated, protozoan trophozoites cannot be expected to survive for more than 48 hours. Although they can survive for that long they "round" up and become more and more difficult to recognize as time passes.

3-2. Preservation of Specimens:

a. **Need for Preservation.** In the event a liquid or mushy specimen cannot be examined within 2 hours, or a formed specimen cannot be examined by the next morning, a portion of the specimen will have to be preserved for later examination. Specimens that are collected in the field during surveys or specimens that must be sent to other laboratories for identification must be properly preserved.

b. **Selecting Preservatives.** A single solution is not available for preserving all of the diagnostic forms (helminth eggs, larvae, and whole worms and protozoan cysts and trophozoites) and, at the same time, provide material that can be both concentrated and permanently stained. To insure that adequate material is available for diagnostic work, more than one preservative procedure is needed. One of the procedures should provide material that can be reliably concentrated, and another should provide material that can be permanently stained.

3-3. Merthiolate-Iodine-Formaldehyde (MIF) Preservation:

a. **Advantages and Disadvantages.** The MIF solution is particularly recommended when only one procedure can be performed. It is good for such cases because it preserves trophozoites, cysts, eggs, and larvae. In addition to preserving the elements, it provides a specimen that can be concentrated with excellent results. The procedure for collecting and preserving specimens with MIF solution is not complicated, but many patients have difficulty following the simplest directions without individual instructions. In situations where patients must collect and deliver specimens to the laboratory, it is best to have trained personnel add the MIF solution to the specimen at the laboratory. Most cysts and many trophozoites can be identified in wet mounts (MIF and others), but identification of trophozoites is difficult and should be confirmed in permanently stained films. Unfortunately, satisfactory permanent films cannot be made from MIF preserved material. Slightly different formulations of MIF are recommended for staining in wet mounts as opposed to those used to preserve specimens. The MIF procedure for staining wet mounts is discussed in chapter 5.

b. **Reagents:**

(1) *Stock "MF" Solution (Stable).*

Distilled water	50 ml
Formaldehyde (USP)	5 ml
Tincture of merthiolate (1:1,000)	40 ml
Glycerin	1 ml

Mix. Store in brown bottle. Prepare fresh after 3 weeks. **Note:** Tincture of Merthiolate, No. 99, 1:1,000. Eli Lilly and Co., Indianapolis, Indiana must be used. It contains eosin, the necessary stain ingredient.

(2) *Lugol's Iodine Solution.*

Iodine crystals (powdered)	5 g
Potassium iodide	10 g
Distilled water	100 ml

Dissolve the potassium iodide in the distilled water. Add the iodine crystals slowly and shake until dissolved. Filter. Store in a brown bottle. Prepare fresh after 3 weeks.

c. **Procedure:**

(1) Add 0.15 ml Lugol's solution to 2.35 ml MF stock solution immediately before use. Prior addition of the Lugol's causes a dense precipitate to form and the iodine does not properly stain the protozoa.

(2) Thoroughly mix a portion of feces about the size of a large pea (0.3 to 1g) in the MIF solution (approximately one part feces to two or three parts preservative). Do not use too much feces. If more feces are desired, use proportionately increased amounts of MIF solution.

(3) The specimen may then be concentrated or a direct wet preparation can be made from a drop of the sedimented feces taken from the top layer of the sediment.

3-4. Formalin Preservation:

a. **Advantages and Disadvantages.** Formalin is not the perfect preservative, but it is satisfactory for collecting specimens to be examined for eggs, larvae, and cysts. It is simple to prepare, stable, and does not have to be mixed with anything prior to being used. For routine purposes where both helminths and protozoa are sought, 5% formalin is preferred; however, when only helminths are the primary concern, 10% formalin is best. *Ascaris* eggs and sometimes *Trichuris* eggs will continue to develop in 5% concentrations, but this should not interfere with identification.

b. **Reagents:**

(1) *Formalin, 10%.*

Formaldehyde (commercial)	10 ml
Water (tap or distilled)	90 ml

Mix thoroughly. Commercial formaldehyde is approximately 40% concentration by volume, which means that 10% formalin is actually about 4% formaldehyde.

(2) *Formalin, 5%.*

Formaldehyde (commercial)	5 ml
Water (tap or distilled)	95 ml

Mix thoroughly.

c. **Procedure:**

(1) *Specimens for Routine Diagnosis.* Mix 1 portion of feces in about 3 volumes of formalin. The specimen can then be concentrated by the formalin-ether procedure (chapter 5), or a direct wet preparation can be made from a drop of the sedimented feces taken from the top layer of the sediment. The specimen will remain in good condition for months or even years.

(2) *Reference Specimens.* When material is stored for future study, it should be checked frequently for evaporation. Protozoan cysts and helminth larvae are best maintained if the preservative is changed at 6-month intervals. For best long-term preservation, use neutral formalin.

3-5. Polyvinyl Alcohol (PVA) Fixative:

a. **Advantages and Disadvantages.** Polyvinyl alcohol (PVA) fixative preserves trophozoites and cysts indefinitely. It is particularly useful for preserving fresh specimens (feces or other materials) for shipment to central laboratories. Permanently stained films can be prepared from the preserved material at any time. A distinct advantage of PVA fixative is that permanent stained smears can be made of organisms occurring in fluid specimens. The solution serves as an adhesive as well as a preservative and prevents the loss of organisms during the staining procedure. The PVA solution is stable and can be kept for long periods in tightly stoppered bottles. When stored in small quantities it will harden or jell after a time. A time limit of 3 months should be indicated on the predispensed collection vials to be sure of satisfactory results.

b. **Sources of Supply for PVA.** Polyvinyl alcohol powder, produced by E. I. DuPont de Nemours and Company, is designated Elvanol. The original grade, Elvanol 90-25, and other grades of high hydrolysis, medium viscosity polyvinyl alcohol (71-24, 71-30) have been found satisfactory. The PVA powder and PVA fixative solution can be purchased from Delkote,

Inc., 76 So. Virginia Ave., Penns Grove, N.J. 08069. When ordering PVA powder, specify the pretested powder for use in PVA fixative. The PVA powder is also produced by Monsanto Chemical Co., 6670 E. Flotilla St., Los Angeles, Calif. 90032. Their product is designated Gelvatol Resin. The grade 3-60 has been found satisfactory for use in the preparation of PVA fixative.

e. **Reagents:**

(1) *Modified Schaudinn's Fixative.*

Mercuric chloride crystals	4.5 g
95% ethyl alcohol	31.0 ml
Glacial acetic acid	5.0 ml

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

(2) *PVA Mixture.*

Glycerol	1.5 ml
PVA powder	5.0 g
Distilled water	62.5 ml

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 125 ml flask. Add the distilled water, stopper, and leave at room temperature for 3 hours to overnight. Swirl mixture occasionally to mix.

(3) *PVA-Fixative Solution.* Heat a waterbath (or large beaker of water) to 70C—75C. Adjust heat to maintain this temperature range. Place the loosely stoppered flask containing the PVA mixture in the bath for about 10 minutes; swirl frequently. When the PVA powder appears to be mostly dissolved, pour in the fixative solution, restopper, and swirl to mix. 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. Remove the flask from the waterbath and let cool. Store the PVA-fixative in a screw-capped or glass-stoppered bottle. The solution can be somewhat cloudy and still be satisfactory.

d. **Procedure:**

(1) *General Considerations.* To obtain full advantage of PVA solution as a preservative, fresh specimens should be thoroughly mixed with the solution before the organisms lose their characteristic morphology. Feces or other materials, can be preserved with PVA-fixative on slides or in vials. It is most commonly mixed in vials, especially in the collection of routine diagnostic specimens. Fixing material directly on the slide is convenient when the quantity of specimen is small, especially with the small amounts of material obtained by sigmoidoscopy or aspiration. Such material can be examined directly in saline, the coverslip removed, and PVA-fixative solution added to the saline suspension and spread into a film for future staining. The PVA films are permeable to all commonly employed stains.

(2) *Preservation in Vials.* If sufficient material is available, fixation in vials is preferable. Thoroughly mix a quantity of specimen in a vial containing 3 or more parts of PVA-fixative solution. If the specimen in the vial jells, reliquefy it by heating it in a waterbath before making films. If the specimen rejells after the second liquefication it should be discarded.

(3) *Fixation on Microscope Slides.* Place a drop of dysenteric stool or other material on a slide and mix with 3 drops of PVA-fixative. Spread the mixture (do not smear like a blood film) over approximately one-third of the slide. Take care to extend the smear to the edge of the slide. Allow the film to dry for several hours or overnight. Drying can be speeded by using a 37C incubator. Dried films will deteriorate within 2 months if they are not stained.

3-6. Shipment of Specimens.

a. It is frequently necessary to mail specimens to laboratories for diagnosis or consultation. When such specimens are sent through the mail, they should be preserved if possible. Nevertheless, even if they are preserved, postal regulations *require* that laboratory and clinical specimens be packed in special containers. Special precautions must be taken to prevent the material from leaking during transit. Current regulations from the United States Postal Manual governing shipment of harmful matter must be followed at all times. Full responsibility for violations of the law rests with the mailer.

b. Double mailing containers consisting of a heavy cardboard outer cylinder and a metal inner cylinder are recommended for shipping parasitologic specimens. They are available in several different sizes. Seal all vials with

adhesive tape, and then dip them in melted paraffin to prevent leakage. Wrap the sealed vials in a suitable absorbent packing material (cotton, toilet tissue, etc.), and place them in the inner cylinder. Wrap all identifying information around the inner cylinder, and slide it into the outer cylinder. As a precautionary measure, place a label on the inside cylinder in case the outer cylinder is damaged during transit.

c. Prepared slides such as stained fecal smears or mounts of helminths do not have to meet the same postal requirements and can be mailed in cardboard holders, slide boxes, or other suitable containers. However, the double cylinders used for vials are excellent for shipping slides because they offer good protection.

d. Be certain that all necessary data is enclosed with the specimens. Put the request slips where they will be least likely to be damaged if a specimen vial does leak. Put them around the inner cylinder rather than around the vials. The data must include source of specimen, any preservative added, and any special instructions. State the type of examination wanted, whether it is "routine" or for specific parasites which require special methods.

Chapter 4
EXAMINATION OF SPECIMENS

4-1. Macroscopic Examinations. Examine all unpreserved stool specimens macroscopically to note the color, consistency, and any abnormality. This examination includes looking for blood, mucus, and adult helminths or their parts. In some cases, particularly in some tapeworm infections, macroscopic examination reveals organisms which would be overlooked if only a microscopic examination was made.

a. **Consistency:**

(1) Hard, resists puncture.
(2) Formed, can be punctured.
(3) Soft, can be cut.
(4) Mushy, can be reshaped.
(5) Loose, shaped to container.
(6) Diarrheic, flows.
(7) Watery, pours.

b. **Appearance (Color).** All components of the fecal specimen contribute to stool color. The normal light or dark brown color is due to the reduction of bile constituents.

(1) Black or tarry appearance can be caused by medications that include iron, bismuth, or charcoal. It can also be due to digested blood from the upper part of the gastrointestinal tract.
(2) Dark brown, normal.
(3) Brown, normal.
(4) Pale brown, normal.
(5) Yellow, usually due to unchanged bilirubin or from the consumption of milk.
(6) Green, due to unchanged bilirubin, the consumption of green vegetables, or use of calomel.
(7) Gray, caused by the presence of excess fats or deficiency of bile components.
(8) Others, red, purple, etc.

c. **Mucus.** Mucus is normally present in very small amounts. Readily visible white patches on the stool specimen suggest abnormal amounts of mucus. This is indicative of intestinal irritation. Bloody mucus in loose or liquid specimens is highly suggestive of amebic ulcerations in the large intestine. Always carefully examine patches of mucus, particularly if blood tinged, for trophic amebae.

d. **Blood.** Some parasites, especially hookworms, cause intestinal bleeding. When blood is present in large amounts, the color of the stool specimen can be red, gray, or black. At times the amount of blood passed is too small to be seen. A blood detection test will reveal such small amounts and should be performed on all specimens.

(1) *Guaiac Test for Occult Blood:*

(a) *Reagents:*

1. Saturated Guaiac Solution.

Guaiac crystals	10 g
Ethyl alcohol (95%)	50 ml

Mix well. Stable 1 month.

2. Hydrogen Peroxide (3%). (Keep in refrigerator.)

3. Glacial Acetic Acid.

(b) *Procedure:*

1. Smear small amount of feces on filter paper.

2. Put 2 drops of Guaiac solution on feces smear.

3. Put 2 drops of glacial acetic acid on feces smear.

4. Put 2 drops of 3% hydrogen peroxide on feces smear.

5. Do the same test on a drop of dilute blood for a control.

(c) *Interpretation:*

1. Immediate deep blue color—4+.
2. Deep blue color in 30 seconds—3+.
3. Blue color in 1 to 3 minutes—2+.
4. Slowly developing faint blue—1+.
5. No blue color in 5 minutes—Negative.
6. Readings of 1+ and negative indicate no occult blood.

(d) *Interfering Substances.* A diet including rare meat within 48 hours of the test will cause the test to be positive. Ferrous fumarate and ferrous carbonate can produce false positive results. Other iron preparations do not interfere.

e. **Examination for Helminths:**

(1) Examine the entire surface of fecal specimens for macroscopic parasites and parts

of parasites. Frequently pinworms will be seen on the specimen. Tapeworm proglottids are sometimes seen on the surface but they are usually distributed throughout the specimen.

(2) If tapeworms are suspected, or the patient has been treated and the physician requests that specimens be examined for worms or the scolex of a tapeworm, the entire specimen should be examined. Two methods are generally used to separate macroscopic worms from feces: (1) straining through wire sieves, and (2) sedimentation.

(a) *Straining Through Sieves.* Straining is reliable and requires very little time. When sieves of the proper mesh are available, it is the preferred method. Two sieves should be used. One should have a mesh coarse enough to pass medium sized worms but fine enough to stop the larger fecal debris (10-20 mesh). The other sieve should have a mesh size small enough to stop the smaller worms and tapeworm scoleces (40-50 mesh).

1. Break up and comminute the specimen in water.

2. Dilute the specimen with more water and pour the suspension through the nested sieves.

3. Wash the material that is retained on the sieves in running water.

4. Transfer the material caught on the sieves to a pan with a dark bottom.

5. With the aid of a good light examine the material for white or pink appearing worms and parts of worms.

(b) *Sedimentation.* This is also a simple and reliable method for recovering worms from feces. It takes more time to use this method than does straining, but it only requires a sedimenting vessel and a dark bottom pan for the examination. Tall slender vessels with a capacity of about 500 ml are adequate sedimenting flasks. Conical flasks are best but standard graduated cylinders are adequate.

1. In a sedimenting flask, stir the fecal mass with sufficient water to form a suspension. Add water to fill flask and allow to stand for 30 minutes. Frequently stir the surface film of the suspension during this time.

2. After standing for 30 mintues carefully decant and discard supernant fluids.

3. From a height sufficient to produce a brisk stirring effect, pour another volume of water into the sediment in the flask.

4. Repeat washing and sedimenting procedure until supernant fluid is clear.

5. Transfer a portion of the washed sediment into a shallow dark bottom pan or dish and use a good light to search for worms. A hand lens is helpful for locating worms such as male pinworms or small tapeworm scoleces.

4-2. Microscopic Examination. The miscroscope is essential for the correct diagnosis of parasitic infections. In most cases the adult helminths that infect man are macroscopic, but the parasite elements that are passed from the body are microscopic. All of the protozoan parasites that infect man are microscopic. The microscope is needed not only to find parasites or their products, but it is also needed for morphological differentiation.

a. **Microscope:**

(1) A good, clean, properly aligned binocular microscope is needed to examine specimens for parasites. The instrument should be equipped with at least three objectives: low-power—10X (16 mm); high-dry—44X (4 mm); and oil-immersion—97X (1.8 mm). A 50X or 60X oil-immersion lens is desirable for scanning stained slides for protozoa. Oculars of 10X or 10X wide-field are satisfactory. In some cases 5X or 6X oculars will facilitate examination.

(2) Clean the objectives and oculars frequently. Dust and gummy excess immersion oil will interfere with the observation of structural details of the parasites. Remove all oil from the stage and oil-immersion objective when an examination is finished. Oil left on the lens will form a film that is difficult to remove. It will sometimes seep into the objective causing a cloudy or hazy appearance. Always use a good quality, low viscosity immersion oil. Cedar oil, mineral oil, or similar oils can damage both the slide preparation and the microscope. Cedar oil will produce a hardened film over the lens, and mineral oil is particularly bad about seeping into the objective. Never use xylene or other similar solvents for periodic cleaning; use small amounts of benzene. Some lens are sealed in place with a glue that is soluble in these

solvents; therefore, excessive use will ruin the objectives of some microscopes.

b. **Illumination:**

(1) Most microscopes that are equipped with attached illuminators are satisfactory for parasitology work, especially if a voltage regulator is included. The best type of lamp to use with a microscope that does not have an illuminator attached is one with a condenser system, regulatory iris diaphragm, and separate removable filters (preferably ground white glass and blue glass). It should also be adjustable so that it can be raised or lowered and focused at different angles. A bright light (100-watt bulb) is usually used in that type of lamp. Some other lamps that use 75- or 100-watt bulbs and filters are also satisfactory. The small box-type lamps that are used as substage illuminators do not give adequate light for work with protozoa. Use blue clear glass and white ground filters with any of the bulb-type light sources because the bright yellow light obtained without them makes it very difficult to see the minute details necessary for species identification.

(2) Adjust the microscope and illuminator by placing the lamp about 6 to 8 inches away and directly in front of the microscope. Remove the filters, if possible, and direct the beam of light into the center of the mirror. Use the flat side of the mirror. The concave side focuses the light in the plane of the condenser rather than on the specimen, giving an inferior image. Adjust the mirror so that the object to be examined appears brightly illuminated to the naked eye. The microscope field should be filled with light.

(3) Raise the substage condenser to the upper range of travel. The optimal position is obtained by removing the ocular; with the substage iris diaphragm fully open, adjust the condenser so that the back lens of the objective is filled with light. Generally, with high-dry, or oil-immersion, the condenser must be moved to the top position. With some microscopes, it may be necessary to lower the condenser slightly to fill the field with light when using the low-power objective. Do not use the substage condenser to reduce the intensity of the light. If the light is too bright after it is aligned, use additional white, ground-glass filters or the voltage regulator if it is available.

c. **Calibration of Ocular Micrometer:**

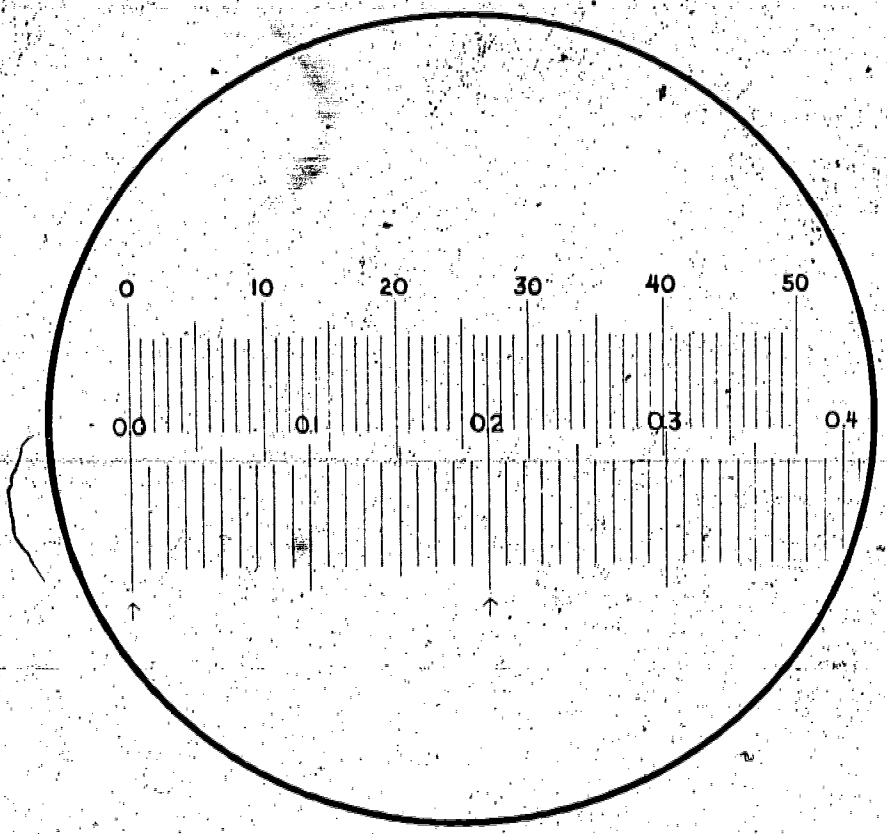
(1) Size is a very important consideration in identifying parasites or their diagnostic stages. A rough estimate can be made by comparing the objects with a human red blood cell (7.5μ to 8μ). To accurately measure an organism, a calibrated micrometer disc is required. The most satisfactory type is a disc with a line scale divided into 50 spaces. It is placed in the ocular so that it is always in focus.

(2) The units on the ocular micrometer are arbitrary. The exact value of the divisions varies from disc to disc and with different combinations of objectives and oculars. The value of the divisions must be calculated with each microscope used and for each combination of lenses: low, high-dry, and oil-immersion. To do this, the ocular micrometer units are compared with a scale of known dimensions.

(a) Remove the ocular from the microscope and unscrew the top or bottom lens, as directed. Place the micrometer disc on the diaphragm within the ocular with the engraved side down. Replace the lens and return the ocular to the microscope. Keep both the micrometer and lens clean and free of dust which will interfere with observations.

(b) Place the stage micrometer (slide with calibrated scale of 0.1 mm and 0.01 mm divisions) on the stage and focus on some portion of the scale.

(c) Look through the microscope and adjust the field until the 0 line on the ocular micrometer is exactly superimposed upon the 0 line on the stage micrometer (figure 4-1).



Ocular Micrometer - Top Scale

Stage Micrometer - Bottom Scale

Figure 4-1. Calibration of the Ocular Micrometer. (From Melvin and Brooke, 1969)

(d) Without moving the stage micrometer locate a second point to the extreme right where two lines are exactly superimposed. This second set of superimposed lines should be as far as possible from the 0 lines, but the distance will vary with the objective and microscope.

(e) Each of the large divisions of the stage micrometer equals 0.1 mm. Determine the total distance (in millimeters) 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: Suppose 49 ocular units (small) equal 8 stage units (large) or 0.8 mm.

(f) Calculate the number of millimeters that is measured by 1 small ocular unit.

Example: 49 ocular units = 0.8 mm

1 ocular unit = $\frac{0.8 \text{ mm}}{49}$

1 ocular unit = 0.0163 mm

(g) Measurements of protozoa and other small organisms or structures are given in microns rather than millimeters. Multiply the millimeter determinations by 1,000 to obtain the number of microns measured by 1 small ocular unit.

Example: 0.0163 mm × 1000 = 16.3 microns (μ).

(h) For ready reference, the values for 1 to 50 units can be recorded in a chart (table 4-1). Record the calibrations of the ocular micrometer obtained using the 10X oculars with each of the objectives. The lines of the stage micrometer will increase in magnification while those of the ocular micrometer will remain constant. Center the thinner ocular line on the broader stage micrometer line for accurate calibration at the higher powers.

(i) Keep the chart handy and the size of any microscopic object can be quickly determined by measuring it with the ocular micrometer, noting the particular lens combinations, and then referring to the chart of the ocular micrometer calibrations.

Table 4-1. Ocular Micrometer Calibrations. (Adapted from Melvin and Brooke, 1969)

Ocular magnification 10X		
Low Power Obj. 16 mm. Units = Microns	High Dry Obj. 4 mm. Units = Microns	Oil-Immersion Obj. 1.8 mm. Units = Microns
1	1	1
2	2	2
3	3	3
4	4	4
5	5	5
6	6	6
7	7	7
8	8	8
9	9	9
10	10	10

d. **Searching the Wet Smear:**

(1) To cover a smear adequately with a microscope and at the same time avoid searching the same area more than once, search the smear as if it were ruled. Start at one corner and systematically examine the entire slide, taking a swath the width of the field each time.

(2) With the 16 mm (low-power) objective, a smear under a cover glass 22 × 22 mm requires 11 round trips totaling 200 or more fields. With the 4 mm objective it takes four times as many round trips (44), involving more than 3,000 fields.

(3) A careful, systematic search is very important. Search each wet preparation systematically with the low-power objective, and then carefully review a number of selected fields with the high-dry objective. The above procedure should be followed when examining all wet preparations. It is a well-known fact that most mistakes in the examination for protozoa are due to failure to find them rather than failure to identify them.

e. **Searching the Stained Smear:**

(1) Examine permanently stained coverslipped smears with the 4 mm objective and 10X ocular lens combination. To use the same objective on smears that are not coverslipped, spread them with a thin film of immersion oil. The oil film prevents the light from reflecting off the particulate matter in the smear and distorting the image. A better practice for scanning uncoverslipped smears is to use the 97X oil-immersion objective in combination with a 5X or 6X ocular. As organisms are easily overlooked, check several fields with the higher power (10X ocular and 97X or similar objective) oil-immersion combination before submitting a final report on a stained smear. Also, use the higher power combination to verify the structural details of all cysts and trophozoites found.

(2) Examine both thick and thin areas of a smear. Although there may be more organisms in the thick areas than in the thin, they are usually easier to find in the latter. However, the thin portions are sometimes poorly stained. Then the thicker portions are of greater diagnostic value. Also the organisms are frequently not evenly distributed in a smear so be sure to examine several areas of the smear. When an organism is easily found and identified, search the smear carefully for other organisms because multiple infections are common.

Chapter 5
ROUTINE PROCEDURES FOR FECES AND URINE

SECTION A—INTRODUCTION

5-1. General Considerations:

a. The laboratory methods used to demonstrate the diagnostic stages of parasites are many and diverse. They all have disadvantages and only a small number have general adaptability. Many factors must be considered before a decision can be made as to which procedures are best to use.

b. What are the most common and important parasites in the area? How many specimens will there be each day? What facilities are available? Is it important to discover all infections or only the heavier ones? Are quantitative results desired?

c. Procedures suited to the most primitive conditions and others suited to the better equipped laboratories are included. Also only those having applications practical to the clinical laboratory are included. Their reliability was the last and most important factor considered before a procedure was selected.

SECTION B—TEMPORARY WET MOUNTS

5-2. Fresh Unpreserved Specimens:

a. **Saline Preparation.** Make it a matter of routine to examine direct saline preparations of fresh unpreserved feces. It is particularly useful for detecting motile protozoan trophozoites and helminth larvae. Even though not all of the morphological features on which the identification is made can be observed in such smears, it is very good to study their characteristic motility.

(1) *Reagent: Physiological Saline.*

Sodium chloride (NaCl) ..... 0.85 g
Distilled water (H_2O) ..... 100.00 ml
Dissolve the sodium chloride in the water.

(2) *Preparation:*

(a) Place a small drop of saline on the center of a clean slide.

(b) With an applicator stick transfer about 2 mg of feces (1 mg of feces is approximately 1 cubic mm and the average applicator stick is about 2 mm in diameter) to the saline. Avoid non-fecal elements unless schistosome eggs or amebae are especially indicated, then select any flecks of mucus and blood. Without spreading the drop of saline, stir and mix in the feces until almost the entire sample is evenly suspended. Remove any gross pieces of fibrous material or sand. Newspaper print can usually be read through such a preparation.

(c) Cover with a 22 × 22 mm coverglass.

(d) If the preparation is not satisfactory in all respects, discard it. Do not waste time on a preparation that can only yield doubtful results if negative.

(3) *Discussion.* If too little fluid is used and the material does not cover the glassed area add more saline at the edge of the preparation. The feces will not be evenly mixed with the diluent, but the preparation will be easier to examine than if vacant spots are present. On the other hand, do not use so much diluent that the coverslip floats on the suspension. In case too much diluent is used, touch a piece of facial tissue or other absorbent material to the edge of the coverslip to remove the excess.

b. **Iodine Stains:**

(1) If structures resembling cysts are seen, prepare and examine an iodine preparation. Prepare the iodine mount just as the saline mount but substitute iodine solution for saline or simply run iodine stain under the coverslip of the saline mount. The latter has two advantages: (1) it does not require a separate preparation, and (2) the fecal material is gradually stained so that areas of various intensity are present.

(2) Several iodine solutions can be used to stain protozoan cysts. Gram's iodine or Lugol's solution are used by some, but a 1% aqueous solution of potassium iodide saturated with iodine crystals (modified D'Antoni's) is preferable.

(a) *Modified D'Antoni's Iodine:*

1. Reagents.

Potassium iodide ..... 1.00 g
Iodine crystals (powdered) ..... 1.50 g
Distilled water ..... 100.00 ml

2. Instructions:

a. Dissolve the potassium iodide in the distilled water.

b. Add powdered iodine crystals to the potassium iodide solution.

c. Put solution in a brown bottle and store in a dark place. Agitate occasionally.

d. The solution is ready to use after 4 days. Decant a small amount into a dropper bottle for use. Change solution in dropper bottle daily.

e. The stock solution remains good for several weeks (so long as an excess of iodine crystals remains in the bottle).

3. Discussion:

a. In a correctly stained cyst, the glycogen, if present, appears reddish brown, the cytoplasm appears yellow, and the chromatin stains brown or black. The location of the karyosomes can be more easily determined, and the character of the nucleus is more distinct in iodine preparations, but the chromatoid bodies are less visible than in saline mounts.

b. Stain that is too concentrated is absorbed so rapidly that the entire cyst becomes a uniform dark brown color. Stains that are too low in concentration are not adequately absorbed, consequently the cysts tend to blend into the pale lemon-yellow background.

(b) *Lugol's Iodine Solution:*

1. Reagents.

Potassium iodide	10.00 g
Iodine crystals (powdered)	5.00 g
Distilled water	100.00 ml

2. Instructions:

a. Stock Solution. Mix ingredients and filter into a brown bottle. Store in the dark. Solution will remain satisfactory for several months.

b. Working Solution. Dilute the stock solution with 5 parts of distilled water. Prepare fresh working solution from stock every 10 days.

3. Discussion. The iodine solution that is used in the Gram's stain is Lugol's iodine diluted one to fifteen. Some parasitologists consider Gram's iodine too dilute for parasitology and Lugol's too strong. If you use Gram's iodine for parasitology, make sure that it is fresh and do not dilute it any further when making the smear. Do not add it to a saline direct smear. Make the smear directly in the Gram's iodine. The comments concerning the use of D'Antoni's iodine also apply to the use of other iodine solutions.

c. **Vital Stains.** The nuclei of trophozoites are indistinct or entirely invisible in saline preparations. Iodine solutions kill and distort the trophozoites making it very difficult to identify them. Therefore, use vital stains to bring out the nuclear characteristics of living trophozoites.

(1) *Quensel's Solution:*

(a) *Stock Reagents:*

1. Sudan III, Saturated Alcoholic Solution.

Sudan III powder	1.60 g
Ethyl alcohol (80%)	100.00 ml

a. Add the stain to the alcohol and shake thoroughly.

b. Let the solution stand at least several hours or overnight to be sure it is saturated. If all of the stain dissolves, add stain powder until no more goes into solution.

c. Filter or decant the solution into a screw-capped or glass-stoppered bottle.

2. Methylene Blue Solution (Saturated).

Methylene blue powder (medicinal)	3.50 g
Distilled water	100.00 ml

a. Add the stain to the water and shake thoroughly.

b. Let the solution stand for a few hours, shake at intervals. If all of the stain goes into solution, add stain powder until no more goes into solution.

c. Filter or decant the solution into a screw-capped or glass-stoppered bottle.

3. Cadmium Chloride Solution.

Cadmium chloride (c.p.)	10.00 g
Distilled water	100.00 ml

a. Dissolve the cadmium chloride crystals in the water.

b. Store in a screw-capped or glass-stoppered bottle.

(b) *Quensel's Stain Solution.*

Sudan III, saturated alcholic solution	20.00 ml
Methylene blue solution	30.00 ml
Cadmium chloride solution	50.00 ml

1. Mix the Sudan III and methylene blue.

2. Add the mixture to the cadmium chloride in an Erlenmeyer flask.

3. Gently shake the mixture for 15 to 20 minutes. A voluminous flocculent precipitate develops and the fluid becomes almost colorless.

4. Filter. Note: The precipitate can be more easily removed from the paper (Step 7) if the material is filtered so that the residue (precipitate) is collected at the bottom of the filter cone rather than spread over a large area.

5. Remove all excess liquid from the precipitate by placing the filter paper with the precipitate upon several other sheets of filter paper. Leave overnight.

6. Transfer as much of the precipitate as possible to another filter. Rapidly pour through 25 to 30 ml of distilled water.

7. Quickly transfer the washed precipitate to a flask containing 250 ml of distilled water.

8. After the precipitate dissolves, fine crystals of cadmium chloride may form a few days later. If so, filter the solution before using.

(c) *Discussion.* Prepare wet preparation of fecal material or culture sediment in a drop of the Quensel's solution. The amebae trophozoites will be stained pale blue with their nuclei a deeper blue shade within 10 to 20 minutes. The stained nuclei will present the same morphologic characteristics as they do in permanently stained hematoxylin preparations. Food inclusions will also be stained. After 30 minutes to one hour, the organisms become overstained and then cannot be identified. Occasionally organisms fail to stain and generally *Dientamoeba fragilis* nuclei do not stain well. The use of a warm stage sometimes aids in staining *Dientamoeba*. *Blastocystis hominis* stains very well, but flagellates, ciliates, and living cysts do not stain.

(2) *Buffered Methylene Blue (Nair's Stain):*

(a) *Reagents:*

1. *Stock Solution A.*

Acetic acid ..... 1.50 ml

Dilute to 1000 ml with distilled water. Mix well and store in screw-capped or glass-stoppered bottle.

2. *Stock Solution B.*

Sodium acetate ($NaC_2H_3O_2$) ..... 16.40 g

or

Sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) ..... 27.2 g

Dilute either to 1000 ml with distilled water. Mix well and store in screw-capped or glass-stoppered bottle.

3. *Acetate Buffer Solution (pH 3.6).*

Stock Solution A ..... 46.30 ml

Stock Solution B ..... 3.70 ml

Mix solution A and solution B and dilute to a total of 100 ml with distilled water.

4. *Stain Solution.*

Methylene blue ..... 60 mg

Acetate buffer (pH 3.6) ..... 100 ml

Mix well and store in screw-cap or glass-stoppered bottle.

5. *Discussion.* Prepare wet preparation of fecal material or culture sediment in a drop of methylene blue stain solution. After a few minutes the amebae trophozoites stain pale blue and their nuclei stain a deeper blue. The morphological characteristics will be the same as in permanently stained hematoxylin preparations. Food inclusions also stain. Occasionally, some organisms fail to stain but the nuclei of *D. fragilis* usually stain with this solution.

d. **Merthiolate-Iodine-Formaldehyde (MIF) Stain.** In addition to staining protozoa cysts, MIF solution will also stain trophozoites in wet preparations. Slightly different formulas are recommended for the MIF stain solution that is used for wet preparations and the MIF preservative solution described in chapter 3.

(1) *Reagents:*

(a) *Lugol's Iodine Solution.*

Iodine crystals (powdered) ..... 5.0 g

Potassium iodide ..... 10.0 g

Distilled water ..... 100 ml

1. Dissolve the potassium iodide in the distilled water.

2. Add the iodine powder slowly and shake until dissolved.

3. Filter and store in a brown bottle away from the light.

(b) *Formaldehyde.*

(c) *Tincture merthiolate, (No. 99, 1:1000 (Lilly).* Tincture of merthiolate, No. 99, 1:1000, (Lilly) (Eli Lilly and Co., Indianapolis, Indiana) must be used, since it contains eosin, one of the stain ingredients.

(2) *MIF Stain Solution.*

Lugol's solution ..... 0.10 ml

Formaldehyde ..... 0.15 ml

Tincture merthiolate (1:1000) ..... 0.75 ml

Mix well.

(a) *Preparation.* As with the other wet preparations, mix 1 to 2 mg of material into a small drop of the MIF solution. Allow a few minutes for the organisms to stain and then examine systematically.

(b) *Discussion.* One ml of stain is sufficient for 25 to 30 fecal preparations. Prepare the working solution of the stain daily. Both cysts and trophozoites are easily detected with the iodine strength recommended. If the strength of the iodine is increased the organisms will stain quicker and darker, but they will be more difficult to locate.

5-3. Preserved Specimens:

a. **Formalin-Preserved Specimens:**

(1) In preserved specimens the formalin replaces the saline used in mounts of fresh feces, and the suspension can be used directly. If the quantity of formalin is more than 2 or 3 times that of the feces, discard the excess before resuspending the material. Thoroughly mix the specimen before taking a sample. Transfer a drop of the specimen to a slide, coverslip, and read. If the suspension is too thick, add a small amount of saline to dilute it to the proper density.

(2) If objects resembling protozoan cysts are seen, make a wet preparation with iodine solution to stain the nuclei. Since the iodine will be diluted by the formalin in the specimen, use a stronger concentration of iodine than is used for fresh specimens. If Lugol's iodine solution is used, dilute the stock solution only 1 to 3 rather than 1 to 5. Determine the need for a more concentrated iodine solution by trial because a too strong solution should be avoided.

b. **MIF Preserved Specimens.** In specimens preserved with MIF solution, the preservative replaces the saline used in mounts of fresh feces. Make the direct examination of the MIF-preserved material just as if it were a saline suspension. If the quantity of MIF solution is more than 2 or 3 times that of the feces, discard the excess before resuspending the material. Thoroughly mix the specimen before taking a sample. Transfer a drop of the specimen to a slide, coverslip, and read. If the suspension is too thick, add a small amount of saline to dilute it to the proper density.

5-4. Concentrated Specimens. Make wet preparations from concentrated specimens the same way as was described for formalin-preserved material. The amount of fluid left in a concentrated specimen determines whether or not additional saline is needed. Often concentrated material, especially that from sedimentation procedures, contains grit or large particles of debris. Avoid getting those in the mount because the coverslip will not lie evenly on the slide.

SECTION C—PINWORM PREPARATIONS

5-5. Introduction. Since the eggs of *E. vermicularis* are usually deposited outside the body on the perianal area rather than within the intestinal tract, anal swabs are used to recover them. The cellulose-tape slide preparation and the vaseline-paraffin swab are the most commonly employed methods for routine purposes.

5-6. Cellulose-Tape Slide Preparation:

a. **Preparation.** Clear cellulose tape, 3/4-inch wide, is best for this purpose. Cloudy or hazy tape interferes with the examination. As illustrated in figure 5-1, anchor one end of a piece of tape, 4-5 inches long, to the underside of a 3 × 1 inch slide. Fold the tape over the near end of the slide and smooth the sticky side down the length of the slide. Attach a paper tab to the free end of the tape for labeling. Unused prepared slides can be stored in the refrigerator for several months.

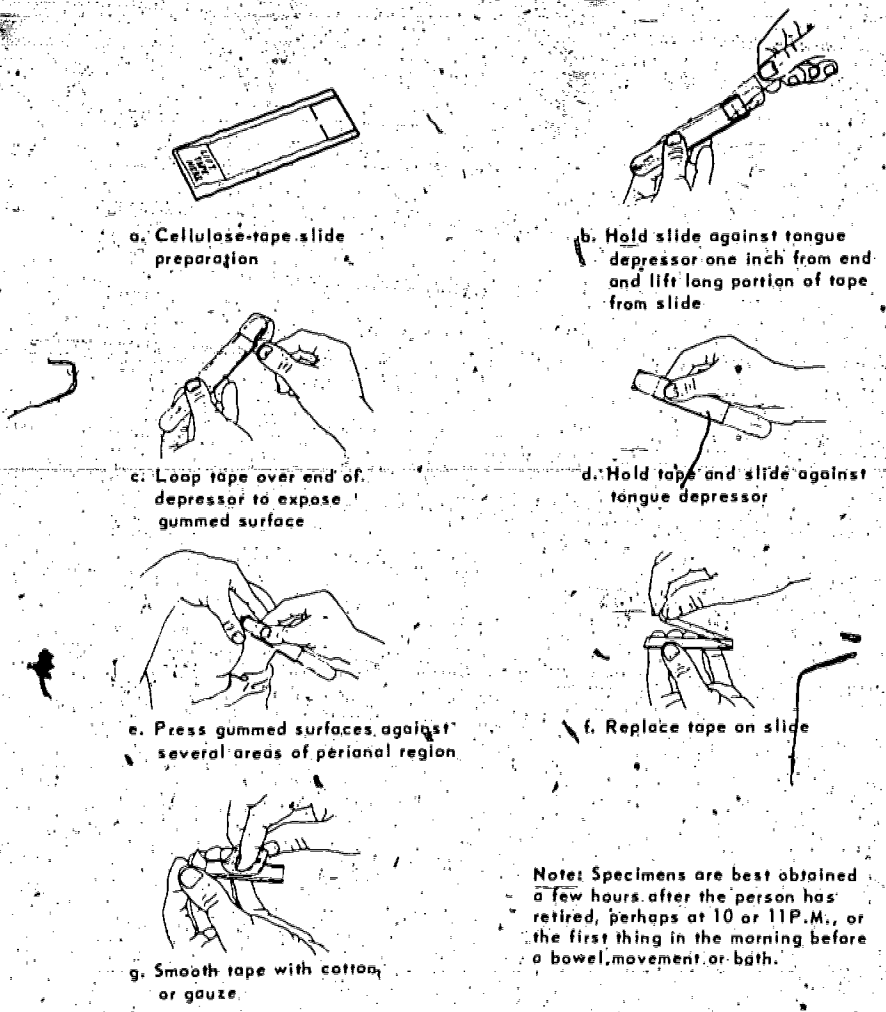


Figure 5-1. Procedure for Recovery of Pinworm Eggs. (From Melvin and Brooke, 1969)

b. **Collection.** (See figure 5-1):

(1) Pull tape back from slide, leaving the anchoring portion on the underside undisturbed, and with sticky side out, loop over a tongue depressor or test tube to hold it steady.

(2) Press the sticky tape surface against the perianal skin. Press open the perianal folds to insure access to eggs concealed in the crevices. Use only the center portion of the tape to decrease the area to be searched.

(3) Smooth the tape back into place with a piece of cloth or cotton. Be sure to wash your hands after collecting the specimen. The eggs will stick to you, and they are infective within a few hours after they are passed.

c. **Examination:**

(1) Lift the tape from the slide and place a drop of toluene or xylene on the slide. Smooth the tape back into position.

(2) Use low-power magnification and reduced light to search the slide for eggs.

(3) Tape preparations can be stored in the refrigerator for several weeks before they are examined without excessive distortion of the eggs. However, do not put toluene or xylene on the slide until it is to be examined because it will cause the eggs to collapse and make them difficult to recognize.

5-7. Vaseline-Paraffin Swab Preparation:

a. **Preparation:**

(1) Dip a cotton-tip swab in a hot mixture of 4 parts vaseline to 1 part paraffin so that the

swab is thoroughly coated. Let the swab cool. If the mixture is too hot, the swab will have to be dipped twice to coat it so that the surface is smooth and not fibrous.

(2) Put the coated swab in a 100 × 13 mm tube and plug it with cotton. These swabs can be stored for long periods in cool places.

b. **Collection:**

(1) Rub the swab gently over the perianal surface, spreading open the perianal folds to reach eggs hidden in the crevices.

(2) Replace swab in tube and wash your hands thoroughly.

c. **Examination:**

(1) Fill tube with xylene until the swab is completely covered. Let the swab soak in the xylene for 5 minutes.

(2) Discared the swab and centrifuge the xylene for 1 minute at 2,000 rpm.

(3) Carefully remove the supernatant with a pipet or suction pump. Transfer the sediment to a slide and examine for typical eggs with low-power magnification and reduced light. Do not use a coverslip as it will unnecessarily spread the specimen over a larger area. A slide with small painted circles is convenient to keep the drop of sediment from spreading.

SECTION D—CONCENTRATION PROCEDURES

5-8. Introduction. Protozoa can be found in direct preparations when they are present in large numbers. However, in most cases, do not rely on direct preparations alone. Concentration procedures are much more reliable for most elements. Concentration procedures, when properly performed, insure a much higher percentage of positive findings, for both helminths and protozoa.

5-9. Sedimentation:

a. **Gravity Sedimentation:**

(1) *Discussion.* The simplest procedure for the recovery of eggs, larvae, and cysts is simple sedimentation of fecal material. The concentration is not marked, but the use of a large fecal sample is of great help when only small numbers of eggs are present. It is one of the most efficient methods for detecting schistosome eggs. It is particularly well suited for use in remote areas where limited resources are available.

(2) *Procedure.*

(a) Place a sample of feces about the size of a large marble or walnut (approximately 10-15 g) in a 250 ml beaker, and fill it one-quarter full with tap water. Use physiological saline rather than water when schistosomes are suspected. Schistosome eggs hatch in a short time in water.

(b) Thoroughly emulsify the feces with a tongue depressor or other suitable instrument.

(c) Add sufficient water to fill the beaker three-quarters full.

(d) Strain the suspension through four layers of wet gauze into a sedimentation glass or cone-shaped graduate of 250 ml to 500 ml capacity.

(e) Let the suspension settle for 1 hour. During that time gently stir the surface film two or three times to release organisms that become trapped in the surface film due to surface tension.

(f) Pour off two-thirds of the water without allowing any of the sediment to escape.

(g) Add fresh water to the flask so that the sediment becomes completely resuspended and let it settle for 45 minutes with periodic stirring of the surface film.

(h) Repeat the washing until the supernatant fluid is relatively clear. Generally two or three washings are sufficient.

(i) After pouring off the last wash water insert the tip of a long capillary pipet into the *top* layer of the sediment and remove a small sample.

(j) Place the material on a microscope slide, coverslip, and examine it as any other wet preparation. Should the preparation be too thick, dilute it with water or saline. Make iodine preparations when cysts are present, or suspected.

(k) Examine additional samplings from the middle and bottom layers of the sediment.

b. **Centrifugal Sedimentation:**

(1) *Discussion.* This procedure is essentially the same process as the gravity sedimentation, except that centrifugation is faster. It is also moderately effective for recovering eggs, larvae, and cysts. It is rapid, reveals the organisms in a living condition, does not require

expensive reagents, and samples a large quantity of feces. Like the gravity sedimentation it is particularly efficient for the recovery of schistosome eggs, and it also has some of the same shortcomings. It does not result in a marked concentration, and there is usually considerable debris present. Centrifugation is the recommended procedure for concentrating *Schistosoma haematobium* eggs in urine.

(2) *Procedure for Feces:*

(a) Place a sample of feces about the size of a large marble or walnut (approximately 5-10 g) in a 250 ml beaker. Fill the beaker one-fourth full with tap water. Use physiological saline rather than water when schistosomes are suspected.

(b) Thoroughly emulsify the feces with a tongue depressor or other suitable instrument.

(c) Strain the suspension through four layers of wet gauze into a 50 ml conical centrifuge tube.

(d) Centrifuge at 1,000 to 1,500 rpm for 1 minute.

(e) Carefully pour off the supernatant fluid without disturbing the sediment.

(f) Add fresh water to the tube so that the sediment becomes completely resuspended and centrifuge again at 1,000 to 1,500 rpm for 1 minute.

(g) Repeat the washing until the supernatant fluid is relatively clear. Usually three to four washings are satisfactory.

(h) After pouring off the last wash water insert the tip of a long capillary pipet into the *top* layer of the sediment and remove a small sample.

(i) Place the material on a microscope slide, coverslip, and examine it as any other wet preparation. Should the preparation be too thick, dilute it with a small amount of water or saline. Make iodine preparations when cysts are present, or suspected.

(j) Remove a sample from the *bottom* layer and handle it in the same manner.

(3) *Suggestions:*

(a) The fecal suspension can be prepared directly in the centrifuge tube instead of in the beaker, but take care to get all of the fecal sample into suspension. Even so, it is not recommended that it be tried with firm specimens.

(b) Do not exceed 1,500 rpm because some types of eggs collapse easily at higher speeds.

(4) *Procedure for Urine:*

(a) Fill a 15 ml conical centrifuge tube with the urine specimen.

(b) Centrifuge at 1,000 to 1,500 rpm for 1 minute.

(c) Quickly invert the centrifuge tube to pour off the supernatant urine; then stand it upright. This will leave a small amount of urine with any sediment that is present in the conical tip of the tube.

(d) Resuspend the spun-down sediment in the residual fluid.

(e) Pipet or pour one drop of the suspension onto a microscope slide.

(f) Examine for schistosome eggs under low power. Also examine the specimen for *T. vaginalis* with the low-power objective, but carefully observe any apparently nonmotile, suspicious objects under the high-dry objective.

c. **Formalin-Ether Sedimentation:**

(1) *Discussion.* This concentration procedure is reasonably efficient for recovering most helminth eggs, larvae, and protozoan cysts. Use this procedure to concentrate any fecal specimens that have been preserved with formalin.

(2) *Procedure for Fresh Fecal Specimens:*

(a) Transfer 2 to 5 g (2 to 5 ml) of feces to a paper cup or beaker. The more fibrous the stool, the more stool specimen you will have to use (up to about 5 g).

(b) Add approximately 3 ml of water and comminute to a uniform suspension.

(c) Add another 10 ml to the fecal suspension in the cup.

(d) Mix; then pour through one to two layers of wet gauze into a 15 ml conical centrifuge tube. Leave the coarse debris in the cup by crimping the lip and pouring the suspension through the slit thus formed.

(e) Fill the tube and balance with another tube; then centrifuge at 2,000 to 2,500 rpm for 1 minute.

(f) Carefully pour off the supernatant fluid. There should be about 0.5 ml to 1.0 ml of sediment in the tube at this point.

(g) Break up the sediment and resuspend it in fresh water. Centrifuge the tube and

decant as before. Repeat this step if the supernatant fluid is not relatively clear.

(h) Break up the sediment, and then add 10% formalin to half fill the tube and mix.

(i) Allow the suspension to stand at this point for at least 5 minutes. This step is important to allow the material to fix properly. The formalin-feces mixture can be stoppered at this point and saved until a later time.

(j) Add ether to half fill the remaining space in the tube. Stopper the tube with a rubber stopper, and shake vigorously for a full minute. Remove the stopper with care, well away from any fire.

(k) Centrifuge at 1,500 rpm for 1 minute. There will be three layers above the sediment: formalin, plug of fecal debris, and a topmost layer of ether.

(l) Loosen the plug of fecal debris with an applicator, and pour off all except the sediment.

(m) Clean the walls of the tube with a cotton swab to prevent the adhering debris from settling down into the sediment.

(n) Mix the remaining sediment with the small amount of fluid that drains back from the sides of the tube. Add more water dropwise if necessary to adjust the suspension to the proper density for examination.

(o) Transfer a small drop of the suspension to microscope slide, coverslip, and examine as you would any wet preparation.

(p) Transfer a small drop of the suspension to a drop of the desired iodine solution when protozoan cysts are present or suspected. Again, coverslip and examine as before.

(q) Examination of the specimen can be delayed. Add 1 or 2 ml of 10% formalin to the sediment and stopper the tube. Formalized sediments can be kept indefinitely. Remove the excess formalin before making the mounts to prevent diluting of the concentration.

(3) *Procedure for Formalin-Preserved Specimens:*

(a) Thoroughly mix the formalized specimen.

(b) Strain a sufficient amount of specimen through one to two layers of wet gauze into a conical 15 ml centrifuge tube to yield from 0.5 to 1.0 ml of sediment.

(c) Add tap water if necessary, mix, and centrifuge at 2,000 to 2,500 rpm for 1 minute.

(d) Decant the supernatant fluid and wash the sediment with tap water if the supernatant fluid is not reasonably clear.

(e) When the supernatant fluid remains relatively clear, break up the sediment, add 10% formalin to half fill the tube, and mix.

(f) Add enough ether to half fill the remaining space in the tube. Stopper the tube with a rubber stopper, and shake vigorously for 1 minute. Remove the stopper with care well away from any fire.

(g) Centrifuge at 1,500 rpm for about 1 minute. There will be three layers above the sediment: formalin, plug of fecal debris, and a topmost layer of ether.

(h) Loosen the plug of fecal debris with an applicator and pour off all except the sediment.

(i) With a cotton swab remove the debris adhering to the sides of the tube.

(j) Mix the remaining sediment with the small amount of fluid that drains back from the sides of the tube. Add more water, if necessary, dropwise, to adjust the suspension to the proper density for examination.

(k) Transfer a small drop of the suspension to a microscope slide, coverslip, and examine as you would any wet preparation.

(l) Transfer a small drop of the suspension to a drop of the desired iodine solution when protozoan cysts are present or suspected. Coverslip the specimen and examine as before.

(m) Examination of the specimen can be delayed. Add 1 or 2 ml of 10% formalin to the sediment and stopper the tube. Formalized sediments can be kept indefinitely. Remove the excess formalin before making the mounts to prevent diluting of the concentration.

d. **Merthiolate-Iodine-Formaldehyde Concentration (MIFC):**

(1) *Discussion.* This procedure is rapid, simple, and preserves and concentrates all stages of intestinal parasites including protozoan trophozoites. A very high percentage of the positive cases tested are detected when the MIFC Procedure is carefully followed. It is the concentration procedure of choice for all facilities that can obtain the few necessary reagents. Try to concentrate specimens within one week

after they have been preserved with MIF solution. It has been reported that specimens preserved for more than one week are not as efficiently concentrated as specimens more recently preserved.

(2) *Reagents:*

(a) *Stock "MF" Solution (Stable).*

Distilled water	50 ml
Formaldehyde (USP)	5 ml
Tincture of merthiolate (1:1,000)	40 ml
Glycerin	1 ml

Mix. Store in brown bottle. **Note:** Tincture of Merthiolate, No. 99, 1:1,000, Eli Lilly and Co., Indianapolis, Indiana must be used. It contains eosin, the necessary stain ingredient.

(b) *Lugol's Iodine Solution.*

Iodine crystals (powdered)	5 g
Potassium iodide	10 g
Distilled water	100 ml

1. Dissolve the potassium iodide in the distilled water. Add the iodide crystals slowly and shake until dissolved.

2. Filter. Store in a brown bottle.

3. Prepare fresh every three weeks.

(3) *Procedure for Fresh Feces:*

(a) Add 0.3 ml Lugol's solution to 4.7 ml MF stock solution immediately before use. Prior addition of the Lugol's causes a dense precipitate to form, and the iodine does not properly stain the protozoa.

(b) In a paper cup, thoroughly mix 1 to 2 g (1 to 2 ml) of feces with the MIF solution to a uniform suspension. The more fibrous the feces, the more you will have to use (up to about 2 g).

(c) Add another 8 ml of MIF solution to the fecal suspension in the cup. (Maintain proper ratio of reagents: 9.4 ml of MF solution to 0.6 ml of Lugol's iodine solution.)

(d) Mix, then pour through one to two layers of wet gauze into a 15 ml conical centrifuge tube. Leave the coarse debris in the cup by crimping the lip and pouring the suspension through the slit that is formed.

(e) Fill the tube with MIF solution and centrifuge at 1,000 to 1,500 rpm for 1 minute.

(f) Carefully pour off the supernatant fluid. There should be about 0.5 ml to 1.0 ml of sediment in the tube at this point.

(g) Break up the sediment and resuspend it in 10 ml of fresh MIF solution. Let the tube stand for 5 minutes to permit complete fixation of the material.

(h) Add ether (2 to 3 ml) to half fill the remaining space in the tube. Stopper the tube with a rubber stopper and shake vigorously for a full minute. Carefully remove the stopper well away from any fire. Should the ether remain sharply separated after shaking, add several drops of water and reshake.

(i) Centrifuge the tube at 1,500 rpm for 1 minute. Three layers will normally form above the sediment: clear MIF solution, a plug of solids, and ether at the top.

(j) Loosen the plug of solids with an applicator and pour off all except the sediment.

(k) Clean the walls of the tube with a cotton swab to prevent the adhering debris from settling down into the sediment.

(l) Mix the remaining sediment with the small amount of fluid that drains back from the sides of the tube. Add more MIF solution dropwise, if necessary, to adjust the suspension to the proper density for examination.

(m) Transfer a small drop of the suspension to a microscope slide, coverslip, and examine as you would any wet preparation.

(4) *Procedure for MIF-Preserved Specimens:*

(a) Estimate the quantity of sediment in the preserved sample and strain through one to two layers of wet gauze a portion of the specimen containing about 0.5 ml to 1.0 ml of the sediment.

(b) Add fresh MIF solution (mix 9.4 ml of MF solution and 0.6 ml of Lugol's iodine) to two-thirds fill the centrifuge tube.

(c) Add ether to half fill the remaining portion of the tube (at least 2 ml). Stopper with a rubber stopper and shake vigorously for 1 minute. If ether remains sharply separated after shaking, add several drops of water and reshake.

(d) Remove the stopper well away from any fire and let the tube stand for 1 or 2 minutes.

(e) Centrifuge the tube at 1,500 rpm for 1 minute. Three layers will normally form above the sediment: clear MIF solution, a plug of solids, and ether at the top.

(f) Loosen the plug of solids with an applicator and pour off all except the sediment.

(g) Clean the walls of the tube with a cotton swab to prevent the adhering debris from settling down into the sediment.

(h) Mix the remaining sediment with the small amount of fluid that drains back from the sides of the tube. Add more MIF solution dropwise if necessary to adjust the suspension to the proper density for examination.

(i) Transfer a small drop of the suspension to a microscope slide, coverslip, and examine as you would any wet preparation.

5-10. Flotation:

a. **Concentrated Brine Flotation:**

(1) *Discussion.* This method of concentration is simple and efficient for the recovery of helminth eggs other than operculate and schistosome eggs. The brine distorts protozoan cysts beyond recognition. The procedure is especially recommended for hookworm screening in places that have limited supplies and equipment.

(2) *Reagents.* Stir sodium chloride (table salt is satisfactory) into hot or boiling tap water until it no longer goes into solution. After cooling check the solution with a hydrometer to make sure the specific gravity is at least 1.20. Reheat the solution and add more sodium chloride when the specific gravity is below 1.20. Filter the solution before using.

(3) *Procedure:*

(a) Transfer 1 to 2 grams (1 to 2 ml) of feces to 2 ml of brine in a paper cup and comminute.

(b) Fill a 1-inch diameter vial with brine, empty it into the cup, and mix thoroughly.

(c) Pouring through a crimp in the cup's brim, fill the vial with the suspension.

(d) Allow the suspension to stand 30 to 60 minutes undisturbed.

(e) Bend a small wire loop so that the loop is at right angles to the stem. Flame the loop; let it cool; then gently slide it under the surface film and remove 2 or 3 loopfuls of the surface material to a clean microscope slide.

b. **Zinc Sulfate-Centrifugal Flotation:**

(1) *Discussion.* The procedure is not suitable for fatty stools, it does not float infertile ascaris or infertile trichuris eggs, and it does not float the eggs of most trematodes. Also, the tapeworm eggs that are operculated are not floated. It does float most other helminth eggs and larvae and protozoan cysts. Fecal debris is effectively separated from the parasite elements. The method is recommended for survey work where large numbers of specimens are to be examined for roundworm eggs and protozoan cysts.

(2) *Reagent, Zinc Sulfate Solution, Specific Gravity 1.18-1.20:*

(a) Add 331 g of zinc sulfate (USP) to 1 liter of warm tap water. A technical grade of zinc sulfate can be used as long as you filter the solution before using.

(b) After the zinc sulfate is thoroughly dissolved, check the specific gravity with a hydrometer. It should be from 1.18 to 1.20. Add water or zinc sulfate as needed to adjust the specific gravity when it does not fall within proper limits.

(3) *Procedure:*

(a) Transfer 0.3 to 0.5 g (1/3 ml to 1/2 ml) of feces to a paper cup containing 1 ml of water. Use more feces when the stool is very fibrous or watery.

(b) Comminute the specimen to an even suspension.

(c) Fill a 13 × 100 mm test tube with water and add all of it to the fecal suspension in the cup.

(d) Crimp the edge of the cup and pour the suspension through one to two layers of gauze into a test tube in order to remove the coarse material that will not go into suspension.

(e) Centrifuge the suspension for 1 minute at 2,500 rpm.

(f) Pour off the supernatant fluid.

(g) Add 1 ml of zinc sulfate solution to the sediment and thoroughly mix by flicking the tube or stirring with an applicator.

(h) Fill tube to within 1-2 mm of the rim with zinc sulfate solution.

(i) Centrifuge the suspension for 1 minute at 2,500 rpm. Use a centrifuge with vertical cups, not a slant head model. Allow the centrifuge to come to a stop without any interference or vibration. It must be properly balanced.

(j) Carefully place the tube vertically in a rack without shaking or spilling.

(k) Make a wire loop approximately 5 mm in diameter so that the loop is at right angles to the stem.

(l) Flame the loop and allow it to cool.

(m) Slide the loop gently under the surface film and remove 2 or 3 loopfuls of material to a microscope slide.

(n) Examine the loop of material using the low-power objective. Do not use a coverglass for the preliminary study. Helminth eggs can be identified without further preparation. When objects resembling protozoan cysts are observed, add a drop of iodine, coverslip, and examine carefully with the high-dry objective.

5-11. Cellophane-Covered Thick Smear:

a. **Discussion.** The cellophane-covered thick smear (kato thick smear) takes little technician time, is simple and does not require a centrifuge. Relatively large amounts of specimens (approximately 50 mg as compared to only 2 mg examined in an ordinary direct smear) are directly examined after being cleared. The procedure is suitable for routine laboratory work or for large surveys for helminth eggs. It is *not* satisfactory for protozoa or for helminth larvae. Also, it does not work well with watery stools or with feces that contain large amounts of gas.

b. **Reagents and Materials:**

(1) *Malachite Green Solution.*

Malachite green (crystals)	0.3 g
Distilled water	10.0 ml

Mix. Store in screw-capped bottle.

(2) *Wetting Solution.*

Glycerine	100 parts
Distilled water	100 parts
Malachite green solution	1 part

Mix. Store in screw-capped bottle.

(3) *Cellophane.* Cut wettable cellophane of medium thickness (40 to 50 microns) into 22 × 30 mm strips. Soak the strips for at least 24 hours in the above wetting solution. Strips soaked in the wetting solution are usable as long as they are damp with the wetting solution.

c. **Procedure:**

(1) Place 50 to 60 mg feces (about the size of a drop of water) in the center of a clean slide. When the specimen contains a large amount of fibrous material, force a portion of feces through a piece of stainless steel bolting cloth of about 100 mesh size. Then transfer approximately 50 mg of the screened feces to the center of a clean slide.

(2) Cover the sample on the slide with a cellophane strip.

(3) Turn the slide over on a paper towel.

(4) Firmly press the slide over the covered sample forcing it to spread evenly to the edge of the cellophane strip. When the selected sample is too large, continue to press the slide until the excess is extruded beyond the strip. If the sample is obviously too large, make another slide.

(5) Turn the slide over with the cellophane strip up and allow it to dry (clear) at room temperature for 1 hour. To speed the process, put the slide in a 40C dry incubator or under the heat of a 50-watt light bulb for 20 to 30 minutes.

(6) Examine the smear under low magnification, focusing up and down through its entire thickness. Use higher magnification if necessary to positively identify any suspicious objects.

d. **Precautions:**

(1) The clearing process will not begin until the cellophane-covered smear is turned up so that it can dry.

(2) The most effective clearing time has to be determined for the local conditions.

(3) Thin-shelled eggs such as hookworm become invisible when smears are cleared too long.

Chapter 6
CULTIVATION AND HATCHING PROCEDURES

SECTION A—CULTIVATION OF PROTOZOA

6-1. Introduction. Protozoa can frequently be cultured from fresh specimens. Refrigerate specimens that cannot be cultured immediately; however, organisms are rarely isolated from specimens that are several hours old. Negative cultures are not conclusive evidence that a patient is free from infection. Cultural procedures do not take the place of careful microscopic examinations. Culture methods can at times be used to good advantage in conjunction with the microscopic examination. When growth does occur, it is still necessary to identify the organism on the basis of morphology as the various media are not species specific. Of the media available, the modified Boeck and Drbohlav medium and Balamuth's medium are very good for intestinal protozoa, especially for *Entamoeba histolytica*. Also, the NIH trypticase-liver serum medium (TLS) is very useful in the diagnosis of *Trichomonas vaginalis* infections. The coccidia, *Balantidium coli*, and *Giardia lamblia* are not usually recovered with the routinely used media.

6-2. Cultures for Intestinal Protozoa:

a. **Modified Boeck and Drbohlav's Medium:**

(1) *Discussion.* This medium is recommended for routine use where cultures are employed to isolate intestinal protozoa. It supports the growth of most intestinal protozoa, especially *E. histolytica*. It requires only materials that are commonly available in laboratories.

(2) *Reagents:*

(a) *Modified Locke's Solution:*

Sodium chloride (NaCl)	8.00 g
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.20 g
Potassium chloride (KCl)	0.20 g
Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	0.01 g
Sodium phosphate (Na_2HPO_4)	2.00 g
Sodium bicarbonate ($NaHCO_3$)	0.40 g
Potassium phosphate (KH_2PO_4)	0.30 g
Distilled water	1,000.00 ml

1. In the order listed, add the above chemicals to the distilled water. Mix each one until it is dissolved before adding the next.

2. Boil for 10 minutes. A precipitate will form.

3. Cool to room temperature. Filter the solution through filter paper.

4. Sterilize in an autoclave for 15 minutes at 15 lb (121C).

(b) *Egg Slants.*

Fresh eggs	4
Sterile Locke's solution	50 ml

1. Wash four fresh eggs with soap and water, rinse in 70 percent ethyl alcohol.

2. Break the eggs into a 250 ml flask containing a few glass beads (use a blender if available).

3. Add the sterile Locke's solution and emulsify.

4. Filter the mixture through gauze into a dispenser.

5. Dispense into sterile test tubes (15 × 125 mm) so that a short butt and a 4 to 5 cm slant is formed.

6. Inspissate. When an inspissator is not available, use an autoclave.

a. In autoclaves that have a jacket, allow the pressure to reach 15 lb. Place the tubes in the chamber in the desired slanted position. Close all exhaust valves and the door of the chamber. Allow the steam from the jacket to enter the chamber as fast as possible until the pressure gauge registers 15 lb. Hold at that pressure for 15 minutes. Cut off the steam supply and with all doors and valves still tightly closed, allow the pressure to drop gradually.

b. In autoclaves without jackets, place the tubes in the chamber and close all exhaust valves and the door. When the drain at the bottom of the autoclave has no valve, stopper it with a rubber stopper. Turn on the steam, allowing the pressure to reach 15 lb. The mixture of air and steam will not be above 85C. Hold at 15 lbs pressure for 15 minutes. Turn off the steam and let the autoclave cool gradually.

7. After the slants cool, cover them to a depth of 1 cm with sterile Locke's solution.

8. Sterilize the tubes in the autoclave at 15 pounds pressure for 15 minutes (temperature 121C). Make certain that the pressure is released very slowly.

9. Test the medium for sterility by incubating at 37C for 24 hours. Then place the tubes in a refrigerator until needed.

(c) *Rice Starch.* Spread a few grams of rice powder evenly in a large tube and heat in a hot air oven for 2 1/2 hours at 150C. Keep the tube in a horizontal position during sterilization. When larger amounts are needed, sterilize the starch in the autoclave at 121C for 20 minutes.

b. **Egg Yolk Infusion Medium (Balamuth):**

(1) *Discussion.* This is an all-liquid, egg-yolk infusion medium to which liver is added. It is good for isolating most intestinal protozoa; furthermore, it can be stored up to 3 months without marked deterioration.

(2) *Reagents:*

(a) *Liver Extract Solution.*

Wilson's dry extract of liver (powder) ..... 5 g
Distilled water ..... 100 ml

1. Dissolve the liver extract powder in the distilled water and heat to the boiling point.

2. Filter through ordinary filter paper and autoclave at 15 pounds (121C) for 20 minutes.

(b) *Stock Buffer Solutions (1 M Solutions):*

1. Dibasic Phosphate Solution.

K_2HPO_4 ..... 87.09 g
Distilled water ..... 500.00 ml

Dissolve the K_2HPO_4 in part of the distilled water in a 500 ml volumetric flask and then add water to make 500 ml of solution.

2. Dihydrogen Phosphate Solution.

KH_2PO_4 ..... 13.61 g
Distilled water ..... 500.00 ml

Dissolve the KH_2PO_4 in part of the distilled water in a 500 ml volumetric flask and then add water to make 500 ml of solution.

(c) *Working Buffer Solution (M/15 Solution).*

Dibasic phosphate
(K_2HPO_4) stock buffer ..... 4.3 parts
Dihydrogen phosphate
(KH_2PO_4) stock buffer ..... 0.7 parts

Mix the stock buffers and dilute 1:14 with distilled water to obtain the M/15 solution.

(d) *Egg Yolk Infusion.*

Fresh eggs 4
Sodium chloride solution (0.8%) ..... 125 ml
Working M/15 buffer solution
Distilled water ..... 20 ml

1. Place four fresh eggs in cool water. Heat water and boil for 15 minutes.

2. Mix the yolks of the four hard-boiled eggs with 125 ml of 0.8% sodium chloride. Use an electric blender if available.

3. Heat the mixture in a covered double boiler for 20 minutes after it reaches 80C. Add 20 ml distilled water to make up for evaporation.

4. Filter through a Büchner funnel under reduced pressure, using several layers of number two filter paper.

5. Autoclave the filtrate for 20 minutes at 15 pounds pressure.

6. Cool the yolk filtrate to less than 10C; then refilter with the Büchner funnel.

7. Mix equal amounts of M/15 buffer solution and yolk filtrate to complete the yolk infusion.

(e) *Rice Starch.* See Modified Boeck and Drbohlav's Medium for preparation instructions.

(3) *Preparation of Medium:*

(a) Mix one part of the liver extract solution with nine parts of the yolk infusion.

(b) Transfer 7 to 10 ml of the medium to large screw-capped tubes.

(c) Autoclave the medium at 15 pounds (121C) for 20 minutes. Store in the refrigerator. Remove any sediment that accumulates in the medium by filtering and reautoclaving it before inoculation.

c. **Inoculation and Examination of Cultures for Intestinal Protozoans:**

(1) For each specimen to be cultured, add approximately 30 mg (1 wireloop full) of sterile rice starch to two tubes of medium and then warm the tubes to 37C just prior to inoculation.

(2) To one of the tubes add 250 units of penicillin and 250 units of streptomycin for each ml of fluid in the tube. (Example: Each of the antibiotics contains 5,000 units per 1 ml. Therefore, 0.05 ml of each contains 250 units. One drop is approximately 0.05 ml).

(3) Whenever liquid or semisolid specimens require culturing, transfer 0.5 ml of the materi-

al to each tube of medium. Do not add any more air with the specimen than necessary. Mix the specimen with the liquid portion of the medium, taking care not to introduce air bubbles.

(4) To culture formed feces, transfer a portion of the specimen, the size of a small pea, to the medium. Mix the material with the liquid portion of the medium by carefully rubbing the material against the side of the tube. Select any mucus, blood, or abnormal-appearing material in the feces for inoculation.

(5) Incubate all tubes at 37C for 24 hours.

(6) Prepare two wet preparations of the sediment from each tube. Leave one of the slides unstained, and prepare a vital stain using Quensel's or buffered methylene blue solutions with the other slide.

(7) Transfer to fresh medium, about half of the sediment of all cultures that show no trophozoites or an insufficient number for positive identification. Do not add antibiotics to any of these secondary cultures.

(8) Incubate both the original cultures with the transfers at 37C for 48 hours and then examine them microscopically. Mix the sediment of all positive and questionable cultures in vials with PVA-fixative. Prepare permanently stained films of the PVA-fixed sediments and carefully examine them under the oil-immersion objective.

(9) To maintain amebae in cultures make transfers to fresh medium every 2 or 3 days. Observe aseptic precautions because the introduction of certain bacteria into the culture medium kills the amebae.

6-3. Culture For Shipping Intestinal Protozoa. The media used for routine cultivation of intestinal protozoa in the laboratory are not suitable for shipping specimens. However, by enriching Balamuth's egg-extract medium with dextrose, trypticase, and sodium thioglycollate you can prepare a medium in which organisms can survive up to 4 days. The medium permits viable organisms to be shipped to other laboratories for identification.

a. **Reagents:**

(1) *Modified Balamuth's Medium.* Prepare as directed for Balamuth's Egg Yolk Infusion Medium, but omit the liver extract solution.

(2) *Enriched Locke's Solution.*

Modified Locke's Solution	100 ml
Dextrose	110 mg
Thioglycollate	50 mg
Trypticase (BBL)	2 g

Add the dextrose, thioglycollate, and trypticase to the modified Locke's solution and mix well.

b. **Preparation of Medium:**

(1) Mix five parts of Balamuth's medium (without liver extract solution) with one part of enriched Locke's solution.

(2) Pipet 6 ml of the medium to screwcap tubes (approximately 15 × 125 mm).

(3) Autoclave at 15 pounds pressure for 15 minutes.

(4) Cool and store in refrigerator. The medium is good for 3 months when it is kept refrigerated.

c. **Inoculation Procedure:**

(1) Add 250 units of penicillin and 250 units of streptomycin for each ml of fluid in the tube. (Example: Each of the antibiotics contains 5,000 units per 1 ml. Then, 0.05 ml of each contains 250 units. One drop is approximately 0.05 ml.)

(2) Transfer a portion of the specimen, the size of a small pea (about 0.5 ml), to the medium. Also, select any mucus, blood, or abnormal appearing material in the feces for inoculation. Mix the specimen with the medium by carefully rubbing the material against the side of the tube.

(3) Screw the cap on tightly, seal with tape and paraffin, and ship the specimen in a double mailing container (or suitable substitute) to the laboratory. Do not incubate.

d. **Culture Procedure:**

(1) When the specimen is received in the laboratory, remove the supernatant fluid.

(2) Transfer about half of the sediment to a second tube of medium. Replace the fluid in the first tube with fresh medium. Do not add antibiotics. Also, do not make the transfer if the amount of sediment is very small as may be the case with sigmoidoscopic specimens.

(3) Incubate the tubes at 37C and examine them for motile organisms daily.

(4) Organisms usually can be detected after 24 hours of incubation in positive specimens if the storage or transit time was not more than 1 day. Cultures kept at room temperature for longer periods have to be incubated longer before growth is apparent. For example, speci-

mens in the holding medium left at room temperature for 3 days must be incubated for 5 days before they are discarded as negative. Although organisms have been recovered after 4 days at room temperature, best results are obtained with specimens not more than 1 to 2 days old.

6-4. NIH Trypticase-Liver-Serum Medium (TLS) Modified for Trichomonas Vaginalis. There are a number of different media now in use for the culture of *Trichomonas vaginalis*. Some are commercially available but most have to be prepared locally. There is no universally accepted "best medium;" however, the one given here is considered to be adequate.

a. **Reagents:**

(1) *Solution A.*

Trypticase (BBL)	4.0 g
Sodium chloride (NaCl)	1.4 g
Dextrose	2.0 g
Distilled water	200.0 ml

(a) Dissolve the reagents in the distilled water.

(b) Heat the solution to 100C.

(2) *Solution B.*

Liver extract (Difco Bacto)	10.0 g
Sodium chloride (NaCl)	1.4 g
Tryptone	1.0 g
Potassium chloride (KCl)	80.0 mg
Calcium chloride ($CaCl_2$)	80.0 mg
Sodium bicarbonate ($NaHCO_3$)	80.0 mg
Distilled water	300.0 ml

(a) Mix 10 g of liver extract in 200 ml of distilled water.

(b) Heat mixture at 56C for 1 hour.

(c) Increase temperature to 80C and filter through paper.

(d) Adjust volume to 200 ml.

(e) Add 1.4 g NaCl, 1.0 g tryptone, 80 mg KCl, 80 mg $CaCl_2$, and 80 mg $NaHCO_3$.

(f) Mix well and continue to heat with mixing until dissolved.

(g) Filter the broth through paper again.

(3) *Sterile Serum (20 ml):*

(a) Aseptically collect 20 ml of any type serum. Do not use serum to which preservatives have been added.

(b) Heat the serum at 56C for 30 minutes in a water bath to inactivate the complement.

(4) *Reducing Agent (Sodium Thioglycollate):*

(a) Dissolve 0.4 g of sodium thioglycollate in 10 ml of distilled water.

(b) Dispense 2 ml of the solution to five small screw-capped vials and sterilize in the autoclave.

(c) Seal the tubes with paraffin and store at room temperature.

(d) Prepare fresh solution weekly.

(5) *Antibiotics.*

Chloramphenicol	250.0 mg
Nystatin	1250.0 mg
Sterile saline (physiological)	100.0 ml

(a) Mix the chloramphenicol and nystatin in a small amount of the saline until they are dissolved.

(b) Make up final volume of the solution to 100 ml.

b. **Basic Medium:**

(1) Mix 120 ml of solution A with 200 ml of solution B.

(2) Adjust the pH to 6.0 with 5M HCl or 5M NaOH.

(3) Add the 20 ml of sterile inactivated serum.

(4) Add 15 ml of the antibiotic solution to the medium.

(5) Dispense 9 ml of the basic medium into sterile 16 × 150 mm screw-capped tubes.

(6) Incubate the tubes for 24 hours at 37C to check for sterility.

(7) Store the tubes in a refrigerator at approximately 4C.

(8) Discard unused medium after 4 weeks.

c. **Culture Procedure:**

(1) Remove tubed medium from the refrigerator.

(2) Add 0.2 ml of the reducing agent (sodium thioglycollate) to the tube.

(3) When wet preparations of vaginal exudates are not requested, place the exudate directly into the medium by breaking cotton swabs off in the medium tube or by rinsing the vaginal pipet in the medium.

(4) When wet preparations and cultures are required on the same specimen of vaginal exudate, rinse the swabs in about 1 ml of sterile

saline or Ringer's solution, examine a drop of the solution for the wet prep, pour the rest of the material into the culture medium, and then break off the swabs in the medium.

(5) To culture urine specimens for *Trichomonas vaginalis*, spin the specimen at slow and pour the sediment into the medium.

(6) Incubate the specimen at 35C to 37C.

(7) Examine a drop of the medium from the bottom of the tube each day.

(8) Incubate the culture for 5 days before issuing a negative report. ("No organisms recovered.") Organisms will be found in most positive cases within 3 days, but a few will not be detected until the fifth day.

6-5. Maturing Coccidia Oocysts. Man occasionally passes oocysts of *Eimeria* species that were ingested with animal tissue even though no infection exists. Therefore, the generic identification of oocysts found in a stool specimen has to be made. Such identification of the immature oocysts of *Eimeria* species and *Isospora* species is difficult. However, when the oocysts are matured, the genera can be differentiated on the basis of the number of sporocysts present and the number of sporozoites in each sporocyst.

a. **Reagent: Potassium Dichromate Solution.**

Potassium dichromate ($K_2Cr_2O_7$) crystals ..... 2 g
Distilled water ..... 100 ml
Dissolve the crystals in the water. Store in a glass-stoppered bottle.

b. **Preparation:**

(1) Emulsify a portion of feces in about 2 volumes of potassium dichromate solution.

(2) Pour the specimen into a petri dish to a depth of about one-fourth inch.

(3) Cover the petri dish and leave at room temperature for 2 to 4 days. Add distilled water when necessary to prevent the suspension from drying up.

c. **Examination:**

(1) Examine a drop of the sediment on a glass slide under low and high power for the organisms. Stains are not required, but the use of reduced light gives the best results.

(2) When the organisms are too few to be detected in this manner, concentrate the material by centrifugation. Transfer the entire contents of the petri dish to a 15 ml conical centrifuge tube and spin at 2,000 rpm for 1 to 2 minutes. Examine a drop of the sediment as described above. Adjust the density of the slide preparation with saline as necessary to observe the organisms.

SECTION B—RECOVERY AND HATCHING OF HELMINTH LARVAE

6-6. Introduction. Eggs of helminths are frequently so scarce in a specimen that they are very difficult to find even when concentration procedures are employed. However, if the eggs are hatched, the active motile larvae are much easier to concentrate and find. Also, some eggs are indistinguishable while the larvae of most species are more easily differentiated.

6-7. Filter Paper Method (Harada-Mori's Method). This is the simplest procedure available to hatch and isolate larvae of helminths for identification. It is particularly effective for hookworms, strongyloides, and trichostrongyles.

a. **Preparation:**

(1) Cut a strip of filter just wide enough to easily enter a 16 × 150 mm test tube or sufficiently wide to keep it from going more than half-way down the conical part of a 15 ml conical centrifuge tube. Cut the strip slightly shorter than the tube so that it will not touch the stopper or plug.

(2) Smear about 0.5 g of fresh feces (a quantity about the size of a small pea) on the center portion of the filter paper so that the smeared feces is about one-sixteenth of an inch thick. The smear should be about one-quarter of an inch wide and one to one and a quarter inches long.

(3) Place 3 ml of tap water in the 16 × 150 mm test tube or the 15 ml conical centrifuge tube.

(4) Put the filter paper strip into the tube so that the lower end of the strip is well into the water, but the feces itself is not in the water.

(5) Incubate the tubes in a dark place at room temperature.

(6) Do not cap the tube until the third day so that the water can carry soluble toxic materials up the filter paper strip away from the eggs. Then cap the tube lightly to prevent larvae from escaping.

b. **Precautions:**

(1) Keep the water level high enough to reach the filter paper strip at all times.

(2) Be careful when handling cultures. Filariform larvae of hookworms and strongyloides produce infections by penetrating intact skin. Such larvae frequently migrate up the sides of the culture tubes and will be found in the drops of condensed moisture.

(3) Free-living nematodes are sometimes found in these preparations when the fecal specimen has been contaminated with soil. When free-living nematodes are suspected, they can be differentiated from filariform larvae of animal parasites by adding 0.1 ml of concentrated hydrochloric acid per 3.0-ml of water in the culture. The free-living nematodes will be killed almost instantly, but the infective larvae of animal parasites will live for about 24 hours.

(4) Refrigeration of stool specimens prior to culturing inhibits hatching of eggs, particularly those of hookworm.

c. **Examination:**

(1) Examine the cultures at the end of 3 days and again at the end of 10 days.

(2) After the third day remove a drop of water from the botton of the tube with a Pasteur pipet.

(3) Search the drop with the lowest power of the microscope for actively motile larvae.

(4) Kill any larvae found by adding a drop of iodine solution or 10% formalin.

(5) Change to the magnification required to observe the identifying characteristics.

(a) Take particular care to distinguish between rhabditiform and filariform larvae to avoid erroneous diagnosis.

(b) After 3 days of incubation, cultures prepared from freshly passed feces can contain rhabditiform larvae of hookworms, trichostrongyles, and or filariform larvae of strongyloides.

(6) After incubating for 10 days, carefully remove the filter paper strip with forceps.

(7) Fill the tube with tap water.

(8) Centrifuge the tube for 2 minutes at 1,500 rpm.

(9) Immediately draw off the supernatant fluids and examine the sediment for actively motile filariform larvae.

(10) Kill any larvae found by adding a drop of iodine solution or 10% formalin.

(11) Switch to the magnification required to observe the identifying characteristics. At the end of 10 days any nematode larvae (that frequently parasitize man) that hatch in this culture are expected to be developed to the filariform stage. Investigate the possibility of free-living nematodes in cultures containing rhabditiform larvae after 10 days; however, do not exclude free-living adults of strongyloides.

6-8. Baermann Apparatus for Recovery of Nematode Larvae. The Baermann technique is probably the simplest method to recover strongyloides larvae from light infections. A good concentration of living strongyloides larvae can be obtained from a large volume of feces, making the procedure very useful for diagnosis as well as therapy followup. The apparatus can also be used to recover other larvae from feces, tissue, or soil by slightly modifying the procedure. In any case the surface area of a specimen is greatly increased, thus allowing larvae to escape.

a. **Preparation:**

(1) Set up a 4-inch glass funnel in a ring stand as shown in figure 6-1.

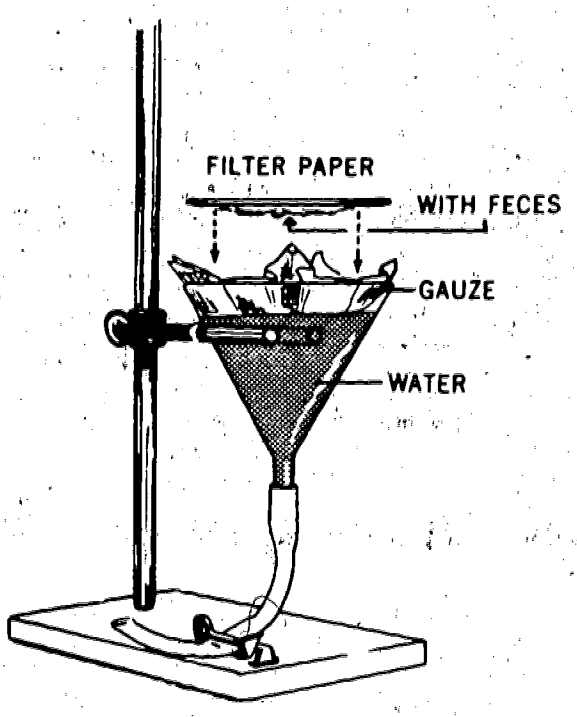


Figure 6-1. Baermann Apparatus.

(2) Cover the top of the funnel with four layers of gauze. Push the gauze down about

one-half inch around the inside edge of the funnel to form a platform in the funnel. If the gauze offers too little support for the material to be tested, use a circle of wire gauze covered with two thicknesses of regular gauze.

(3) Fill the funnel with lukewarm water to a level that just covers the gauze.

(4) Spread the fecal specimen in a thick layer over a circle of filter paper that will just rest on the gauze in the funnel.

(5) Place the filter paper, feces side down, on the gauze. (See figure 6-1.) Place other material to be tested directly on the moist gauze. Add tap water to feces that are too hard to spread, and with a spatula make a firm paste of the specimen that is workable.

(6) Incubate the set-up from room temperature to 40C.

(a) Incubate fresh feces for 1 to 2 hours.

(b) Incubate soil samples or tissue specimens for 8 to 12 hours.

(7) Draw off 10 cc of water from the bottom of the funnel and centrifuge at 1,500 rpm for 2 minutes.

(8) Examine the sediment for larvae.

b. **Examination:**

(1) Kill any larvae found by adding a drop of iodine solution or 10% formalin.

(2) Change to the magnification required to observe the identifying characteristics.

6-9. Hatching of Schistosome Eggs. Viable schistosome eggs contain a fully developed miracidium when passed from the body. Upon exposure to fresh water for a few hours the miracidium escapes from the egg shell and seeks a suitable molluscan host. Miracidia demonstrate phototropism which aids them in their search for their molluscan hosts because it helps guide them to shallow water where the molluscs are more concentrated. This characteristic of phototropism also makes it possible to concentrate the miracidia after hatching them in the laboratory. The procedure is very useful for indirectly demonstrating small numbers of viable eggs present in a fecal sample.

a. **Preparation:**

(1) Boil approximately 1,500 ml of tap water for 10 minutes to make it chlorine-free. Chlorine in high enough concentration will kill the miracidia.

(2) Cool the water to room temperature.

(3) Comminute thoroughly approximately 5 to 10 g of feces (a sample the size of one or two large walnuts) in a 250 ml beaker one-fourth filled with water. The exact sample size does not matter, but it should be ample.

(4) Fill the beaker three-fourths full and stir to mix the suspension.

(5) Strain the material through two layers of wet cheesecloth into a sedimentation glass or cone-shaped graduate.

(6) Allow the suspension to settle for 1 hour.

(7) Pour off the supernatant fluid without allowing any sediment to escape.

(8) Resuspend the sediment in 200 ml of the dechlorinated water and allow to settle for another hour. If the supernatant fluid is very cloudy at this time, wash the fecal material again. If the water is too cloudy, the miracidia will not concentrate properly; they will be found throughout the fluid.

(9) Again pour off the supernatant fluid.

(10) Resuspend the sediment in a small quantity of water and pour it into a 500 ml sidearm flask. If a sidearm flask is not available, pour the suspension into a 500 ml Erlenmeyer flask.

(11) Fill the flask almost to the lip with water.

(12) Cover the sidearm flask with paper towels so that light only reaches the sidearm. When using an Erlenmeyer flask, cover all of it except for the anterior one-half inch of the neck containing water.

(13) Incubate the flask at room temperature for several hours or overnight in the light.

b. **Examination.** The eggs will hatch, liberating the miracidia, which will swim to the light, thus collecting in the sidearm or in the neck of the flask depending upon which type is used. The organisms concentrated in a small area are easily visible against a dark background.

(1) Place a black matte object behind the sidearm or the neck of the flask and look for minute, white organisms swimming in a straight course. They tend to collect around the margins of the container.

(2) Carefully check with a hand lens before reporting "No organisms recovered." Often

there are very few miracidia present, but they can be found by searching carefully.

(3) Free-living ciliates, such as various species of the genus *Colpoda*, can quickly appear in contaminated water and must be differentiated from miracidia. To make the distinction, transfer a few organisms to a slide and examine under the microscope. While observing the organisms, add a drop of weak iodine solution or dilute methylene blue. Miracidia will become motionless in a short time. Figure 6-2 illustrates the morphological characteristics of a miracidium and a free-living ciliate.

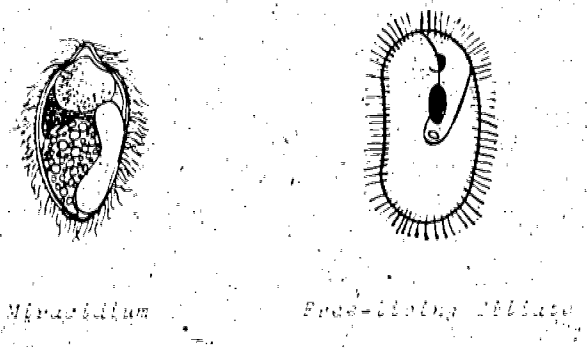


Figure 6-2. Typical Miracidium and Free-Living Ciliate ×200.

Chapter 7
PERMANENT STAINS FOR PROTOZOA

7-1. Introduction. Most trophozoites and cysts are very difficult to positively identify without being stained. In fact no trophozoite should be reported as positively identified without having been examined in a stained preparation. Do not restrict the use of permanently stained smears to confirming the identification of organisms already found in wet preparations. Small organisms that are frequently overlooked on other preparations can be found and identified upon the careful examination of properly stained smears. The smears make permanent records that can be reexamined if questions arise at a later date.

7-2. Preparation of Smears:

a. **Slides.** Make smears for permanent stains on 3 × 1 inch slides that have been cleaned in a solution of one part acetone and four parts methanol. Slides that have a frosted end are best. Write the identifying information on the slide with an ordinary lead pencil. When using nonfrosted slides, clearly mark them with a diamond pencil to insure positive identification. Never use other types of markers that will wash off during fixing or staining.

b. **Routine Smears Fixed in Schaudinn's Solution:**

(1) *Making the Smear.* Streak the specimen back and forth on the slide with an applicator stick to a thin even smear. An absolutely uniform smear cannot be obtained, but the specimen should be as evenly distributed as possible. Therefore, if necessary, dilute any hard, dry specimens with saline. Smears that are too thick stain poorly and will be too dense to examine. Very thin smears destain too rapidly, and when organisms are scarce, they can be missed or require prolonged searching to be found. The "just right" smear appears quite thin and very smooth. With a little practice the correct density will be quickly found.

(2) *Fixation.* Immediately after the smear is made on a slide, completely immerse it in Schaudinn's fixative solution for 2 to 3 hours. Do not allow the smear to dry at any time after it is made until it is mounted. This is very important because any slide that does dry is ruined. Slides that cannot be stained and mounted immediately after they are prepared can be left in the fixative solution for several hours or even days when necessary. When it is necessary to reduce the fixing time, heat the Schaudinn's solution to 56C and fix the smears for 3-5 minutes. Protozoan cysts and trophozoites are generally not distorted. However, *Chilomastix* and *Trichomonas* tend to round up and often appear atypical.

(3) *Schaudinn's Fixative Solution.*

Mercuric chloride	70 g
Distilled water	1000 ml
Ethyl alcohol, 95%	500 ml
Glacial acetic acid	150 ml

(a) In a 2-liter container dissolve the mercuric chloride in the water by heating and occasional stirring.

(b) Allow the solution to cool to room temperature. Excess mercuric chloride will crystallize out.

(c) Decant the clear solution from the crystals.

(d) Mix two parts of the mercuric chloride solution with one part of 95% ethyl alcohol. This stock solution will keep almost indefinitely if tightly stoppered to prevent evaporation.

(e) Add 5 ml of glacial acetic acid to each 95 ml of the above stock solution just prior to use. The acidified solution deteriorates and must be prepared fresh each time it is used.

c. **Smears Fixed with PVA-fixative.** Feces or other materials can be fixed with PVA-fixative either directly on slides or in vials for making smears later. Specimens collected for routine diagnosis are usually fixed in vials and smeared later. However, when small amounts of material are obtained by sigmoidoscopy or other special techniques, it is best to fix them directly on the slide to keep from losing them. Such material can be examined directly in saline, the coverslip removed, PVA-fixative added, and the material spread into a film for future staining. Smears fixed with PVA are permeable to all commonly used stains.

(1) *Fixation on Microscope Slides.* Add 3 volumes of PVA-fixative for each volume of material to be fixed directly on the slides. See paragraph 3-5c, for PVA-fixative preparation. Mix the materials and spread (do not smear) over approximately one-third of the slide. Extend the smear to the edge of the slide. Allow the film to dry for several hours or overnight. Drying can be speeded by using a 37C incubator. Do not attempt to stain the smear until it is thoroughly dry. The smear can wash off the slide if it has not been completely dried. Stain the smear not later than 2 months after it has been prepared to prevent deterioration.

(2) *Smears from PVA-Fixed Specimens.* When preparing smears from PVA-fixed specimens in vials, carefully mix the specimen before putting any on the slide. If the specimen in the vial has jelled, reliquefy it by heating it in a waterbath. Discard any specimens that rejell after the second liquefication. After the specimen is mixed, transfer 2 or 3 drops to a clean slide and proceed as for smears fixed directly on slides.

7-3. Staining Procedures:

a. **Heidenhain's Iron-Hematoxylin Stain:**

(1) *Introduction.* The Heidenhain's iron-hematoxylin stain is the "classical" procedure to which all others are compared. Excellent results can be obtained if the procedure is carefully followed, especially with regard to the destaining of the organisms. The original procedure is very long, but it can be shortened considerably by heating the components. Smears fixed with PVA-fixative require different times in the various solutions but stain as well as smears fixed in plain Schaudinn's solution.

(2) *Stain Reactions.* Properly stained organisms are bluish or grayish with black nuclear structures. Chromatoid bodies of amebae cysts and inclusions, such as bacteria or red blood cells, in the cytoplasm of trophozoites stain black. Leukocytes, *Blastocystis*, and some yeasts and molds can cause confusion unless the observer is trained in the differential morphology of the protozoa because they have similar staining characteristics. Background material usually stains blue gray. Poor fixation rather than a poor stain is usually the cause of unstained organisms.

(3) *Reagents:*

(a) *Iodine Alcohol.* Prepare a stock solution by adding sufficient iodine crystals to 70% ethyl alcohol to make a dark, concentrated solution. For the working solution, dilute some of the stock with 70% ethyl alcohol until a strong tea-colored solution is obtained. The exact concentration is not important, but the solution must not be too strong because the iodine will stain the protozoa and interfere with the other staining.

(b) *Mordant (Iron Alum).*

Ferric ammonium sulfate (violet crystals) ..... 4 g
Distilled water ..... 100 ml

1. Dissolve the ferric ammonium sulfate crystals in the distilled water.

2. Prepare this solution just before use.

3. Store ferric ammonium sulfate crystals in a refrigerator.

(c) *Stock Hematoxylin.*

Hematoxylin (powder or crystals) ..... 10 g
Ethyl alcohol, 95% ..... 100 ml

1. In a 100 ml volumetric flask dissolve the hematoxylin in a small amount of 95% ethyl alcohol.

2. Add 95% ethyl alcohol to make 100 ml.

3. Allow stain solution to ripen for 6 weeks before diluting it for staining.

4. When stock stain is ripe, it is a deep orange-brown, clear solution.

(d) *Hematoxylin Staining Solution (0.5%).*

Stock hematoxylin solution (ripened) ..... 5 ml
Distilled water ..... 95 ml

1. Mix 5 ml of the stock hematoxylin solution with 95 ml of distilled water to make the 0.5% staining solution.

2. This dilute aqueous solution is not stable and must be prepared fresh each day or when needed.

(e) *Destaining Solution (2% Iron Alum Solution).*

Ferric ammonium sulfate solution ..... 1 part
Distilled water ..... 1 part

Dilute one part 4% ferric ammonium sulfate solution [mordant, reagent (b)] with one part distilled water.

(f) *Clearing Solution (Carbol-Xylene).*

Carbolic acid (phenol) ..... 1 part

Xylene ..... 3 parts

1. Liquefy phenol crystals by heating the jar in a water bath. Loosen the jar lid before heating.

2. Do not use liquid phenol solutions that contain water.

3. Add one volume of liquefied carbolic acid (phenol) to three volumes of xylene.

(g) *Alcohol-Lithium Carbonate Solution.*

Lithium carbonate ..... 2 g
Distilled water ..... 100 ml
Ethyl alcohol, 70% ..... as required

1. Agitate the lithium carbonate powder in the distilled water until no more will go into solution (saturated solution);

2. Immediately prior to use add a few drops of saturated lithium carbonate solution to each 100 ml of 70% ethyl alcohol in the staining dish.

(h) *Ethyl Alcohol.* Using either absolute or 95% ethyl alcohol and distilled water, prepare sufficient amounts of 50%, 70%, and 95% alcohol.

(4) *Procedure for Schaudinn's-Fixed Smears:*

(a) Smear and fix slides according to paragraph 7-2b. Do not allow smears to dry.

(b) Transfer slides to iodized alcohol [7-3a(3)(a)] for 5 minutes. (The iodine precipitates the excess mercury from the Schaudinn's fixative as mercuric iodide.)

(c) 50% alcohol ..... 3 minutes.

(d) Tap water ..... 3 minutes.

(e) Transfer slides to 4% iron alum mordant [7-3a(3)(b)] for 10-20 minutes at 40-50C, or 12-24 hours at room temperature.

(f) Water (distilled or tap) ..... 2 minutes.

(g) Water (distilled or tap) ..... 2 minutes.

(h) Stain slides in 0.5% hematoxylin [7-3a(3)(d)] for 5-10 minutes at 40-50C, or 12-24 hours at room temperature.

(i) Wash slides in two changes of tap water for a total of 5 minutes.

(j) This is the most critical step and must be performed carefully. Transfer the slides to the destaining solution [7-3a(3)(e)] (2% iron alum solution). At intervals of 1/2-1 minute, depending on the destain solution used, rinse the slides in water and examine them with a microscope. Do not allow the slides to dry. Repeat the process until the desired results are obtained. The total destaining time can vary from 1 to 5 minutes. The destaining process is completed when the structural details of the organisms present are visible. When large numbers of organisms are not present, destain the smears until the background material is more gray than black. Experience and practice are necessary to get consistently good results.

(k) Wash the slides in running tap water or several changes of water for 15-30 minutes to stop the destaining action. The stain will gradually fade unless the smears are thoroughly washed.

(l) Transfer the slides to the alcohol-lithium carbonate solution [7-3a(3)(g)] for 3 minutes. Lithium carbonate intensifies the blueness of the stain.

(m) 95% alcohol ..... 5 minutes.

(n) Carbol-xylene clearing solution, [7-3a(3)(f)] ..... 3 minutes.

(o) Xylene ..... 3 minutes, or until no beads of water can be seen on the slides.

(p) Coverslip with any suitable mounting medium dissolved in xylene or toluene.

b. **Modified Heidenhain Stain for PVA-Fixed Smears:**

(1) *Introduction.* The procedure for staining dried PVA-fixed smears is essentially the same as for other types of smears. The main difference is the variation in time required for certain steps. The staining reactions are also the same as for the original Heidenhain iron-hematoxylin stain.

(2) *Reagents.* This staining procedure uses essentially the same reagents as are used in the original Heidenhain iron-hematoxylin stain. The only difference is that a saturated solution of picric acid is used for destaining rather than a 2% iron alum solution. To prepare the saturated aqueous picric acid solution, add approximately 2 g of picric acid cyrstals to 100 ml of water. Shake thoroughly; let stand for several days, shaking at intervals. If all crystals dissolve, add more. After 3 to 4 days, some undissolved picric acid should remain in the flask. Filter before using.

(3) *Procedure:*

(a) Smear and fix slides according to paragraph 7-2c. Let dry completely.

(b) Place the slides in iodized alcohol [7-3a(3)(a)] for 20 minutes.

(c) 50% alcohol ..... 10 minutes.

(d) Tap water ..... 5 minutes.

(e) Then transfer slide to 4% iron alum mordant [7-3a(3)(b)] for 8-12 hours.

(f) Water (distilled or tap) ..... 2 minutes.

(g) Water (distilled or tap) ..... 2 minutes.

(h) Stain slides in 0.5% hematoxylin [7-3a(3)(d)] for 8-12 hours or overnight.

(i) Wash slides in two changes of tap water for a total of 5 minutes.

(j) This is the most critical step and must be performed carefully. Transfer the slides to the saturated picric acid destaining solution [7-3b(2)]. After about 10 minutes, rinse the slides in water and examine them with a microscope. Do not allow the slides to dry. Repeat the process at 2 minutes intervals until the desired results are obtained. The total destaining time can vary from 10 to 20 minutes. The process is completed when the structural details of the organisms present are visible. When large numbers of organisms are not present, destain the smears until the background material is more gray than black. Experience and practice are necessary to get consistently good results.

(k) Wash the slides in running tap water or several changes of water for 30 minutes to stop the destaining action. The stain will gradually fade unless the smears are thoroughly washed.

(l) Transfer the slides to the alcohol-lithium carbonate reagent [7-3a(3)(g)] for 10 minutes. Lithium carbonate intensifies the blueness of the stain.

(m) 95% alcohol ..... 10 minutes.

(n) Carbol-xylene clearing reagent [7-3a(3)(f)] ..... 10 minutes.

(o) Xylene ..... 10 minutes.

(p) Coverslip with any suitable mounting medium dissolved in xylene or toluene.

c. **Iron-Hematoxylin-Phosphotungstic Acid:**

(1) *Introduction.* The iron hematoxylin stain used with phosphotungstic acid for destaining is a good short staining procedure for routine use. The destaining is self-limiting and does not require microscopic observation for control as does the classical procedure. The stain reaction is similar to that of Heidenhain's stain, but the slides are bluer. This procedure gives very good results with fresh smears fixed in Schaudinn's solution, but it is not recommended for PVA-fixed material.

(2) *Reagents.* This staining procedure uses the same reagents as the original Heidenhain iron-hematoxylin stain except for the destaining agent. A 2% phosphotungstic acid solution is used instead of an iron alum solution. It is prepared by dissolving 2 g of phosphotungstic acid crystals in each 100 ml of distilled water.

(3) *Procedure:*

(a) Smear and fix the slides as directed in paragraph 7-2b.

(b) Transfer the slides to iodized alcohol [paragraph 7-3a(3)(a)] for 5 minutes.

(c) 50% alcohol ..... 3 minutes.

(d) Tap water ..... 3 minutes.

(e) After washing, transfer slides to 4% iron alum mordant [paragraph 7-3a(3)(b)] for 5 minutes.

(f) Rinse slides in tap water for 1 minute.

(g) Stain in 0.5% hematoxylin [paragraph 7-3a(3)(d)] for 1 minute.

(h) Wash in tap water for 1 minute.

(i) Destain in 2% phosphotungstic acid [paragraph 7-3c(2)] for a minimum of 2 minutes.

(j) Wash in running tap water or several changes of water for a total of 5 minutes.

(k) Transfer slides to the alcohol-lithium carbonate reagent [paragraph 7-3a(3)(g)] for 3 minutes.

(l) 95% alcohol ..... 3 minutes.

(m) Carbol-xylene clearing reagent [paragraph 7-3a(3)(f)] ..... 5 minutes.

(n) Xylene ..... 3 minutes.

(o) Coverslip using any suitable mounting medium dissolved in xylene or toluene.

d. **Trichrome:**

(1) *Introduction.* The trichrome staining procedure is quick and easy to perform. It gives very good results for routine purposes. Overstaining and differentiation are not necessary to bring out the morphologic details of the parasites. Also, mordanting is not necessary before staining. However, for best results, destaining is recommended because it gives better differentiation of cellular detail. This stain gives good results with smears fixed with either plain

Schaudinn's or PVA-fixative solution. Both procedures are given below.

(2) *Stain Characteristics:*

(a) The cytoplasm of properly fixed and stained organisms is blue-green, tinged with purple. Occasionally, *Entamoeba coli* cysts stain more purplish than cysts of other species. The nuclear chromatin, chromatoid bodies, and ingested red cells and bacteria stain red or purplish red. Other ingested particles, such as yeasts or molds, generally stain green, but variations in color of ingested particles do occur. Background material usually stains green, thus contrasting with the protozoa.

(b) Cysts that do not stain and those that stain predominantly red are usually associated with incomplete fixation. Organisms that stain pale green are associated with degenerate forms, although understained or overstained organisms can also appear green.

(c) Eggs and larvae stain red and contrast strongly with the green background. In contrast to smears stained with hematoxylin, trichrome-stained smears have a transparency which enables embedded protozoa to be identified in somewhat thicker preparations.

(3) *Reagents.* The solutions used in the trichrome procedure are the same as those used for the Heidenhain stain except for the stain and the destaining solution. The trichrome stain solution is stable and can be used repeatedly. Replace the lost volume by adding stock solution. However, the stain will be weakened when a large number of smears are stained in a short period of time. Its strength can be restored by allowing it to evaporate in the open air for 3 to 8 hours. Simply leave the cover off the staining dish overnight.

(a) *Trichrome Stain.*

Chromotrope 2R	0.60 g
Light Green SF	0.15 g
Fast Green FCF	0.15 g
Phosphotungstic acid	0.70 g
Acetic acid (glacial)	1.00 ml
Distilled water	100.00 ml

1. Put the dry stains into a clean flask.

2. Add the glacial acetic acid, shake to mix. Allow the mixture to stand for 30 minutes.

3. Add the distilled water. Shake to mix thoroughly.

4. Good stain is deep purple, almost black.

(b) *Destaining Solution (Acid Alcohol).*

Acetic acid (glacial)	0.5 ml
Ethyl alcohol, 90%	100.0 ml

Add 0.5 ml of glacial acetic acid to each 100 ml of 90% ethyl alcohol used to fill the staining dish.

(4) *Staining Procedure for Schaudinn's Fixed Smears:*

(a) Smear and fix slides as outlined in paragraph 7-2b. Do not allow smears to dry.

(b) Transfer slides to iodized alcohol [paragraph 7-3a(3)(a)] for 5 minutes.

(c) 70% ethyl alcohol ..... 1 minute.

(d) Pass slides through a second 70% ethyl alcohol bath for 1 minute.

(e) Stain slides in trichrome [paragraph 7-3d(3)(a)] for 2-8 minutes.

(f) Destain smears in acidified alcohol [paragraph 7-3d(3)(b)] for 10-20 seconds or until stain barely runs from smear. Prolonged destaining (over 20 seconds) will cause the organisms to be poorly differentiated.

(g) Rinse slides in two changes of 95% ethyl alcohol. If several slides are being stained, change the first alcohol rinse frequently to prevent the slides from being destained excessively.

(h) Carbol-xylene clearing solution [paragraph 7-3a(3)(f)] ..... 1 minute.

(i) Transfer to xylene for 1 minute or until refraction at interface of smear and xylene ends.

(j) Coverslip with any suitable mounting medium dissolved in xylene or toluene.

(5) *Staining Procedure for PVA-Fixed Smears:*

(a) Prepare slides as instructed in paragraph 7-2c. Let dry completely.

(b) Place slides in iodized alcohol [paragraph 7-3a(3)(a)] for 10 minutes.

(c) 70% Ethyl alcohol ..... 5 minutes.

(d) 70% Ethyl alcohol ..... 5 minutes

(e) Stain smears in trichrome [paragraph 7-3d(3)(a)] for 8 minutes.

(f) Destain in acidified alcohol [7-3d(3)(b)] for 10-20 seconds or until stain barely runs from smear. Prolonged destaining (over 20

seconds) will cause the organisms to be poorly differentiated.

(g) Rinse slides in 95% ethyl alcohol. If several slides are being stained, change the alcohol rinse frequently to prevent the slides from being destained excessively.

(h) 95% Ethyl alcohol ..... 5 minutes.

(i) Carbol-xylene clearing solution [paragraph 7-3a(3)(f)] ..... 10 minutes.

(j) Transfer to xylene for 10 minutes or until refraction at interface of smear and xylene ends.

(k) Coverslip with any suitable mounting medium dissolved in xylene or toluene.

e. **Chlorazol Black E (Kohn's):**

(1) *Introduction.* Chlorazol black E stain is a simple procedure in which fixation and staining take place in a single solution. Destaining is not necessary. Chlorazol black stains protozoa in smears or in tissues. It gives good results with fresh smears but it is not recommended for PVA-fixed material.

(2) *Stain Characteristics.* Protozoa in fresh smears stain green to gray-green, but organisms in older material will be gray to black. Nuclei, chromatoid bodies, karyosomes, and cell membranes appear dark green to black. Ingested red cells vary from pink to black. Cysts of *Entaboeba coli* stain pink or green, and rarely, *E. histolytica* cysts stain faintly pink. Also, *E. histolytica* trophozoites stain the same in smears and tissues.

(3) *Reagents:*

(a) *Phosphotungstic Acid (1% Solution).*

Phosphotungstic acid crystals ..... 1 g
Distilled water ..... 100 ml

1. Dissolve the phosphotungstic acid crystals in a small amount of water in a 100 ml volumetric flask.

2. Add water to the mark.

(b) *Basic Solution.*

Ethyl alcohol, 90% ..... 170 ml
Methyl alcohol ..... 160 ml
Acetic acid, glacial ..... 20 ml
Phenol, liquid ..... 20 ml
Phosphotungstic acid, 1% ..... 12 ml
Distilled water ..... 618 ml

1. Put about half of the distilled water into a 1000 ml volumetric flask.

2. Add the alcohols and acids and mix.

3. Add distilled water to the mark and mix.

(c) *Stock Stain Solution.*

Chlorazol black E dye ..... 5 g
Basic solution ..... 1000 ml

1. Grind the 5 g of chlorazol black E dye in a mortar for 3 minutes.

2. Add a small amount of basic solution and grind until a smooth paste is formed.

3. Add more solution and grind for 5 minutes.

4. Allow particulate matter to settle a few minutes and pour off the liquid into a separate, dry, clean container.

5. Add more basic solution, and continue grinding and mixing until all the dye appears to be in solution.

6. Add any remaining basic solution to the stain.

7. Bottle and cap the stain solution and put it aside for 4 to 6 weeks to ripen.

8. A black sediment settles out within a few days, leaving a black cherry-colored liquid that is the fixative-stain.

9. Filter the stain through Whatman #12 filter paper before using.

10. Keep the filtered stain in a stoppered bottle.

(4) *Deterioration of Stain.* The optimum staining dilution and staining time must be determined for each batch of stock stain prepared. The stock stain can be kept indefinitely, and stain dilutions can be used repeatedly. However, repeated use "wears out" the stain dilution. When slides appear visibly red rather than greenish-black at the end of the staining period, discard the stain dilution. Approximately 20 slides can be stained in a 50 ml coplin jar of stain before it must be discarded. The deterioration of the stain depends on use and not on time. Stain dilutions will give satisfactory results for at least 30 days if the number of slides stained is not excessive.

(5) *Procedure for Staining Smears:*

(a) *Determination of Stain Dilution and Staining Time.* Prepare the following series of dilutions and stain trial smears for the times given. From those trial smears, determine the optimum dilution and time for routine staining with the stock stain concerned. More than one

dilution-time combination is often satisfactory, and the choice of which to employ depends on the laboratory schedule and urgency of diagnosis. Usually the 1:2 dilution for 2 hours or the 1:3 dilution for 4 hours or overnight are best but, for best results, determine what is best for your stain.

Stock Stain [Reagent 7-3e(3)(c)]	Basic Solution [Reagent 7-3e(3)(b)]	Hours
Undiluted	---	2-3
1	1	2-4 to overnight
2	1	2-4
1	2	2 to overnight
1	3	4 to overnight

(b) *Staining Steps for Smears:*

1. Place freshly prepared smears in fixative-stain dilution as determined above [paragraph 7-3e(5)(a)] to be the best combination of dilution and time for your stain. Restain the slides in fresh stain if they appear red rather than greenish-black.

2. Ethyl alcohol, 95% .. 10-15 seconds.

3. Transfer slides to carbol-xylene clearing solution [paragraph 7-3a(3)(f)] that is modified to be 1 part phenol to 2 parts xylene rather than 1:3. Clear slides for 5 minutes.

4. Xylene ..... 5 minutes or until refraction at interface of smear and xylene ends.

5. Coverslip with any suitable mounting medium dissolved in xylene or toluene.

(6) *Procedure for Staining Protozoa in Tissue.* Cut tissue sections 5 to 7 microns in thickness. Prepare them using the usual histological procedures for fixation, embedding, and sectioning. Treat the sections in xylene and alcohols to remove the paraffin and prepare them for staining.

(a) *Determination of Stain Dilution and Staining Time.* To stain protozoa in tissue, use the same method to determine the best stain dilution and time as used for protozoa in smears. Substitute distilled water for the basic solution used to dilute the stock stain. The staining time required for tissues usually runs about twice as long as for smears. The most common satisfactory time is about 6-7 hours with the optimum dilution; however, for best results, determine what is best for your stain.

Stock Stain Undiluted	Basic Solution ---	Hours 4-6
1	1	4-8 or overnight
2	1	4-8
1	2	4 to overnight
1	3	8 to overnight

(b) *Staining Steps for Tissues:*

1. Place tissue slides in the proper fixative-stain dilution as previously determined [paragraph 7-3e(6)(a)] to be the best combination of dilution and time for your stain. Restain the slides in fresh stain if they appear red rather than greenish-black.

2. Ethyl alcohol, 95% .. 10-15 seconds.

3. Transfer slides to carbol-xylene clearing solution [paragraph 7-3a(3)(f)] that is modified to be 1 part phenol to 2 parts xylene rather than 1:3. Clear slides for 10 minutes.

4. Xylene ..... 10 minutes or until refraction at interface of smear and xylene ends.

5. Coverslip with any suitable mounting medium dissolved in xylene or toluene.

f. **Acid Fuchsin, Fast Green Stain (Lawless):**

(1) *Introduction.* The acid fuchsin, fast green stain is a simple procedure where fixation and staining take place at the same time. Destaining is not required. Smears stained with this rapid procedure can be retained for reference since they do not fade for several months or even years. Good results are obtained with fresh smears but this stain is not recommended for PVA-fixed material.

(2) *Stain Characteristics.* The staining characteristics of this stain are similar to those seen in smears prepared with hematoxylin, and the same criteria are employed for identification of intestinal protozoa. Nuclear and chromatoid substances stain almost black, and cytoplasm with the usual inclusions stains well in both cysts and trophozoites of all species.

(3) *Acid Fuchsin, Fast Green Stain Solution.*

Acetone	50.0 ml
Acetic acid, glacial	50.0 ml
Formaldehyde, USP	10.0 ml
Schaudinn's solution [paragraph 7-2b(3)]	890.0 ml
Acid fuchsin	2.5 g
Fast green, FCF	1.0 g

(a) Mix the four solutions and add the two dyes.

(b) Agitate until the dyes are completely dissolved.

(c) Store the prepared stain in a tightly stoppered brown bottle.

(d) Staining solution is stable indefinitely.

(4) *Staining Procedure:*

(a) Prepare a thin smear of fresh material on a clean glass slide. Do not let it dry.

(b) Immediately cover the slide with the fixative-stain. Make sure it is completely covered.

(c) Gently heat the stain-covered smear with a low flame, such as that from an alcohol lamp or even lighted applicator sticks. Stop heating the slide when steam is first observed. Two or three passes of the flame are usually all that is necessary. *Do not boil or allow the solution to flame.*

(d) Immediately wash gently with tap water.

(e) Place slide in 50% ethyl alcohol for 30 seconds.

(f) 70% Ethyl alcohol ..... 1 minute.

(g) 95% Ethyl alcohol ..... 1 minute.

(h) Carbol-xylene clearing solution [paragraph 7-3a(3)(f)] ..... 2 minutes.

(i) Xylene ..... 3 minutes, or until refraction at interface of smear and xylene ends.

(j) Coverslip with any suitable mounting medium dissolved in xylene or toluene.

Chapter 8
PERMANENT MOUNTS OF HELMINTHS

8-1. Introduction. At times, it is necessary to examine the whole worm. Helminths are sometimes passed from the anus, mouth, or other body openings. You can bet they will be brought to the laboratory for identification. Helminths found at autopsy, especially those found in unexpected places in the body, will have to be identified. Also, those passed following treatment must be examined and identified occasionally. Whatever the source of the material, permanent mounts must usually be prepared before the parasites can be identified.

8-2. Recovery of Helminths:

a. **Fecal Specimens.** Feces for the recovery of helminths may be normally passed, purged, or collected following chemotherapeusis. The fresher the specimen the better, especially following chemotherapeusis. Detailed procedural instructions are given in paragraph 4-1e.

b. **Biopsied Material.** It is very easy to damage helminths in biopsy material. About the only way to handle them is to put the material into saline and tease or tear it apart very gently, watching all the while with a dissecting microscope. Never cut the tissue; you will invariably cut the worm.

c. **Autopsy Material.** Most helminths will remain alive and identifiable for a few days after the host dies if the remains are kept cold. Of course the majority of helminths infecting man are associated with the gastrointestinal system, but several others such as filarial worms, tapeworm larvae, and *Paragonimus* will be found in other places.

(1) *Gastrointestinal Tract.* First, expose the entire viscera. Tie off the gut at the esophageal and rectal ends before removing it from the body cavity. Open the intestine lengthwise and collect samples of the contents from several levels. Label each sample and keep it separated from the others. Set those samples up in sedimenting flasks as for feces (paragraph 4-1e). Grossly examine the entire gut and its contents for large worms such as *Ascaris* and tapeworms. With more care, visually examine the colon for smaller worms such as *Trichuris* or pinworms and the small intestine for hookworms. Do not forcibly detach helminths that are securely attached to the gut wall. Put a piece of the gut with the attached worms in a dish of saline and leave it in the refrigerator overnight. That will cause many of them to relax, and they can then be more easily removed, or they may dislodge themselves. Scrape the mucosa from sections of the duodenum and jejunum, shake it in saline, and examine the material in a petri dish with a dissecting microscope for *Strongyloides*. To recover other minute worms such as heterophyid flukes, that may be deeply embedded, shake the desired sections of the gut in lukewarm saline, and examine the liquid in a petri dish with a dissecting scope. Do not give up the first time if no parasites are found; try it three or four times over a period of 2-3 hours. Carefully open and examine the gallbladder and liver passages for such trematodes as *Clonorchis*, *Opisthorchis*, *Fasciola*, and *Dicrocoelium*. They can be the same color as the tissue in which they are found so care must be used in looking for them. As a matter of routine, prepare representative blocks of the intestine for histological study.

(2) *Extraintestinal Locations.* It is necessary to have some idea what helminth to look for or what organ is involved. Very few parasites will be found if you go blindly searching, not knowing what to look for nor where to look. A thorough knowledge of the life histories of the helminths is the first requirement for a successful examination. To look for the adults of *Schistosoma mansoni*, or *S. japonicum*, remove the liver with all its attachments to the intestinal tract, and carefully perfuse the mesenteric and rectal venules with citrate solution. Examine the perfusate for adult schistosomes. Also, open the intrahepatic portal vein and look for the worms. To find *S. haematobium* and occasionally *S. mansoni*, remove the urinary bladder together with the other pelvic organs and examine their associated blood vessels. While exploring the liver, note any firm, well-defined yellow lesions. Mash a

small piece of the material on a slide and check it with a microscope for the typical eggs of *Capillaria hepatica*. Examine the liver and other abdominal viscera for large and small cystic tumors that can contain parasites such as larval tapeworms or *Paragonimus*. Although *Paragonimus* sometimes develops in unexpected parts of the body, it will usually be found encysted in the lungs. Carefully dissect any enlarged or inflamed lymph nodes especially those in the pelvic region and groin when looking for the filarial worms *Wuchereria* and *Brugia*.

8-3. **Cleaning Helminths.** After removing the parasites from the host carefully wash them in saline. Use a soft brush to gently remove all adhering mucus and debris that cannot be removed by shaking the container. You will find a dissecting scope very useful at this stage. Choose entire specimens for further study when available.

8-4. **Relaxing Helminths.** Various methods can be used to relax helminths making it possible to observe the morphological details. The technique of choice depends upon the kind and size of the particular parasite.

a. **Trematodes.** Frequently trematodes are so packed with eggs that internal structures cannot be observed. They can usually be made to discharge some of the eggs by putting them in distilled water for a few minutes. When they do not discharge some eggs in 15-30 minutes, add a few drops of glacial acetic acid. Then relax very small trematodes (flukes) on a slide. Transfer them with several drops of saline to a slide and place a coverslip over the flukes. Touch a piece of absorbent material to the saline at the edge of the coverslip. As the saline is drawn from under the coverslip, pressure will be exerted on the parasites. Do not remove too much saline because excess pressure will be applied to the parasites causing them to be distorted or even ruptured. At the same time run fixative under the coverslip at one end with a dropper, and draw liquid from under the opposite end of the coverslip with the absorbent material. Add fixative and withdraw the fluid from under the coverslip at the same rate to maintain constant pressure on the flukes and consequently not to damage them. To relax larger trematodes, place them in a jar half-filled with saline and shake vigorously for several minutes. Depending upon their size, place them individually or in groups on large glass slides. Again depending upon size, cover them with a large cover glass or another slide. The larger the worms, the greater the pressure required to adequately flatten them; therefore, you have to decide which is appropriate in each case, cover glass or slide? Run hot fixative between the slides and carefully add more pressure, if required, to flatten the worms properly. Gently tie the slides together and then put them in a petri dish. Then pour fixative into the dish until both slides are completely submerged. Do not tie the slides together too tightly as that will distort or rupture the parasite. From time to time separate the slides slightly and force fixative between them to make certain that all parts of the worm come in contact with the fixative. After being pressed in fixative for several hours to overnight, the worm will retain the desired flattened shape. Trematodes die and degenerate quite rapidly so do what relaxing is necessary and get on with fixing them.

b. **Cestodes.** Cestodes (tapeworms) are more hardy and degenerate more slowly than trematodes; thus the relaxing process can be done more leisurely. The best way to relax tapeworms is to rapidly twirl them in steaming 10% formalin. It is very easy to do especially with the small ones. Heat 10% formalin in a wide, shallow container until it steams. Do not boil. Remove container from the heat and rapidly twirl the worms in the steaming 10% formalin with a blunt instrument such as a glass rod or an applicator stick. The worm will lengthen noticeably as it relaxes while being twirled. Cut the larger tapeworms into workable size sections before twirling them in the heated formalin. As soon as the worms have obviously died and relaxed in the heated formalin, transfer them to 10% formalin at normal room temperature. Large tapeworms that are to be kept whole have to be handled differently. Leave them in a dish of saline in the refrigerator overnight. Then carefully wrap the worm about strips of plate glass or around a glass jar. Completely cover the worm with heavy paper toweling or gauze that has been

soaked in 10% formalin. After about 30 minutes do any rearranging necessary and then completely cover the specimen with formalin.

c. **Acanthocephalans.** Acanthocephalans will invert their spiny proboscis when they are detached from the intestinal wall. The number and location of the spines are very important for their identification. To make the worms relax and evert their proboscis, transfer them to a container of tap water and put it in the refrigerator. Check them periodically after they have been chilled overnight. The worms die and swell as they take up tap water. The swelling causes the proboscis to be everted. Worms that have not relaxed after 48 hours of soaking and chilling are not likely to do so.

d. **Nematodes.** Most of the nematodes are relatively extended when found and will remain that way and require no special relaxing. Those that are loosely coiled or fairly active when found can frequently be relaxed and extended. Probably the most effective way to accomplish that is to twirl or shake them in glacial acetic acid for a few minutes. Others, such as the trichostrongyles, that are tightly coiled when found are very difficult to extend. Drop them into hot water to kill them. Then tease them into an extended position and hold them extended while glacial acetic acid is poured over them. Hold them extended for several minutes. They will still coil somewhat but some of them will remain sufficiently extended to be identified after they are mounted.

8-5. Fixing Helminths. The proper fixation of helminths is essential for permanent preservation and identification of specimens. Fixation stops metabolic processes and hardens the tissues. It prevents regressive changes from taking place; thus cytological and histological elements are preserved, making microscopic morphological study possible. There are many fixatives and fixation procedures available. A few of the simpler, more commonly used ones will be described here.

a. **Fixatives:**

(1) *AFA (Alcohol-Formol-Acetic Acid).*

Ethyl alcohol, 95%	50 ml
Formaldehyde	10 ml
Acetic acid, glacial	5 ml
Distilled water	45 ml

Mix reagents and store in tightly-stoppered bottle.

(2) *Bles'.*

Ethyl alcohol, 70%	90 ml
Formaldehyde	7 ml
Acetic acid, glacial	3 ml

Mix reagents and store in tightly-stoppered bottle.

(3) *Formalin, 10%.*

Formaldehyde	10 ml
Distilled water	90 ml

Mix reagents and store in tightly-stoppered bottle.

(4) *Gilson's.*

Nitric acid, concentrated	18 ml
Acetic acid, glacial	4 ml
Mercuric chloride	20 g
Ethyl alcohol, 60%	100 ml
Distilled water	880 ml

Mix reagents until mercuric chloride is dissolved. Store in tightly-stoppered bottle.

b. **Procedures:**

(1) *Trematodes.* Trematodes in general are very delicate. They have to be fixed soon after they are found to prevent degeneration. For best results, fix them while they are still alive. Heated AFA or Gilson's fixative gives very good results. Worms fixed with either take the standard stains quite well. Gilson's contains mercury; therefore, do not use metal containers or instruments when using it to fix specimens. Leave the specimens in the fixative solution overnight. Transfer those fixed in AFA directly to 70% ethyl alcohol where they can be left indefinitely. Those fixed in Gilson's contain mercury, which has to be precipitated before they can be stained. The surest way to accomplish that is to precipitate the mercury by transferring the specimens from Gilson's to 10% iodized alcohol [paragraph 7-3a(3)(a)] and allowing them to remain there with occasional agitation for 12-24 hours, depending on the thickness of the worm. Next, transfer the specimen to plain 70% ethyl alcohol where it can be left indefinitely. When caught with neither AFA nor Gilson's on hand, use heated 5% formalin to fix the worms. When the 5% formalin has cooled, transfer the worms to 10% formalin where they can be left indefinitely. Remember to wash formalin-fixed specimens in

running tap water for several hours to remove the formalin before staining.

(2) *Cestodes.* Cestodes (tapeworms) are more durable than trematodes, but they still should be fixed while they are alive. Twirl small to medium worms in steaming 10% formalin; then transfer them to fresh, cool, 10% formalin. They can be left there indefinitely. Large worms can be handled in the same manner by cutting them into representative sections before fixing. When it is desirable to preserve large worms intact, wrap them around a cylinder or about a strip of plate glass and immerse the whole thing in heated 10% formalin. Pour off the formalin after it cools and add fresh 10% formalin that has not been heated. Wash the worms in running tap water for several hours before staining.

(3) *Acanthocephalans.* Acanthocephalans (thorny-headed worms) should be thoroughly relaxed before they are fixed. However, go ahead and put them in fixative after about 48 hours even when the proboscis has not been everted. Drop them into steaming 5% formalin. After the formalin cools, transfer the worms to fresh 10% formalin that has not been heated. They can be left in the 10% formalin indefinitely. Wash the worms in running tap water for several hours before staining.

(4) *Nematodes.* Nematodes have a cuticle that is very impervious. It makes them difficult to fix or stain. Small, delicate nematodes such as *Strongloides* and small nematode larvae require a mild fixative like Bles' [paragraph 8-5a(2)]. It is good for all elements of the very delicate worms because it does not clear them too much. Heat the Bles' fixative to about 60-65C, and then drop the worms into the solution individually. After the fixative cools, transfer the worms to 70% ethyl alcohol. They can be left there indefinitely. Large, robust worms such as *Ascaris* are difficult to do much of anything with. They are too large to clear and examine as is done with most other helminths. The internal structure of *Ascaris* can best be studied by dissecting the worms. The best way to fix them for dissection is to soak them in heated 5% formalin for 30 minutes and then transfer them to 10% formalin that has not been heated. Nematodes that are left in 5% formalin too long frequently swell and rupture. They can be kept in the 10% formalin indefinitely. To properly fix medium size nematodes for clearing, place them in glacial acetic acid for about 10 minutes. Then transfer them to 70% ethyl alcohol where they can be kept indefinitely. Never let any nematode or any other helminth completely ruin for the lack of the proper fixative. When the specific fixative cannot be obtained, use 10% formalin. It will almost always give at least fair results.

8-6. Staining and Mounting of Trematodes, Cestodes, and Acanthocephalans. Staining and mounting procedures vary, depending upon the species and size of the helminths. Trematodes, cestodes, and acanthocephalans readily take up the routinely employed stains. When these helminths are properly stained, they are much easier to identify because their internal organs can be observed in detail. All of these worms react in much the same way with the same stains. There will be greater differences in staining reactions between different sized worms than there will be between of different species. Two basic stains are included, both of which will give adequate results. They are Semichon's aceto-carmine, and Delafield's hematoxylin. Delafield's hematoxylin gives slightly better differentiation, but it will fade in an acid medium or upon prolonged exposure to light while Semichon's aceto-carmine does not fade.

a. **Semichon's Aceto-Carmine Stain:**

(1) *Reagents:*

(a) *Stock Stain.*

Acetic acid, glacial ..... 100 ml
Distilled water ..... 100 ml
Carmine (alum lake, certified) ..... qs

1. Mix the acetic acid with the distilled water and add carmine powder in excess; that is, add carmine powder until no more will immediately go into solution.

2. Stopper and place the flask in a boiling water bath for 15 minutes.

3. Cool the solution and filter.

4. Kept in a tightly stoppered bottle, the stock stain is stable indefinitely.

(b) *Acidified Alcohol.*

Ethyl alcohol, 70% ..... 100 ml
Hydrochloric acid (HCl), concentrated ..... 1 ml

Add 1 ml concentrated hydrochloric acid to 100 ml of 70% ethyl alcohol and mix.

(c) *Alcohol, Ethyl (30%, 50%, 70%, 80%, 95%, and 100%).*

(d) *Methyl Salicylate, Cedarwood Oil or Xylene.*

(e) *Mounting Medium Dissolved in Xylene or Toluene.*

(2) *Staining and Mounting Procedure:*

(a) Helminths fixed in Gilson's solution or other fixatives that contain mercury must be treated with iodine prior to staining. This should have been done before they were stored in 70% alcohol; however, if it was not done at that time, transfer them to 70% iodized alcohol [paragraph 7-3a(3)(a)] for 12-24 hours before staining.

(b) Specimens that have been fixed and stored in formalin have to be washed in running tap water overnight. After washing, transfer the specimen to glacial acetic acid for 15 minutes. The acid removes some cytoplasmic elements making the worms more receptive to the stain. Wash again in water, this time for 1 hour. Then transfer through 30%, 50%, and 70% ethyl alcohol for 30 minutes each. The specimens are then ready to be stained.

(c) Specimens that have been fixed in AFA or Bles' solution and stored in 70% ethyl alcohol are ready for staining in Semichon's aceto-carmine stain.

(d) Mix Semichon's aceto-carmine stock solution [paragraph 8-6a(1)(a)] with an equal volume of 70% ethyl alcohol. Transfer specimens directly from 70% alcohol preservative to the staining solution and allow to stain overnight to 24 hours. Some cestodes require longer staining times than most trematodes and have to be stained for 48 hours.

(e) Wash in 3 changes of 70% alcohol for 5 minutes each. When working with very small helminths, it is best to process them in a small dish such as a watch glass or a small porcelain evaporating dish that has a round bottom. The organisms are sometimes so small that a binocular dissecting scope has to be used when the solutions are changed. Carefully pipet off the different solutions at each step leaving only a sufficient amount in the bottom of the dish to prevent specimens from drying.

(f) Destain specimens in 70% acidified alcohol [paragraph 8-6a (1)(c)] until the specimens are a delicate pink color. The internal structures will be visible by strong transmitted light. Specimens of average size usually require about 30 minutes to destain. Change the destaining solution if it becomes deep red in color.

(g) Wash in three changes of 70% alcohol for at least 10 minutes each, or until the alcohol no longer acquires a pinkish color.

(h) Dehydrate in successive changes of 80, 95, and 100% alcohol for 30 minutes each. If absolutely necessary, substitute 95% alcohol for the 100% alcohol in this step and the next step.

(i) Start clearing process in 100% alcohol plus methyl salicylate or cedarwood oil, i.e., equal parts of 100% alcohol and methyl salicylate or equal parts of 100% alcohol and cedarwood oil. In the event, neither methyl salicylate nor cedarwood oil is available, use a mixture containing equal parts of 100% alcohol and xylene.

(j) Clear in methyl salicylate or cedarwood oil for 1 hour. Again, if necessary, clear specimens in xylene. Xylene clears the specimens quite adequately, but it leaves them very stiff and brittle.

(k) Before mounting, leave the specimens in a mixture containing equal parts of the clearing agent and the appropriate mounting medium. Use a mounting medium that has a xylene or toluene diluent such as Permount, Balsam, or any of several others currently available.

(l) Carefully position and mount the specimen using the chosen mounting medium.

b. **Delafield's Iron-Hematoxylin Stain:**

(1) *Reagents:*

(a) *Ammonium Alum.*

Aluminum ammonium sulfate
[$AlNH_4(SO_4)_2$] ..... qs
Distilled water ..... qs

1. Add aluminum ammonium sulfate crystals to hot distilled water, stirring continuously, until no more crystals will dissolve.

2. Let the solution cool. Excess alum will crystalize.

3. Pour off the clear solution.

4. Store the saturated solution in a glass-stoppered bottle. The solution will keep indefinitely.

(b) *Stock Hematoxylin Stain.*

Hematoxylin crystals	4 g
Ethyl alcohol, 95%	25 ml
Ammonium alum, reagent [paragraph 8-6b(1)(a)]	400 ml
Methyl alcohol, acetone free	100 ml
Glycerin	100 ml

1. Dissolve the hematoxylin crystals in the 95% ethyl alcohol and add the ammonium alum solution.

2. Place in a container, covered only very loosely to keep out dust. Leave the container exposed to sunlight for 2 weeks to oxidize the hematoxylin to hematin.

3. Mix the glycerin and methyl alcohol and the solution to the oxidized stain.

4. Filter before using.

(c) *Acidified Alcohol.*

Ethyl alcohol, 70%	100 ml
Hydrochloric acid (HCl), concentrated	1 ml

Add 1 ml concentrated hydrochloric acid to 100 ml of 70% ethyl alcohol and mix.

(d) *Alcohol, Ethyl (30%, 50%, 70%, 80%, 90%, and 100%).*

(e) *Alcohol-Lithium Carbonate Solution, Reagent* [paragraph 7-3a(3)(g)].

(f) *Methyl Salicylate, Cedarwood Oil or Xylene.*

(2) *Staining and Mounting Procedure:*

(a) Worms fixed in Gilson's solution or other fixatives that contain mercury must be treated with iodine prior to staining. This should have been done before they were stored in 70% alcohol; however, if it was not done at that time, transfer them to 70% iodized alcohol [paragraph 7-3a(3)(a)] for 12-24 hours. Then transfer the specimens through 70, 50, and 30% ethyl alcohol for 30 minutes each and also wash in distilled water for 30 minutes. The specimens are then ready to be stained.

(b) Specimens that have been fixed in AFA or Bles' solution and stored in 70% ethyl alcohol have to be transferred through 50 and 30% ethyl alcohol for 30 minutes each and then washed in distilled water for 30 minutes before they are ready for staining.

(c) Specimens that have been fixed and stored in formalin have to be washed in running tap water overnight. After washing, transfer the specimens to glacial acetic acid for 15 minutes. The acid removes some cytoplasmic elements, making the worms more receptive to the stain. Wash again in water, this time for 1 hour. The specimen is then ready to be stained.

(d) Dilute stock Delafield's iron-hematoxylin [paragraph 8-6b(1)(b)] with distilled water just prior to use. Use 1 part stock stain [paragraph 8-6b(1)(b)] and 9 parts distilled water. Transfer specimens directly from water bath to the staining solution and allow to stain overnight to 24 hours. Some cestodes will require the full 24 hours to completely stain, but most specimens will stain adequately overnight.

(e) Wash in three changes of distilled water to remove adhering stain. When working with very small worms it is best to process them in a small dish such as a watch glass or a small porcelain evaporating dish that has a round bottom. The organisms are sometimes so small that a binocular dissecting scope has to be used when the solutions are changed. Carefully pipet off the different solutions at each step, leaving only a sufficient amount in the bottom of the dish to prevent specimens from drying.

(f) Pass through successive changes of 30%, 50%, and 70% ethyl alcohol for a minimum of 30 minutes each. Large specimens should be left longer in each solution.

(g) Destain in acidified alcohol [paragraph 8-6b(1)(c)] until the specimens assume a delicate, light, reddish-purple color. If the alcohol becomes deeply colored, it will have to be changed.

(h) Wash for 10 minutes in each of three changes of 70% alcohol, or until the alcohol no longer acquires a bluish color.

(i) Neutralize in alcohol-lithium carbonate solution [paragraph 8-6b(1)(e)] for 1 hour or until the specimens become bluish-purple. Should lithium carbonate not be available, you can get by with ammoniated alcohol. Prepare it by adding 1 drop of concentrated ammonium hydroxide to 250 ml of 70% ethyl alcohol.

(j) Dehydrate successively in 80%, 95%, and 100% ethyl alcohol for 30 minutes each. If absolutely necessary, use 95% alcohol in place of 100% alcohol in this step and the next step.

(k) Start clearing process in 100% alcohol plus methyl salicylate or cedarwood oil; that is, equal parts of 100% alcohol and methyl salicylate or equal parts of 100% alcohol and cedarwood oil. If neither methyl salicylate nor cedarwood oil is available, use a mixture

containing equal parts of 100% alcohol and xylene.

(l) Clear in methyl salicylate or cedarwood oil for 1 hour. Again, if necessary, clear specimens in xylene. Xylene will adequately clear the worms, but it leaves them very stiff and brittle.

(m) Before mounting, leave the specimens in a mixture containing equal parts of the clearing agent and the appropriate mounting medium. Use a mounting medium that has a xylene or toluene diluent such as Permount, Balsam, or any of several others currently available.

(n) Carefully position and mount the specimen using the chosen mounting medium.

8-7. Clearing, and Mounting Nematodes. The cuticle of nematodes is very impervious, and the usual methods of fixation and dehydration are not satisfactory for preparation of permanent mounts. Simple clearing without staining reveals most structural details required for identification. The cleared worms can then be mounted in glycerin-jelly, making very good semipermanent preparations.

a. **Reagents:**

(1) *Glycerin-Alcohol.*

Glycerin	5 ml
Ethyl alcohol, 70%	95 ml

Mix and store in a well stoppered bottle. Solution is good indefinitely.

(2) *Glycerin-Jelly With Phenol.*

Bacto-gelatin	10 g
Distilled water	60 ml
Glycerin	70 ml
Phenol (melted crystals)	0.5 ml

(a) Bring water to boil and add gelatin stirring until it is dissolved.

(b) Add glycerin and mix thoroughly.

(c) Add liquid phenol and mix thoroughly.

(d) Dispense glycerin-jelly in 10 to 25 ml quantities. Stopper and store in refrigerator.

b. **Procedure:**

(1) Medium to large nematodes that have been fixed and stored in formalin can best be cleared after some of the cytoplasmic material has been removed. Wash worms overnight in running tap water. Transfer the worms to glacial acetic acid for 15 minutes. Then transfer the specimens through 30% and 50% ethyl alcohol for 1 hour each. The worms are then ready to go into the glycerin-alcohol clearing solution.

(2) Small, delicate nematodes that have been fixed and preserved in formalin need not go through the acetic acid because they clear so easily. Transfer them through 30% and 50% ethyl alcohol, and they are ready to go into the glycerin-alcohol clearing solution.

(3) Nematodes that have been fixed in Bles' solution or glacial acetic acid and then preserved in 70% ethyl alcohol are ready to go directly into the glycerin-alcohol clearing solution.

(4) Use a shallow clear bottom or black container to carry out the clearing process. The clearing process is facilitated by using a clearing dish that is similar in size to the worm.

(5) Pour a considerable volume of the glycerin-alcohol over the worms.

(6) Place the container in a 37C incubator. Refill the container with glycerin-alcohol for 2 or 3 days, depending upon the size of the worms to be cleared.

(7) At the end of 4 to 5 days all of the alcohol should have evaporated, leaving the worm in pure glycerin. The worm should be cleared so that the internal structures can be easily observed with a microscope.

(8) If the worm is to be mounted in glycerin-jelly for further study or reference, heat the glycerin-jelly in a water bath until the jelly becomes completely liquid.

(9) Transfer the cleared specimen to a clean slide, cover with the glycerin-jelly, position the worm for optimum observation of details, and carefully coverslip to exclude air.

(10) Let the slides stand until the glycerin-jelly hardens. With a razor blade or scalpel blade trim excess jelly from around coverslip.

(11) Seal the preparation with a ringing compound such as a quick drying enamel or asphaltum. A second application will usually insure that the mount is well sealed.

(12) Glycerin-jelly mounts that are properly sealed will last for several months or even years.

(13) If the preparation becomes damaged or starts to dry up, gently heat the mount in a hot water bath to melt the glycerin-jelly and remount the worm.

8-8. Mounting Helminth Eggs and Larvae:

a. **Double Coverslip Method:**

(1) *Reagents:*

(a) *Formalin, 10%.*

(b) *Mounting Medium.* Any good mounting medium such as Permount, Balsam, or Clarite is suitable.

(2) *Procedure:*

(a) Mix the fecal specimen thoroughly with a large volume of saline and strain it through a filter made of four layers of cheesecloth to remove the larger particles.

(b) Concentrate the specimen by the sedimentation method and suspend the sediment in 10% formalin.

(c) Place a small drop of the formalinized specimen on the center of a 22 mm square coverslip. Cover the specimen with an 18 mm round coverslip or a small piece of broken coverslip. It is not significant so long as the covering coverslip is sufficiently smaller than the first coverslip to allow at least a 2 mm overlap on all sides.

(d) Place a moderate-sized drop of mounting medium on the center of a slide. The exact amount of medium to use has to be determined by experience.

(e) Invert the coverslips so the smaller one is down and carefully place the preparation on the mounting medium.

(f) Dry in a flat position until the mounting medium has hardened.

(g) Properly prepared specimens will last several months to years.

b. **Glycerin-Jelly Mounts:**

(1) *Reagents:*

(a) *Formalin, 10 percent.*

(b) *Ringing Compound.* Any fast drying enamel or asphaltum.

(c) *Glycerin-Jelly (with Chrom-Alum).*

Bacto-gelatin	15 g
Glycerin	50 ml
Chromium potassium sulfate $[CrK(SO_4)_2 \cdot 12H_2O]$	1 g
Distilled water	250 ml

1. Bring water to boil and add the chromium potassium sulfate.

2. Stir in the gelatin until it is dissolved.

3. Add the glycerin and mix thoroughly.

4. Dispense the glycerin-jelly solution in 10 to 25 ml quantities before it hardens. Stopper and store in refrigerator.

(2) *Procedure:*

(a) Mix the fecal specimen thoroughly with a large volume of saline and strain it through a filter made of four layers of cheesecloth to remove the larger particles.

(b) Concentrate the specimen by sedimentation, and suspend the concentrate in an equal volume of 10% formalin. Allow the specimen to fix for several hours.

(c) Concentrate the specimen again.

(d) Heat the glycerin-jelly in a water bath until the jelly becomes liquefied.

(e) Resuspend the concentrate in an equal volume of liquefied glycerin-jelly.

(f) Return the material to the heated water bath until the mounts are prepared. If it cools, the material will jell.

(g) As the liquid evaporates, replace the fluid lost with liquefied glycerin-jelly. About twice weekly over a period of 2 to 3 weeks is usually sufficient.

(h) When the specimen becomes suspended in pure glycerin-jelly, it is time to prepare the slide mounts.

(i) Thoroughly mix the specimen in the glycerin-jelly.

(j) Transfer about 2 drops of the suspension to a glass slide. While the medium is still liquefied, apply a coverslip.

(k) Set the slides aside at room temperature until the glycerin-jelly congeals. With a scalpel or razor blade, trim away any excess medium around the coverslip.

(l) Seal the mount with a ringing compound. A second application usually insures that the mounts are well sealed.

Chapter 9
IDENTIFICATION OF INTESTINAL PARASITES

SECTION A—INTRODUCTION

9-1. Presentation. The diagnostic stages of intestinal parasites are differentiated on the basis of specific morphological features which can be seen microscopically. The characteristics commonly used to distinguish species of many of the parasites are presented in tables for the microscopist's ready reference. The descriptions given do not include all of the morphological characteristics of the various stages; therefore, supplemental references will be useful in some cases.

9-2. Scope. Some of the parasites listed occur only infrequently or accidentally in man; however, their presence is just as important as the more common organisms. For example, *Entamoeba polecki* is rarely found in human feces; however, it closely resembles *Entamoeba histolytica* and *Entamoeba coli*, and unless the technician is aware of its existence, he can mistake it for these species. Likewise, several helminth species of lower animals that occasionally parasitize man are included. For example, *Trichostrongylus* eggs are easily confused with hookworm eggs, and *Hymenolepis diminuta* eggs can be confused with *Hymenolepis nana* eggs.

SECTION B—PROTOZOA

9-3. General Considerations:

a. The intestinal protozoa of man belong to four groups: amebae (Sarcodina), flagellates (Mastigophora), ciliates (Ciliophora), and coccidia (Sporozoa). In addition to the protozoa found strictly in the intestinal tract, the closely related organisms found in the mouth *(Entamoeba gingivalis* and *Trichomonas tenax)* and those found in the genitourinary tract *(Trichomonas vaginalis)* will be considered. With the exception of the coccidia and *Giardia lamblia*, which inhabit the small intestine, other intestinal protozoa live in the colon.

b. The coccidia (*Isospora* species) differ from the other protozoan species in being obligatory tissue parasites. The growth stages in the host cells are similar to those of malaria. Oocysts, and occasionally sporocysts, are passed in the feces and are the diagnostic stages.

c. Organisms belonging to the three other groups have two stages, trophozoites and cysts, in their life cycle, except the ameba, *Dientamoeba fragilis*, and the trichomonad flagellates, which are only known to have a trophozoite stage. Those living in the intestine are identified by the diagnostic trophozoites and cysts that are passed in the feces.

d. The features listed in the Tables of Differential Morphology of Protozoa are those commonly used for identifications. Not all of the characteristics listed can be seen in a single type of preparation. Features or structures that can only be demonstrated by special tests or that are very difficult to see are frequently not included.

e. Wet mounts can be either unstained (saline or formalin) or stained (iodine, buffered methylene blue, Quensel's stain, merthiolate-iodine-formaldehyde [MIF], or other temporary stains). Iodine is primarily a cyst stain and is usually used to prepare specimens to be examined for protozoa. Buffered methylene blue and Quensel's stains are used to stain living trophozoites. MIF will stain both stages, but the specimens are less satisfactory for trophozoites than for cysts. Scaled drawings of the intestinal and closely related protozoa are presented.

9-4. Amebae. Species identification of trophozoites can rarely be made from single features, such as nucleus or cytoplasm, or from a single organism. Several features and several organisms have to be examined. Cysts are less variable and can usually be more easily identified than trophozoites. Any time Charcot-Leyden crystals are found, be on the alert for parasites, especially amebae *(Entamoeba histolytica)* and some of the nematodes.

Material in chapter 9 was adapted from Brooke and Melvin, 1969. *Morphology of Diagnostic Stages of Intestinal Parasites of Man.* PHS Publication No. 1966.

a. **Trophozoites.** The characteristics used to distinguish trophozoites of intestinal amebae are outlined below. Table 9-1 gives characteristics visible in different types of preparations and table 9-2 lists the differential morphological characteristics which are illustrated in figure 9-1.

(1) *Motility* — progressive or nonprogressive.

(2) *Cytoplasm:*

(a) Appearance — finely granular, coarsely granular, or vacuolated.

(b) Inclusions — erythrocytes, bacteria, molds.

(3) *Nucleus:*

(a) Number present.

(b) Peripheral chromatin—present or absent. If present, the distribution along the nuclear membrane and the size of the granules are important.

(c) Karyosome—location and size.

(4) *Size.* Sizes overlap, but they can be used as a secondary distinguishing feature. However, size is the chief criterion for distinguishing *Entamoeba histolytica* from *Entamoeba hartmanni.*

b. **Cysts.** The characteristics used to distinguish cysts of intestinal amebae are outlined below. Table 9-1 gives characteristics visible in different types of preparations and table 9-3 lists the differential morphological characteristics, which are also illustrated in figure 9-1.

(1) *Nucleus:*

(a) Number present.

(b) Peripheral chromatin—present or absent. If present, the distribution of the granules along the nuclear membrane is important.

(c) Karyosome—location and size.

(2) *Cytoplasm:*

(a) Chromatoid bodies—present or absent. If present, the shape is important.

(b) Glycogen—appearance.

(3) *Size.* Sizes overlap, but they can be used as a secondary distinguishing feature. Size is the major criterion for distinguishing *Entamoeba histolytica* from *Entamoeba hartmanni.*

(4) *Shape.* Shapes vary, but they can be useful as a secondary distinguishing feature.

Table 9-1. Characteristics of Amebae as Seen in Different Types of Preparations. (From Brooke and Melvin, 1969)

	Unstained		Temporary Stains		Permanent Stains
Characteristic	Saline	Formalin	Iodine (Cysts)	Buffered Methylene Blue (Trophs)[1]	
Trophozoites					
Motility	+	−		−	−
Cytoplasm					
appearance	+	+		+	+
inclusions (rbc, bacteria)	+	+		+	+
Nucleus	−	+[2]		+	+
Cysts					
Nuclei	−	+	+		+
Chromatoid bodies	+	+	+[3]		+
Glycogen	−	−	+		− (vacuole present)

[1] Quensel's stain may be substituted for buffered methylene blue.
[2] Nuclei of trophozoites are visible in formalin-fixed material, but are usually not sufficiently distinctive for species identification.
[3] Chromatoid bodies are more easily seen in unstained wet mounts than in iodine preparations.

Table 9-2. Differential Morphology of Amebae: Trophozoites. (Adapted from Brooke and Melvin, 1969)

SPECIES FROM INTESTINE	SIZE (DIAMETER OR LENGTH)	MOTILITY	NUCLEUS			CYTOPLASM	
			NUMBER	PERIPHERAL CHROMATIN	KARYOSOMAL CHROMATIN	APPEARANCE	INCLUSIONS
Entamoeba histolytica	10-60. Usual range: 15-20.	Progressive with hyaline, finger-like pseudopods.	1 Not visible in unstained preparations.	Fine granules, usually evenly distributed and uniform in size.	Small, discrete. Usually centrally located, but occasionally eccentric.	Finely granular.	Red blood cells, occasionally. Noninvasive organism may contain bacteria.
Entamoeba hartmanni	5-12. Usual range: 8-10.	Usually nonprogressive	1 Not visible in unstained preparations.	Similar to E. histolytica.	Small, discrete, often eccentric.	Finely granular.	Bacteria.
Entamoeba coli	15-50. Usual range: 20-25.	Sluggish, nonprogressive, with blunt pseudopods.	1 Often visible in unstained preparations.	Coarse granules, irregular in size and distribution.	Large, discrete, usually eccentric.	Coarse, often vacuolated.	Bacteria, yeasts, other materials.
Entamoeba poleckí	10-25. Usual range: 15-20.	Usually sluggish, similar to E. coli. Occasionally in diarrheal specimens, motility may be progressive.	1 May be slightly visible in unstained preparations. Occasionally distorted by pressure from vacuoles in cytoplasm.	Usually fine granules evenly distributed. Occasionally, granules are irregularly arranged. Chromatin sometimes in plaques or crescents.	Small, discrete, eccentric. Occasionally large, diffuse, or irregular.	Coarsely granular, may resemble E. coli. Contains numerous vacuoles.	Bacteria, yeasts.
Endolimax nana	6-12. Usual range: 8-10.	Sluggish, usually nonprogressive with blunt pseudopods.	1 Visible occasionally in unstained preparations.	None.	Dirge, irregularly shaped, blotlike.	Granular, vacuolated.	Bacteria.
Iodamoeba bütschlii	8-20. Usual range: 12-15.	Sluggish, usually nonprogressive.	1 Not usually visible in unstained preparation.	None.	Large, usually central, surrounded by refractile, achromatic granules. These granules are often not distinct even in stained slides.	Coarsely granular, vacuolated.	Bacteria, yeasts, or other material.
Dientamoeba fragilis	5-15. Usual range: 9-12.	Pseudopodia are angular, serrated, or broad lobed and hyaline, almost transparent.	2 (In approximately 20% of organisms only 1 nucleus is present.) Nuclei invisible in unstained preparations.	None	Large cluster of 1-8 granules.	Finely granular, vacuolated.	Bacteria.
SPECIES FROM MOUTH							
Entamoeba gingivalis	5-30. Usual range: 10-20.	Pseudopodia vary from long and lobose to short and blunt.	1 Not usually visible in unstained preparations.	Fine granules similar to E. histolytica.	Small, well-defined, centrally located.	Finely granular.	WBCs and epithelial cells, at times bacteria and spirilla, rarely red blood cells.

Table 9-3. Differential Morphology of Amebae: Cysts. (Adapted from Brooke and Melvin, 1969)

SPECIES FROM INTESTINE	SIZE	SHAPE	NUCLEUS: SOMATIC	NUCLEUS: PERIPHERAL CHROMATIN	NUCLEUS: KARYOSOMAL CHROMATIN	CYTOPLASM: CHROMATOID BODIES	CYTOPLASM: GLYCOGEN
Entamoeba histolytica	10–20 Usual range: 12–15	Usually spherical.	Mature cyst: four nuclei. Immature cyst: with 1 or 2 chromatoid seen.	Peripheral chromatin present. Fine, uniform granules, evenly distributed.	Small, discrete, usually centrally located.	Present. Elongate bars with bluntly rounded ends.	Usually diffuse. Concentrated mass often present in young cysts. Stains reddish brown with iodine.
Entamoeba hartmanni	5–10 Usual range: 6–8	Usually spherical.	Mature cyst: four nuclei. Immature cysts with 1 or 2 often seen.	Similar to E. histolytica.	Similar to E. histolytica.	Present. Elongate bars with bluntly rounded ends.	Similar to E. histolytica.
Entamoeba coli	15–35, Usual range: 15–25	Usually spherical, ovoid, ellipsoidal, oval, triangular or other shapes.	Mature cyst: usually eight nucleated cysts with 16 or more are seen. Immature cysts with 2–4 more often seen.	Peripheral chromatin present, coarse granules irregular in size and distribution, but often appear heavier than in trophozoites.	Large, dissacts, usually seven tri, but may be normally centrally located.	Present, but less frequently seen than in E. histolytica. Usually splinter like with pointed ends.	Usually diffuse, but occasionally well defined mass in immature cysts. Stains reddish brown with iodine.
Entamoeba polecki	5–15 Usual range: 7–10	Spherical or oval	Mature cysts: four nuclei, rarely visible in unstained preparations.	Usually fine granules evenly distributed.	Usually small and/or central.	Present. Many small bodies with angular or pointed ends, or few large ones. May be oval, rod like or irregular.	Usually small, diffuse masses. Stain reddish brown with iodine. A dark area called "inclusion mass" (possibly concentrated cytoplasm) is often also present. Mass does not stain with iodine.
Endolimax nana	5–10 Usual range: 6–9	Spherical, oval, ellipsoidal.	Mature cysts: four nuclei with less than 4 nuclei seen.	None	Large, clear like, usually central.	Occasionally granules of small oval mass seen, but bodies as seen in Entamoeba spp. are not present.	Usually diffuse. Concentrated mass seen occasionally in young cysts. Stains reddish brown with iodine.
Iodamoeba bütschlii	5–20 Usual range: 10–12	Oval, ellipsoidal, triangular, or other shapes.	Mature cysts: one nucleus.	None	Large, usually eccentric, ovoid to irregular, sometimes granules on one side of karyosome. Indistinct in iodine preparations.	Occasionally granules present, but chromatoid bodies as seen in Entamoeba spp. are not present.	Compact, well defined mass. Stains dark brown with iodine.
SPECIES FROM MOUTH							
Entamoeba gingivalis	Not known cyst stage.						

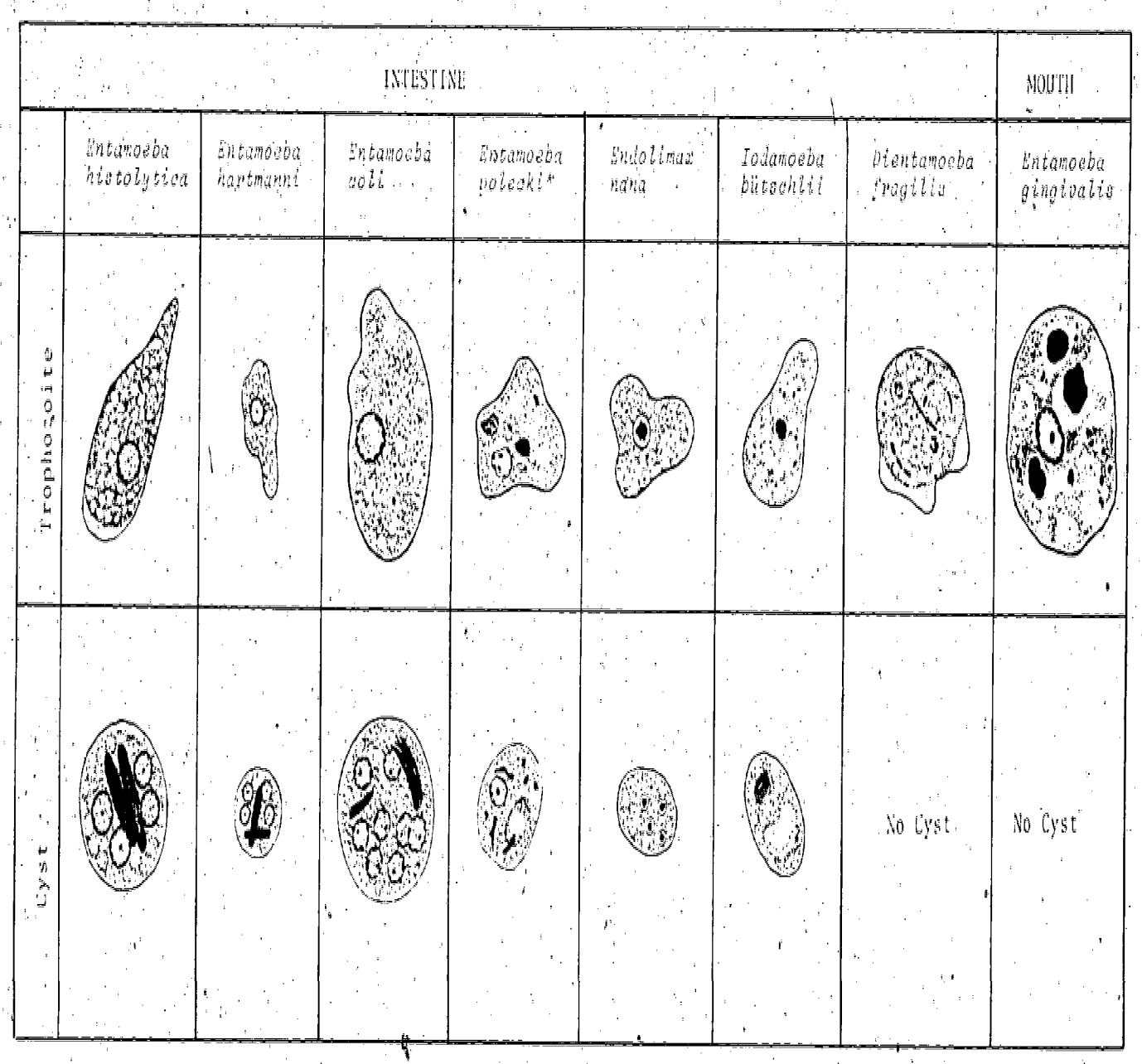


Figure 9-1. Intestinal Protozoa and Related Species in Man: Amebae. Iron-hematoxylin stain. ×1,000. (Adapted from Brooke and Melvin, 1964, 1969)

9-5. Flagellates. As with amebae, not all of the characteristics can be seen in a single type of preparation, and both unstained and stained preparations are usually necessary. Nuclei are not visible in unstained saline mounts, but they can be seen in material fixed with formalin. Most of the other features can be seen in either saline or formalin unstained mounts. Structures of cysts can usually be seen easily in iodine preparations. Quensel's and buffered methylene blue solutions do not stain the flagellate trophozoites. When fresh specimens are used, trophozoites can be readily distinguished by their characteristic motion in saline mounts. Neutral red dye (1:3,000) will stain the trophozoites to some extent when it is added to the mounting solution. Table 9-4 lists the structures or characteristics of both the trophozoites and cysts that can be seen in different preparations.

a. **Trophozoites.** The characteristics used to distinguish trophozoites of flagellate species are outlined below. Table 9-4 gives characteristics that are visible in different types of preparations. Table 9-5 gives the diagnostic characteristics of flagellate trophozoites and drawings of them are presented in figure 9-2.

(1) *Motility* — fluttery, jerky, or rotary.

(2) *Shape* — varies somewhat between species.

(3) *Nucleus:*

(a) Number present is important.

(b) The appearance of the nucleus is not commonly used as a major distinguishing characteristic.

(4) *Flagella.* Although the number and location of flagella vary with the species, they are often difficult to see and count. Therefore, the number and location of flagella are not practical diagnostic features for routine identification.

(5) *Special Features.* Features such as undulating membrane, sucking disk, and prominent cytostome are very important.

b. **Cysts.** The characteristics used to distinguish cysts of flagellate species are outlined below. Table 9-4 gives characteristics that are visible in different types of preparations. Table 9-6 gives the diagnostic characteristics of flagellate cysts and drawings of them are presented in figure 9-2.

(1) *Shape* — varies somewhat between species.

(2) *Size* — varies between species, but it is not a major identifying characteristic.

(3) *Nucleus:*

(a) Number present is important.

(b) Position of nuclei in cyst is important.

(4) *Fibrils.* Presence of fibrils is significant.

9-6. Ciliates:

a. Only one ciliate species, *Balantidium coli* (figure 9-3), parasitizes man. It is also the largest protozoan parasitizing man. The trophozoites and cysts are easy to detect in saline mounts of fresh feces. The size, shape, and motility of the trophozoites are readily recognized. Although the cysts are not as easy to identify, they present no real diagnostic problem. In specimens fixed with formalin, trophozoites can be somewhat distorted, but they are usually recognizable. Formalin does not significantly alter the appearance of the cyst.

b. Properly stained preparations of *B. coli* are difficult to obtain. It is of little consequence, however, as most of the structural details can be seen in unstained mounts. Table 9-4 indicates the structures or characteristics that can be seen in different types of preparations. The common diagnostic characteristics are listed in Table 9-7.

9-7. Coccidia:

a. The species of coccidia that parasitize the intestine of man belong to the genus *Isospora* (figure 9-3). The diagnostic stages are often difficult to locate in feces. They are easily overlooked because they are almost transparent. To see them, the lighting must be correct, and the focusing must be done carefully. Table 9-4 presents the structures or characteristics that can be seen in different types of preparations. Stained preparations are of little or no value in demonstrating the organisms, and unstained wet mounts are generally satisfactory. Iodine or MIF mounts are helpful in some cases. The diagnostic characteristics are listed in Table 9-7.

b. The immature oocysts of *Isospora* that parasitize man are difficult to distinguish from those of *Eimeria* which parasitize lower ani-

mals. *Eimeria* oocysts will be passed out in feces when man ingests them with animal tissue; therefore, mature oocysts are usually required to make a positive identification. A procedure for maturing coccidia oocysts is given in paragraph 6-5. Mature oocysts of *Eimeria* contain four sporocysts with two sporozoites each while those of *Isospora* contain two sporocysts with four sporozoites each. Drawings of the immature and mature oocyst states of *Isospora* are in figure 9-3.

Table 9-4. Characteristics of Intestinal Flagellates: Cilliates, and Coccidia in Different Types of Preparations. (From Brooke and Melvin, 1969)

	Unstained		Temporary Stains		Permanent Stains
Characteristic	Saline	Formalin	Iodine (Cysts)	Neutral Red[1] (Trophs)	
Flagellates					
Trophozoites					
Motility	+	−		+	−
Shape	+	+		+	+ (may be distorted)
Nucleus	−	+		+	+
Flagella	±	−		+	±
Other Features[2]	+	+		+	+
Cysts					
Shape	+	+	+		+
Nuclei	−	+	+		+
Fibrils	±	+	+		+
Ciliate (B. coli)					
Trophozoites					
Motility	+	−		+	−
Macronucleus	+	+		+	+
Cilia	+	+		+	+
Cysts					
Macronucleus	+	+	±		+
Coccidia (Isospora)					
Oöcysts	+	+	+		± (usually distorted)

[1] Neutral red dye in methocel solutions.
[2] The undulating membrane of Trichomonas may not be visible in all cases.

Table 9-5. Differential Morphology of Intestinal and Related Flagellates: Trophozoites. (Adapted from Brooke and Melvin, 1969)

SPECIES FROM INTESTINE	SIZE	SHAPE	MOTILITY	NUMBER OF NUCLEI	NUMBER OF FLAGELLA*	OTHER FEATURES
Chilomastix mesnili	6-24μ Usual range; 10-15μ	Pear-shaped	Stiff, rotary	1 Not visible in unstained mounts	3 anterior, 1 in cytostome.	Prominent cytostome extending 1/3-1/2 length of body. Spiral groove across ventral surface.
Giardia lamblia	10-20μ Usual range, 12-15μ	Pear-shaped	"Falling leaf"	2 Not visible in unstained mounts	4 lateral, 2 ventral, 2 caudal.	Sucking disk occupying 1/2-3/4 of ventral surface.
Enteromonas hominis	4-10μ Usual range, 8-9μ	Oval	Jerky	1 Not visible in unstained mounts	3 anterior, 1 posterior.	One side of body flattened. Posterior flagellum extends free posteriorly or laterally.
Retortamonas intestinalis	4-9μ Usual range, 6-7μ	Pear-shaped or oval	Jerky	1 Not visible in unstained mounts	1 anterior, 1 posterior.	Prominent cytostome extending approximately 1/2 length of body.
Trichomonas hominis	5-14μ Usual range, 7-8μ	Pear-shaped	Nervous, jerky	1 Not visible in unstained mounts	3-5 anterior, 1 posterior.	Undulating membrane extending length of body. Posterior directed flagellum bordering undulating membrane extends beyond the posterior margin of membrane.
SPECIES FROM UROGENITAL TRACT						
Trichomonas vaginalis	7-23μ Usual range 12-14μ	Pear-shaped	Nervous, jerky	1 Not visible in unstained mounts	3-5 anterior, 1 posterior.	Undulating membrane extends about half the length of the body. Posterior directed flagellum bordering undulating membrane does not extend beyond the posterior margin of membrane.
SPECIES FROM MOUTH						
Trichomonas tenax	5-12μ Usual range, 6.5-7.5μ	Pear-shaped	Nervous, jerky	1 Not visible in unstained mounts	3-5 anterior, 1 posterior.	Undulating membrane does not extend full length of the body.

*Not a practical feature for identification of species in routine fecal examinations.

Table 9-6. Differential Morphology of Intestinal and Related Flagellates: Cysts. (Adapted from Brooke and Melvin, 1969)

SPECIES FROM INTESTINE	SIZE	SHAPE	NUMBER OF NUCLEI	OTHER FEATURES
Chilomastix mesnili	6-10μ Usual range, 8-9μ	Lemon shape with anterior hyaline knob or "nipple"	1 Not visible in unstained preparations.	Cytostome with supporting fibrils. Usually visible in stained preparation.
Giardia lamblia	8-19μ Usual range, 11-12μ	Oval or ellipsoidal	Usually 4. Not distinct in unstained preparations. Usually located at one end.	Fibrils or flagella longitudinally in cyst. Occasionally may be slightly visible in unstained cysts. Deep staining fibers or fibrils may be seen lying laterally or obliquely across fibrils in lower part of cyst. Cytoplasm often retracts from a portion of cell wall.
Enteromonas hominis	4-10μ Usual range, 6-8μ	Elongate or oval	1-4, usually 2 lying at opposite ends of cyst. Not visible in unstained mounts.	Resembles E. nana cyst. Fibrils or flagella are usually not seen.
Retortamonas intestinalis	4-9μ Usual range, 4-7μ	Pear-shaped or slightly lemon-shaped	1 Not visible in unstained mounts.	Resembles Chilomastix cyst. Shadow outline of cytostome with supporting fibrils extends above nucleus.
Trichomonas hominis	No cyst			
SPECIES FROM UROGENITAL TRACT				
Trichomonas vaginalis	No cyst			
SPECIES FROM MOUTH				
Trichomonas tenax	No cyst			

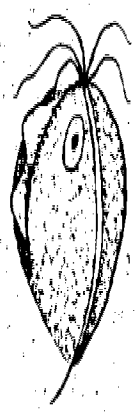
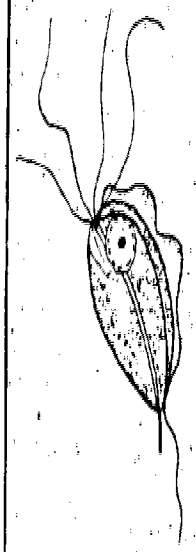
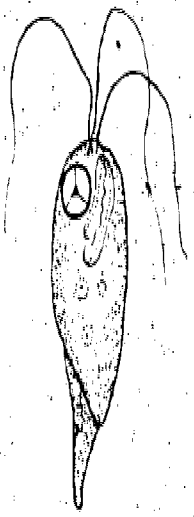
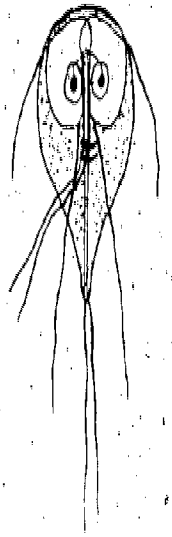
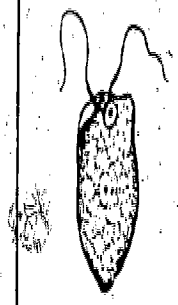
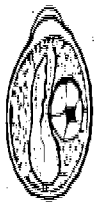
UROGENITAL TRACT	ORAL CAVITY	INTESTINE				
Trichomonas vaginalis	*Trichomonas tenax*	*Trichomonas hominis*	*Chilomastix mesnili*	*Giardia lamblia*	*Enteromonas hominis*	*Retortamonas intestinalis*
						
No Cyst	No Cyst	No Cyst				

Figure 9-2. Intestinal Protozoa and Related Species in Man: Flagellates. Iron-hematoxylin stain. ×1,500. (Adapted from Brooke and Melvin, 1964, 1969)

Species	Size (Length)	Shape	Motility	Number of Nuclei	Other Features
Balantidium coli					
Trophozoite	50-70μ or more. Usual range, 40-50μ.	Ovoid with tapering anterior end.	Rotary, boring.	1 large, kidney shaped macronucleus. 1 small subspherical mircronucleus immediately adjacent to macronucleus. Macronucleus occasionally visible in unstained preparation as hyaline mass.	Body surface covered by spiral, longitudinal rows of cilia. Contractile vacuoles are present.
Cyst	45-65μ Usual range, 50-55μ.	Spherical or oval.	——	1 large macronucleus visible in unstained preparations as hyaline mass.	Macronucleus and contractile vacuole are visible in young cysts. In older cysts, internal structure appears granular.
Isospora species (I. belli and I. hominis)	Oocyst: 25-30μ Usual range, 28-30μ. Immature oocyst not usually seen in I. hominis.	Ellipsoidal.	Non-motile.		Mature oocyst contains 2 sporocysts with 4 sporozoites each. I. belli: usual diagnostic stage is immature oocyst with single granular mass (zygote) within. I. hominis: mature sporocysts, singly or in pairs, are usually passed in feces. Oocyst wall not apparent.
	Sporocyst: I. belli 12-14μ I. hominis 14-16μ.	Round or oval.			

Table 9-7. Differential Morphology of the Ciliates and Coccidia Species Found in Stool Specimens of Humans. (Adapted from Brooke and Melvin, 1969)

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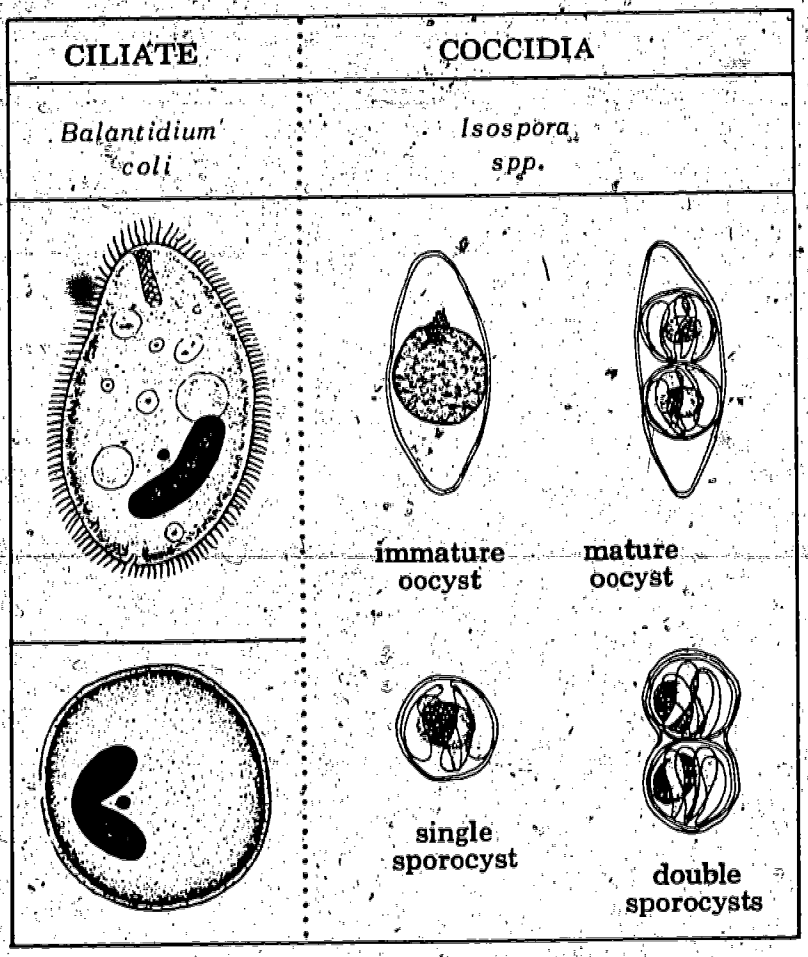


Figure 9-3. Intestinal Protozoa and Related Species in Man: Ciliates (Iron-hematoxylin stain. ×450.) and Coccidia (Unstained. ×1000.). (Adapted from Brooke and Melvin, 1964, 1969)

SECTION C—HELMINTHS

9-8. General Considerations:

a. The common intestinal helminths that parasitize man belong to three groups: nematodes (roundworms), cestodes (tapeworms), and trematodes (flukes). The diagnostic stages of helminths are more easily detected by most technologists than the diagnostic stages of protozoa. The distinguishing features of these helminths are comparatively larger than those of protozoa. Also, the features of these helminths demonstrate little variability; therefore, they are more readily identified. Laboratory diagnosis of human intestinal helminth infections is usually made from eggs, or in some cases, larvae, or body segments (proglottids); however, occasionally it will be necessary to identify adult helminths.

b. The eggs and larvae of intestinal helminths are best seen in unstained wet mounts. Neither temporary nor permanent stains are needed to clearly observe the characteristics that are used to identify the eggs. Those characteristics are:

(1) Size.

(2) Shape.

(3) Stage of development when passed in feces.

(4) Thickness of the eggshell.

(5) Color.

(6) Presence of structure such as mammillated coat, operculum, spine, or plugs.

9-9. Intestinal Nematodes (Roundworms) Found in Humans:

a. **Nematodes Commonly Found in Humans:**

(1) *Enterobius vermicularis:*

(a) *Geographical Distribution. E. vermicularis*, the pinworm or seatworm, is cosmopolitan in distribution; but it is more common in cool or temperate regions than in strictly tropical areas. It is the most common nematode infecting humans in the United States. It is most frequently found in small children who live in crowded conditions.

(b) *Adult Morphology.* The adult parasites (figure 9-4) are ivory white in color, rigid, and bristlelike, with the posterior third of the body attenuated and terminating in a fine, rather sharp point. As with most nematodes, the male is smaller than the female. The male is about 2 mm long with its tail sharply curved so that its body resembles a question mark. The female is about 10 mm long and usually packed with eggs.

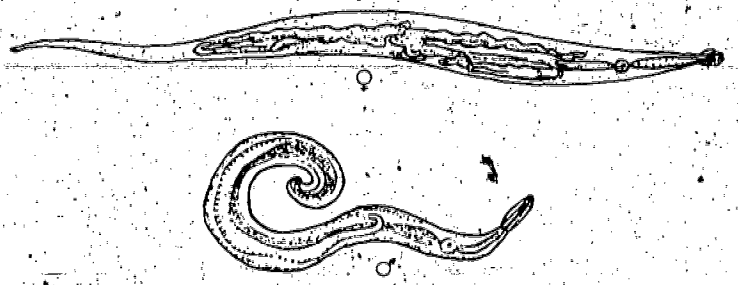


Figure 9-4. Adult Pinworm: Male and Female. ×10.

(2) *Ascaris lumbricoides:*

(a) *Geographical Distribution.* Ascaris is found all over the world except in areas that are very cold and dry. It is most prevalent in tropical areas, but it is a common parasite in cooler climates as well.

(b) *Adult Morphology.* This is the largest intestinal roundworm that infects man (figure 9-5). Fully mature females measure from 20 to 45 cm in length and are about 5 mm in diameter. The males are roughly two-thirds as long and somewhat smaller in diameter. They localize in the small intestine and when freshly passed, they are light flesh colored and quite relaxed. The anterior extremity is bluntly pointed and has three fleshy lobes arranged in a triangular pattern when observed in end view with a dissecting scope. The posterior extremity is tapered and more finely pointed. The posterior extremity of the male is sharply recurved bearing two spicules on the inner margin of the curvature. Spontaneous elimination of the adult worms is not uncommon. They are sometimes regurgitated with vomitus, and they sometimes actively migrate from the stomach through the esophagus and creep out the mouth or nostrils. Any heavy-bodied roundworm in excess of a few centimeters in length that is passed by humans is almost certainly *A. lumbricoides.*

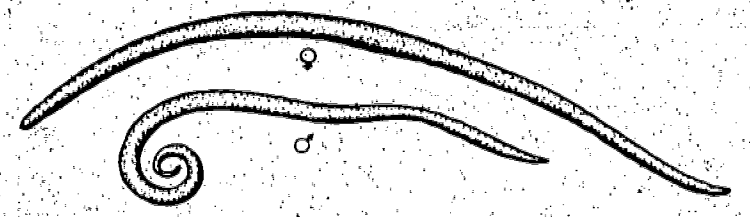


Figure 9-5. Adult Ascaris: Male and Female. ×1/2.

(3) *Trichuris trichiura:*

(a) *Geographical Distribution. Trichuris* (whipworm) infections are widely distributed about the world, but they are prevalent only in warm, moist, temperate climates. The worm is frequently present in individuals that are infected with *Ascaris* and hookworm.

(b) *Adult Morphology.* The worms (figure 9-6) are whiplike in shape. The anterior two-thirds of the worm is slender and threadlike, whereas the posterior one-third is thick and fleshy. The anterior of the worm is delicately but firmly threaded into the mucosa of the cecum. The male measures 30 to 45 mm in length. The posterior end is heavy-set and curled into a full circle. The female measures 35 to 50 mm in length. The body is bluntly rounded on the posterior end and not sharply curved.

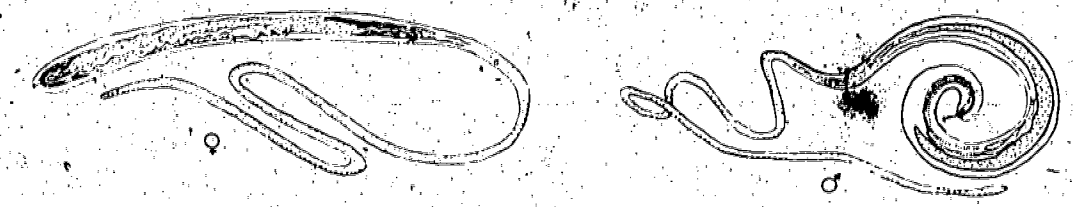


Figure 9-6. Adult Trichuris: Male and Female. ×3.

(4) *Hookworms:*

(a) *Geographical Distribution.* There are two species of hookworms of major importance and one species of lesser importance to man. *Ancylostoma duodenale* (Old World), and *Necator americanus* (New World) are hookworms that are normal parasites of man while *Ancylostoma ceylanicum* is a hookworm that is normally a parasite of cats and dogs but is occasionally found in humans. *A. duodenale* is found principally in Southern Europe, Northern Africa, China, and Japan. *Necator americanus* is found in the southern United States, Central America, the West Indies, and South America east of the Andes, as well as in Central and South Africa, Southern Asia, and Polynesia. The principal factor controlling the distribution of these parasites is temperature. *N. americanus* eggs are quickly killed at temperatures below 45F, whereas those of *A. duodenale* survive at considerably lower temperature. *A. ceylanicum* is found in humans in Southeast Asia and Brazil and probably throughout the Far East and elsewhere in tropical and semitropical climates of the world. In some localities it is a very common parasite of humans, but its range is usually overlapped by *A. duodenale* and *N. americanus*, both of which are more frequently found in man, even in areas where *A. ceylanicum* is most common.

(b) *Adult Morphology.* Adult hookworms (figure 9-7) are fairly stout, gently tapering to both ends. They range in length from about 8 mm to 12 mm by about 0.30 mm to 0.50 mm in width. The males are slightly smaller than females, and they have a copulatory bursa. The living worms are pinkish or cream-gray in color. The species of hookworm that infect man can be differentiated from each other as adults (table 9-8 and figure 9-8).

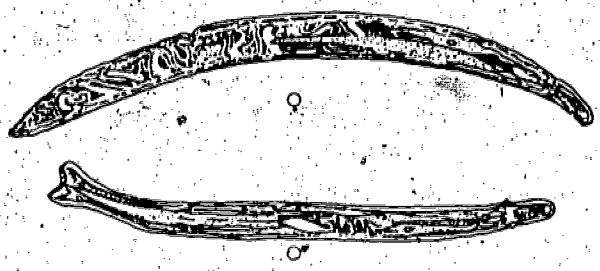
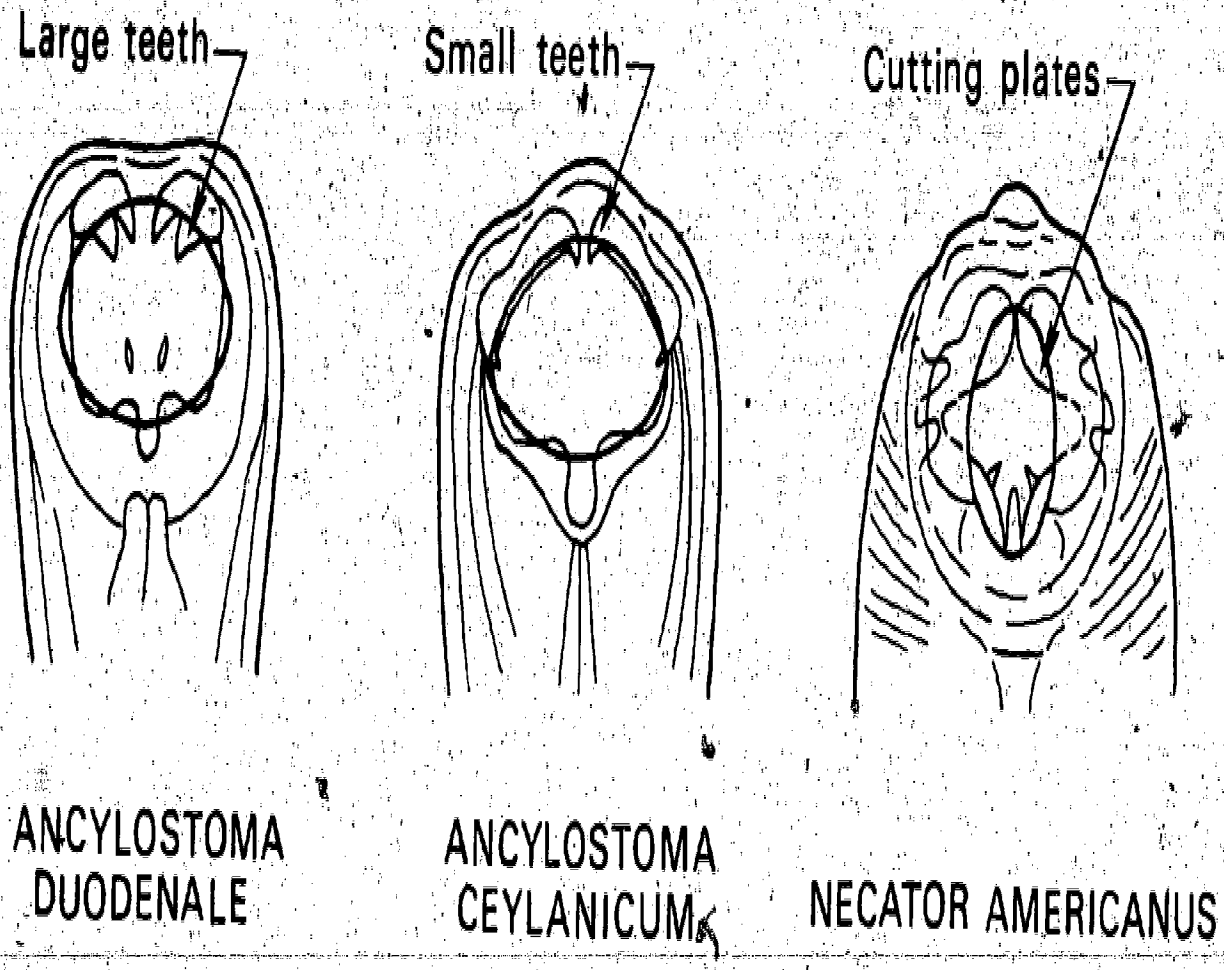


Figure 9-7. Adult Hookworms: Male and Female. ×10.



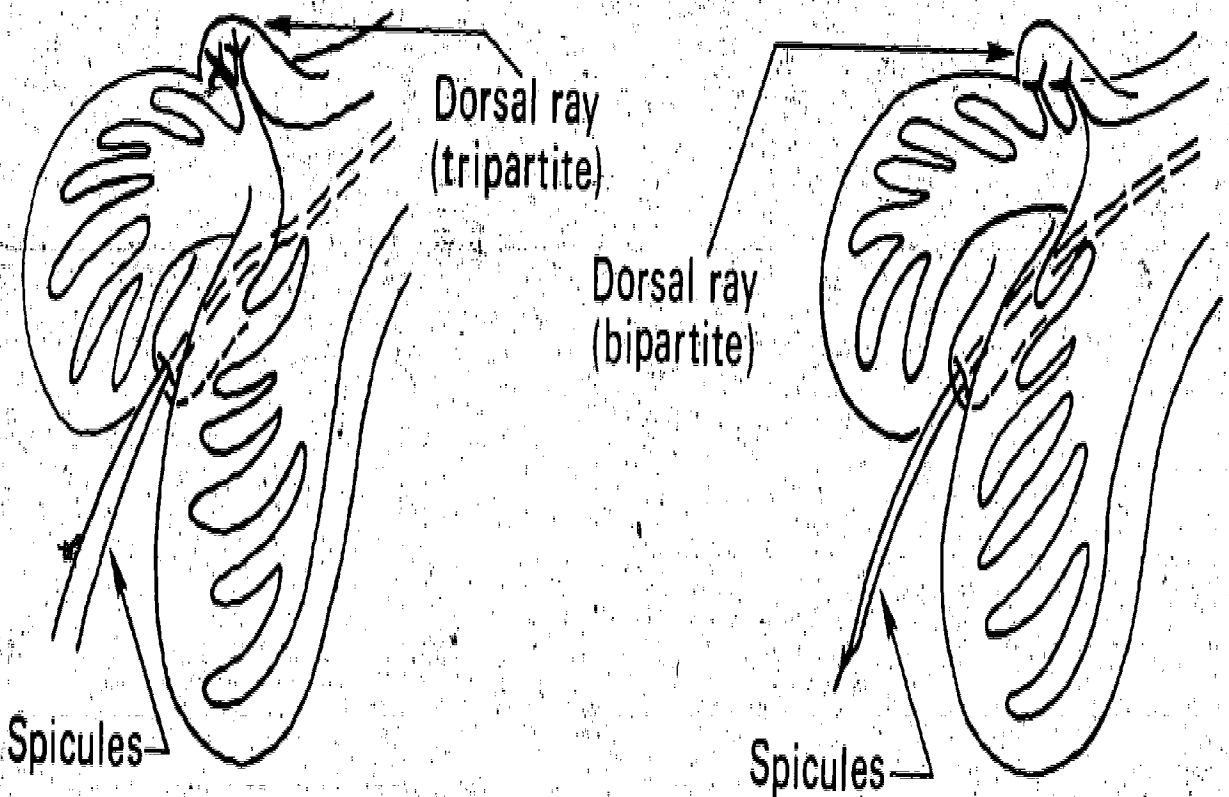


Figure 9-8. Buccal Capsules and Copulatory Bursae of Ancylostoma ceylanicum, Ancylostoma duodenale, and Necator americanus. × 100.

Table 9-8. Characteristics of Mature (Adult) Hookworms. ×100.

	A. duodenale	A. ceylanicum	N. americanus
Approximate size, mm.	Male, 10; Female, 12	Male, 8; Female, 10.5	Male, 8; Female, 10
Position of head	Anterior end continues in same curve as body	Same as A. duodenale	Anterior end strongly reflexed dorsally
Buccal capsule	Four conspicuous curved ventral teeth, one pair small teeth deep in capsule	One pair large teeth at the anterior edge of mouth, below or behind them on each side turned toward the middle line there is a very small tooth, the tip of which is visible below the large one. The oral aperture is transversally oval	Two ventral semilunar plates, two poorly developed dorsal plates, one median dorsal tooth, and a pair of short triangular lancets deep in capsule
Copulatory bursa	Dorsal ray divided in distal third, each division ends in three digitations (tripartite)	Copulatory bursa is very similar to A. duodenale	Dorsal ray divided at base, each division ends in two digitations (bipartite)
Copulatory spicules	Two hair-like spicules	Similar to A. duodenale	Spicules fused at tip into a barb
Vulva	In posterior half	Similar to A. duodenale	In anterior half

(5) *Strongyloides stercoralis:*

(a) *Geographical Distribution.* In general, the distribution of *Strongyloides* parallels that of the human hookworms. Its distribution pattern is much more spotty than that of hookworms, and fewer people are infected. It is found mainly in tropical and subtropical areas. Cases are rarely found in Europe. In the United States, *S. stercoralis* is endemic along the Gulf Coast of Florida, Louisiana, Mississippi, and Alabama.

(b) *Adult Morphology. Strongyloides* has a complicated life cycle. It can have a free-living cycle or a parasitic cycle. In the parasitic cycle there are only females, no males. The adult parasitic female is parthenogenetic (produces viable eggs without fertilization by a male). There are both males and females in the free-living stage. The parasitic female (figure 9-9), inhabits the mucosa of the small intestine. It is typically filariform, measuring about 2 mm long by 30μ wide. The esophagus is approximately one-third of the total body length, continuing into the midgut without a bulb. The anterior quarter is more slender than the rest of the body; the anus is located a short distance from the pointed tail. The free living male and female are rhabditoid; the female closely resembles the rhabditoid larval stage, and the male is about 0.7 mm long by 40 to 50 microns in diameter.

(6) *Trichostrongylus:*

(a) *Geographical Distribution. Trichostrongylus* is considered an incidental parasite of man as ruminants are the natural final hosts of the parasites. However, *Trichostrongylus* sp. are found in man in many areas of the world, including Africa, Iran, Iraq, India, Armenia, Siberia, Indonesia, Japan, Korea, China, Formosa, Australia, and occasionally the United States. In fact, *T. orientalis* is more commonly found in man than in other animals in Japan and Korea.

(b) *Adult Morphology.* The worms are delicate and threadlike, and they have a reddish color when alive. They reach a length of almost 10 mm, and they are about 75-120 microns wide. A buccal capsule and dental apparatus is lacking. The males have a relatively large copulatory bursa. In the female the vulva is situated a short distance behind mid-body, with the anus located near the posterior extremity.

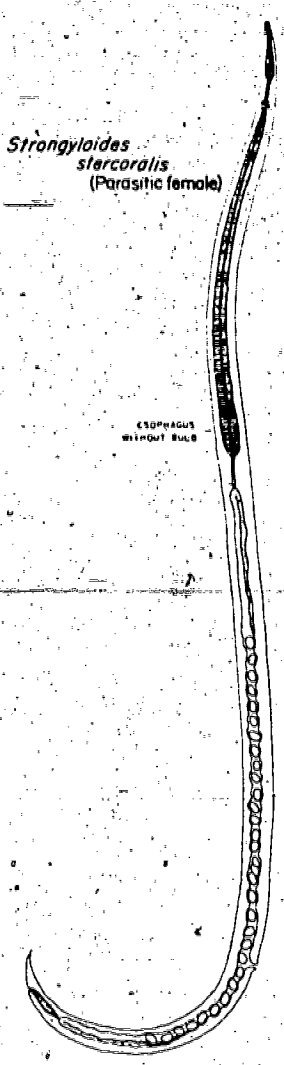


Figure 9-9. Parasitic Adult Female Strongyloides stercoralis. ×75.

b. **Nematodes of Other Animals Occasionally Found in Humans.** Humans are occasionally infected with a parasite that naturally infects other animals. When a human is infected with an animal parasite, it is known as a zoonotic infection, and the parasite is considered to be an incidental parasite of humans. Some of these parasites have a limited geographical distribution, whereas others are quite widespread. It is necessary to be aware of such parasites and always be on the alert for them.

(1) *Ternidens deminutus.* The natives of Southern Rhodesia, Malawi, and Mozambique are frequently found to be infected with *Ternidens deminutus.* The parasite resembles a hookworm; but the buccal capsule is terminally

located, and the buccal cavity is guarded on the inside by a double row of stiff bristles. The eggs of *Ternidens* can be distinguished from hookworm eggs only because they are larger. However, the eggs of *Ternidens* and those of *Trichostrongylus* are within the same size range. *Ternidens* eggs average 84 by 51 microns, whereas the maximum size of hookworm is about 70 by 40 microns. When it is necessary to differentiate *Ternidens* from *Trichostrongylus*, the eggs can be hatched to obtain the rhabditoid larvae. The rhabditoid larvae of *Trichostrongylus* as seen in figure 9-10 has a small bead-like knob on the tip of its tail that is not present on the larvae of *Ternidens*.

(2) *Physaloptera caucasica*. *P. caucasica* is the only species of the genus *Physaloptera* that is known to parasitize man. It is a natural parasite of some monkeys. It has been reported from man in the Caucasus, tropical Africa, Southern Rhodesia, India, Panama, and Columbia. The adults resemble young ascarids, from which they can be distinguished because they have two lips, each provided with dental processes. The eggs have thick, smooth shells, and they are fully embryonated when passed in feces. They measure 44 to 65 microns by 32 to 45 microns. Infertile *Physaloptera* eggs resemble fertile decorticated *Ascaris* eggs; however, fertile *Physaloptera* eggs contain a fully developed larva, whereas *Ascaris* eggs never contain a larva when passed in feces.

(3) *Capillaria philippinensis*. *C. philippinensis* is the only intestinal capillarid known to parasitize man. Several thousand cases have been reported, all to date from the Philippines. The small adult worms measure less than 5 mm. They are found embedded in the mucosa near the junction of the large and small intestines. The males have caudal alae and long, nonspiny spincule sheaths. In the females the anterior half of the body contains esophagus and esophageal gland, and the posterior half contains intestine and reproduction organs. Females are reported to produce ova with shells and embryos without shells, as well as typical bioperculate eggs resembling *Trichuris* eggs. All stages of development are found in man, and all stages have been recovered from feces. The eggs of *T. trichiura* measure 50 to 54 by 22.5 microns, whereas the bioperculate eggs of *C. philippinensis* measure about 36 to 45 by 21 microns making them easily distinguishable.

(4) *Capillaria hepatica*. *C. hepatica* is a delicate, threadlike parasite that lives in the parenchyma of the liver of mammals, especially rodents. The female worms pass eggs directly into the liver tissues, causing fibrosis. As the worms and eggs accumulate in the liver, considerable damage results. To make a positive diagnosis, a liver biopsy must be performed to recover the worm or the typical eggs. Morphologically, the eggs are similar to those of *T. trichiura*. They measure 51 to 61 by 30 to 35 microns, which is slightly larger than those of *T. trichiura*. The eggs of *C. hepatica* are more barrel-shaped, and the shells appear velvety because they are finely pitted with minute pores. The eggs are occasionally found in the feces of human patients. Do not confuse them with *T. trichiura*. The presence of the eggs in feces does not mean that the patient has *C. hepatica;* it only means that the patient has recently eaten infected liver.

c. **Diagnosis:**

(1) The usual diagnostic stage of these nematodes is the egg, except for *Strongyloides stercoralis*, in which the first or second stage (rhabditiform) larva, is the usual diagnostic stage. Occasionally rhabditiform larvae of hookworms are present in feces and must be distinguished from those of *Strongyloides*. Hookworm-like eggs recoverd from patients who have been in areas where trichostrongyles, or *Ternidens* are prevalent in humans have to be carefully examined and measured to differentiate between hookworms, *Trichostrongylus*, and *Ternidens*.

(2) The rhabditiform larvae of hookworm, *Strongyloides*, and *Trichostrongylus* (figure 9-10) are differentiated by the length of the buccal cavity, the appearance of the genital primordium, the size of the esophagus, and the appearance of the tip of the tail. In rare cases, third stage (filariform) larvae will be found in feces, or they can be obtained by culturing specimens containing eggs or rhabditiform larvae. Filariform larvae differ from rhabditiform larvae in size and in the appearance of the esophagus. The filariform larvae are about twice as long as the rhabditiform larvae and they lack the bulbed configuration of

esophagus which is characteristic of the rhabditiform larvae. The filariform larvae of *Strongyloides* (figure 9-11) are distinguished from those of hookworm by the appearance of the tip of the tail and the length of the esophagus.

(3) Characteristics of the nematode eggs found in man are listed in table 9-9, while those of the larvae are given in table 9-10. Drawings of the nematode adults with their relative sizes are presented in figure 9-12. Figure 9-16 at the end of the chapter shows nematode eggs along with other helminth eggs and some common pseudoparasites and artifacts.

(4) Unlike some of the other helminths, intestinal nematodes shed eggs more or less continuously, and they are usually present in every fecal specimen from an infected individual. *Strongyloides* larvae, however, are passed irregularly and will not be found in every specimen.

Table 9-9. Characteristics of Nematode Eggs. (Adapted from Brooke and Melvin, 1969).

SPECIES	SIZE	SHAPE	COLOR	STAGE OF DEVELOPMENT WHEN PASSED	SPECIFIC FEATURES AND VARIATIONS
Enterobius vermicularis	55μ x 26μ Usual range: 50-60μ x 20-32μ	Elongate, asymetrical with one side flattened, other side convex.	Colorless shell.	Embryonated. Contains C-shaped or tadpole-like embryo.	Smooth, thin egg shell with one flattened side. Occasionally may contain fully developed larva. (More readily found on anal swabs than in feces.)
Ascaris lumbricoides fertile egg	60μ x 45μ Usual range: 45-70μ x 35-50μ	Round, or oval with thick shell.	Brown or yellow brown shell.	1 cell, separated from the shell at both ends.	Mammillated albuminous coat or covering on outer shell. Covering is occasionally lost and the decorticated eggs have a colorless shell with gray or black internal material.
Infertile egg	90μ x 40μ Usual range: 85-95μ x 35-45μ	Elongate, occasionally triangular, kidney-shaped or other bizarre forms. Shell often very thin.	Brown shell.	Internal material is a mass of irregular globules and granules that fills the shell.	Mammillated covering thin or missing in many cases.
Hookworms					
Ancylostoma duodenale and Necator americanus	60μ x 40μ Usual range: 57-76μ x 35-47μ	Oval or elliptical with a thin shell.	Colorless shell with greyish cells.	4- to 8-cell stage.	Occasionally, eggs may be in advanced cleavage (16 or more cells) or even embryonated. Rhabditiform larvae may be present if the specimens are old. Species identification cannot be made on eggs alone; therefore, eggs should be reported simply as "hookworm eggs."
Trichostrongylus spp.	90μ x 40μ Usual range: 75-115μ x 40-50μ	Elongate with one (or both) end more pointed than hookworm.	Colorless shell with greyish cells.	May be in advanced cleavage or morula stage.	Egg resembles hookworm egg but is larger and more pointed at the ends.
Strongyloides stercoralis	50μ x 30μ	Oval or elliptical.	Colorless shell with greyish cells.	Developed beyond 4-cell stage.	Egg resembles hookworm but is larger, and though similar to Trichostrongylus in size and appearance, it is more broadly rounded at the poles.
Rhabditoid nematodes	50μ x 35μ Usual range: 45-65μ x 32-42μ	Round or oval.	Colorless shell.	Developed to larval stage.	Eggs resemble decorticated, round or oval Ascaris eggs but they contain a developed larva. The shell is thick and colorless.
Trichuris trichiura	51μ x 22μ Usual range: 49-65μ x 20-29μ	Elongate, barrel shaped with a polar plug at each end.	Yellow to brown shell. "Plugs" are colorless.	1 cell or unsegmented.	Polar plugs are distinctive. Occasionally are presented in a vertical or slanted position and may not be readily recognized. A gentle tap on the coverslip will usually reorient the egg. On rare occasions, atypical eggs lacking polar plugs may be seen.
Capillaria hepatica	70-65μ x 30-35μ	Barrel-shaped with polar "plugs."	Brown shell; colorless "plugs."	Unsegmented.	Thick shell with polar plugs. Distinctly radially pitted. Has velvety appearance. Longer than Trichuris.
Capillaria philippinensis	30-35μ x 21-16μ	Elongate, barrel shaped with polar "plugs."	Yellow to brown shell; colorless "plugs."	Unsegmented.	Females produce ova with shells and embryos without shells as well as typical biopercolate eggs resembling, but smaller than, Trichuris.

Table 9-10. Differential Morphology of Larvae of Intestinal Nematodes. (Adapted from Brooke and Melvin, 1969)

	RHABDITIFORM LARVA (First Stage. Has bulbed esophagus.)			FILARIFORM LARVA (Third Stage. Lacks prominent bulb in esophagus.)		
SPECIES	SIZE	GENITAL PRIMORDIUM	BUCCAL CAVITY	SIZE	LENGTH OF ESOPHAGUS	TIP OF TAIL
Strongyloides stercoralis	225μ x 16μ Usual range, 200-300μ x 16-20μ	Prominent. Is an elongate, tapered, or pointed structure located along ventral wall about midway the body length	Short, about 1/3 to 1/2 as long as the width of the anterior end of the body	550μ x 20μ Usual range, 500-550μ x 20-24μ	Extends approximately 1/5 length of body	Notched
Hookworm	250μ x 17μ Usual range, 200-300μ x 14-17μ	Inconspicuous. Rarely distinct. When seen, is small, located nearer the tail than that of Strongyloides.	Long. Approximately as long as the width of the body	500μ Usual range, 500-700μ x 20-24μ	Extends about 1/4 length of body	Pointed

Trichostrongylus larva is similar to hookworm larva but it is characterized by having a small knob at the tip of the tail.

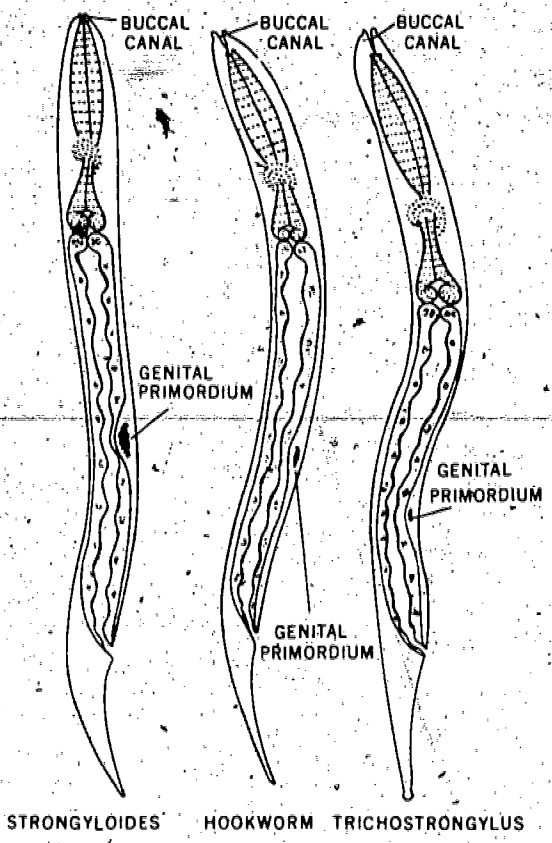


Figure 9-10. Rhabditiform Larvae. ×450.

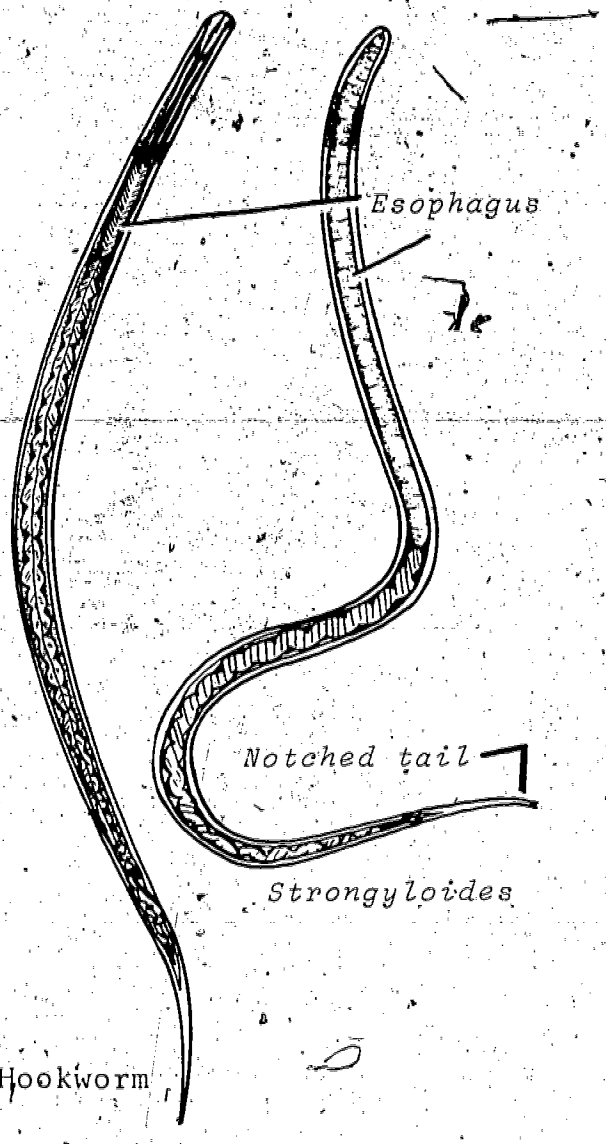


Figure 9-11. Filariform Larvae. ×200.

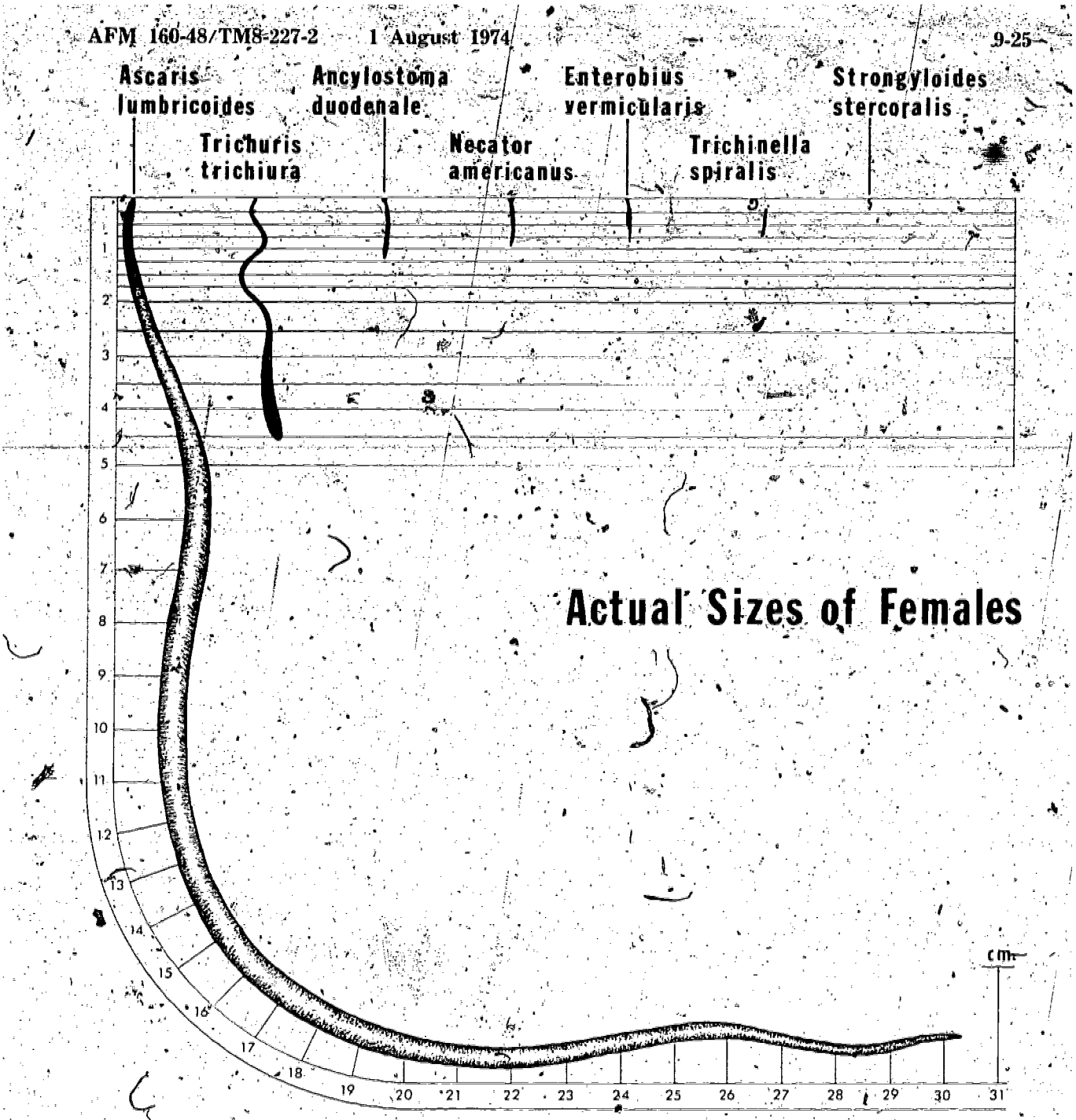


Figure 9-12. Relative Sizes of Intestinal Roundworms.

9-10. Cestodes (Tapeworms) Found in Humans:

a. **Cestodes Commonly Found in Humans:**

(1) *Diphyllobothrium latum.* This parasite is commonly known as the fish tapeworm because fish are the final intermediate hosts. The worm (Strobila) can be up to 35 feet long and have as many as 4,000 proglottids. *D. latum* is a common parasite in parts of Europe, Russia, Japan, the Philippines, Australia, South America, and North America. Dogs, wolves, bears, and a few other carnivores in addition to man can be hosts for this worm.

(2) *Taenia saginata* This is the largest tapeworm that infects man. It is usually about 15 feet long, but it can be up to 75 feet long. There are usually 1,000 to 2,000 proglottids. *T. saginata* is widely known as the beef tapeworm because cattle are the normal intermediate hosts. It is distributed worldwide, but is most prevalent in countries of southwestern Europe, Africa, and South America.

(3) *Taenia solium. T. solium* is found in all areas of the world where people eat improperly cooked pork. Man is the only definitive host for the adult worm, and swine are the usual intermediate hosts. The anterior part of the strobila is filiform, and the proglottids are much wider than long. Gradually the proglottids become square, and the last of the proglottids changes from square to oblong. Frequently several worms will be present, and occasionally many will be present.

(4) *Hymenolepis nana. H. nana* is found all over the world, but it is most prevalent in warm climates, especially where the summers are hot. It is common in Europe, Russia, Latin America, India, and the southern United States. *H. nana* is the most commonly found tapeworm in America. The parasite is known as the dwarf tapeworm of man; however, rats and mice are also acceptable hosts. The worm is about 2 inches long, but it sometimes has as many as 200 proglottids.

b. **Cestodes of Other Animals Occasionally Found in Humans:**

(1) *Hymenolepis diminuta.* This tapeworm is a common parasite of rats, mice, and other rodents; therefore, it is commonly known as the rat tapeworm. It is occasionally found in humans, usually children under 12 years of age. It has been reported from humans in most areas of the world. *H. diminuta* is small in comparison with some of the other tapeworms; nevertheless, it is considerably larger than *H. nana*, which it closely resembles. It can be up to 20 inches long and have 2,000 proglottids.

(2) *Dipylidium caninum.* This is a common parasite of both dogs and cats. It is occasionally found in humans, especially children. It has been found most frequently in humans from European countries, North America, the Antilles, the Philippines, China, South Africa, and Australia. The individual proglottids resemble pumpkin seeds in shape, and each one contains two sets of genital organs which open into two genital pores situated opposite each other in the middle of the lateral margins. The gravid proglottids are passed singly or in short chains, and sometimes they disintegrate leaving 300-400 egg capsules, each of which contains 5-15 eggs.

(3) *Bertiella.* Two species of *Bertiella* have been found in humans. *B. studeri*, a parasite of anthropoid apes, has been found in humans, usually children, in the islands of Mauritius, Sumatra, Java, the Philippines, the West Indies, and India. The other species, *B. mucronata*, a parasite of monkeys and chimpanzees, has been found in humans in Brazil, Cuba, Argentina, Paraguay, and once from a child in the United States. Both of these parasites are of the short-proglottid type as is *Hymenolepis.* The uterus extends across the width of the proglottid between the excretory canals. The genital pore alternates positions from one proglottid to the next. The wide, egg-filled proglottids are usually shed in groups. The eggs are frequently discharged before the gravid proglottids are expelled. The eggs somewhat resemble those of *D. caninum*, but the inner shell surrounding the embryophore is bottle or flask shaped rather than spherical.

(4) *Inermicapsifer madagascariensis.* This tapeworm is primarily a parasite of wild rodents in East Africa. In that area it is occasionally found in humans, but it has spread to other areas of the world where its rodent hosts are not present. In those areas outside of Africa, which include Cuba, Madagascar, the Comoros, Mauritius, and Venezuela, it is considered to be a human parasite. The strobila is long and slender, measuring up to 40 cm long and 2.5 mm

wide. The scolex has four unarmed suckers and no rostellum. The proglottids near the scolex are wider than long; those in the middle of the strobila are almost square; and the gravid segments that are filled with egg capsules are longer than wide (3 mm by 2.5 mm). The genital openings are unilateral, situated in the middle of the lateral margin of the proglottid. The uterus develops in the whole of the proglottid, gradually breaking down until the proglottid is generally filled with egg capsules similar to *D. caninum*. The proglottid is filled with 150 to 175 egg capsules, each containing from 4 to 10 eggs. The eggs are about 35 to 50 microns in diameter and they contain a hexacanth embryo that measures about 11 microns in diameter.

(5) *Raillietina*. Parasites belonging to the genus *Raillietina* are occasionally found in humans. Most of the species that have been found in humans are normally parasites of rodents or monkeys. Members of the genus *Raillietina* are difficult to differentiate from *Inermicapsifer*. *Raillietina* does have an armed rostellum as opposed to *Inermicapsifer*, whose scolex is unarmed. Also, the genital pore is situated in the anterior third of the lateral margin of the proglottid of *Raillietina*, and it is in the middle of the lateral margin of *Inermicapsifer*. Both *Raillietina* and *Inermicapsifer* resemble *Dipylidium* in that the gravid proglottids disintegrate into egg capsules each containing several eggs. The eggs are large and ovoidal, and they contain a hexacanth embryo. The cases found in humans have been mostly in children, and they have been reported from Japan, Formosa, Thailand, Indonesia, Australia, Cuba, and Ecuador.

c. **Diagnosis:**

(1) In certain of the cestodes, *(Hymenolepis nana, Hymenolepis diminuta,* and *Diphyllobothrium latum),* the egg is the usual diagnostic stage. In other species, *(Taenia* and *Dipylidium caninum),* eggs can be present in feces, but proglottids as illustrated in figure 9-13 are the usual diagnostic stage. Except in the case of *D. latum*, the cestode eggs are embryonated when they are passed. The embryo is a six-hooked, spherical structure called an oncosphere.

(2) The unsegmented eggs of *D. latum* can resemble the eggs of the hookworm, infertile *Ascaris*, or *Paragonimus westermani* and must be distinguished from them. Occasionally, portions of the chain of proglottids are passed, and the species can be identified by the characteristic appearance (figure 9-13) of the proglottids.

(3) The eggs of *Taenia saginata* and *Taenia solium* are identical. When the characteristic eggs are found, report them as "*Taenia* species." The diagnostic stage usually recovered, however, is the gravid proglottid rather than the egg. Proglottids, either singly or in short chains, become detached from the strobila and are passed with the feces. *T. saginata* proglottids occasionally migrate out of the anus independent of defecation. Specific identification is based on the number of lateral uterine branches in a gravid proglottid. A gravid proglottid of *T. saginata* has 15 to 20 lateral branches on each side, while *T. solium* has only 7 to 13 branches. They can be seen in uncleared segments, but they are more distinct in stained and cleared proglottids. Use a hand lens or dissecting scope to observe the structural details as seen in figure 9-13. Less frequently under ordinary circumstances, but quite often after treatment, scoleces (figure 9-13) are obtained for species identification.

(4) Normally, *Dipylidium caninum* is also frequently diagnosed by finding proglottids rather than eggs in fecal specimens. The eggs, when found, are usually contained in packets, or capsules of 5 to 15 or more eggs. In rare cases, the scolex will be obtained for diagnosis.

(5) The eggs of the six most common cestode parasites found in humans are described in table 9-11. Gravid proglottids and scoleces are described in tables 9-12 and 9-13, respectively. Drawings of the eggs are presented in figure 9-17.

Species	Size	Shape	Color	Stage of Development When Passed	Specific Features and Variations
CESTODES					
Taenia saginata Taenia solium	35μ Usual range, 31-43μ.	Spherical or sub-spherical with thick striated shell.	Walnut brown.	Embryonated. 6-hooked oncosphere is present inside a thick shell.	Thick, striated shell. Eggs of T. solium and T. saginata are indistinguishable and species identification should be made from proglottids or scoleces. "Taenia" spp. should be reported if only eggs are found.
Hymenolepis nana	47μ x 37μ Usual range, 40-60μ x 30-50μ.	Oval or sub spherical. Shell consists of 2 distinct membranes. On inner membrane are two small "knobs" or poles from which 4 to 8 filaments arise and spread out between the two membranes.	Colorless, almost transparent.	Embryonated. 6-hooked embryo contained in egg.	Polar filaments.
Hymenolepis diminuta*	72μ Usual range, 70-86μ x 60-80μ.	Round or slightly oval. Striated outer membrane and thin inner membrane with slight poles. Space between membranes may appear smooth or faintly granular.	Yellow.	Embryonated. 6-hooked oncosphere inside shell.	Resembles H. nana but lacks polar filaments. Poles are rudimentary and often hard to see.
Dipylidium caninum*	35-40μ Usual range, 31-50μ x 27-48μ.	Spherical, subspherical or oval. 5-15 eggs (or more) are enclosed in a sac or capsule.	Colorless.	Embryonated.	Eggs are contained in a sac or capsule which ranges in size from 58μ x 45μ to 60μ x 170μ. Occasionally, capsules are ruptured and eggs are free.
Diphyllobothrium latum	66μ x 44μ Usual range, 58-76μ x 40-51μ.	Oval or ellipsoidal with an inconspicuous operculum at one end and a small "knob" at the other end.	Yellow to brown.	Unembryonated. Germinal cell is surrounded by a mass of yolk cells which completely fills inner area of shell. Germinal cell is usually not visible.	Egg resembles hookworm egg but has a thicker shell and an operculum.

*Usually found in lower animals, only occasionally found in man.

Table 9-11. Differential Morphology of Cestode Eggs. (From Brooke and Melvin, 1969)

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SPECIES	SIZE (in mm.)	SPECIAL FEATURES
Diphyllobothrium latum	2-4 x 10-12; "broader than long"	Rosette-shaped, coiled uterus; no lateral gonopore
Taenia saginata	16-20 x 5-7; "longer than broad"	15 or more main uterine branches; single lateral gonopore
Taenia solium	10-12 x 5-7; "longer than broad"	13 or less main lateral uterine branches; single lateral gonopore
Hymenolepis nana	0.15-0.3 x 0.8-0.9; "broader than long"	Uterus irregular, sac-like; single lateral gonopore
Hymenolepis diminuta	0.75 x 2.5; "broader than long"	Uterus irregular, sac-like; single lateral gonopore
Dipylidium caninum	10-12 x 2.5-3.0; "longer than broad"	Two gonopores on each segment; uterus with polygonal egg sacs

Table 9-12. Differential Characteristics of Gravid Proglottids.

Table 9-13. Differential Morphology of Tapeworm Scoleces. (Adapted from Brooke and Melvin, 1969)

SPECIES	SIZE	SHAPE	SUCKERS NO.	SUCKERS APPEARANCE	OTHER
Taenia solium	Approximately 1mm in diameter	Globular or rounded	4	Cup-like	Double row of 25-30 large and small brown chitinous hooks arranged around a rostellum (small projection) at the top of the scolex.
Taenia saginata	1 to 2mm in diameter	Rounded or slightly pyriform	4	Cup-like	Does not have a rostellum or hooks.
Diphyllobothrium latum	2 to 3mm in length x 1mm wide	Almond-shape or spatulate	2	Grooves	Does not have a rostellum or hooks. Deep grooves (suckers) are located dorsally and ventrally on the scolex, but often appear to be lateral.
Dipylidium caninum	0.35 x 0.37mm	Rhomboid or rounded	4	Oval, cup-like	Prominent conical or ovoid rostellum with 50 to 150 small thorn-shaped hooks arranged in several rows (1-7 rows). Rostellum may be retracted into a depression at the upper margin of the scolex.
Hymenolepis nana	0.25mm in in diameter	Globular	4	Cup-like	Rostellum is present and is equipped with a single row of 24 to 30 hooks. Rostellum is retractable.
Hymenolepis diminuta	Very small, less than 0.25mm	Rounded or club-shaped	4	Cup-like	The scolex has an apical invagination cavity into which the unarmed rudimentary rostellum is frequently retracted.

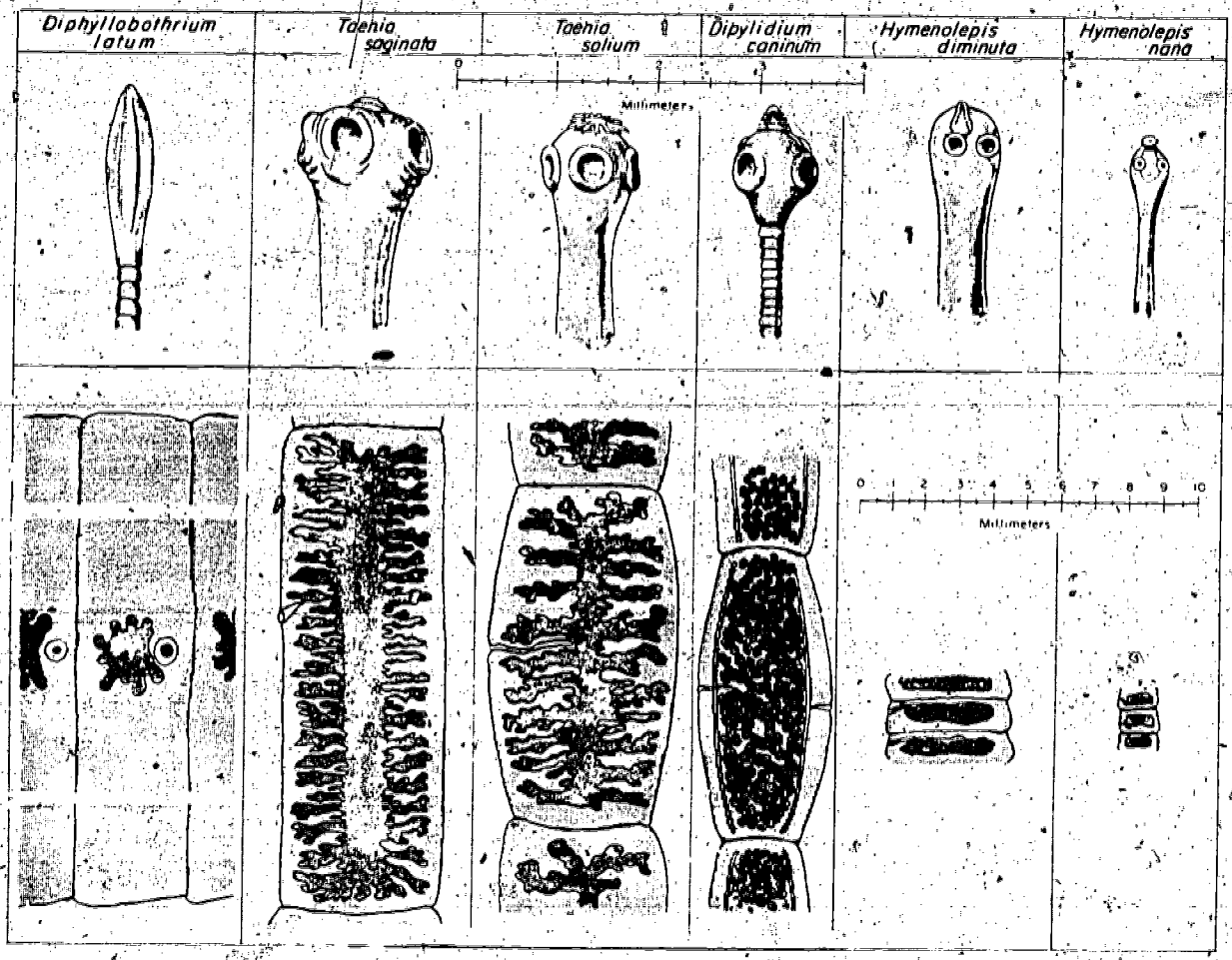

Figure 9-13. Scoleces and Gravid Proglottids of the Cestode Parasites Commonly Found in Humans. ×10.

9-11. Trematodes Found in Humans:

a. **Schistosomes (Blood Flukes):**

(1) *Schistosoma mansoni.* This organism, also known as Manson's blood fluke, causes the disease variously known as Manson's schistosomiasis, intestinal schistosomiasis, or schistosomiasis mansoni. It is found chiefly in eastern and central Africa, the Middle East, the Caribbean area, and South America as far south as Brazil. Man is the definitive host, and snails of several genera are intermediate hosts. Adult male and female *S. mansoni* are shown in figure 9-14. The male fluke is about 12 mm long and possesses six to nine testes. The body of the male is flattened and folded ventrally to form the sex canal in which the female is held. The female is longer than the male (16 mm as compared with 12 mm), threadlike, and has an anteriorly located ovary. The outer surface of the adult *S. mansoni* is coverd with minute sensory papillae, known as tuberculations. Eggs are usually laid in the small blood vessels of the lower intestine. From here they break into the lumen of the intestine to be passed with the feces. The egg ranges in size from 114 to 175 microns long by 45 to 73 microns wide. It is narrow and rounded at the anterior end with a more broadly rounded posterior end. The most noticeable morphological feature is the long, sharp, lateral spine on the posterior third of the

egg. The eggs have a nonoperculate shell. When mature, they are yellow brown in color, transparent, and contain a ciliated miracidium that is fully developed. Laboratory diagnosis of *S. mansoni* depends upon finding the characteristic eggs in fecal specimens. The number of eggs you find in a direct fecal smear parallels, to some degree, the severity of the infection. Appropriate concentration methods, such as sedimentation or acid-ether-formalin concentration, are recommended. Complement-fixation, slide flocculation, and fluorescent antibody tests are available at reference laboratories for use when you do not find eggs in fecal specimens from suspected cases.

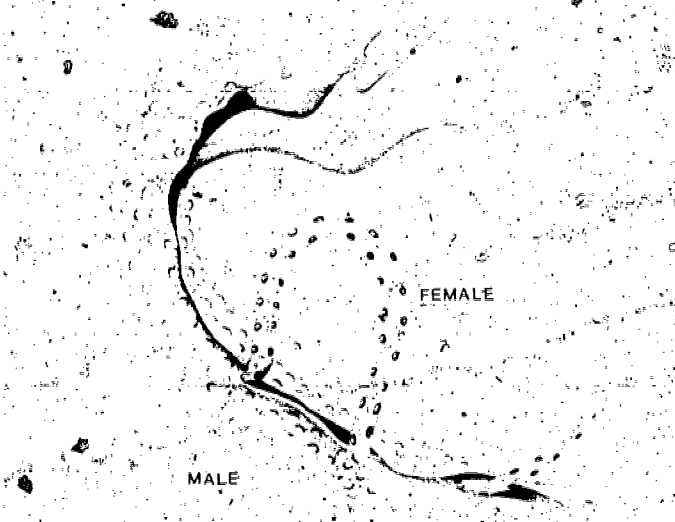

Figure 9-14. Schistosoma mansoni: Adult Male and Female. ×10.

(2) *Schistosoma japonicum.* This parasite is frequently referred to as the Oriental blood fluke. *S. japonicum* is restricted to the Far East: Japan, China, Formosa, and the Philippines. Many mammals—including dogs, cats, horses, cattle, rats, and mice—act as reservoir hosts. Snails of the Genus *Oncomelania* are the intermediate hosts. The adult male of *S. japonicum* is 12 to 20 mm long. The integument is not tuberculated, but it is covered with minute spines, especially in the locality of the suckers and the gynecophoral canal. There are seven testes in the male *S. japonicum*. The female is about 26 mm long, and the integument is also covered with minute spines. The ovary is just below the center of the body, and the vitelline (yolk-producing) glands are limited to the lateral margins of the distal quarter of the body. The uterus consists of a long, straight tube containing up to 50 eggs, which are normally laid in the blood vessels of the small intestine. The eggs then break into the intestine to be passed with the feces. The eggs are smaller than the eggs of the other species of schistosomes. They measure about 70 to 100 microns by 45 to 80 microns. You can see a wartlike thickening near the posterior end when the egg is positioned properly.

(3) *Schistosoma haematobium.* This parasite is also known as the vesical blood fluke. The disease caused by *S. haematobium* is sometimes called vesical schistosomiasis, schistosomiasis haematobia, urinary bilharziasis, or schistosomal hematuria, depending upon the geographical location where the cases are found. The disease is widespread in Africa and surrounding islands. Occasionally, cases have been found in the southern tips of Europe and India. Man is the only definitive host for *S. haematobium*, and snails of the Genus *Bulinus* are the most common intermediate hosts. Morphologically, *S. haematobium* closely resembles *S. mansoni*; however, adults of *S. haematobium* are slightly larger than *S. mansoni*. The male *S. haematobium* has only four or five testes, whereas *S. mansoni* has six to nine testes. The male is covered with minute integumentary tuberosities; but in the female, the tuberosities are usually confined to the extremities. Eggs are usually laid in the blood vessels of the urinary bladder and passed in the urine, but they may be passed in feces too. The eggs are 112 to 170 microns long by 40 to 70 microns wide. They are spindle-shaped, rounded anteriorly, and conical at the posterior extremity. The posterior end terminates in a pointed spine. The egg is nonoperculate, has a thin transparent shell, and is yellow-brown in color. When passed in feces, the egg normally contains a fully developed miracidium. Examine both feces and urine for eggs when *S. haematobium* is suspected. The techniques used to examine feces in cases of *S. mansoni* and *S. japonicum* are also satisfactory for *S. haematobium*.

b. **Liver FLukes.** Unlike the schistosomes, all liver (and intestinal) flukes are hermaphrodi-

tic. None of the liver flukes are found exclusively in man. The two species of greatest general interest are *Fasciola hepatica* (the sheep liver fluke) and *Clonorchis sinensis* (the oriental liver fluke). However, interest in other species discussed in this section is increasing rapidly as more is learned about them.

(1) *Fasciola hepatica.* The sheep liver fluke is found in all sheep-raising countries, including the United States. Humans, sheep, and cattle are the definitive host, while snails belonging to the Family Lymnaeidae are the intermediate hosts. The egg is one of the largest produced by a helminth that infects man. It is oval in shape and measures about 80 by 150 microns. The egg is yellow brown and has an operculum. Eggs of *F. hepatica* are impossible to differentiate from those of *Fasciolopsis buski;* therefore, you should report them as "egg morphologically resembling the Genus *Fasciola* and *Fasciolopsis.*" The only way for you to positively identify the parasite as *F. hepatica* is to recover the typical *F. hepatica* eggs from uncontaminated bile collected by the attending physician. This will give you a positive identification because *F. buski* inhabits the intestine and not the bile ducts. False fascioliasis occurs when someone ingests liver infected with *F. hepatica.* The eggs are then passed in the feces of the person eating the liver. This can be ruled out if the patient is kept on a liver-free diet for 3 days or more. If the patient continues to pass eggs, he probably has a true infection.

(2) *Clonorchis sinensis. C. sinensis* is known as the Chinese liver fluke. There are several authors who feel that the Genus *Clonorchis* has characteristics sufficiently like *Opisthorchis* to classify the species *sinensis* under the latter genus. Therefore, you may see the term "*Opisthorchis sinensis*" used in some references. This is the most important liver parasite of man in parts of China, Japan, Formosa, and Indochina. There is no evidence that the infection has become established in any region outside the China Sea area. Frozen fish and dried or pickled fish shipped from endemic areas probably account for infection in persons who have never visited areas where the parasite is found. There are many reservoir hosts for this parasite, including the dog and cat. The adult worm lives in the bile passages and occasionally in the pancreatic duct. It is a flat, transparent, flabby worm that is somewhat spade shaped. It is relatively small (10 to 25 mm in length by 3 to 5 mm in width). The worm has large branched testes which are situated one behind the other in the posterior third of the body. Laboratory diagnosis is based on the recovery of typical eggs from feces. The eggs are small and ovoid with a distinct operculum that fits into a rimmed extension of the shell. They have moderately thick, light yellow-brown shells, and they measure about 29 microns by 16 microns.

(3) *Opisthorchis felineus. O. felineus* is widely distributed in eastern and southeastern Europe and Asiatic USSR, and it is reported to be common in Vietnam. The worm is smaller than *Clonorchis* (10 mm by 2.5 mm as compared with 20 mm by 4 mm); otherwise, they are very similar. The eggs of the two worms are also very similar. They are slightly narrower than those of *Clonorchis.* The egg of *O. felineus* is 30 microns by 11 microns, whereas that of *C. sinensis* is about 29 microns by 16 microns. A snail is the first intermediate host, and cyprinid fresh water fish are the second intermediate host. In addition to man, many fish-eating mammals are infected. Clinical aspects and diagnostic procedures are essentially the same as those of clonorchiasis.

(4) *Opisthorchis viverrini. O. viverrini* is closely related to *O. felineus.* As many as 75 percent of the people from some areas of northeastern Thailand have been found to be infected with the parasite. The eggs are slightly smaller than those of *Clonorchis* and *O. felineus.* They measure 26 microns by 13 microns. It is very difficult to differentiate the eggs of *Clonorchis, Opisthorchis,* and the intestinal fluke *Metagonimus* from each other.

(5) *Dicrocoelium dendriticum. D. dendriticum* is a parasite commonly found in the bile duct of sheep and other herbivorous animals. The parasite is widely distributed in Europe, North Africa, Northern Asia, and some other areas in the Orient. *D. dendriticum* has frequently been reported when actually the patient was not infected at all. This happens quite often when people eat sheep liver that has been infected with the eggs, which are then passed in the feces just as in false fascioliasis. However, the parasite does infect man and is frequently

reported form Europe, Asia, and Africa. The eggs are asymmetrically ovoidal, thick-shelled, dark brown in color, have a broad convex operculum, and measure 38 to 45 microns by 22 to 30 microns. They contain a mature miracidium when passed in the feces of the definitive host.

c. **Intestinal Flukes.** In this country, intestinal flukes are of little medical importance. There are no known intestinal flukes that are strictly human parasites. A number of species parasitize humans as well as other animals. In certain localities, this condition constitutes a serious medical problem because the other animals form a larger reservoir of infection. The best example of this is *Fasciolopsis buski* in southeastern Asia, where it is a common parasite of pigs and humans.

(1) *Fasciolopsis buski.* This fluke is called the giant intestinal fluke. It is most common in India, China, and the southwest Pacific. It is found in man, swine, and occasionally in dogs. *Fasciolopsis buski* is the largest and best known of the intestinal flukes of man. It measures over an inch in length and about a half inch in width. The worm is leaflike in appearance and has a spinose integument. The ventral sucker (acetabulum), located near the anterior end, is three to four times as large as the oral sucker. *F. buski* has highly branched testes, which occupy the posterior three-fifths of the body. The egg is practically identical to that of *F. hepatica*. It is operculate and measures 130 microns to 140 microns by 80 microns to 85 microns. Diagnosis is based on the recovery of typical eggs from feces and on the physician's clinical findings.

(2) *Heterophyes heterophyes. H. heterophyes* is found in Egypt, Palestine, and the Orient. Suitable hosts include dogs, cats, rabbits, and a few other mammals, as well as man. Snails of the Genus *Pironella* are first intermediate hosts, and fresh water fish are second intermediate hosts. The adult worm is very small. It measures approximately 0.4 mm by 1.5 mm. The oral sucker is ventrally located, but it is only about one-third as large as the acetabulum. The adult worm is covered with minute spines, which are set closely together. The egg is small (30 microns by 17 microns), operculate, brownish in color, and contains a well-developed ciliated miracidium. It is very difficult to differentiate the eggs of this parasite from *Opisthorchis* sp., *C. sinensis*, or *M. yokogawai*.

(3) *Metagonimus yokogawai. M. yokogawai* is a common parasite of the Far East and the Balkan States. Man and several other mammals are suitable definitive hosts. The infective metacercariae of *M. yokogawai* excyst in the small intestine of the definitive host and attach themselves to cells in the mucosal crypts and grow to maturity. Normally the eggs are passed into the intestinal lumen and pass out with feces. However, the worms may penetrate so deeply into the intestinal wall that some eggs can get into the general blood circulation, as in *H. heterophyes* infections. The egg is operculate, brownish in color, measures 28 microns by 17 microns, and contains a well developed ciliated miracidium. Diagnosis is based on the recovery of characteristic heterophyid eggs in feces. More than likely you will not be able to differentiate the eggs of *M. yokogawai* from those of *H. heterophyes*, and you can expect to have difficulty in differentiating them from those of *Clonorchis* and *Opisthorchis*. Experts differentiate them by extensive study of morphological differences and also study of life-cycle forms.

(4) *Echinostoma.* There are a number of members of the Genus *Echinostoma* that have been reported from humans. They are intestinal flukes that average under 1 cm in length and 2mm in width. They are distinguished by a collarette of spines on a disk surrounding the oral sucker. The most important species is *Echinostoma ilocanum*, which has been reported from the Philippines, the Celebes, and Indonesia. Other species of *Echinostoma* have been found in humans in the same area and a few other places in Asia. Diagnosis is difficult because the eggs are unembryonated and appear very similar to those of *Fasciolopsis*. The eggs vary in size in the different species, and some overlap the size range of *Fasciolopsis*. The eggs of *Echinostoma ilocanum* are straw-colored. They have an operculum similar to *Fasciolopsis*, and they measure 83 to 116 microns long by 58 to 69 microns wide.

(5) *Gastrodiscoides hominis. G. hominis* inhabits the cecum and ascending colon of its host. The pig is the most common reservoir host,

but monkeys from several areas have also been found to be infected. It is a common human parasite in Northeast India but it has been found in other parts of India, Vietnam, the Philippines, and Russia. The parasite has a very wide acetabulum at its posterior edge, which gives it the appearance of a very shallow bowl with a handle, the handle being the anterior end and the bowl, the posterior. The living worm is reddish and measures about 5 to 10 mm in length by 4 to 6 mm in width. The worm passes unembryonated operculate eggs. They are oval and measure 150 to 170 microns long by 60 to 70 microns wide. The eggs of this parasite are much more elongate than the other nonembryonated operculate eggs. It is distinct from the *Fasciolopsis*-type eggs because of its spindle shape.

d. **Lung Fluke (Paragonimus westermani).** The only lung fluke of man is *Paragonimus westermani*. Humans are commonly infected with this parasite in Japan, Korea, Formosa, the Philippines, and Thailand. It is endemic in small foci of Africa and northern South America. *P. westermani* has been recovered from wild felines in India, Malaysia, Indonesia, and Thailand. A very similar or identical fluke is found in cats, dogs, pigs, and wild carnivores in Africa and North and South America. Morphologically the adult is plump and ovoidal in cross section, about 12 mm long and one-third as wide. The worm is reddish brown when alive. Its surface is covered with large spines. The eggs of *P. westermani* are relatively large, measuring approximately 80 to 120 microns in length by 45 to 60 microns in width. The eggshell is relatively thick and golden brown in color. The eggs have a flat-shaped operculum, and they are unembryonated when passed. Specific diagnosis is easily made when the eggs of *P. westermani* are recovered from rusty or blood-tinged sputum, feces, pleural aspirates, or from peritoneal abscesses. In suspected cases where eggs cannot be recovered, intradermal tests and complement-fixation tests may prove to be very helpful.

e. **Diagnosis:**

(1) The diagnostic stage of the trematode species parasitic in man is the egg. The characteristics of the eggs of the species commonly found in humans are described in table 9-14. The eggs of *Clonorchis*, *Opisthorchis*, *Heterophyes*, and *Metagonimus* closely resemble each other, and for this reason, they are difficult to identify. The characteristics described are not always apparent. The eggs of *Fasciola hepatica* and *Fasciolopsis buski* are also very similar, and it is very difficult if not impossible to differentiate them.

(2) The eggs of *Schistosoma haematobium* are ordinarily passed in urine, and the eggs of *Paragonimus westermani* are ordinarily passed in sputum. Eggs of both, however, are occasionally found in feces.

(3) The eggs of trematodes are usually found at irregular intervals in feces; therefore, it is necessary to examine several specimens to demonstrate their presence. Drawings of the eggs are presented in figure 9-18.

(4) Although the eggs are the usual diagnostic stage in trematode infections, adult worms are sometimes passed by patients or recovered during postmortem examinations. The diagrammatic illustrations in figure 9-15 will aid in identifying such worms that have been mounted and stained for study.

Table 9-4. Differential Characteristics of Trematode Eggs.

SPECIES	SIZE (in microns)	SPECIAL FEATURES
Schistosoma japonicum	70-100 x 55-80; average 85 x 60	Nonoperculate; inconspicuous, lateral, curved spine, often not seen; contains miracidium
Schistosoma mansoni	114-175 x 45-73; average 155 x 65	Nonoperculate; conspicuous lateral spine; contains miracidium
Schistosoma haematobium	112-170 x 40-70; average 150 x 60	Nonoperculate; conspicuous terminal spine; contains miracidium
Clonorchis sinensis	27-35 x 12-20; average 29 x 16	Distinctly operculate; contains asymmetrical miracidium; light bulb-shaped; shoulders
Opisthorchis felineus	Average 30 x 11	Distinctly operculate; contains asymmetrical miracidium; shoulders not pronounced
Opisthorchis viverrini	Average 26 x 13	Distinctly operculate; contains asymmetrical miracidium
Dicrocoelium dendriticum	38-45 x 22-30; Average 42 x 26	Asymmetrically ovoidal, thick shelled, dark brown; they have a broad convex operculum, and contain a mature miracidium
Fasciola hepatica	130-150 x 65-90; average 140 x 80	Indistinctly operculate; immature; yolk granules concentrated around nuclei of cell
Fasciolopsis buski	130-140 x 80-85; average 135 x 82	Indistinctly operculate; immature; yolk granules evenly distributed in yolk cells
Heterophyes heterophyes	28-30 x 15-17; average 30 x 17	Distinctly operculate; contains symmetrical miracidium; shoulders not pronounced
Metagonimus yokogawai	27-29 x 16-18; average 28 x 17	Distinctly operculate; contains symmetrical miracidium; shoulders not pronounced
Echinostoma ilocanum	85-115 x 58-70; average 90 x 60	Indistinctly operculate; immature when passed
Gastrodiscoides hominis	150-170 x 60-70; average 160 x 65	Distinctly operculate; immature; rhomboidal, tapering toward poles
Paragonimus westermani	80-120 x 45-60; average 95 x 55	Distinctly operculate, golden brown; immature; shoulders distinct but not pronounced

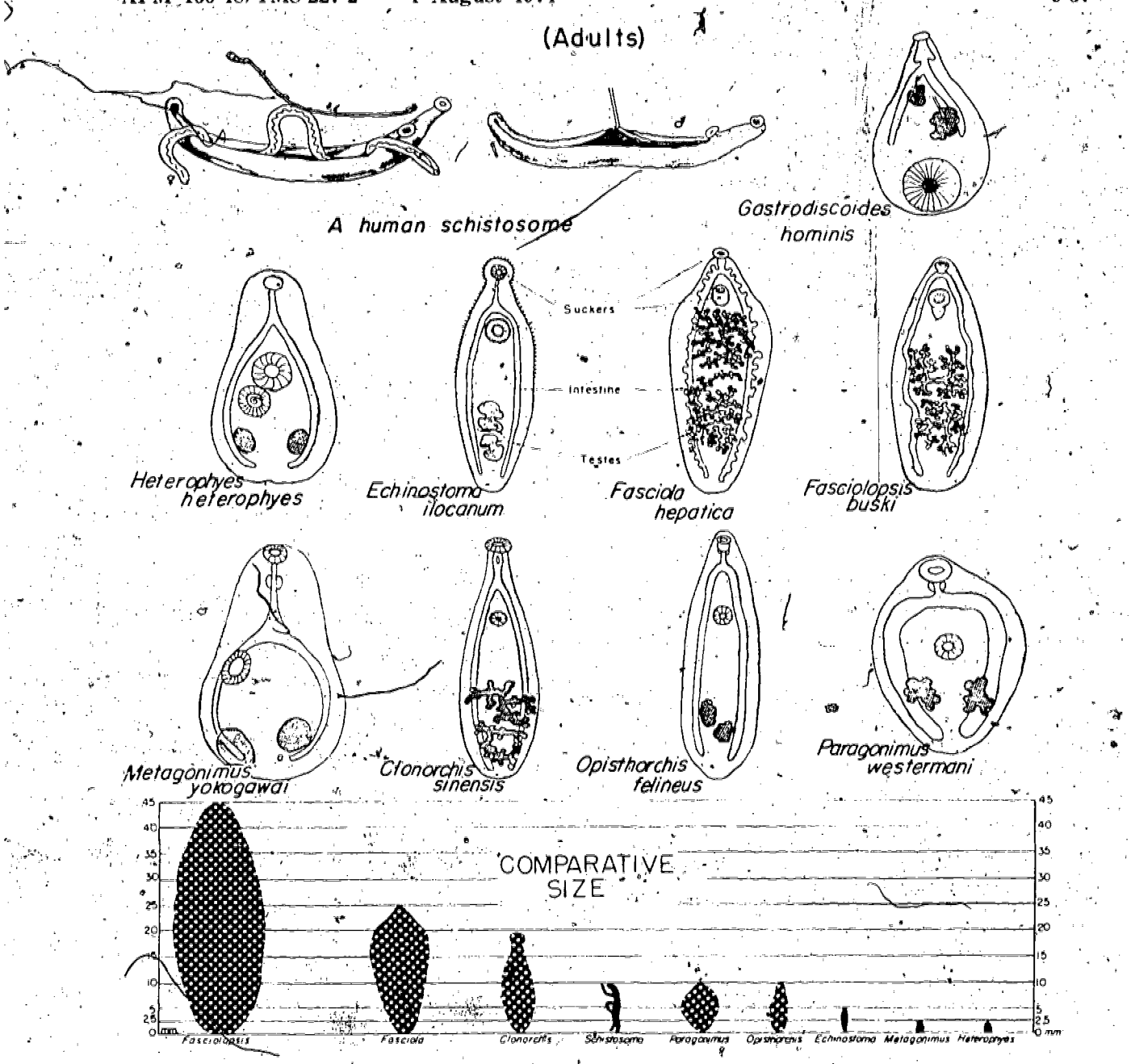

Figure 9-15. Differential Morphology of Adult Trematodes Infecting Humans. ×450.

9-12. Pseudoparasites and Artifacts. Objects which can be incorrectly identified as parasites fall into several broad categories. These include cellular elements which are normally shed into the intestine from various points in the digestive tract, remnants of debris originating from solids ingested by the patient in food or drink, yeasts and fungi which are normal inhabitants of the intestinal tract, and contaminants introduced into the specimen after it has been passed by the patient. Some commonly confused objects are illustrated in figure 9-19 and figure 9-20.

a. **Cellular Elements From the Intestinal Tract:**

(1) Various cellular elements originating from the intestinal tract may be present, and these often closely resemble intestinal protozoan cysts and trophozoites. In certain situations, such as in dysenteric stools or stools from patients suffering from chronic disease of the intestinal tract, these cells may be found in large numbers. Cells commonly encountered are columnar epithelial cells from the gut, squamous cells from the anal mucosa, and leukocytes, including large endothelial macrophages. Careful observation of structural characteristics of the nuclei, their relative size in relation to the cellular cytoplasm, and the nature of cytoplasmic inclusions are the most diagnostic criteria for distinguishing these cells from protozoans.

(2) In fresh preparations, leukocytes may exhibit pseudopodial activity and motility. Careful observation, under varying intensities of light will usually reveal the presence of uniform-sized, highly refractile granules easily distinguishable from characteristic protozoal inclusions. In hematoxylin-stained preparations, polymorphonuclear leukocytes may exhibit nuclei closely approximating those present in amebic cysts. On close study, it will generally be seen that these nuclei are larger in relation to the cytoplasm than in amebic cysts. They also tend to be unequal in size, irregular in outline, and stain more intensely; they fail to show uniformity of internal morphology corresponding to karyosome bodies and even distribution of inner peripheral chromatin.

(3) Large endothelial macrophages containing ingested red cells may be mistaken for the trophozoites of *E. histolytica*. In the nucleus of the macrophage, the absence of the karyosome body, and the presence of fine, rather evenly distributed network of chromatin interspersed with a scattering of relatively large, deeply staining particles usually facilitate recognition.

b. **Substances Ingested with Food or Drink:**

(1) Numerous substances that are ingested can remain comparatively unaltered in their journey through the intestinal tract. They are eliminated in such configurations as to be easily confused with whole parasites, portions thereof, or stages in development of parasites. Among these are cysts of normally free-living protozoans; eggs or adults of free-living nematodes; eggs, whole larvae, body parts, or whole adult insects; the indigestible cell walls or other portions of plant and animal tissues; and pollen grains.

(2) Free-living protozoa that live in fecal material are known as coprozoic species. Cysts of these normally free-living forms may pass through the body unchanged. Or they may be ingested, and after passing through the body, excyst when they are exposed to room temperature. In this manner, both cysts and trophozoites of protozoans which are foreign to the intestine of man may be recovered in fecal examinations.

(3) Soil nematodes are common parasites of the roots of plants. In the case of vegetables which are eaten raw, the possibility of ingestion of the worms or their eggs and their ultimate recovery in fecal examinations is apparent. While it is known that the ingestion of the eggs of certain species of *Diptera* can give rise to intestinal myiasis, the recovery of either dead or viable dipterous larvae must be carefully interpreted in the light of all possible factors before it can be concluded that larvae present are responsible for this type of parasitic infection. Certain species of oviporous *Diptera* seek feces upon which to deposit their living young, and specimens left unprotected against flies following passage may immediately have maggots deposited upon them. Unprotected fecal specimens often serve as habitats of choice for oviposition by oviporous species. Under optimal conditions of temperature these eggs soon hatch and result in maggot infestation of specimens. Since no adult arthropods are known to be associated with intestinal parasitism in man, the recovery of portions of, or whole, insects is always due to accidental ingestion and subsequent expulsion rather than to parasitism.

(4) Vegetable cells, starch granules, and animal cells from food are of many shapes and sizes and can resemble cysts, eggs, or helminths. Starch granules are frequently quite spherical, and if undigested, they are composed of successive concentric rings of gray, homogeneous material. Potato starch cells often occur in clumps of sacklike, fairly uniform aggregates suggestive of egg packets of *D. caninum*. In iodine-stained preparations, undigested starch

will stain blue while partially digested starch particles assume a pinkish hue.

(5) The undigested, individual spindle-shaped vesicles of citrus fruit, such as oranges and grapefruit, are wormlike in outline and can easily be mistaken for adult pinworms. Close examination of the interiors reveals that these sacs are hollow and structureless. Fibrous plant hairs and lint fragments can also be mistaken for nematodes. Ingested pollen grains can be mistaken for helminth eggs. Their lack of characteristic yolk granules or developing embryos and the absence of a clearly delineated shell serve to distinguish them from eggs.

c. **Normal Nonparasitic Inhabitants of the Intestinal Tract:**

(1) Perhaps the greatest sources of confusion and error in the identification of objects seen in microscopic examination of feces are yeasts and fungi. These are normal inhabitants of the intestinal tract. *Blastocystis hominis*, a harmless intestinal commensal, is the yeastlike organism most easily mistaken for a protozoan cyst. It is spherical in outline, and its gradations in size correspond to those of the larger amebae of man. It is characterized by a spherical central mass which is devoid of morphological structures; the outer wall within which the nuclei are contained is relatively thick and surrounded by a thin capsule. In iodine-stained preparations, the central mass does not stain, but the thickened outer zone takes on a yellowish-brown color. Within this latter area the nuclei, usually about 3-7 in number, are evenly distributed and very darkly stained. Most of the other species of yeast cells encountered are ovoid in shape, and they too fall within the range of the various protozoan cysts.

(2) Yeast cell nuclei are solid; they lack internal morphological structure, and stain blue-black with hematoxylin stains. The best criterion for separating yeasts from parasitic protozoa is the examination of a series of these cells for budding forms.

d. **Contaminants.** The necessity for collecting fecal specimens in clean, covered, disposable containers cannot be overemphasized. Glassware in which specimens are processed and mixing devices must be scrupulously clean and free of all extraneous matter. Equally important precautions apply to the care which must be exercised in the selection of diluents to be added to specimens during examination. Failure to exercise these precautions can result in the introduction of all manner of objects which will lead to erroneous, embarrassing, laboratory errors. Objects which can be introduced include, but are by no means limited to, cysts and trophozoites of free-living amebae, free-living ciliates, yeasts, pollen, and fly maggots.

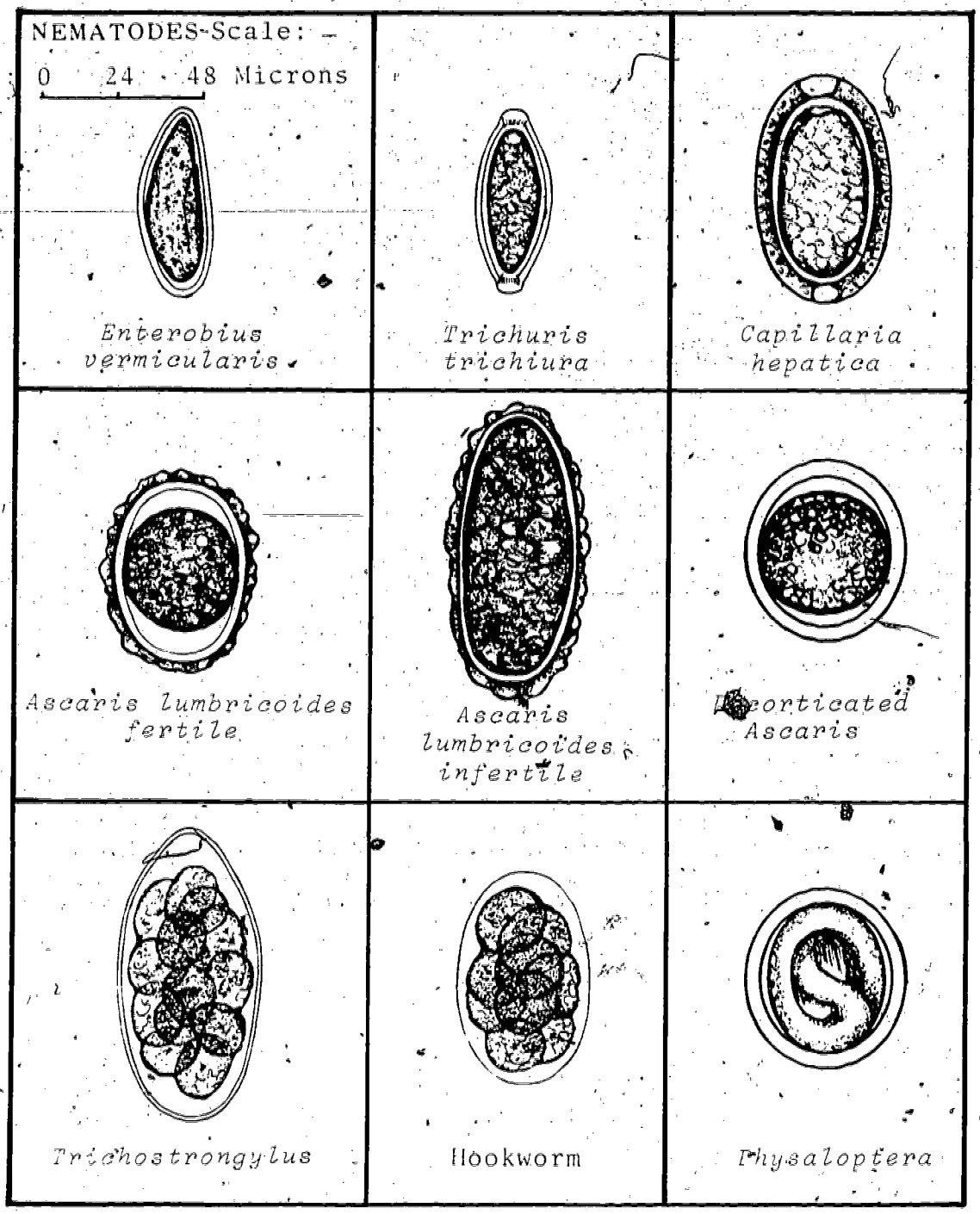

Figure 9-16. Nematode Eggs Found in Humans. ×450. (Adapted from Brooke and Melvin, 1964, 1969)

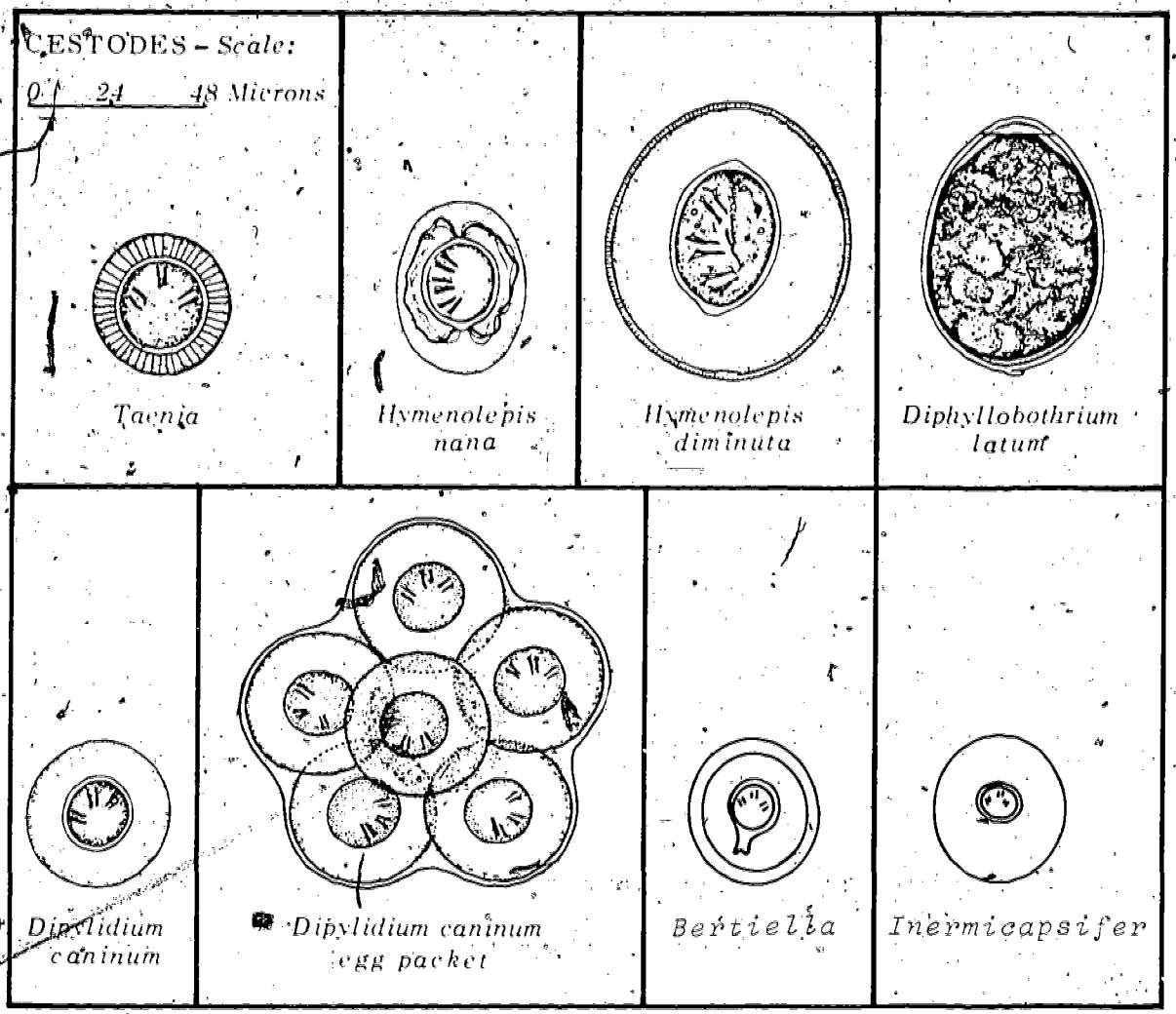

Figure 9-17. Cestode Eggs Found in Humans. ×450. (Adapted from Brooke and Melvin, 1964, 1969)

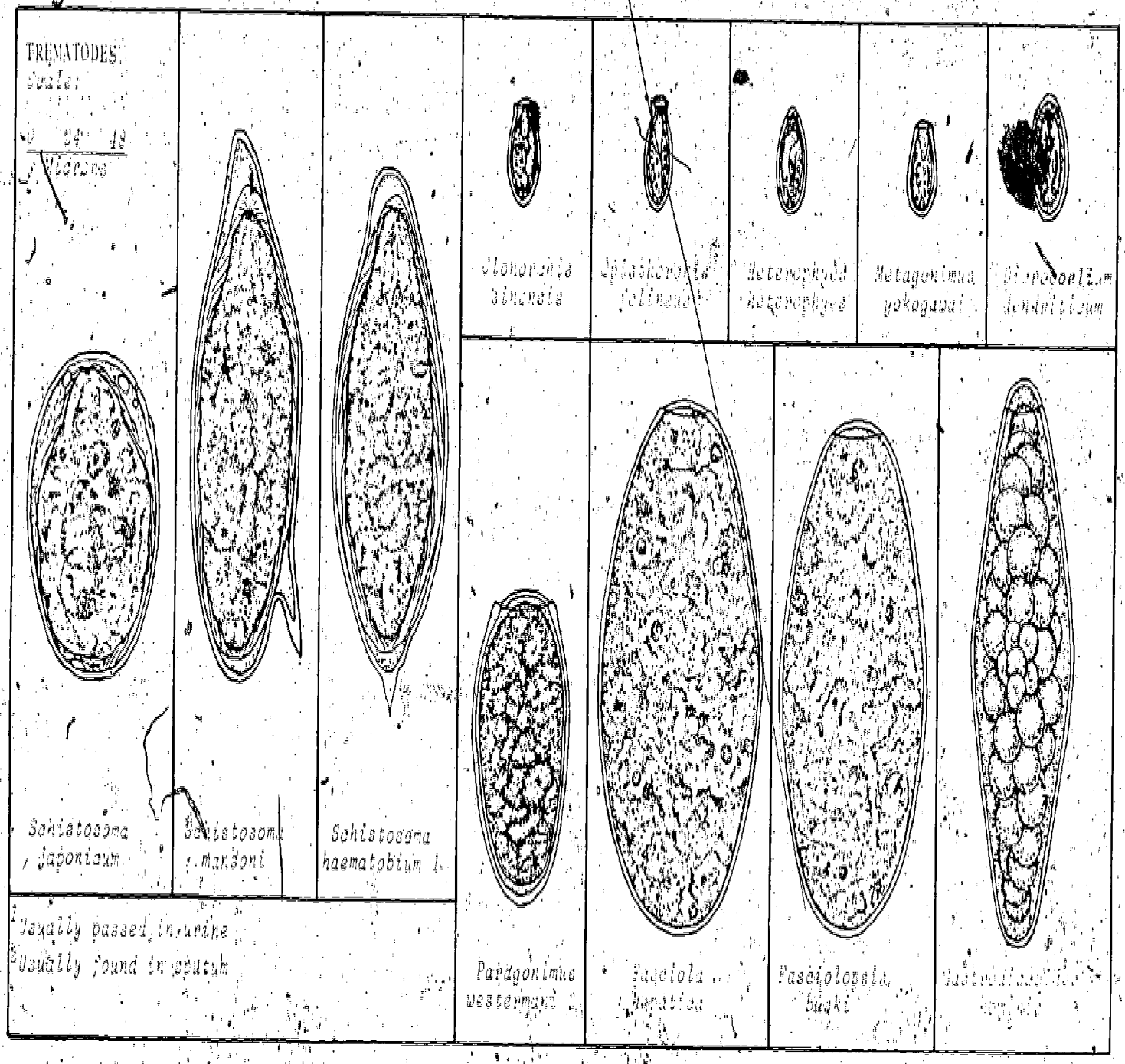

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Figure 9-18. Trematode Eggs Found in Humans. ×450. (Adapted from Brooke and Melvin, 1964, 1969)

AFM 160-48/TM8-227-2 1 August 1974

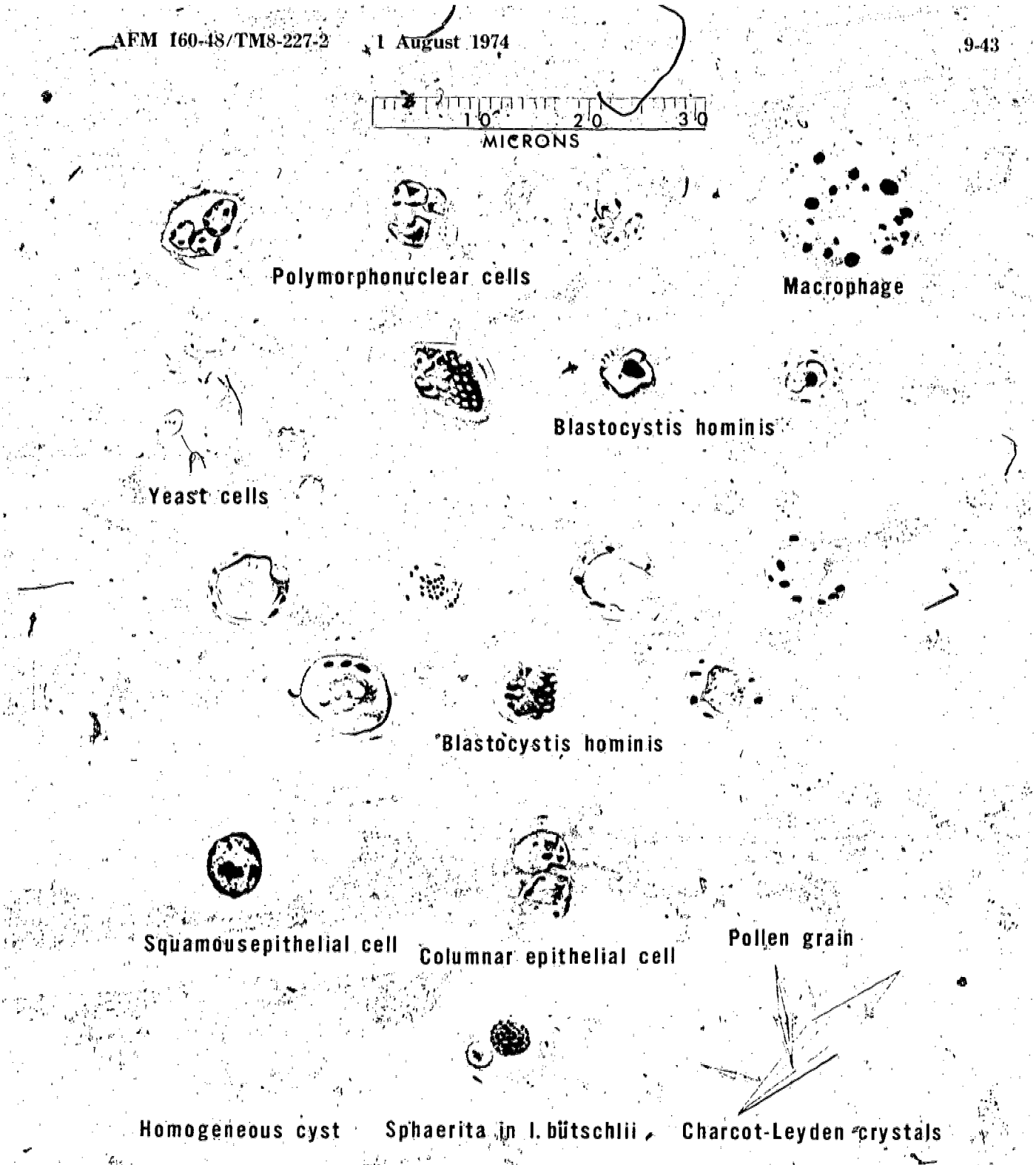
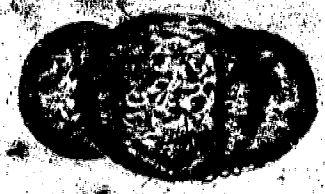


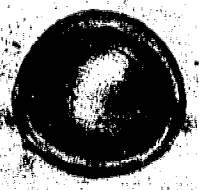
Figure 9-19. Objects in Feces Resembling Protozoans. ×1,000.

Food remains, resembles Ascaris and Taenia

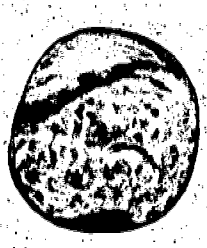
Vegetable cell, resembles helminth egg



Pollen grain (pine)

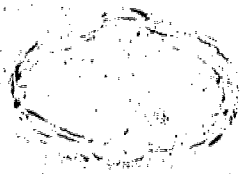


Vegetable cell, resembles helminth egg



Vegetable hair, resembles nematode larva

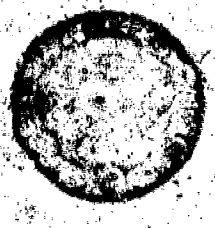
Food remains, lacks definite internal structure



Pollen grain without internal structure

Food remains, resemble helminth egg

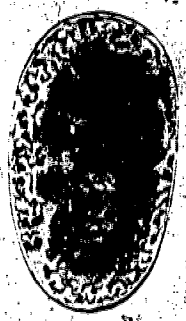
Figure 9-20. Objects in Feces Resembling the Eggs of the Helminths that Parasitize Humans. ×450.



Pollen grain resembles Ascaris or Taenia

Diatom and unknown food remains

Unknown food remains, resembles infertile Ascaris



Mite egg, resembles nematode egg

Pseudoparasite (beaver body) from crayfish

Heteroderma marioni, plant nematode egg

Figure 9-20. Objects in Feces Resembling the Eggs of the Helminths that Parasitize Humans. (Continued)

PART TWO
PARASITES OF BLOOD AND OTHER TISSUES

Chapter 10
FILARIAE AND DRACUNCULUS

10-1. Considerations for Diagnosis:

a. The laboratory diagnosis of filarial infections is dependent upon finding and identifying the characteristic microfilariae; adults are rarely recovered. The microfilariae of all species except *Onchocerca volvulus* and *Dipetalonema streptocerca* usually appear for some period during each 24 hours in the circulating blood. The time of day when blood is taken from the patient must take into account the type of periodicity (time of day at which microfilariae are found in circulating blood) characteristic of the species present in the area where the patient acquired the infection. Table 10-1 lists the major differentiating characteristics, including periodicity, of the microfilariae. *Wuchereria bancrofti* microfilariae usually circulate in the blood at night (nocturnal) but in some of the South Pacific islands they demonstrate practically no periodicity. *Brugia malayi* microfilariae are also nocturnal though not as strong in some areas.

b. Microfilariae of *Wuchereria bancrofti* and *Brugia malayi* can be found in fluid aspirated from a hydrocele, or an enlarged lymph node and on some occasions in the urine. Microfilariae of *Onchocerca* and *Dipetalonema streptocerca* are most often recovered from skin snips teased in saline. *Onchocerca* microfilariae can also be found in fluid aspirated from skin nodules.

c. Living microfilariae can be easily seen in fresh blood preparations with the low power of a microscope. They are continuously lashing and writhing, moving the red blood cells. Species identification is practically never possible on living unstained microfilariae. Identifications can best be made from stained, thick or thin blood films as for malaria. When very few microfilariae are circulating, concentration methods have to be employed to find the organisms.

d. It must be emphasized that the microfilariae of *Wuchereria bancrofti* and *Brugia malayi* do not occur in the circulation early after infection, and frequently they do not appear in the blood in very old infections. It has rarely been possible to demonstrate circulating microfilariae in Americans with early symptoms of filariasis. Almost all of the morphological characters used to differentiate species of microfilariae are shown in figure 10-1.

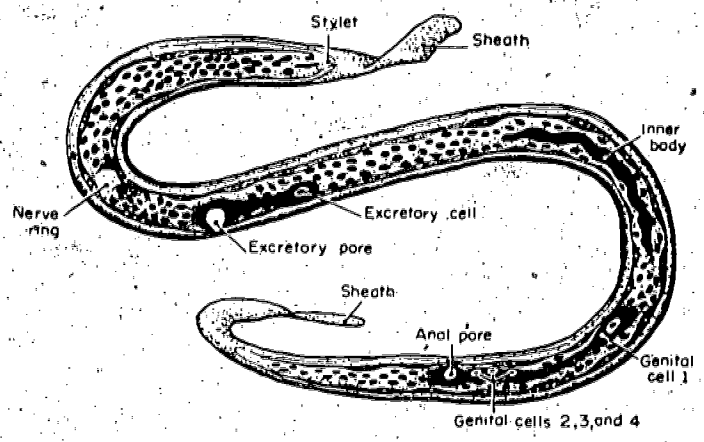


Figure 10-1. Characteristics of a Typical Microfilaria. ×1,000.

e. The clarity of morphological characteristics depends upon good fixation and proper staining; however, the anatomical features of some species are more easily stained than others. Microfilariae of *B. malayi* are much more difficult to stain well than are microfilariae of *W. bancrofti*. Dilute Giemsa stain brings out the sheath of *W. bancrofti* microfilariae, but it fails to stain that structure in *Loa loa* microfilariae. Also, the reaction of living microfilariae to methylene blue can be used to separate the microfilariae of *W. bancrofti* and *L. loa* since the former absorbs the stain while the latter does not. The morphological characteristics of the different species are illustrated in drawings of the microfilariae in figure 10-2.

10-2. Routine Laboratory Procedures. Microfilariae can be demonstrated by several methods. These include the examination of fresh blood for motile organisms, examination of thin and thick stained blood films, and various concentration procedures. Concentration procedures should be employed before suspected cases are given up on. Large amounts of blood are easily examined with concentration procedures, greatly enhancing the chances of finding microfilariae in low grade infections.

a. **Fresh Blood Preparations:**

(1) Standard methods of finger puncture or venipuncture can be used to obtain the blood specimen. Cleanse the puncture site with lintless material, such as a good grade of gauze, since strands of lint are easily mistaken for microfilariae by inexperienced technicians.

(2) For routine screening in the laboratory mix a moderate drop of blood with one or two drops of physiological saline on a clean glass slide; spread the preparation and cover it with a 22 × 50 mm coverslip.

(3) With the aid of the low power objective search the entire preparation. Use reduced light to more easily view the microfilariae as they thrash about.

(4) For survey work where standardized procedures must be followed, draw the blood up with a 20 µl pipet; mix it with two drops of physiological saline, and examine as above. A blood volume of 20 µl mixed with two drops of saline and covered with a 22 × 50 mm coverslip gives a moderately thick preparation, yet one thin enough for the technician to be able to see any microfilariae present.

b. **Stained Blood Films.** This procedure offers various advantages. A relatively large sample of blood is obtained. It serves as a convenient method of forwarding specimens to a central laboratory for confirmation or for holding the specimen for examination at a later time. It is an especially good method for performing field surveys on large numbers of individuals in endemic areas. Staining reveals structures which make differentiation possible while microfilariae in unstained preparations do not show morphological details sufficiently well to permit identification of species. Stained slides also serve as permanent records of the specimen upon which the diagnosis was made.

(1) *Giemsa-Stained Blood Films:*

(a) *Reagents:*

1. Buffered Water, Stock Solutions.

a. Sodium Dibasic Phosphate, M/15 (Stock Solution A).

Sodium dibasic phosphate (Na_2HPO_4) ..... 9.5 g
Distilled water ..... 1000.0 ml

In a 1000 ml volumetric flask dissolve the phosphate salt in a portion of the distilled water and then dilute to the mark with the remaining distilled water.

b. Sodium Monobasic Phosphate, M/15 (Stock Solution B).

Sodium monobasic phosphate
($NaH_2PO_4 \cdot 2H_2O$) ..... 9.2 g
Distilled water ..... 1000.0 ml

In a 1000 ml volumetric flask dissolve the phosphate salt with a portion of the distilled water and then dilute to the mark with the remaining distilled water.

2. Buffered Water, Working Solution pH 7.2.

Sodium dibasic phosphate,
M/15 (Solution A) ..... 72.0 ml
Sodium monobasic phosphate,
M/15 (Solution B) ..... 28.0 ml
Distilled water ..... 900.0 ml

Both stock solutions and working solutions are stable for several months when stored in pyrex glass-stoppered bottles in the refrigerator.

3. Giemsa Stain.

Giemsa powder, CP ..... 0.6 g
Glycerin, CP [$C_3H_5(OH)_3$] ..... 50.0 ml
Methyl alcohol, absolute (acetone-free) ..... 50.0 ml

a. Place a small amount of dry stain in a clean mortar.

b. Add a small amount of the glycerin and grind thoroughly.

c. Pour off the mixture into a clean flask and repeat the process until all the stain has been ground with glycerin.

d. Rinse the mortar and pestle with the remaining glycerin and pour into flask.

e. Place the glycerin-dye mixture in 55-60C water bath for 6-8 hours. Shake periodically.

f. Rinse mortar and pestle with a portion of the methyl alcohol. Hold the alcohol including that used to rinse the mortar and pestle and add it to the glycerin-dye mixture after it has been removed from the water bath and allowed to cool to room temperature.

g. Stopper. Allow to age 2 weeks.

h. After aging, filter into chemically clean, dry, glass bottles.

(b) *Procedure for Thin Smears:*

1. Prepare thin smears from a small drop of blood in the same manner as for a WBC differential.

2. Allow the smear to thoroughly air-dry.

3. Fix in absolute methyl alcohol for 1 minute.

4. Stain in standard Giemsa stain for 30-45 minutes. (Prepare the stain by mixing 1 ml of stock Giemsa stain with 50 ml of pH 7.2 buffered water.)

5. Differentiate for 10 minutes in the buffered water.

6. Air-dry the slide at room temperature.

7. Search the slides under low power and check the morphological characteristics under oil immersion.

8. The body of the microfilariae, the excretory cell, and the "G" cells will stain azure. The anal and excretory pores will be reddish pink and the sheath, if present, will be stained light pink. The sheath of *Loa loa* will not stain with Giemsa stain.

(c) *Procedure for Thick Smears:*

1. To prepare a thick film, puncture the finger in the usual manner. As the blood wells up, bring a clear slide into contact with the drop of blood. Rotate the slide with a circular motion until an even circle of blood of uniform thickness about the size of a dime has been made on the slide. (Practice is necessary to judge the proper thickness. Smears that are too thick will crack and peel off during the processing.)

2. Air-dry thick films for several hours to overnight in covered containers.

3. Soak the smears in tap water for 10 minutes to lake the RBCs. Allow laked smears to air-dry.

4. Fix the smears in absolute methyl alcohol for 10 minutes and then proceed just as for thin smears.

(2) *Delafield's Hematoxylin Stain.* The hematoxylin stains are the most efficient for demonstrating the presence or absence of a sheath on microfilariae. Since the presence or absence of a sheath is one of the most diagnostic characteristics in the differentiation of human microfilariae, it is desirable to utilize hematoxylin stain.

(a) *Reagents:*

1. Fixative Solution. Equal parts of 95% ethyl alcohol and ether.

2. Delafield's Hematoxylin. Procedure for preparing stain is outlined in paragraph 8-6b(1)(b).

(b) *Procedure for Thin Smears:*

1. Prepare thin smears from a small drop of blood in the same manner as for a WBC differential.

2. Allow the smear to air-dry thoroughly.

3. Fix smears in alcohol-ether mixture for 10 minutes and allow to air-dry.

4. Stain for 10-15 minutes in diluted Delafield's hematoxylin. (Dilute one part of stain [paragraph 8-6b(1)(b)] with nine parts of distilled water.)

5. Wash the smears in running tap water until the color changes form violet to blue. That usually takes about 30 minutes.

6. Air-dry smears and mount with suitable mounting medium.

(c) *Procedure for Thick Smears:*

1. To prepare a thick film, puncture the finger in the usual manner. As the blood wells up, bring a clean slide into contact with the drop of blood. Rotate the slide with a circular motion until an even circle of blood of

uniform thickness about the size of a dime has been made on the slide. (Practice is necessary to judge the proper thickness. Smears that are too thick will crack and peel off during the processing.)

2. Air-dry thick films for several hours to overnight in covered containers.

3. Soak the smears in tap water for 10 minutes to lake the RBCs. Allow laked smears to air-dry.

4. Fix slides in the alcohol-ether mixture for 10 minutes and again allow to air-dry thoroughly.

5. Stain for 10-15 minutes in diluted Delafield's hematoxylin. (Dilute one part of stain [paragraph 8-6b (1)(b)] with nine parts of distilled water.)

6. Wash the smears in running tap water until the color changes from violet to blue. That usually takes about 30 minutes.

7. Air-dry smears and mount with suitable mounting medium.

c. **Concentration of Microfilariae.** In cases where few microfilariae are in the circulating blood, concentration procedures in which the erythrocytes are laked out is a most efficient method for their recovery. In suspected cases of filarial infection one of the concentration procedures should be performed when more direct methods of examination are negative.

(1) *Knott's Method.* Dilute formalin is used to hemolyze the red blood cells and preserve the microfilariae. The disadvantages of laking blood with dilute formalin is that the microfilariae are killed, and their presence is not revealed by their thrashing movements. However, it has the advantage of killing, fixing, and preserving the organisms so that they can be readily shipped, stained, or even stored.

(a) Draw 1 ml of blood by venipuncture and mix it with 10 ml of a 2 percent solution of formalin. (Add sufficient volume of distilled water to 2 ml of formaldehyde solution, USP to make a total volume of 100 ml.)

(b) Allow the mixture to stand for 5 minutes.

(c) Centrifuge for 5 minutes at 1,500 rpm.

(d) Pour off the supernate without disturbing the sediment. With a capillary pipet transfer a portion of the sediment to a clean slide.

(e) Apply a coverslip and examine the entire preparation under low power. Confirm findings under high dry.

(f) When microfilariae are found, prepare a thick smear of the remaining sediment, remove the coverslip from the wet preparation you have prepared, and make it into a thick smear.

(g) Thoroughly air-dry the smears and proceed to stain them with Giemsa as outlined, beginning with step 10-2b(1)(c)4, or with Delafield's hematoxylin as outlined, beginning with step 10-2b(2)(c)4, to bring out the morphological characteristics for identification.

(2) *Method Using Saponin for Hemolysis.* Saponin solution hemolyzes the red blood cells, removing a large portion of the sediment, thus increasing the chances of finding microfilariae when there are only a few in the circulating blood. Saponin solution does not kill the microfilariae; therefore, they remain actively motile, making it very simple to locate them.

(a) Draw 1 ml of blood by venipuncture and add it to a centrifuge tube containing 10 ml of 2 percent saponin solution (Dissolve 2 g of saponin powder in a small amount of physiological saline in a 100 ml volumetric flask. Dilute to the mark with physiological saline. Refrigerate solution and discard after 2 weeks or before at first sign of contamination.)

(b) Mix thoroughly and let stand until hemolysis is complete.

(c) Centrifuge at 1,500 rpm for 10 minutes.

(d) Pour off the supernatant fluid without disturbing the sediment. With a capillary pipet transfer a portion of the sediment to a clean glass slide.

(e) Apply a coverslip and search the entire preparation for actively motile microfilariae under the low power of the microscope. Check suspicious objects under high dry.

(f) When microfilariae are found, prepare a thick smear of the remaining sediment, remove the coverslip from the wet preparation, and make it into a thick smear.

(g) Thoroughly air-dry the smears and proceed to stain them with Giemsa as outlined, beginning with step 10-2b(1)(c)4, or with Dela-

field's hemotoxylin as outlined, beginning with step 10-2b(2)(c)4, to bring out the morphological characteristics for identification.

10-8. Laboratory Diagnosis of Onchocerca Volvulus and Dipetalonema Streptocerca:

a. The microfilariae of *O. volvulus* and *D. Streptocerca* rarely, if ever, get into the bloodstream. They are found in the superficial lymphatic spaces and connective tissue of the skin. *O. volvulus* can be diagnosed either by demonstration of the microfilariae in fluid aspirated from a skin nodule containing a pair of adult worms or by their recovery from small sections of skin. Some nodules contain only dead worms or unmated adults; therefore, failure to find the microfilariae does not indicate absence of infection. Also, microfilariae of *O. volvulus* can be found in skin when there are no palpable nodules. The adults of *D. streptocerca* live in cutaneous connective tissue but do not form characteristic nodules like *O. volvulus*.

b. To recover the microfilariae of *O. volvulus*, remove a thin skin shaving from an area near any nodules that are present. With a needle point, lift a portion of skin to form a small cone. Cut the skin off at the base of the cone with a razor blade. The skin shaving should be about 0.5 cm in diameter, and the cut should be so shallow that the area does not even bleed. Use the same method for taking skin snips for the recovery of *D. streptocerca* except that the skin snip must be deep enough to include dermal tissue. Snips that are deep enough to include dermal tissue will usually induce some slight bleeding. After the skin specimen is collected, tease it apart in a drop of saline, and examine it microscopically for microfilariae.

c. To positively identify the microfilariae they must be stained to demonstrate their morphological characteristics. While the preparation is still wet, add a small drop of plasma, serum, or egg albumin and mix thoroughly. Air-dry the preparation overnight. To stain the preparation with Giemsa, start the procedure with step 10-2b(1)(c)4 or to stain with Delafield's hematoxylin, start the procedure with step 10-2b(2)(c)4. The microfilariae are illustrated in figure 10-2, and the differentiating characteristics are listed in table 10-1.

Table 10-1. Differential Characteristics of Microfilariae.

SPECIES	FOUND IN	LENGTH (IN MICRONS)	PERIODICITY	SHEATH	TAIL MORPHOLOGY
W. bancrofti	Blood	245-295	Usually Nocturnal	Present	Tapers to delicate point; Nuclei do not extend to tip of tail.
B. malayi	Blood	177-230	Nocturnal	Present	Tapers to delicate point; Two terminal nuclei.
L. loa	Blood	250-300	Diurnal	Present	Tapers gradually; Nuclei continuous into tail.
M. ozzardi	Blood	185-200	None	Absent	Tapers gradually; Nuclei do not extend to tip of tail.
D. perstans	Blood	190-200	None	Absent	Tapers gradually; Bluntly rounded; nuclei to tip of tail.
D. streptocerca	Skin	180-240	None	Absent	Slender hooked tail; Bluntly rounded; nuclei to tip of tail.
O. volvulus	Skin	150-287 or 285-368	None	Absent	Tapers gradually; Nuclei do not extend to tip of tail.

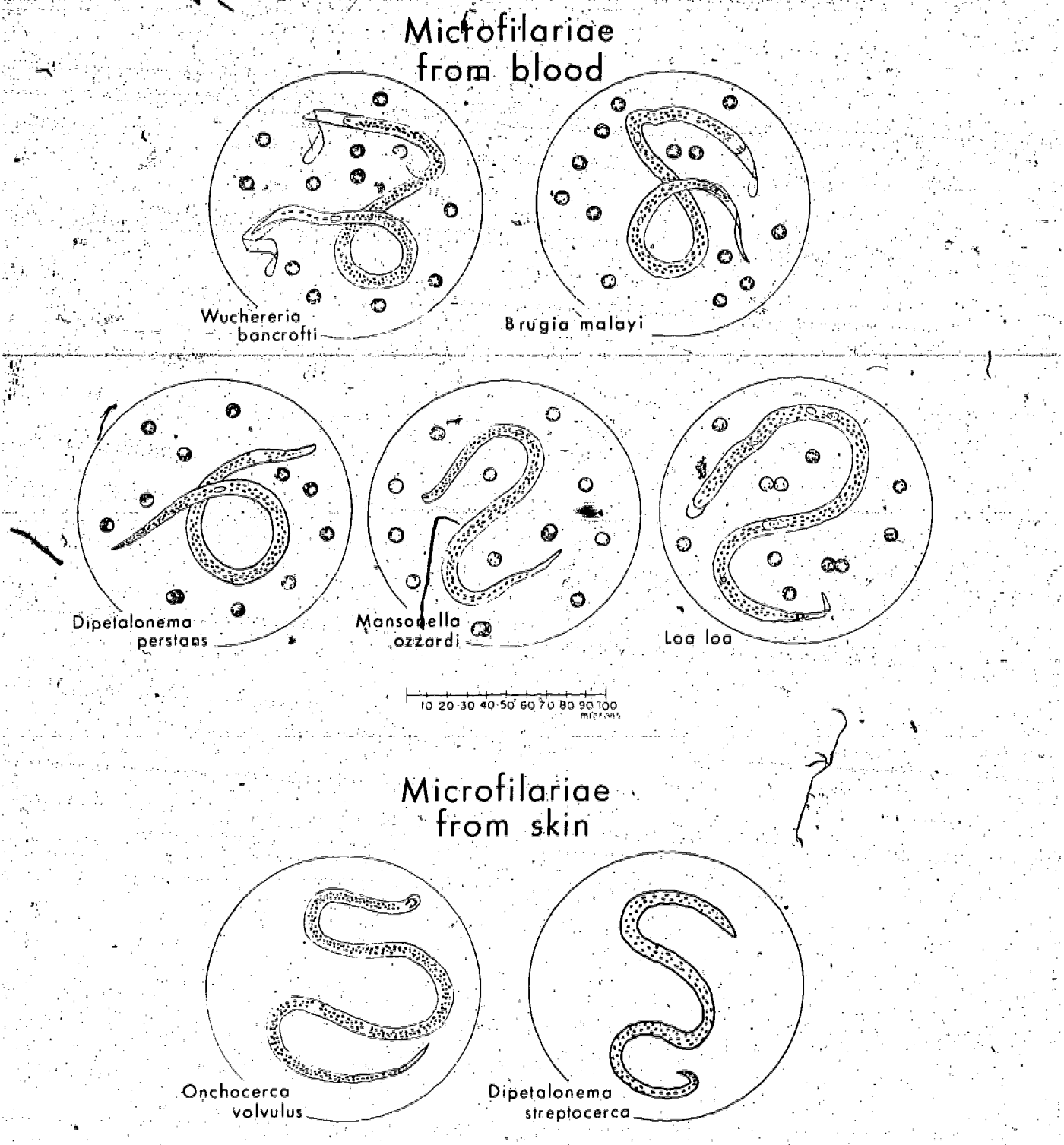


Figure 10-2. Microfilariae Found in Humans. Giemsa's stain. ×600.

10-4. Identification of Canine Microfilariae. Use the same procedures to recover microfilariae from the blood of other animals that are recommended for the recovery of microfilariae from the blood of humans. The same morphological characteristics that are used to differentiate the species of human filariids are used to differentiate the species of canine filariids. There are two species of canine filariids commonly found in the United States; *Dirofilaria immitis*, the disease producing heartworm of dogs, and *Dipetalonema reconditium*, a parasite of the subcutaneous tissues which is not known to produce disease. Both of these parasites are found in many of the same areas of the country making it necessary to positively differentiate between the two species whenever microfilariae are recovered from the blood of dogs in the United States. Table 10-2 lists the differentiating characteristics of the two species as seen in material fixed on slides. Another canine filariid, *Dirofilaria repens*, must be considered when microfilariae are found in the blood of dogs that have spent several months in Europe, Asia, or South America. This parasite of the subcutaneous tissues is not known to produce significant disease. The microfilariae of *D. repens* are very similar to those of *D. immitis*, and the measurements of their lengths and widths overlap considerably. The help of an expert parasitologist will be needed to make a positive differentiation. In some parts of Asia and Africa at least two members of the genus *Brugia* can be found in dogs. However, the microfilariae of these parasites are sheathed, while those of *Dirofilaria immitis*, *Dirofilaria repens*, and *Dipetalonema reconditium* are not sheathed. This is an obvious difference which makes it easy to separate the two groups.

10-5. Shipment of Specimens for Microfilariae Identification. Specimens containing microfilariae can be sent to reference laboratories when assistance is desired. They can be prepared for shipment in any of the following ways:

a. Thick films prepared directly from blood or prepared from concentrated sediments can be shipped, but remember to fix the slides before mailing.

b. Whole blood can be shipped. Send 5 ml of blood, anticoagulated with 1 ml of sodium citrate (2% solution in physiological saline), or send heparinized blood. Microfilariae will remain alive for several days when treated in this manner.

c. Microfilariae preserved by addition of 10 ml of 2 percent formalin to 1 ml of blood, as in the Knott's concentration procedure, can be shipped without fear of deterioration.

10-6. Immunological Tests. Immunological tests of all kinds have been employed, but skin tests and complement-fixation tests have received the most attention. The antigens usually employed are derived from *Dirofilaria immitis*, the heartworm of dogs. The tests, of course, are not species specific, and techniques have not been standardized; however, results of such tests can be helpful in conjunction with clinical findings. The tests are helpful when testing large groups, as in field surveys, to get an idea of the percentage of the population having had a filariid infection. The results of these tests require very critical evaluations when employed in the diagnosis of individual cases.

10-7. Laboratory Diagnosis of Dracunculus. A positive laboratory diagnosis cannot be made until the female worm causes a blister to form on the skin. Immerse the blister in a small amount of water. That stimulates the discharge of larvae from the female worm and causes the blister to rupture releasing the larvae. Centrifuge the water at 1,500 rpm for 5 minutes. Examine the sediment for actively motile larvae (figure 10-3). Frequently part of the prolapsed worm will protrude from its channel in the skin after the larvae are released. Serological tests have been developed, but they have not been standardized and are of little value.

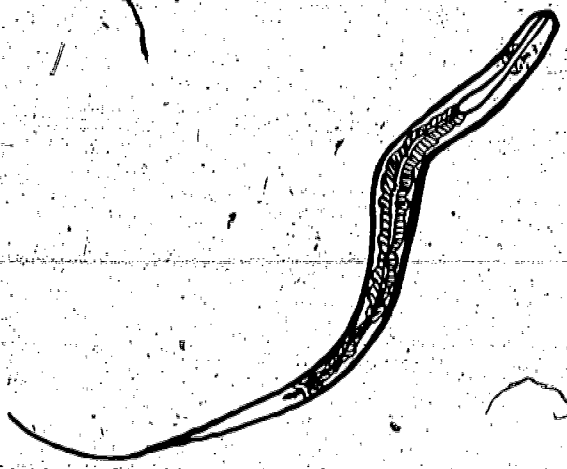


Figure 10-3. Dracunculus Larva. ×200.

Table 10-2. Characteristics for Differentiation of Dirofilaria immitis and Dipetalonema reconditium.

Species	Length (in microns)	*Width (in microns)	Periodicity	General Description
Dirofilaria immitis	286-340 (mean 314)	6.1-7.2 (mean 6.8)	Slightly nocturnal	Anterior end is tapered. The body sides appear smooth and relatively straight. The tail gradually tapers to a fine point. Microfilariae sometimes forms a slight crescent.
Dipetalonema reconditum	258-292 (mean 270)	4.7-5.8 (mean 5.2)	Diurnal	Anterior end is straight without taper, and relatively blunt. The body sides have a wavy appearance along longitudinal axis. The tail forms an imperfect button-hook. Microfilariae assumes a wavy crescent position.

*Width measured at 50-60 microns from anterior end.

Chapter 11
MALARIA

11-1. Introduction. Following injection during the mosquito's bite, the parasites undergo development in the internal organs of man before they eventually are found in the red blood cells. Here they multiply, eventually rupture the cells, and infect more red blood cells. After several complete cycles, the synchronized rupture of cells, followed by the development of increasing numbers of parasites, results in the appearance of the classical malarial "chills and fever" paroxysm. The chill phase starts when the red blood cells rupture. The duration of the complete cycle depends on which of the four human species of *Plasmodium* is present. The four infecting man are *P. falciparum, vivax, ovale,* and *malariae*. The first three species have a cycle of about 48 hours while *P. malariae* has one of 72 hours duration.

11-2. Stages in the Blood. In general, all four species go through similar stages of development during each cycle, but certain stages of *P. falciparum* will be found only within internal organs and not in peripheral blood smears. The morphological characteristics of the stage or stages present are used in species identification. The physician must have an accurate identification of the species present in order to select the correct therapy. Falciparum malaria in particular is a very serious disease that requires more drastic treatment than the other species would.

a. **Merozoite.** This stage is released when the red blood cells rupture and is the infective stage for new red blood cells. It consists of a tiny bit of nuclear chromatin surrounded by a thin layer of cytoplasm.

b. **Small Trophozoite (Ring).** This is the first stage after the merozoite invades a new cell. The cytoplasm develops a vacuole which gives the parasite the appearance of a signet ring with the chromatin as the "stone."

c. **Large Growing Trophozoite.** In this stage the cytoplasm of parasite has grown, but the chromatin has not yet started to divide.

d. **Immature Schizont.** This stage is characterized by division of the chromatin while the cytoplasm remains intact.

e. **Mature Schizont (Segmenter).** This is the last stage before rupture of the red blood cells. A small bit of cytoplasm has divided around each bit of chromatin, thus producing merozoites. Upon rupture of the red blood cell, the merozoites are released, the chill phase of the disease is initiated, new red blood cells are infected, and the cycle is repeated. The number and arrangement of merozoites within the segmenter are characteristics used in species identification.

f. **Gametocytes.** After several cycles, some merozoites develop into *gametocytes* instead of trophozoites. The gametocytes are of two types—microgametocytes and macrogametocytes. These may be likened to male and female sex cells, respectively. These undergo no further development in man. However, when ingested by the mosquito, these forms combine to complete the sexual cycle. Finding the characteristic gametocytes of *Plasmodium falciparum* in the blood is especially helpful in the definitive identification of that species while the gametocytes of other species are of lesser importance.

11-3. The Time to Make Blood Films. An interesting question is, "When is the best time to make a smear for malaria?" Several things have to be considered in the answer. Normally in vivax or malariae infection, parasites of some stage are present in the blood at almost any time. However, if a smear is made at the time of, or immediately following, a chill, most of the parasites are likely to be in the young ring stage and difficult to differentiate as to species. Hence, a more accurate diagnosis can be made after the parasite has attained some growth and acquired more easily differentiated characteristics. The greatest number of ring forms of *Plasmodium falciparum* are present on the day between the chills, before any of the current generation of parasites has left the peripheral

Material in Chapter 11 was adapted from Wilcox, A., 1960. *Manual for the Microscopical Diagnosis of Malaria in Man.* PHS Publication No. 796.

circulation to mature in the internal organs. It is possible in falciparum infections to take a smear just before the chill and find no parasites, since the older stages preceding and including the mature schizont are in the internal organs. However, the period of the rupture of the infected cells is not synchronized in *P. falciparum* as completely as in the other species, and some ring forms may be present at any time. As in the case of *P. vivax* or *P. malaria*, species diagnosis is often practically impossible on a few rings. Therefore, getting the slide at the height of the ring infection, so as to determine ring-form characteristics, is very important. Gametocytes of *P. falciparum*, as is well known, frequently do not appear for several days after the beginning of the symptoms. With all these facts in mind, a favorable time for making the smear would appear to be about halfway between paroxysms. This does not mean that one should wait this long to make the first smear. Diagnosis should be attempted as soon as possible, and repeated smears taken as necessary.

11-4. The Types of Blood Films and Advantages of Each:

a. **The Thin Film:**

(1) The thin film is ideal for the study of the morphology of the individual parasites of the various species of malaria when the infection is sufficiently heavy for parasites to be found without a long search. A thorough working knowledge of the thin blood film, as well as of the different species of plasmodia in their various stages, is most desirable if not absolutely necessary before attempting to identify malaria parasites in a thick film. The thin film has the great disadvantage, however, of failing to reveal many light infections.

(2) To make the thin film most usable for identifying malaria parasites, the greater part of it should be really thin—a single-cell layer with cells well distributed, not overlapping, and with straight, even edges that do not come too close to the edge of the slide for examination. Structural detail of individual parasites is best preserved where cells lie free of each other. Cells parasitized with older parasites are more frequently found along the edges and in the feather ends of the smear; whereas ring forms are usually more evenly distributed. It should be kept in mind that a spreader slide used on one positive individual can transfer parasites to succeeding smears even when wiped between the making of smears, so absolutely clean spreaders should be used.

b. **The Thick Film:**

(1) The thick film is a method by which a relatively large quantity of blood is placed in a small area and stained so that the hemoglobin is dissolved from the red cells and the blood smear is rendered sufficiently transparent for examination by transmitted light. Knowledge of the procedure is indispensable to the person who is to examine the blood for malaria. It reveals comparatively quickly sparse or scanty infections such as occur in new or chronic cases. There are occasions when immediate diagnosis is a matter of urgent necessity, and frequently in those cases no certain information can be deduced from a blood examination by the thin film method, whereas the thick film taken at the same time will reveal parasites. Because of its efficiency in detecting light infections, it gives a much more accurate idea of the incidence of malaria, and because great numbers of slides can be dehemoglobinized and stained at one time, it is particularly well adapted for survey work and for use in laboratories where large numbers of slides for malaria examination are received. The thick film gives an idea of the degree of infection and can be a great help, because of the increased density of parasites, in identifying the type of malaria in cases where only one or two young forms can be found in the thin film. It gives a fairly good idea of the number of leukocytes and shows pigmented white cells more readily than the thin film.

(2) Practice and experience are necessary to become proficient in the interpreting of thick films for they are different in appearance from thin films and may be confusing at first. However, the ease with which parasites can be found in the thick film, after a long, unfruitful search by the thin-film method, is ample recompense for the time spent in learning the technique. It has been repeatedly observed that when a person has learned this technique, he has no desire to return to the thin film for diagnosis.

(3) In nearly all cases parasites may be found in thick films from patients with clinically active malaria, unless they have been reduced to a microscopically undetectable level by antimalarial drugs. However, in persons with extreme susceptibility, symptoms may occur before parasites can be found. In these latter cases, examination of subsequent smears should be made on successive days.

(4) The thick film can be used for examination of blood for trypansomes, microfilariae, the spirochetes of relapsing fever, and for estimation of the percentage of eosinophils.

11-5. Preparation of Blood Films:

a. **Cleaning New and Used Slides:**

(1) For making films, slides must be clear, unscratched, noncorroded, and above all meticulously clean, that is, free from grease, dust, acid, or alkali. When slides are to be cleaned and reused repeatedly, a grade of glass should be used that will stand the cleaning process and the necessary handling without fogging or corroding, and that will not scratch too easily. Scratches in slides will hold the stain and give various false impressions. Corroded slides have this disadvantage as well as that of cutting out part of the light necessary for exact microscopical work. New slides are always preferable, but new slides should never be used without cleaning, no matter how bright or shiny they may look, for there may be left on them traces of oil, put there in the glass polishing process, which will cause difficulty. Thin films will not spread evenly on an oily or dirty surface, and thick films will not adhere to it. There may be on an unwashed new slide traces of soda, lime, or potash from the glass itself, and these particles can change the pH of the stain to such an extent as to make young malaria parasites and parts of older ones invisible. Slides should be held by the edges, because oil from the fingers on their surface will prevent proper adhesion of thick smears. If the pusher slide used to make a thin film "catches" or halts as the blood is pulled over the slide's surface, you can be sure that the slides have not been properly cleaned. The movement should be steady, even and easy.

(2) For cleaning, drop new slides individually into a solution of warm water and one of the good detergents (liquid or liquefied) in the proportion suggested by the manufacturer. Wash surfaces of each slide with a soft brush or cloth, rubbing to remove any foreign matter. Rinse thoroughly in warm running tap water. If the tap water contains excessive chemicals, rinse again in distilled water. Let drain briefly on a clean towel. Place in 95% ethyl alcohol. Dry and shine with a soft, lintless cloth. During the entire process avoid chipping the edges of the slides.

(3) Often it is not considered profitable to clean used slides, but if they are cleaned, drop them individually into dichromate cleaning solution, and let stand overnight or longer. Remove from the solution and rinse repeatedly in warm running tap water until every trace of acid is removed. Then follow the directions given above for new slides.

b. **Obtaining Blood for Films:**

(1) Be sure that blood taken is free from grease, perspiration, or dirt which may be on the skin and that the alcohol used for cleansing never be allowed to mix with the blood. Cleanse the area to be punctured with gauze or cotton soaked in alcohol. Then rub dry with a piece of sterile cotton or gauze (preferably gauze since it does not leave lint on the skin). Alcohol on the skin or needle will fix the red blood cells and interfere with the dehemoglobinization of the red cells, which is part of the staining process for thick films. Hence all alcohol must be wiped away or allowed to evaporate before blood is taken for the smear.

(2) Prick the skin deeply enough to allow the blood to well up in a large drop under gentle pressure but not deeply enough to cause excessive bleeding. This puncture is made with a lancet in the same manner as a puncture is made for a hemoglobin determination or a WBC differential.

c. **Making the Smears:**

(1) It is often desirable to have thick and thin films on opposite ends of the same slide. The thin film, in specimens of this sort, should be made first, using a very small drop of blood, taken near one end of the slide and smeared toward the other end, so that the thinner, better portion of the thin film comes very close to the spot where the thick film is made on the opposite end of the slide. (The very small drop is necessary in order to make the smear very thin

and not too long. If too much blood is used, the film will be too thick or too long, extending into the area intended for the thick film; or the technician will have a tendency to stop short of making the feather ends, piling up the cells and ruining one of the best parts of the smear for finding parasites. This manner of making the smear facilitates staining (when slides are stained standing on end, thick film downward, in a staining dish), makes examination easier by permitting the objective to slip quickly from the thick film to the better portion of the thin film, and leaves an area for labeling at the end of the slide covered by the thicker, poorer portion of the thin film.

(2) For the thick film, near one end of the slide, cover a space about the size of a dime with as much blood as will easily spread over this area without crackling or peeling when dry. This smear may be made in either of two ways: The first method is to touch the undersurface of a slide to the crest of the fresh, large, rotund drop of blood and without losing contact with the drop of blood or touching the skin, move the slide in narrow circles in the blood until a smear of the required size and thickness is made. The second method is to take several average-sized drops of blood quite near each other on the slide, and then with the needle or with the corner of a clean slide, quickly puddle these into one fairly homogenous drop about the size of a dime. One should be careful to take enough blood to make a smear that is several layers of erythrocytes thick and yet not so thick that it will contract and pull loose from the slide in drying. On the other hand, one should not make the smear too thin, or it will have no advantage over the thin film. Never use the second method without smearing the drops together, and do not put them so far apart that the smear has to be spread in a large thin drop in order to bring them together. The ideal thick film is several layers of cells thick in the middle and has a thinner edge of one-cell thickness. Ordinary printing can just be read through the wet center of a well-made thick film when the slide is placed on the printed page. In order to be able to examine the entire thick film with a mechanical stage, place the end of the film about one-quarter inch from the end of the slide.

(3) Lay the slide flat to dry so that the blood may be evenly distributed, and have it well protected from dust and insects. Air-dry without application of excessive heat. If the blood smear is tilted while drying, the greater part of the blood will collect in as much thickened line along one edge of the smear. This may peel off completely in staining and, if not, it will be too thick for ease in examination. Also, the remainder of the film will be so thin as to be valueless for diagnosis of light infections. A covered Petri dish may be used for drying slides in the laboratory. In field work the slides should be inserted, film-side down, in a slidebox, which is held upright. This box should be kept closed and level in an upright position until the slides are dry. Stained dust particles, etc, may cause trouble for the microscopist and slow down examination time. Flies or cockroaches will eat away the blood or contaminate it with bacteria or other organisms. If possible, let the blood smears dry for 8 to 12 hours before staining. Thick films stain most clearly when several hours old. Very fresh smears may not have had time to adhere well to the slide and hence part of the blood may be lost. They frequently show a meshlike, fine, fibrinous arrangement in the background also. This does not interfere materially with diagnosis but does not allow so clear a picture as the same slide will present if it dries for a few hours before staining. However, if a report must be made immediately, the slide may be stained as soon as visibly dry, provided it is gently handled in both the stain and rinsing water. To aid in quick drying, the slide may be placed for a short while in an incubator at 37C. Too long drying of this kind will harden the cells and prevent perfect staining. Direct, excessive heat should never be applied, for like alcohol, it "fixes" the red blood cells.

d. **Shipment of Smears.** When smears are taken outside the laboratory, they should be sent immediately to the person who is to stain them—because of the adverse effect of summer heat or of age on the smears. Slides shipped by mail must be carefully protected from breakage. If shipped in slide boxes, a strip of adhesive or Scotch tape stretched securely over the slides and ends of the box before putting on the top will hold the slides and keep them from rattling about with a likelihood of breakage. The closed

box should be wrapped in corrugated paper, then in wrapping paper. Slides not in boxes should be wrapped when the blood is dry, slide over slide in toilet tissue in small packages. These are then placed with much protective packing in mailing containers to prevent breakage. Broken slides are difficult or impossible to stain and examine.

c. **Handling Precautions.** Thick films may be hardened or partially or totally fixed by age or by summer heat to the extent that they will not give up their hemoglobin. Such films are valueless. No way has been found to make such slides suitable for examination. Old films never take the stain so easily or so brilliantly as fresh ones, but if they are not fixed by age, heat, or alcohol, they may be used for diagnosis provided they are carefully stained. Unstained slides may be successfully kept for a few days during cool or cold weather. Care has to be taken to prevent condensation of moisture on the slides, since this may ruin thin films and cause thick films to be loosened and then lost in the staining process.

11-6. Staining the Film With Giemsa Stain:

a. **Types of Giemsa Stains.** The most dependable stain for malaria parasites, particularly for thick films, is obtained with good quality of Giemsa stain solution diluted with distilled water having a pH of from 7.0 to 7.2. With the exception of those places where large quantities of malaria slides are processed, most laboratories will prefer to use ready-made stain solution. However, stain solutions prepared from the powders are generally more satisfactory. The quality of the stain must be such as to bring out chromatin and cytoplasm equally well when the stain solution is diluted with distilled water buffered to pH 7.0 to 7.2. Not all Giemsa stains that give good cell staining for the differential count will do equally well by malaria parasites. When stains made from American dyes are used, they should be those stains that are certified by the Commission for the Standardization of Biological Stains. Since 1949, two forms of Giemsa stain have been certified by the Commission, that is, Giemsa Stain, Azure A type, and Giemsa Stain, Azure B type. The Azure B type is the one suitable for staining malaria parasites, whereas the Azure A type is not. If stain solution is bought for staining malaria slides, it should be of the Azure B type, also. If the stain solution is made in the laboratory, the best reagent methyl alcohol (neutral, acetone free) and reagent glycerine C.P. (neutral) both from freshly opened bottles should be used. Glycerine in bottles that have been partially emptied often absorbs enough moisture from the air space to affect the quality of Giemsa stain made with it. All glassware used in stain making should be chemically clean and dry.

b. **Preparing Giemsa Stain Solution for Malaria:**

(1) *Giemsa Stain.* Using the Azure B type Giemsa stain which has been certified by the Commission for the Standardization of Biological Stains, prepare a stock stain as outlined in paragraph 10-2b(1)(a)*3*.

(2) *Buffer Solutions and Buffered Water.* Prepare buffered water stock solutions and buffered water working solutions in accordance with the directions in paragraphs 10-2b(1)(a)*1* and 10-2b(1)(a)*2*.

c. **Giemsa Stain Procedures For Malaria:**

(1) Add one part of Giemsa stain to 50 parts buffered water working solution, pH 7.2.

(2) Fix the thin film only for 1 minute with methyl alcohol. Do not let alcohol touch the thick film.

(3) Allow the slide to dry and then place entire slide in staining solution for 45 minutes.

(4) Remove and dip the whole slide three times in buffered water working solution, pH 7.2.

(5) Immerse thick portion only in buffered water working solution, pH 7.2, for 3 to 5 minutes. (The age and thickness of the smear and density of the stain are the controlling factors here. Very fresh smears require little washing. This is true also of films that are not very thick. Older, thicker, or deeply stained films give a clearer picture if washed a longer period of time.) If slides appear too deeply blue after washing, a quick dip in unbuffered distilled water, which is usually acid to some degree, will give a better microscopic picture.

(6) Remove slides from the water and allow to air-dry, standing on end on absorbent paper. Never blot blood films. An electric fan will facilitate the drying, but direct heat is not advised since some of the excess stain that

might flow from the smear may be dried upon it and cloud the background.

(7) Always clean the staining dish after use, as sediment from previous staining may precipitate part of subsequent stain. For maximum staining qualities use the stain only once.

11-7. Rapid Staining Methods. Many times a more rapid method may be desirable. It is seldom that a few extra minutes, to assure better staining, cannot be spared, and longer, better staining may mean the difference in finding or not finding parasites on a difficult slide. Hence, the methods suggested below are not the most rapid but are among the most dependable for really good staining, particularly for technicians not constantly associated with positive malaria slides.

a. **Wright-Giemsa Stain:**

(1) *Preparation of Wright-Giemsa Stain.* To make this stain, dissolve 2 g Giemsa powder in 100 ml of glycerine (C.P. from a freshly opened bottle). This may be done by heating in a water bath at 55C to 60C for 2 hours, mixing well at intervals with a glass stirring rod. Avoid absorption of moisture by covering the mouth of the flask containing the mixture with a double thickness of paper or a layer of foil secured with a rubber band and by removing the flask from the water each time the mixture is stirred. To this mixture add 100 ml Wright's stain solution (aged solution of 2 g powder to 1,000 ml methyl alcohol). Let stand overnight and then add an additional 800 ml of aged Wright's stain solution. Filter and use.

(2) *Staining Procedure with Wright-Giemsa Stain:*

(a) Prepare a solution containing one part Wright-Giemsa stain and nine parts of buffered water working solution, pH 7.2, paragraph 10-2b(1)(a)*2*.

(b) Pour staining solution over the slides in a staining dish.

(c) Stain for 10 minutes.

(d) Flush scum from the top of the dish with buffered water working solution, pH 7.2; then remove slides and wash them for 1 minute by standing in buffered water, pH 7.2.

(e) Air-dry the slides at room temperature.

b. **Rapid Giemsa Stain.** Use the Giemsa stain, Azure B type, certified by the Commission for the Standardization of Biological Stains, that has been prepared as outlined in paragraph 10-2b(1)(a)*3*. To prepare the working stain solution, add two parts of the Giemsa stain to 50 parts of buffered water working solution, pH 7.2 [paragraph 10-2(1)(a)*2*]. This double-strength Giemsa stain does a better job of staining thin smears than does the regular Giemsa stain.

(1) Immerse slides in stain solution for 20 minutes.

(2) Dip the slides in buffered water working solution to wash thin smears. Immerse thick portion only in buffered water working solution for 3 to 5 minutes. The same properties of age and thickness of smear influence this stain as they did in the regular Giemsa stain in paragraph 11-6c(5).

(3) Air-dry the slides at room temperature. Do not blot to dry.

11-8. Identification of the Species of Malaria in Thin Films. The identification of species requires a careful study of all forms, starting with the morphology as seen in thin blood smears. At times, speciation will be difficult, but accuracy and speed are imperative, especially with *P. falciparum*. Table 11-1 summarizes the characteristics of the parasites in thin films; illustrations of the parasites in thin films are presented in figures 11-1, 11-2, 11-3, and 11-4.

a. **Plasmodium vivax (vivax malaria):**

(1) Considering the parasites of *P. vivax* as they appear in stained thin films from peripheral blood, the youngest ring form, consisting of a blue margin of cytoplasm and a rather heavy red dot of chromatin, which may be located centrally or peripherally, is about one-third the diameter of a normal red blood cell. As the parasite grows, the infected red blood cells are enlarged, pale, and may be very bizarre in shape. Also, in correctly stained films Schüffner's dots may be demonstrated in many of the parasitized cells at any stage after this period and are often a very important factor in making diagnosis of this species. Schüffner's dots are small, fairly uniform, pink granules that appear evenly distributed throughout the part of the parasitized cell not occupied by the parasite. As

the parasite grows, the dots often become more pronounced and take a somewhat deeper stain. This stippling is peculiar to *P. vivax* and *P. ovale*, hence of diagnostic value. Best stains are made at about pH 7.2, careful staining being necessary to demonstrate the dots in the maximum number of infected cells. Prolonged washing will obliterate them. As development proceeds, the parasite may continue to show a ringlike appearance in stained films with much thickened cytoplasm and enlarged chromatin mass. However, it may very early exhibit pseudopodial processes indicative of ameboid movement, a characteristic that is very pronounced in this species and that gave rise to the parasite's specific name *vivax*. After 5 or 6 hours the trophozoite begins to show yellowish-brown pigment granules. These are small, angular, or rodlike, and increase in number with the growth of the parasite. In the young forms they frequently cannot be distinguished as separate granules or rods, but exhibit their presence by giving a yellowish tinge to portions of the cytoplasm. As the trophozoite develops, it may assume a great variety of shapes within the enlarged cell, with projecting pseudopodia and one or more vacuoles. Meanwhile both the cytoplasm and chromatin are increasing in amount. In *P. vivax* the fully grown trophozoite is larger than the corresponding stage of the other species. At the end of about 36 to 40 hours the parasite practically fills the entire cell, which may be half again as large as normal or more. It has now completed its vegetative growth and is preparing for reproduction. To this end it draws in its pseudopodia, ceases its movement, and assumes a rather compact form, usually irregular in outline and with cytoplasm mottled in appearance as though unevenly massed. It has a single nucleus, which is compact and usually lies near the periphery of the parasite. This stage may appear much smaller than some of the ameboid forms that have preceded it, and on account of its compactness usually appears stained much more heavily.

(2) Now the division of the chromatin begins and the parasite becomes a schizont. The nucleus divides successively into irregular masses, and the mass of cytoplasm gradually breaks up into strands and portions. The pigment begins to clump in various parts of the cytoplasm. The number of nuclei in the final division is commonly 16, but 12 to 24 is usually given as the range. When the division is complete, the nuclei are smaller, more rounded and to each adheres a more or less rounded mass or circle of cytoplasm, forming individual parasites or merozoites. The pigment at this stage is clumped in one mass, this being a definite sign in *P. vivax* that segmentation is complete. The time required for the parasite to develop from small trophozoite through mature schizont is about 48 hours.

(3) Upon maturity of the parasite, the cell bursts and the merozoites enter new cells and begin another generation. There is a tendency in *P. vivax* for many of the parasites to leave the peripheral circulation in the schizont stage; consequently, although all forms are found, the mature schizonts are not so numerous as the trophozoites that preceded them.

(4) The majority of a brood of parasites attain maturity at about the same time, but the process is not entirely synchronous. Also, there may be two broods of parasites maturing on alternate days. For these two reasons there are often several stages of vivax parasites in the peripheral circulation at the same time.

(5) Gametocytes of *P. vivax* develop in the deep organs. The young forms are not regularly found in the peripheral blood, but when seen, are usually rounded, with homogenous cytoplasm and very often a vesicular area around the chromatin mass. Young males and females are not so easily differentiated as are the mature forms, nor is it easy at times to differentiate this stage from growing trophozoites that may have drawn in their pseudopodia before drying.

(6) The mature microgametocyte is often about the size of a normal red cell; the mature macrogametocyte is distinctly larger. The red cells they occupy are enlarged. The quantity of pigment granules in the mature gametocytes of both sexes is usually greater than in the trophozoite, and the grains and rods usually appear darker in color in the female. They are nearly always distributed rather evenly throughout the cytoplasm of both sexes.

(7) The macrogametocyte possesses a densely blue-staining, generally homogeneous cytoplasm. The nucleus is usually compact and very

rich in deep-red or magenta chromatin. Around this chromatin there is sometimes a colorless, vesicular area. The nucleus is usually situated near the periphery of the parasite.

(8) The microgametocyte contains less cytoplasm than the macrogametocyte; it stains more lightly than that of the female and may be gray blue, greenish blue, pinkish blue, or at times practically colorless. There is a loose nuclear system with reticular distribution of the chromatin. Sometimes the nucleus is round or "stellate", sometimes it extends in a broad spindle across the body. Practically always it is centrally placed and has a light staining quality. There is often in the microgametocyte a large unstained vesicular area around the mass or grains of stained chromatin. This makes the nucleus of the microgametocyte larger than in any other stage of the parasite.

(9) One may encounter difficulty in differentiating between the full-grown trophozoite (just before division of the chromatin) and the slightly immature macrogametocyte. The following points can be of assistance. The cytoplasm of the trophozoite may present a mottled appearance or may even contain one or more small vacuoles. The outline of the trophozoite is usually irregular and may show deep indentations. The pigment of the trophozoite, although scattered through the cytoplasm, is in small golden-brown stains. In the macrogametocyte the cytoplasm is rather homogeneous and contains no vacuoles. The circular or ovoid outline, as a rule is smooth in contour. Its pigment may be more abundant and in larger, darker-brown granules. The full-grown macrogametocyte is usually larger than the mature trophozoite.

b. **Plasmodium malariae (malariae malaria):**

(1) The young trophozoites of *P. malariae* are ring forms and are about the size of those of *P. vivax*, or slightly smaller though they sometimes seem to have a broader circle of cytoplasm than young vivax rings. Double chromatin dots are rare. The vacuole of the ring stage disappears very soon after the parasite begins its growth. The growing trophozoites usually seen in stained films are compact and nonvacuolated; they are angular, round, or ovoid shapes or bands across the infected cell. The band shape is much more frequently found in *P. malariae* than in any other species. The chromatin in the growing or older trophozoite may be rounded or streaky, frequently having a stretched appearance. The pigment is darker than in *P. vivax* often with rounded-appearing grains, which may have a peripheral arrangement, at times opposite the elongated nucleus. The pigment appears early in the growth of the parasite and increases in amount as the parasite ages. During examination, light transmitted through the smear frequently causes a yellowish-appearing edge to the large pigment granules, and this gives a greenish hue to the blue cytoplasm of the compact parasites, which is different from the color of most parasites of *P. vivax*. Because it is a sluggish, slow-growing parasite, the trophozoite of *P. malariae* rarely shows the irregular, tenuous, ameboid form so characteristic of *P. vivax*, but retains the rounded, oval or band shape until maturity. The cells containing malariae parasites are never enlarged. Frequently they even seem smaller than normal and sometimes darker in the early stages.

(2) The immature schizonts of *P. malariae* are often so dark and dense that it may be difficult to differentiate the divisions of chromatin within the heavily pigmented cytoplasm. The divisions are frequently uneven in size and shape. The pigment remains more or less scattered through a good portion of the cytoplasm until shortly before complete division of the chromatin. The red cells containing the schizonts of *P. malariae* have little tendency to withdraw from the peripheral blood as do those containing *P. vivax* schizonts. Very often one will find, at the proper time in the life cycle, great numbers of mature schizonts - many more than are found usually in any of the other species. When schizogony is complete, there are six to 12 merozoites (usually eight), sometimes arranged peripherally around the centrally clumped pigment (rosette formation) but more often in an irregular cluster. The length of the asexual cycle is 72 hours.

(3) *P. malariae* seems to have fewer gametocytes than the other species. They are frequently difficult or impossible to find. The rounded compact trophozoite is very difficult to differentiate from the rare young gametocyte.

However, the young gametocytes are rarely seen in this species. The mature gametocytes of *P. malariae* are smaller than those of *P. vivax*, and there is often not much difference in the size of the sexes. Like those of *P. vivax* they are spherical or oval, and the sexes have the same differences in staining qualities, although the abundant, prominent, dark-brown, coarse pigment grains may make each appear darker than in *P. vivax* particularly the female. Mature macrogametocytes are likely to be larger than the mature trophozoites and may be ovoid in shape.

(4) On occasions there have been demonstrated in the cells containing malariae parasites certain pink-staining dots similar to the stippling in other species. These have been named Ziemann's stippling. The stippling is usually pale and the dots slightly irregular in size, spherical, and less distinct than in *P. vivax*. Also, they are, as a rule, less developed than those found in *P. falciparum*. They seem to be best demonstrated by intensive staining or with stain solution having a pH of about 7.5. Since they have little differential diagnostic significance, it is better to stain at pH 7.0 or 7.2 without regard for these possible dots.

e. **Plasmodium falciparum (falciparum malaria):**

(1) The young trophozoites of falciparum malaria are usually smaller than those of other species and have a delicate, threadlike line of cytoplasm and one or more rather small dots of chromatin. They may be only one-fifth or one-sixth the diameter of the cell. Double chromatin dots are much more frequently found in this species than in the others. The rings may vary in size, but the small size and delicacy of the young ones are leading diagnostic characteristics. The young parasites may at times be quite irregular in form (round, rectangular, flameshaped, or shaped like a narrow band). Flattened marginal forms and bridge forms are more common in *P. falciparum* than in any other species. Multiple infections of the single cells are also more common in this species, though certainly not confined to it.

(2) As growth proceeds, the parasites of falciparum malaria usually retain a ring formation much longer than those of other species. The older rings differ from the very young ones in the slightly increased size and in the increased amount of cytoplasm and chromatin; also, in the fact that they contain traces of pigment, which appears as tiny grains or gives a yellowish tinge to the cytoplasm. These parasites, which correspond in age to the large ameboid forms of *P. vivax*, may be confused, in a diagnosis of smears containing few parasites, with the younger ring forms of *P. vivax* or even of *P. malariae*, since they are within the size range of these. Because falciparum parasites remain as ring forms during much of the early trophozoite growth, and because of the great number of parasites produced in *P. falciparum*, there are likely to be more rings of *P. falciparum* in the peripheral circulation at one time than of any of the other species. Also, unlike the other species, it is the tendency of the parasites of *P. falciparum* to disappear from the peripheral blood while still in the ring form and complete their growth and development in the capillaries of the internal organs. This accounts for the fact that usually only the ring forms of the asexual cycle are found in the peripheral blood. The exact stage at which the ring forms disappear from the circulation varies. Sometimes they disappear very soon after they enter the red blood cells; at other times they remain for hours, attaining considerable size. With the foregoing facts it is safe to state that when only a large number of ring forms are seen, without any older trophozoites or schizonts, the infection is in all probability *P. falciparum*. Many times the rings are of various sizes because *P. falciparum* has a less effective synchronization of the schizogonous cycle than have the other species. Ring forms are found more readily after the chill, the number having a tendency to build up to a height before the parasites return to the internal organs to develop.

(3) The more mature trophozoites and schizonts of *P. falciparum* generally are not found in the peripheral blood, although some strains have shown them more readily than others. In heavy infections rare ones may occur, usually along with large numbers of ring forms. The old trophozoites consist of a small bit of compact, light-staining cytoplasm, a granule of chromatin somewhat larger than in the ring, and usually a dull blur or a small, dense, almost black block of pigment. The parasite at this

stage is very small, sometimes hardly as large in circumference as the older ring forms. The clumping of pigment from this stage onward instead of only in the mature schizont is a falciparum characteristic. In the immature schizonts the chromatin dots and the dark pigment clump are far more noticeable than is the small amount of clear cytoplasm around them. Of the older asexual stages that appear at times in the blood, the mature trophozoites and young schizonts are more frequently seen, while the older, dividing forms appear less often and the mature schizonts least often of all the stages. Usually the number of merozoites given for *P. falciparum* is 8 to 24. Greater numbers have been seen, but it is possible in some instances that more than one parasite reached maturity in a single cell at the same time. Each schizont usually has its own clump of pigment in a double infection. About 48 hours are required for complete schizogony.

(4) The cell parasitized by *P. falciparum* is not altered in size. Sometimes there may be observed in the cells containing the asexual parasites from the older ring onward pink-staining dots called Maurer's spots. These may be of various shapes and sizes generally much coarser than Schüffner's dots and not nearly so numerous. To demonstrate them, overstaining or an alkaline pH of the stain is necessary. Some films show them far better than others. The age of the parasite seems to control the depth of the stain in these spots.

(5) Except in cases of grave infection, the younger forms of falciparum gametocytes are seldom seen in peripheral blood. They range from the earliest, small, compact, rounded form with pigment, through elongate, angular, and oat shapes into what are termed spindle forms. In the rounded and angular forms the pigment is usually scattered through the cytoplasm, and the nucleus may be stretched along one side. As the parasite gets older the chromatin usually has a tendency to pass to the center, and the pigment remains scattered. The long, thin, spindle or lanceolate shapes with pigment scattered to the ends, are, as a rule, the youngest forms seen in the peripheral blood, and they are much rarer than mature forms.

(6) The mature gametocyte assumes a crescentic, or more often, sausage shape, which makes it different from that of any other species. The mature macrogametocyte usually shows a dense blue cytoplasm, a small compact red or magenta mass of chromatin, lying in or near the center, or near one of the poles. The pigment closely adheres to the chromatin in separate grains, surrounding it completely or partially or even covering it completely. The macrogametocyte may be more slender and slightly longer, as well as more deeply stained, than the microgametocyte. The microgametocyte, by comparison, is likely to be broader, shorter, and more sausage shaped, with lighter staining qualities throughout. The cytoplasm in the microgametocyte is usually pale, often grayish blue or pink; the chromatin is in loose granules scattered irregularly, with the abundant, brownish-pigment rodlets and granules through about the central half of the length of the gametocyte. The ends of both mature male and mature female gametocyte are usually clear and free of pigment.

(7) It is usually several days after the appearance of ring forms before gametocytes of *P. falciparum* occur in primary infection. On the other hand, in old or chronic cases in endemic areas, rare ones may be found when no rings can be seen. The mature gametocytes of *P. falciparum* seem to be produced in showers instead of being produced simultaneously and continuously with the asexual forms as in *P. vivax*. The cells containing the falciparum gametocytes stretch as the longitudinal growth proceeds, and frequently in the stained thin film one can see on the concave side of the parasite a faint bow-shaped projecting rim representing the residuum of the infected red cell. Sometimes the remains of the red cell appear as a red zone around the gametocyte.

d. **Plasmodium ovale (ovale malaria):**

(1) *P. ovale* has now been reported from all five continents and some islands of the Pacific, although the strain reported from the United States was definitely of Pacific origin. *P. ovale* seems to be native to a large portion of Africa and is fairly common in the Western portion of that continent. However, the infection rate for this species is always low as compared to the total malaria infection rate in any given area.

(2) The parasite has definite resemblances to both *P. vivax* and *P. malariae*, which may

easily lead to a wrong identification of the species in the hands of persons not well acquainted with the stages and characteristics of all four species. As in *P. vivax*, the parasite has a 48-hour cycle; the infected cell shows Schüffner's stippling and is enlarged. This enlargement is not so great as it is in *P. vivax* although the difference of size could be overlooked by an unobservant or inexperienced worker. As in both *P. vivax* and *P. malariae*, all stages of the parasite appear in the peripheral blood. Like *P. malariae*, the parasite is compact in appearance and has around eight merozoites in the mature schizont stage. There seems to be no definite way to tell the *P. ovale* ring forms alone from those of *P. vivax* or *P. malariae* although most of them have rather large chromatin dots, and some may have the heavy cytoplasmic circle found in malariae rings. Double infection of cells is not unusual. In fact, on some slides frequent instances of double infection with various combinations of rings or of other asexual or sexual forms are seen.

(3) The growing trophozoite of *P. ovale*, like that of *P. malariae*, has a tendency to fill in and have a solid, compact arrangement of chromatin and cytoplasm with very little ameboid appearance and few vacuoles. The parasite is in a slightly enlarged red cell and is accompanied by Schüffner's dots, which become more prominent as the parasite grows older. As it reaches the mature trophozoite stage, it is often a rounded parasite in the center of the cell, and the cell is frequently drawn out into oval, spindle or pear shape with ragged points or fimbriations on one or both ends. The Schüffner's dots in this stage and older stages may follow the ragged, decolorized points to their very ends, giving the impression that they form these points. The parasite at this stage and later stages looks much like *P. malariae*, but its pigment is not so dark and conspicuous.

(4) The immature schizont of *P. ovale* may be larger than that of *P. malariae*, and the cell containing it is frequently distinctive in being oval or elongated with fimbriations. Mature schizonts contain from 6 to 12 merozoites, but 8 is the average number. Often the merozoites are arranged in rosette formation. The individuals are particularly massive, especially in schizonts with low numbers of merozoites. The enlarged cell and Schüffner's dots distinguish this stage of *P. ovale* from *P. malariae*.

(5) The presence of Schüffner's dots and enlarged cells may enable one to distinguish the sexual forms of *P. ovale* from those of *P. malariae*. By measurement these forms of *P. ovale* are somewhat smaller than in *P. vivax*, but there is little to aid one in differentiating them from the latter. Mature gametocytes almost completely fill the host cell.

(6) *P. ovale* has a tendency to have an abrupt drop in the number of parasites from one day to the next, apparently without cause, and for parasites to disappear spontaneously from the blood. Another point of interest is that Negroes, who usually exhibit a racial immunity to *P. vivax*, show no such immunity to *P. ovale* and can be expected to contract *P. ovale* just as readily as do Caucasians.

e. **Mixed Infections.** There are probably many more mixed infections in highly infected areas than are usually determined in blood examinations, and the possibility of a mixed infection is frequently overlooked by the microscopist. First of all, it seems to be the tendency in mixed infections for one species to predominate over the others, and the chance of finding numbers of parasites of one species in a film and of missing the rare parasite of another species is quite possible. The fact is that many technicians look no further when definite parasites of one species are seen. Of course, typical and characteristic forms of the respective species must be found to determine a mixed infection. In thin films, the specifically distinctive forms of *P. falciparum* are the crescent-or sausage-shaped gametocytes. In *P. vivax* they are the large, distinctly ameboid trophozoites in an enlarged cell containing Schüffner's dots. In *P. malariae* they are the pigmented band forms, the heavily pigmented, rounded forms beyond the ring stage, or the mature schizonts in unenlarged cells.

f. **Accompanying Blood Picture.** In malaria, after a few paroxysms, the total number of white cells, as a rule, is below normal, though the count may rise with a complicating factor such as pneumonia. In the stained thin film one frequently finds the picture of secondary anemia in greater or less degree; that is, there may be increased polychromatophilia, central achro-

mia of the red cells, variation in size and shape of red cells, or even normoblasts. Basophilic stippling of red cells is frequently present also. In the early stages of the disease there may be a reduction of the number of neutrophils and an increase in lymphocytes and monocytes. During subsequent attacks there is a transient increase in neutrophils with a display of many immature forms. After the attack the neutrophils decrease again, and the monocytes increase. These latter play a part in combating the infection by ingesting the pigment and damaged red cells. Clumps of malaria pigment in the white cells are almost as certain proof of malaria as the parasites themselves, but when this pigment is present, plenty of malaria parasites can usually be found.

11-9. Identification Of Malaria Parasites In Thick Films:

a. General Appearance of the Blood in Stained Thick Films:

(1) The discussion of the differentiating points of the species of malaria parasites is usually based upon their appearance in the thin film. In this kind of film the cells are spread over the slide in one layer and then by the application of alcohol, either as a separate process or in the stain itself, are fixed so that their outlines and the outlines of the parasites within them are preserved.

(2) In the thick film, on the other hand, hemoglobin is removed from an unfixed thick film of blood during the staining process in aqueous Giemsa stain. Dehemoglobinization increases chances of finding parasites, because the red cells are obliterated, while the stained parasites from a large quantity of blood remain. Species identification is more difficult from thick films, but if unidentifiable parasites are found, the thin films can be studies more extensively. Keep in mind that a prompt, accurate identification of the species involved is imperative.

(3) In the thicker portion of the thick film, the background varies in color from a clear, light blue to a mottled gray blue, depending upon stain factors, age of the smear, and individual blood variations. At the edge, the thinner portion of the smear (frequently only one cell deep and about the width of one or two microscopical fields) often takes a pinkish color. Against the laked background the familiar purple nuclei of the white cells, in varying states of preservation, stand out clearly. Sometimes the cytoplasm of the white cells stains also, but it is ragged and uneven in appearance. The neutrophilic granules are often indistinct or absent, but eosinophilic granules show rather distinctly in their characteristic color. Blood platelets are pinkish violet in color, finely granular in texture, and hazy in outline. They lie singly or in groups and due to their distinctive granular appearance are not likely to be confused with parasites in the thick film. If the blood is taken from a free-flowing puncture, there will be little likelihood of excessive clumping of platelets. Very often, particularly in anemic bloods, the thick film shows in the background nuclear and reticular remains of immature red cells (cellular debris). The thicker the smear, the more evident this is. These remains may be found singly, often filling a space the exact size and shape of the laked cell, or they may appear in blue clouds of fine skeinlike material or stippling. The technician with a thorough knowledge of the stages and species of malaria parasites as they appear in the thin film will have no great difficulty in diagnosing malaria in the thick film or in differentiating species. For the less experienced person, the thin edge of the thick film will be valuable for study. Here the red cells frequently retain a ghostlike outline and one finds characteristics of infected cells and of parasites duplicating those in the thin film. The thin edge is particularly valuable for determining mixed infections. It is well in learning thick film technique to start in this thin edge and work in toward the thicker portion, comparing the typical forms with the less characteristic ones.

	Plasmodium vivax	*Plasmodium malariae*	*Plasmodium falciparum*	*Plasmodium ovale*
Infected cell	Larger than normal, paler, often bizarre in shape. Schüffner's dots often present with all forms beyond young rings; greater number and larger with older parasites. Multiple infection of erythrocyte not uncommon.	About normal or slightly smaller. Sometimes darker in early stages. Ziemann's stippling rarely seen. Multiple infection of erythrocyte rare.	Normal in size. Multiple infection of erythrocytes more frequent than in the other species. Maurer's spots sometimes seen with older rings and asexual stages in over-stained smears or when pH of H_2O is on alkaline side).	Somewhat larger than normal, often with fringed or irregular edge and oval in shape. Schüffner's dots present frequently with older ring forms and other stages beyond ring. Multiple infection common.
Small trophozoite (early rings)	Ring about $\frac{1}{3}$ diameter of red cell with heavy chromatin dot and large cytoplasmic circle, possibly with fine pseudopodia.	Ring form with single, heavy chromatin dot and cytoplasmic circle which is often smaller, thicker, and heavier than that of *P. vivax*, but not always distinguishable from it. Vacuole fills in early.	Small ring $\frac{1}{4}$ diameter of red cell with small threadlike cytoplasmic circle, and one or two small chromatin dots. (Double chromatin dots more frequent than in other species.) Marginal and bridge forms are frequent. May disappear in this stage from peripheral circulation and return to internal organs for development.	Undifferentiated from *P. vivax* and *P. malariae* darker in color and more solid, *with sharper outline* as a rule, than those of *P. falciparum*. Rather large nucleus; Schüffner's dots with greater percentage of ring forms than in *P. vivax* (Giemsa stain).
Growing trophozoite	Same as above with gradual increase in amount of cytoplasm and chromatin. Often with tenuous pseudopodial processes and large vacuoles. Small yellowish-brown pigment rodlets in cytoplasm, number increasing with age of parasite.	Chromatin rounded or streaky; cytoplasm usually in a compact form with little or no vacuole or in a narrow band form across the cell. Round, dark brown, coarse pigment granules may have peripheral arrangement.	This stage remains in the ring form but chromatin and cytoplasm increase to the extent that in size the parasite resembles closely the *small* trophozoite of *P. vivax*. A few pigment granules give a yellowish tinge to the cytoplasm. This is usually the oldest asexual stage seen in peripheral circulation.	Confluent with little vacuolation resembles closely some stage of *P. malariae*, but is somewhat larger and is in enlarged, possibly fimbriated and oval cell, usually with Schüffner's dots. Pigment is lighter in color and less conspicuous than in *P. malariae*, similar to that of *P. vivax*. Parasite increases in size with age.
Large trophozoite	One abundant mass of chromatin, loose, irregular or close compact cytoplasm, with increased amounts of fine brown pigment. Parasite often practically fills enlarged cell.	One mass of chromatin, often elongated, frequently less definite in outline than that of *P. vivax*. Cytoplasm dense, compact with few irregularities of outline; in rounded, oval or sometimes band shape. Rounded pigment granules, larger, darker than in *P. vivax* with great tendency toward peripheral arrangement. Fills or almost fills normal cell.	Stage seldom seen in peripheral blood. Very small, usually solid, with one mass of chromatin, tightly staining, compact, cytoplasm; and with haze of pigment scattered through the cytoplasm or with very dark pigment collected in one small, dense block on clear cytoplasm.	
Immature schizont	Chromatin divided into 2 or more irregular masses; cytoplasm shows varying degrees of separation into strands and particles; pigment shows tendency to collect in parts of the parasite. Not so numerous as preceding trophozoites.	Same as *P. vivax* except that the parasite is smaller and shows fewer divisions of chromatin, in irregular shapes and sizes, as it approaches segmentation. There is more delayed clumping of the conspicuous pigment; less tendency to leave blood than in *P. vivax*.	When found in peripheral blood this stage resembles the same stage of *P. malariae* but is smaller and the pigment is likely to be completely clumped in one small dark mass.	Many of infected cells are definitely of oval shape. Picture is often that of a round parasite in center of an oval cell. Many cells with indefinite fringed outline. Pigment lighter in color and less coarse than in *P. malariae*, more like *P. vivax*.

Table 11-1. Summary of Plasmodium Differentiation on Stained Thin Films. (From Wilcox, 1960)

	Plasmodium vivax	*Plasmodium malariae*	*Plasmodium falciparum*	*Plasmodium ovale*
Mature schizont (segmenter).	Usually 12 to 24 merozoites, each composed of a dot of chromatin and a small mass or circle of cytoplasm. The pigment is in one or two clumps. Parasite practically fills enlarged cell.	6 to 12, usually 8 or 10 merozoites in a rosette or irregular cluster with clumped pigment. Practically fills normal-sized cell. Merozoites, being fewer in number, usually appear more husky than those of *P. vivax*. Many found in peripheral circulation.	8 to 24 or more merozoites, which are very small compared to those of other species. Rarely found in peripheral blood. Usually fills about two-thirds of normal-sized cell.	4-16, usually 8 merozoites in rosette or irregular cluster around mass of pigment. Cell enlarged, possibly oval; contains Schüffner's dots. Smaller than in *P. vivax*. Nuclei and merozoites often larger than in other species.
Macrogametocyte	Rounded or oval, usually regular in outline. Dark blue, homogeneous cytoplasm with no vacuoles; small, compact, dark red, usually eccentric chromatin; abundant, brown pigment scattered through cytoplasm. When grown usually fills or nearly fills enlarged cell.	Cytoplasm and chromatin same as *vivax*. Pigment, abundant, round, dark brown granules coarser than in *P. vivax*. When grown usually fills the normal sized cell. Outline spherical or ovoid. May be confused with mature trophozoites of same species.	Crescentic or sausage-shaped about $1\frac{1}{2}$ times diameter of erythrocyte in length, possibly longer and more slender than microgametocyte. Cytoplasm possibly a deeper blue than in microgametocyte. Usually single dark red chromatin mass near center associated with concentrated aggregation of pigment, darker than in microgametocyte.	Distinguished from *P. malariae* by size of infected cells and by Schüffner's dots. Not easily differentiated from *P. vivax* though somewhat smaller. Mature macrogametocyte fills infected cell, microgametocyte smaller.
Microgametocyte	Small amount of light blue, gray, pink, or almost colorless cytoplasm, containing large, diffuse mass of light red or pink chromatin usually centrally placed, often surrounded by a vesicular area. Abundant dark pigment throughout cytoplasm. When grown, about size of a normal cell. Usually circular in outline.	Same as *P. vivax* except smaller. When grown, fills or almost fills normal sized cell. Pigment more conspicuous than in *P. vivax*.	Often the cytoplasm is paler than in macrogametocyte—grayish blue or pink. Loose, diffuse, light staining granules or threads of chromatin scattered with numerous granules of pigment throughout central half or more of parasite. Parasite possibly broader, shorter, and with more rounded ends than those of macrogametocyte.	
Length of asexual cycle	42 to 48 hours	72 hours	48 hours	48-50 hours (variable).
Stages in peripheral blood	All	All	Usually ring form trophozoites and gametocytes. Other stages rarely found except in heavy infections and acute cases.	All.
Remarks	More stages of growth likely to be seen in one film than in other common species. Gametocytes appear early in cycle.	Parasites are usually more compact with heavy pigment and hence appear more intensely stained than those of other common species. Gametocytes rarer than in other species, appear late.	Parasites frequently more numerous than in other infections. Unlike other species—growth of asexual forms, following the ring stage, takes place in internal organs. Gametocytes produced in waves.	Rarest of the species. Differentiation not possible in thick films except to those comparing it daily with other species. A one-slide diagnosis impossible because of its resemblance to aberrant forms of *P. vivax*.

Table 11-1. Summary of Plasmodium Differentiation on Stained Thin Films. (From Wilcox, 1960)—Continued.

AFM 160-48/TM8-227-2 1 August 1974

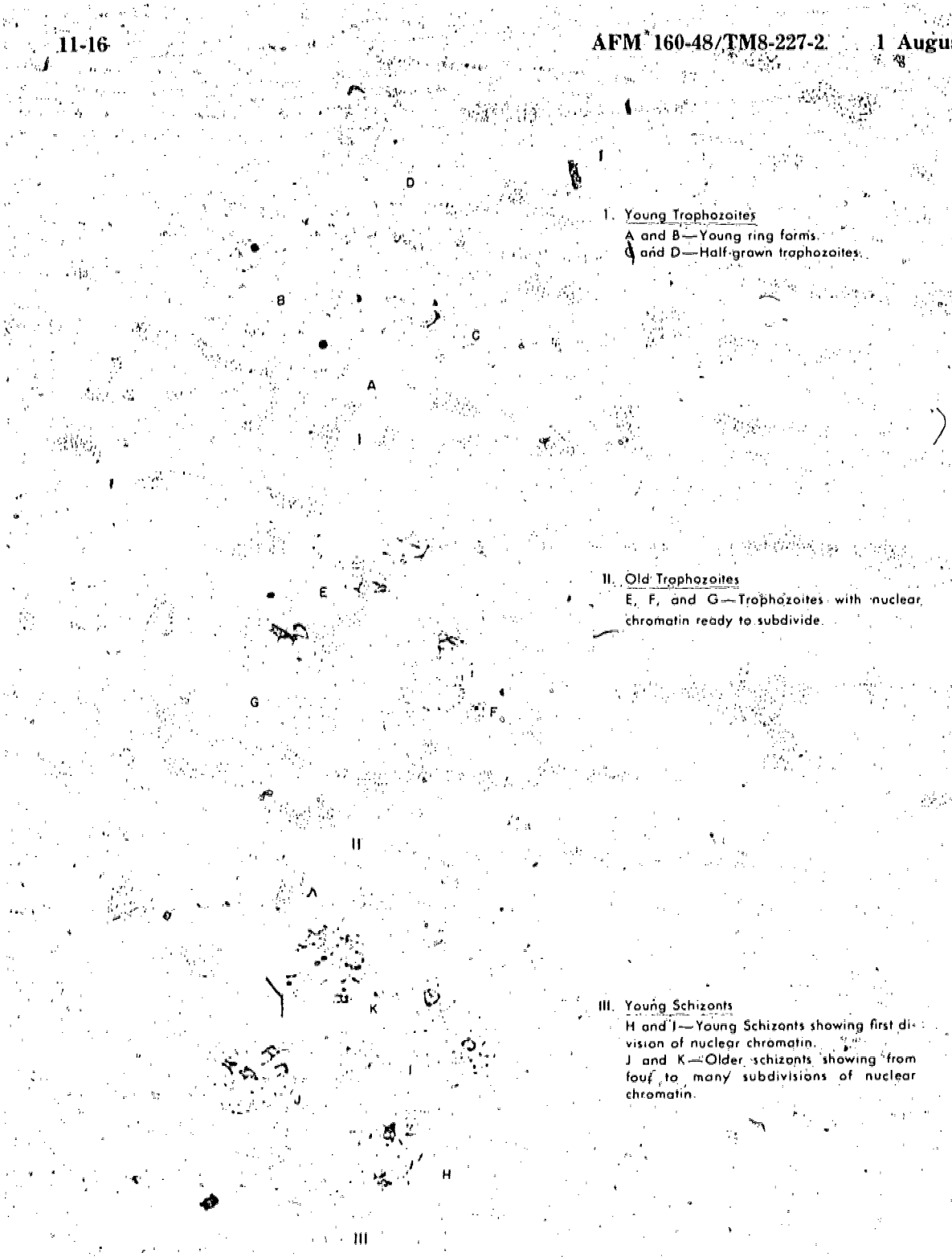


Figure 11-1. Plasmodium vivax in Thin Film. Giemsa's stains. ×2,000.

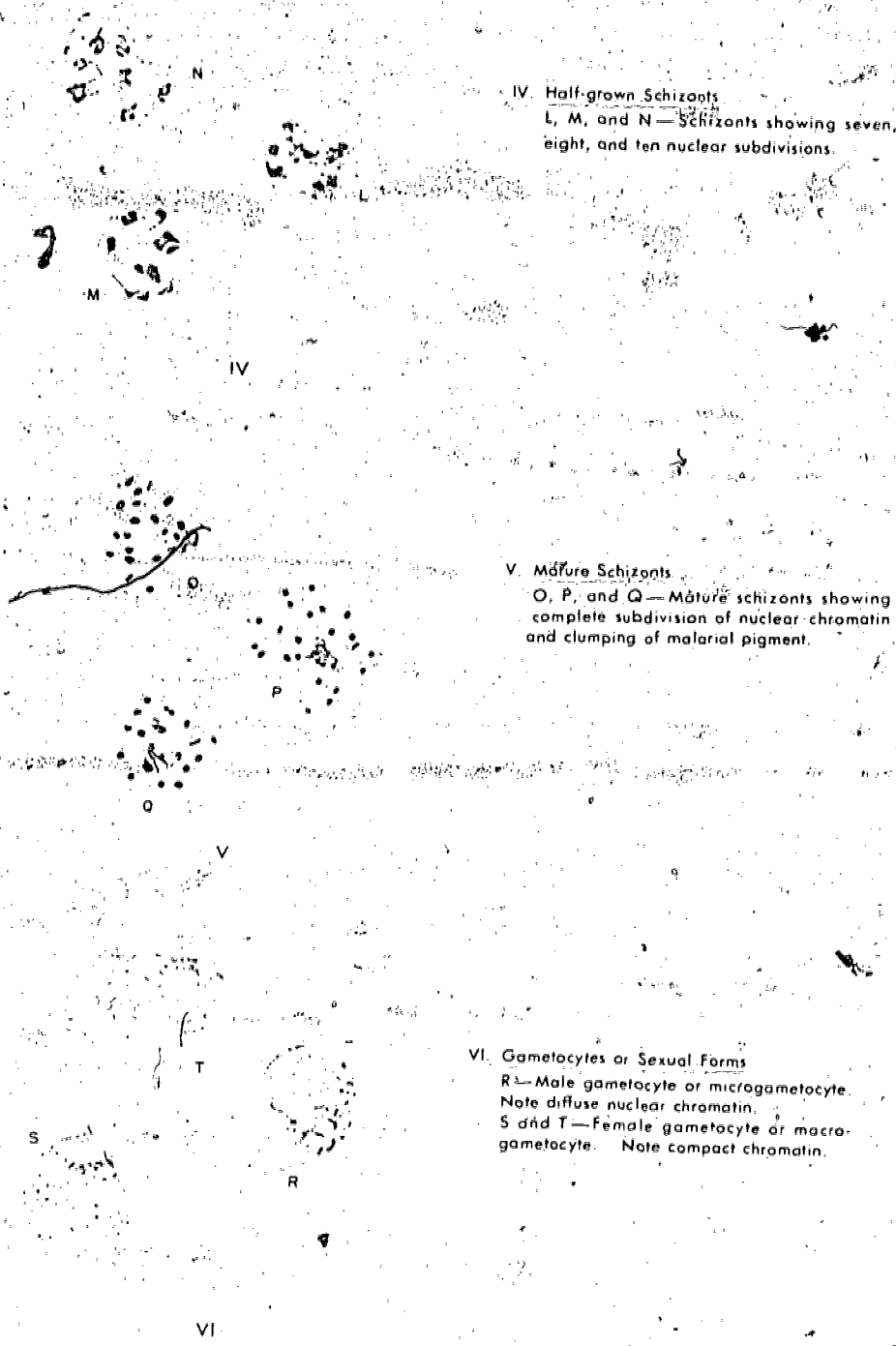


Figure 11-1. Plasmodium vivax in Thin Film—Continued.

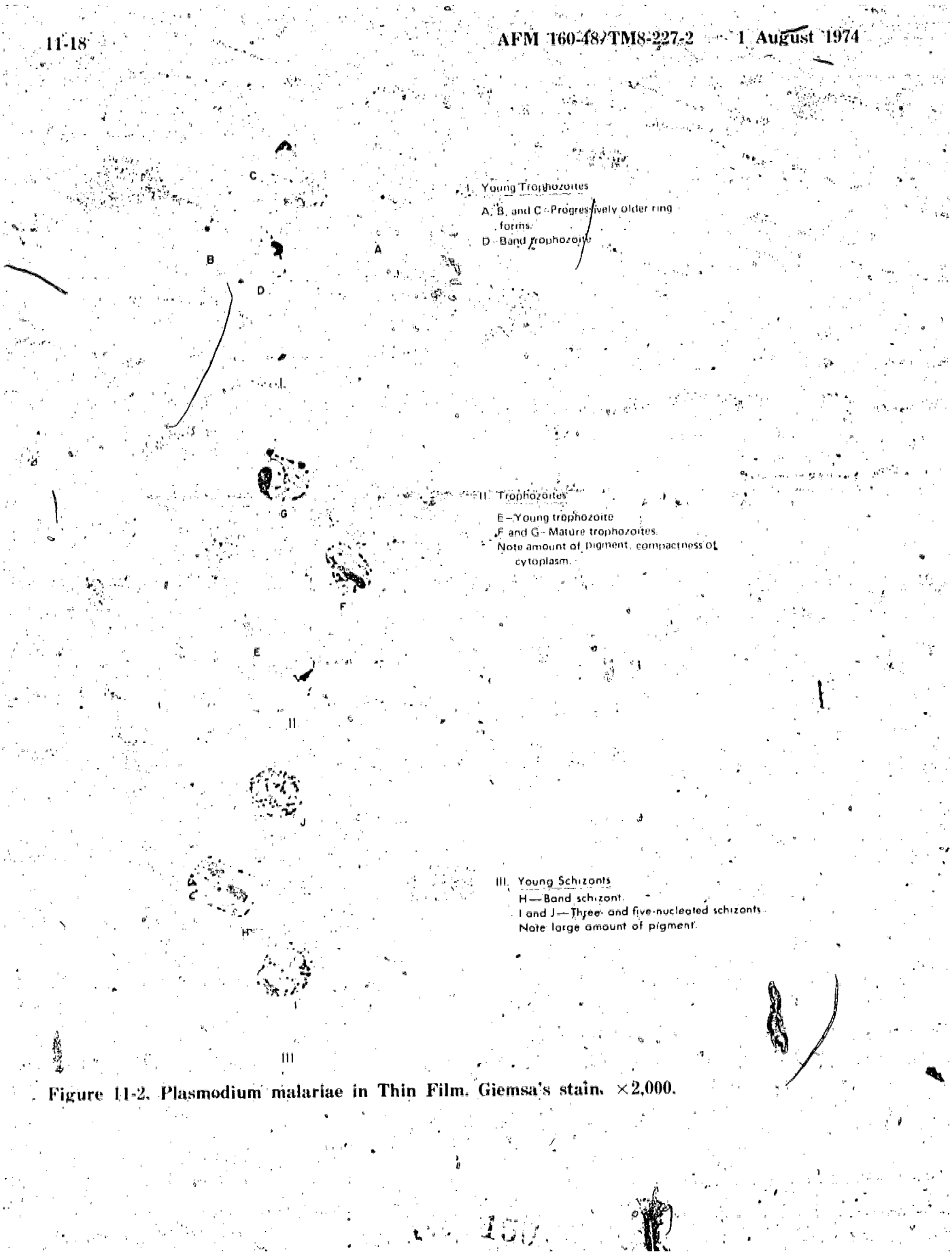
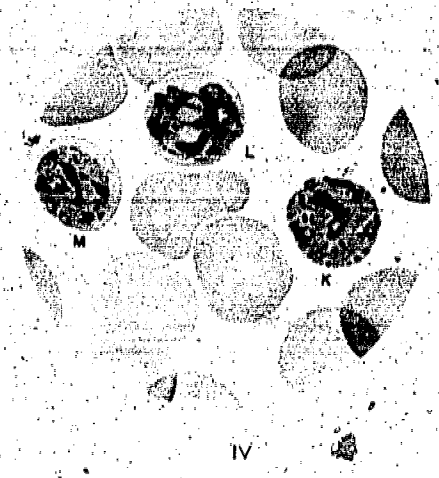
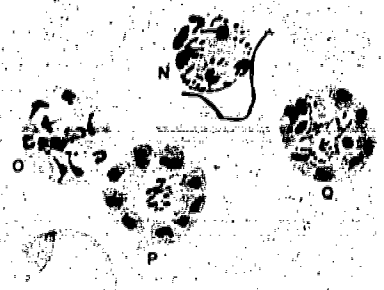


Figure 11-2. Plasmodium malariae in Thin Film. Giemsa's stain. ×2,000.



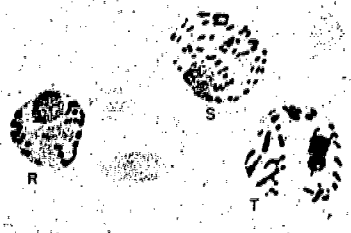
IV

IV. Half-grown Schizonts
K, L, and M—Four- to six-nucleated schizonts. Note amount of pigment.



V

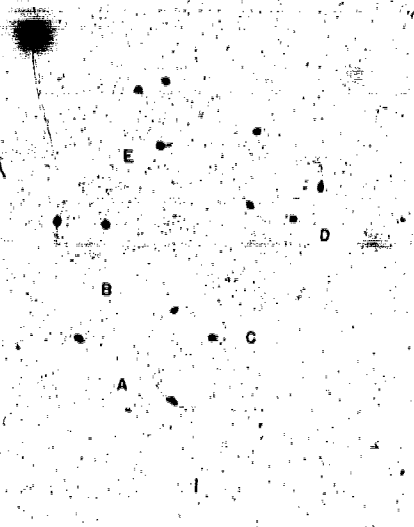
V. Mature Schizonts
N, O, P, and Q—Eight- or ten-nucleated schizonts ready to segment and release merozoites. Note "daisy" forms.



VI

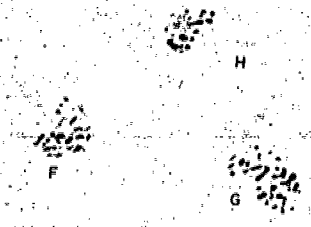
VI. Gametocytes or Sexual Forms
R and S—Male gametocytes or microgametocytes.
T—Female gametocyte or macrogametocyte.

Figure 11-2. Plasmodium malariae in Thin Film—Continued.



I. Trophozoites in Peripheral Blood

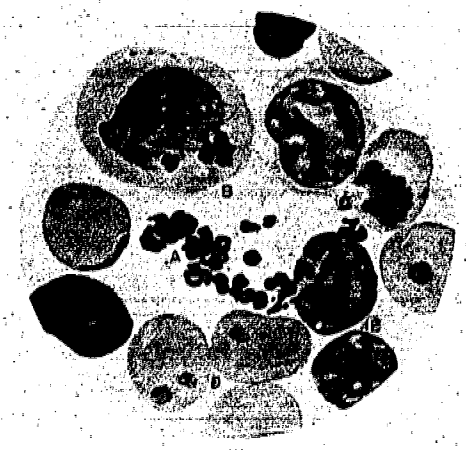
A, B, C, D, and E—Ring forms and young trophozoites.
Note multiple infections of cells and applique forms. These are oldest trophozoite forms normally found in peripheral blood.



II. Gametocytes or Sexual Forms in Peripheral Blood

F—Gametocyte folded over.
G—Microgametocyte or male gametocyte. Note diffuse chromatin.
H—Female or macrogametocyte. Note compact chromatin.

II

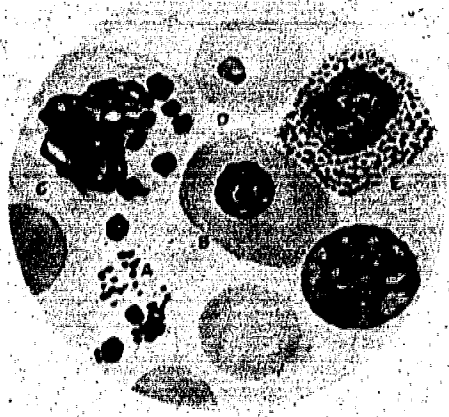


III

III. Impression Smear of Spleen

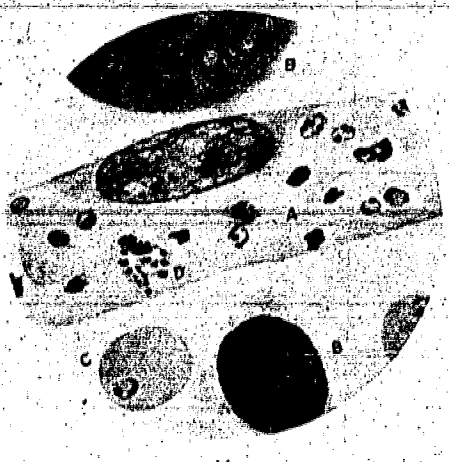
A—Free pigment of splenic pulp.
B—Pigment in macrophage.
C—Half-grown schizont in cell.
D—Parasitized red cells.
E—Lymphocytes.

Figure 11-3. Plasmodium falciparum in Thin Film. Giemsa's stains. ×2,000.



IV

IV. Impression Smear of Bone Marrow
A—Free pigment granules.
B—Nucleated red cell.
C—Pigment in macrophage.
D—Parasitized red cell.
E—Eosinophile.



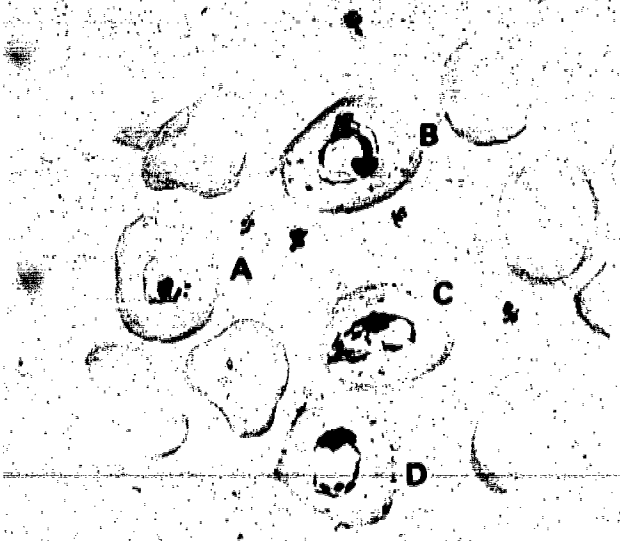
V

V. Impression Smear of Brain
A—Capillary blocked with parasitized erythrocytes.
B—Glial cells.
C—Trophozoite in red cell.
D—Maturing schizont.

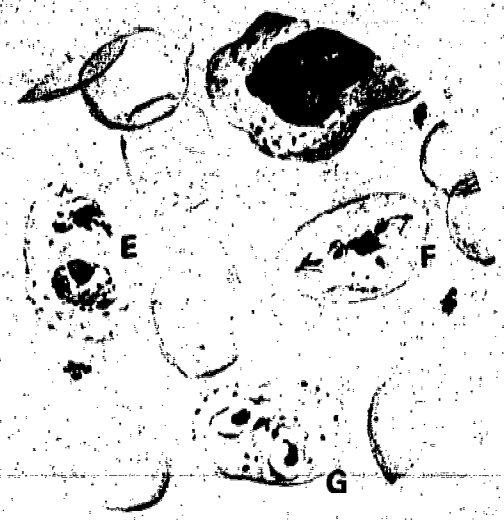
VI

VI. Section of Liver
A—Kupffer cells with pigment.
B—Hepatic cells.
C—Endothelial cell.
D—Free red blood cells.

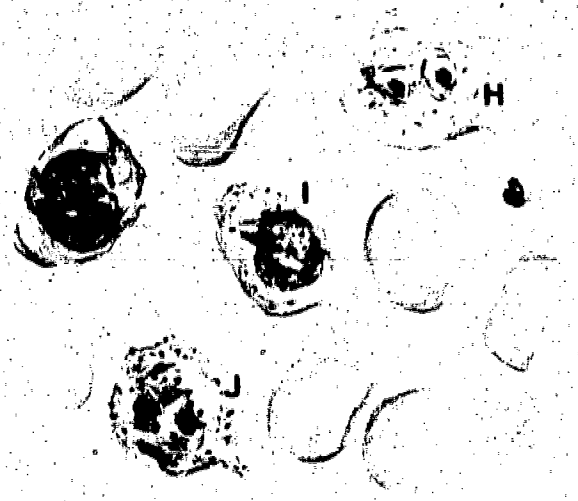
Figure 11-3. Plasmodium falciparum in Thin Film—Continued.



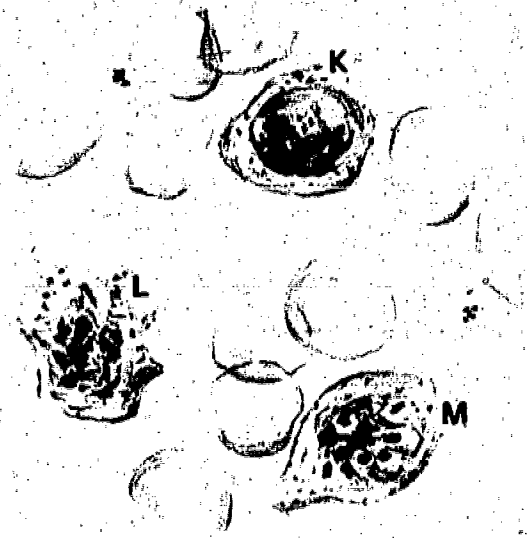
A—Young ring-shaped trophozoite.
B, C, D—Older ring-shaped trophozoites



E—Doubly infected cell: young gametocytes
F—Older ameboid trophozoite
G—Doubly infected cell: trophozoites



H—Doubly infected cell: trophozoites
I—Developing schizont
J—First stage of the schizont



K—Mature gametocyte
L, M-Advanced stages of schizonts

Figure 11-4. Plasmodium oval in Thin Film, Giemsa's stain. ×2,000.

(4) In the thin edge of the film, the ghostlike outlines of the cells parasitized with *P. vivax* often contain rather prominent Schüffner's dots. Leaving the edge of the film and working in toward the thicker, center portion of the film, one finds that separate granules are often no longer visible but that the parasites lie in pink areas suggesting the size and shape of the cells that contain them although no definite cell wall can be seen. In carefully stained films containing *P. vivax* parasites these pink areas are seen often enough to be considered a diagnostic aid. Occasionally in thick films containing older ring stages of *P. falciparum*, pink areas around parasites have been observed. When this applies, Maurer's spots are nearly always demonstrable in the thin films of the same blood. Except in the very thin edge the parasites usually appear without the definite outline of their host cells. The chromatin, cytoplasm, and pigment stain characteristically, but in the thicker portion of the smear the parasites often seem smaller and more shrunken. This may be due in part to the destructive effects of lysis and also to the heaping and crowding of the red cells in the thick smear, which prevent the cells and parasites from flattening and spreading out as they do in the thin film. Also, in the slower drying thick film the parasites have an opportunity to draw in their pseudopodia, which gives them a denser, smaller appearance.

(5) Almost constant focusing is necessary to distinguish all the parasites in the thicker fields, because of their occurrence at varying optical levels. Too much stress cannot be laid upon careful staining, for ease in examination and accuracy of diagnosis are completely dependent upon it.

b. **Appearance of Parasites in Thick Films.** *Plasmodium ovale* is not discussed in this section on thick films. In this type of preparation the parasite is almost impossible to identify because of its resemblance to both *P. vivax* and *P. malariae*. However, an expert technician who deals with these parasites on a daily basis can frequently identify *P. ovale* from thick smears. The characteristics of the other species are summarized in table 11-2, and the parasites are illustrated in thick films in figures 11-5, 11-6, and 11-7.

(1) *Small Trophozoites (Rings).*

(a) In young ring forms the red or purplish red of the chromatin dot first strikes the eye, and the cytoplasm is distinguished subsequently. Young trophozoites may be found in the thick film in the signet ring formation just as in thin films, but are frequently not complete in outline; that is, the chromatin dot with only a portion of the cytoplasmic circle is visible. This portion may be connected to the chromatin dot on both sides in short, straight or curved dashes, giving the impression that the rings are turned sideways or are lying at an angle to the plane of the blood. Often the cytoplasmic dash is visible adjoining only one side of the chromatin dot. This form resembles more or less an "exclamation mark" or "comma" and has been designated by these terms. Sometimes the cytoplasm follows a circular outline but is made up of seemingly disconnected small pieces or fragments and is called an "interrupted ring." At times the cytoplasm is in a solid, semicircular piece and lies completely disconnected from the chromatin dot on the curve of the ring opposite the nucleus. This form might also come under the term "interrupted ring."

(b) When only this ring stage of the parasite is present and the rings are infrequent or rare in the thick film specimens, it is often impossible to differentiate the species. Diagnosis may be facilitated if an older stage of the parasite can be found.

(c) In *P. falciparum* malaria, the young ring forms are very small and delicate, the chromatin dot tiny, and the cytoplasm threadlike. If many small rings are found and no older form of the parasites is in evidence, one can be practically sure that the infection is *P. falciparum*.

(d) The young rings of *P. vivax* malaria are, as a rule, larger than the same stage of *P. falciparum* with a heavier chromatin dot and cytoplasmic circle. Sometimes, even in this stage, there is evidence on the stained smear of ameboid activity shown by tiny pseudopodial processes on the cytoplasmic circle. It is seldom that species diagnosis need be based on young trophozoites alone, however, since older forms of *P. vivax* can usually be found.

(c) The young ring of *P. malariae* is often heavier than that of *P. falciparum* and frequently not so large as that of *P. vivax*. In thick films it often appears as a large chromatin dot with a small concentrated mass or dash of cytoplasm. There is not so great a likelihood of the *P. malariae* ring spreading out or showing ameboid tendencies as in *P. vivax*.

(2) *Large Growing Trophozoites:*

(a) While the round and delicate ring forms of *P. falciparum*, when found in sufficient numbers in the thick film, can often be differentiated from those of *P. vivax* and *P. malariae*, the growing trophozoites of *P. falciparum* present more difficulty in diagnosis of species. These heavy ring forms of *P. falciparum* are about the size of much younger ring forms of *P. vivax* and can be confused with the latter, yet they correspond in age to much larger ameboid parasites of *P. vivax*. The old trophozoite of *P. falciparum* (the form just before the division of the chromatin) is not often found except in heavy infections and is consequently usually accompanied by large numbers of ring forms. When it is found, it is usually very small, compact, and often nonvacuolated. The chromatin dot is larger than that in the young ring; the cytoplasm seems lighter than that of the similar stage of *P. malariae*, and the pigment, even at this stage, is usually clumped in one or two very small, dark or blurred masses.

(b) In thick films showing the growing trophozoites of *P. vivax* one may still encounter ring formations, much enlarged, with increased amounts of delicate, tenuous cytoplasm. There is a great variation in cytoplasmic pattern at this stage, and there is a decided tendency in the older ameboid forms of this species for the cytoplasm to be fragmented and arranged irregularly in a cluster of varying-sized pieces with no visible connection. This cytoplasm is associated with a large, round or irregularly shaped, red or magenta mass of chromatin. The pigment appears as a yellowish haze, or as small light-brown grains or delicate rods on the cytoplasm. The older trophozoite stage of *P. vivax*, just before division of the chromatin, is frequently quite solid in the thick film with a dark-staining appearance often with a more or less regular outline. Its yellow-brown pigment like that of the gametocyte is rather evenly distributed through the cytoplasm. In the thick film these forms cannot be distinguished definitely from macrogametocytes.

(c) Because there is little ameboid activity in *P. malariae*, the growing and older trophozoites of this species, as a rule, appear more compact in thick films than those of *P. vivax*. This is the most common stage seen in smears containing *P. malariae*. The profuse, heavy, dark pigment scattered through the solid cytoplasm gives a dense appearance to a majority of the parasites. The picture often presented in the thick film by the quarter-or third-grown tropozoites of *P. malariae* is so distinctive that when they are present in sufficient numbers, diagnosis is practically certain. In this stage the inconspicuous body of chromatin is often imbedded in a small, tight, rounded, heavily pigmented mass of cytoplasm. At first glance, one does not distinguish the characteristic red, blue, and brown of the parasite, but close examination will reveal the colors. Old trophozoites of *P. malariae* are also often quite compact and dark. They are larger than the ones mentioned above, and hence, the morphological characteristics are more easily seen. They often display the elongated or streaky chromatin and peripheral arrangement of pigment, so characteristic of this stage in the thin film. Band forms of *P. malariae* cannot be certainly identified in thick films except perhaps in the thin edge.

(3) *Immature Schizonts:*

(a) The immature schizonts of all the species of malaria have much the same appearance as in the thin film, except that there is a tendency toward more compactness. There is a division of chromatin material, and the chromatin masses are usually more irregular in appearance in the early stages when they are few in number. These chromatin masses often appear quite dark reddish purple, and are sometimes distinguished with difficulty within the heavy cytoplasm. As the parasite approaches complete segmentation, the nuclear masses appear more regular in shape, and the cytoplasm in the process of dividing may appear somewhat paler and in light wisps, particularly in *P. vivax*. The pigment is gradually collected into fewer and fewer groups as segmentation progresses in *P.*

vivax, but it has a tendency to concentrate late in *P. malariae*. The immature schizonts are probably the ones most often passed over in the thick film by the inexperienced worker. These forms of *P. vivax* and *P. malariae* are (aside from infrequent ring forms) the most difficult on which to differentiate species in the thick film. However, these stages will usually be found accompanied by more readily recognizable forms on which diagnosis may be based. There are times when the immature schizonts of *P. vivax* are definitely larger than those of *P. malariae*, but one cannot depend completely on size. The pink area of Schüffner's stippling around the parasite may help here.

(b) The immature schizont of *P. falciparum*, when found in the peripheral blood, is as a rule even smaller than that of *P. malariae* and very compact. The best differentiating characteristic is the small, rather prominent, dark mass of pigment. Then, too, this form is nearly always found associated with many young ring forms.

(4) *Mature Schizonts.* Mature schizonts of the three species resemble very closely the same stages in the thin film, practically the only difference being the absence of the cell outline and a possible shrinkage of the parasite. The individual merozoites often stand out from the group, each rounded mass of chromatin having its completely differentiated, light-blue zone of cytoplasm. The pigment at this stage is usually clumped in the center or near the edge of the parasite. Often species may be determined by the comparative size of the rounded or oval cluster of merozoites and by the difference in their number. *P. vivax* mature schizonts are not as frequently found as are other stages of this parasite. The mature schizont of *P. malariae* is the stage by which the inexperienced can diagnose this species most readily in the thick film. It regularly has eight merozoites, clearly separated. Frequently only the chromatin divisions and pigment are apparent. It is unusual to find the mature schizont of *P. falciparum* except in severe cases, but in this species the merozoites are smaller than in *P. malariae*, often more numerous than in the other species, and there is a small dark, closely knit mass of pigment.

(5) *Gametocytes.*

(a) In the thick film it is impossible to distinguish definitely the macrogametocytes of either *P. vivax* or *P. malariae* from the rounded or oval mature trophozoites with a single mass of chromatin, rather regular dense cytoplasm, and evenly dispersed pigment. Mature microgametocytes of these two species are more easily determined because of their very large, rounded or stellate nucleus, completely or partially surrounded by a small amount of lightly staining or colorless cytoplasm containing numerous grains of prominent pigment. This nucleus often stains more deeply than it does in the thin films. The parasite is often just a blob of chromatin with a halo of pigment granules. There is no other stage that resembles it closely. Occasionally exflagellation of fully matured microgametocytes takes place on the slide before the thick film dries.

(b) The mature gametocytes of *P. falciparum* (crescents) are easily determined as long as they retain their characteristic elongate or sausage-like shape, though it is often impossible in thick films to differentiate the sexes. In heavier portions of thick films, particularly when the blood dries slowly, mature *P. falciparum* gametocytes assume a rounded shape, a change that would normally take place in the stomach of the mosquito. These forms may be confused with *P. malariae* parasites, but the stages with which they are associated in the blood will aid in diagnosis as will certain characteristics of the form. The pigment is in distinct rodlets often arranged compactly with a clear halo or fringe of blue cytoplasm. Also, a flag or tongue of pinkish- or reddish-staining material is frequently found projecting from one portion, or several parts, of the mass. The nature of this material is not known. It might possibly be the wall of the infected red cell, which wrinkled around the shrunken parasite. At any rate, it is found sometimes in thick films with normally shaped crescents as well as with the rounded or "balled-up" forms. Immature crescents often take an elongated form with sharply pointed ends and with pigment scattered throughout their length. Usually typical crescents will be found in the thinner edges of the films so that species identification is not dependent on the uncertain forms.

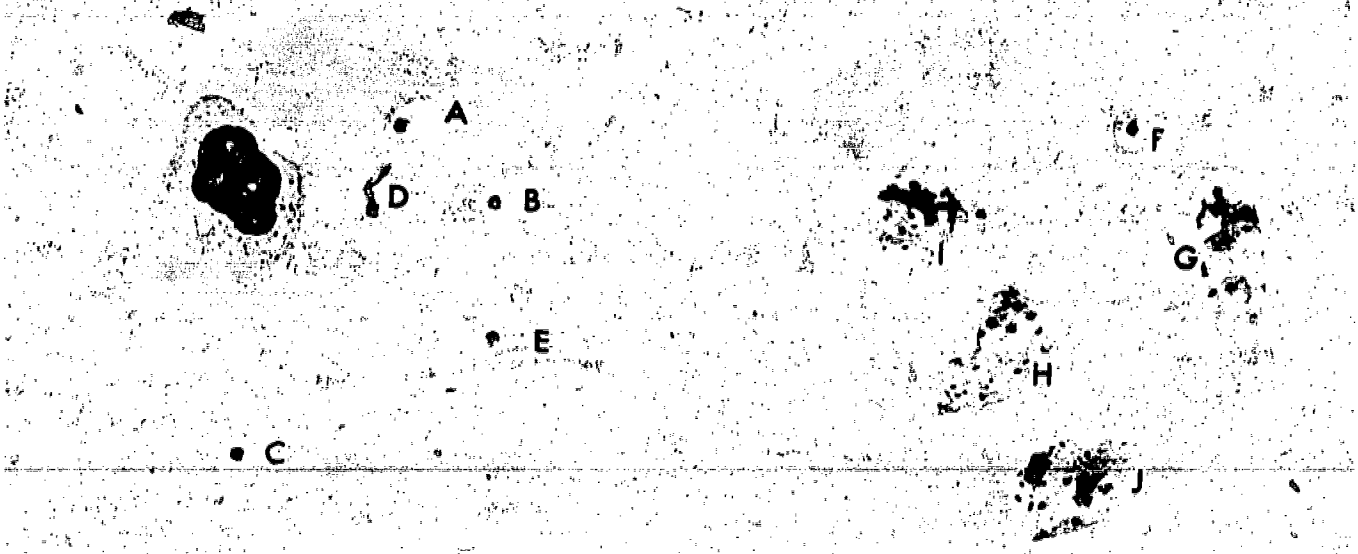
Stage of parasite	*Plasmodium falciparum*	*Plasmodium vivax*	*Plasmodium malariae*	Comments
Small trophozoite (early ring)	Small size rings, with small chromatin dot and delicate, scanty cytoplasm. Frequently rings have double chromatin dots. Tendency toward large number of rings. Many ring forms *with no older stages*—practically certain to be *P. falciparum* infection. (Diagnosis on small number of rings may often be assisted by finding distinguishing gametocyte though this stage is not necessarily present.)	Larger, heavier ring form than same stage in *P. falciparum*, often with variety of cytoplasmic pattern and irregularities in shape. Usually older stages of the parasite can be found also.	Ring is likely to be heavy, with large dot of chromatin and small amount of cytoplasm, which is often "filled in," without a vacuole. Pigment forms early and may appear as single bead or haze in cytoplasm. Rings practically always associated with older forms. The ring phase is brief, so stage is not found as often as older stages.	Ring forms of all the species often not complete circle—may be "swallow" forms, "exclamation mark," "comma" forms, or "interrupted rings." When rings only are present and the number is small, it is practically impossible to differentiate species.
Growing trophozoite	Heavy large ring forms—resemble young rings of *P. vivax*. Sometimes show pigment grains or haze rather clearly in cytoplasm.	Stage usually ameboid in appearance, with large variety of shapes. Cytoplasm frequently fragmented and arranged irregularly in cluster of varying-sized pieces or streamers, about or close to a large chromatin mass. Small yellowish brown pigment granules scattered through parts of the cytoplasm. This is the most characteristic stage of *P. vivax*. Frequently other younger or older stages accompany this one.	Small, usually rounded non-vacuolated forms, "like marbles in a ring." Profuse, round, dark, large-grained pigment. Forms frequently so solid that chromatin seem buried in the mass. This stage and the one that follows are the commonest forms of this parasite seen.	In well-stained films and in films which have been kept for several days before staining, the "ghost" of the enlarged host cell and persistence of Schüffner's stippling or a pink cell area remaining from the stippling, may assist in diagnosis of *P. vivax* parasites of *any stage*, except very young rings.
Large trophozoite	Ring vacuole lost or almost lost. Parasite quite small and compact; cytoplasm often quite pale, irregularly circular or oval. One large chromatin dot. Pigment in blurred mass or very dark clump about size of chromatin. Stage is usually found only when the infection is intense and usually accompanied by numbers of ring-form trophozoites.	Frequently quite solid and dark staining. More or less irregular in outline, possibly with one or more vacuoles. Fine brown pigment scattered throughout the cytoplasm. May be confused with macrogametocyte.	Compact, dark, larger than "growing" stages. Sometimes in thinner portion of the smear spreads to normal size. Profuse, fairly coarse, dark brown pigment—often masking the chromatin. May be confused with "rounded up" *P. falciparum* gametocytes or with gametocytes of *P. malariae*.	On rare occasions evidence of Maurer's spots have been observed in thick films of *P. falciparum*. The infrequently found stages of *P. falciparum* are, of course, more readily found in thick films. Band forms have tendency to become rounded in thick films of *P. malariae*—except perhaps in very thin edge of smear.
Immature schizont	Stage, not often seen, is usually accompanied by large numbers of growing trophozoites when present. Parasite is very small. Contains 2 or more divisions of chromatin and very little cytoplasm (often pale) in which	Irregular or compact clusters of chromatin divisions, often dark reddish-purple in color. Cytoplasm in irregular broken masses and wisps, containing light brown pigment granules which are clumping in spots. Usually accompa-	Much like *P. vivax* of the same stage except that parasites are often smaller with darker, larger pigment granules. Often so compact that internal structure is difficult to define. Usually accompanied by other stages. May be con-	Schizonts are much like thin film forms of same stages—more compact, smaller in thicker portions of smear. This is most difficult stage (except infrequent ring forms) on which to diagnose species.

Table 11-2. Summary of Plasmodium Differentiation on Stained Thick Films. (From Wilcox, 1960)

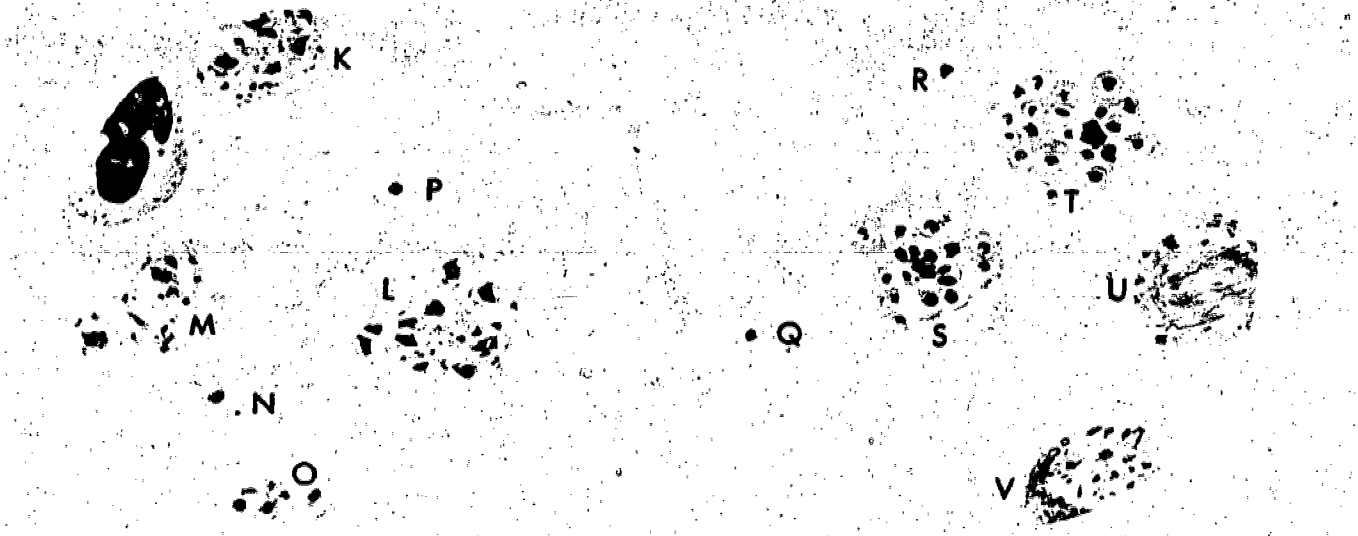
AFM 160-48/TM8-227-2 1 August 1974

Stage of parasite	*Plasmodium falciparum*	*Plasmodium vivax*	*Plasmodium malariae*	Comments
	there is located one or more small, dense blocks of very dark pigment.	nied by other stages. May be confused with same stage of *P. malariae*.	fused with presegmenting schizonts of *P. vivax*.	Schüffner's stippling or a pink infected cell area may identify *P. vivax*.
Mature schizont (segmenter).	Seldom seen except in severe cases. Always associated with many small trophozoites. Usually contains 8 to 24 or more tiny merozoites clustered around a small, very dark, pigment mass.	Usually contains around 16 merozoites (which are individually larger than those of *P. falciparum*) accompanied by a clump of pigment. Stage usually relatively larger than in other species. Nearly always associated with other stages. Not so often found as other stages of *P. vivax*.	Most distinctive stage of malariae in thick film. Often found in large numbers—usually with trophozoites or presegmenting forms or both. About 8 merozoites—each with large chromatin dot and small amount of cytoplasm—may be compact or clearly separated. Frequently the chromatin and pigment only are seen, the chromatin dots being bare and well separated. The dark, heavy pigment is most often concentrated, though sometimes dispersed.	Usually smaller than same stage in thin film. When typical, are among the better stages for species diagnosis.
Young gametocyte	Sometimes long, slender and pointed with pigment scattered to the ends. Usually associated with many trophozoites.	Not easily differentiated from compact growing trophozoites in thick films. When found, is a small, compact, usually rounded, parasite, with one chromatin mass which is often in the center of cytoplasm and frequently has unstained area around chromatin mass. Sex is impossible to determine.	Same as in *P. vivax* except that parasite is even less frequently found and resembles compact trophozoite so closely that positive differentiation is absolutely impossible.	
Mature gametocyte	Differentiation of sex is difficult or impossible. As "crescent" or "sausage" shapes, may be quite diagnostic of species. In thicker portions of smear may take an oval, rounded or somewhat eroded appearance which may be confused with *P. malariae* trophozoites or gametocytes. Oftimes may be distinguished by difference in amount and appearance of pigment or by pink or red "flag" protruding from the edge of the parasite. May be accompanied by ring form trophozoites or appear alone and infrequently. Often appears in "showers".	Macrogametocyte is larger as a rule, than in other species; pigment is light, plentiful, well dispersed through nonvacuolated cytoplasm. *Except in thin edge of film cannot be differentiated from some mature trophozoites of same species.* Microgametocyte often distinguishable as large, dense blob of chromatin (varying from pink to purplish-red) surrounded by or close to a scant amount of pale or colorless cytoplasm in which many pigment granules are more or less evenly dispersed. The chromatin mass is larger than in any other stage, making this form easily distinguished. Other stages of the parasite can usually be found.	As a rule, few in number, somewhat smaller than *P. vivax*, otherwise have the same distinguishing features except that pigment is coarser and darker. May resemble rounded *P. falciparum* gametocytes.	In treated cases the gametocytes often persist beyond the asexual forms. In old cases, rare gametocytes of *P. falciparum* may be found when no other parasites are present.

Table 11-2. Summary of Plasmodium Differentiation on Stained Thick Films. (From Wilcox, 1960)—Continued.



A, B, C—Young rings
D, E—More ameboid young trophozoites
F—Ring stage trophozoite
G—Older ameboid trophozoite in process of development
H—Mature trophozoite
I—Mature trophozoite with chromatin in process of division
J—Young schizont showing early division of chromatin

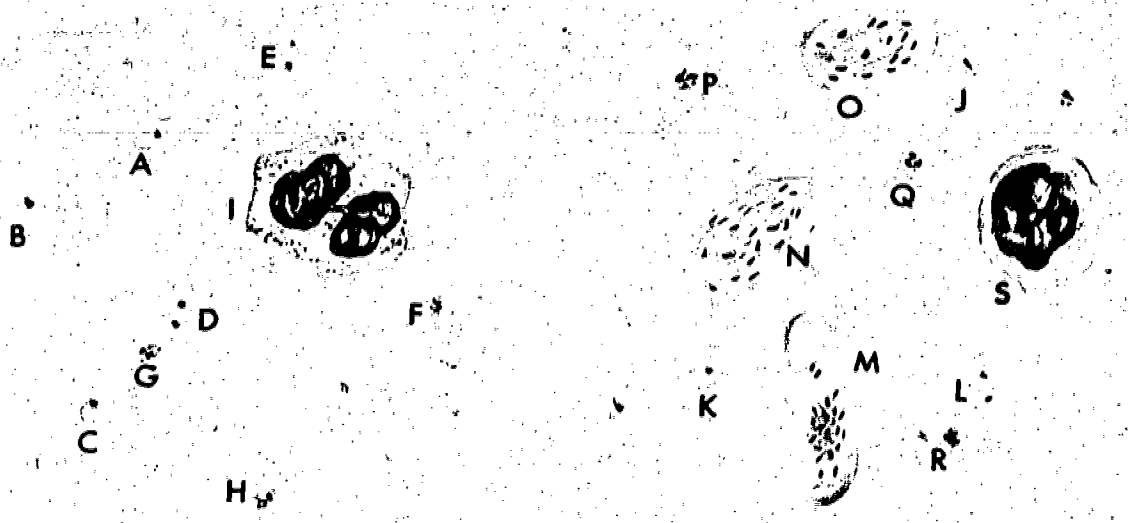


K, L—Maturing schizonts showing several divisions of chromatin
M—Young schizont showing first division of chromatin
N, O, P—Young rings from ruptured schizont
Q, R—Young rings
S, T—Mature schizonts
U—Mature microgametocyte
V—Mature macrogametocyte

Figure 11-5. Plasmodium vivax in Thick Film. Giemsa's stain. ×2,000.

A, B, C—Ring forms
D, E—Developing trophozoites
F—First stage schizont
G, H, I—Blood platelets
J—White blood cell
K, L, M, N—Ring forms
O, P—Platelet
Q—Immature schizont
R—Mature schizont
S—Mature macrogametocyte
T—Mature microgametocyte

Figure 11-6. Plasmodium malariae in Thick Film. Giemsa's stain. ×2,000.



A, B, C—Single nucleus ring forms
D, E—Double nucleus ring forms
F, G, H—Platelets
I—White blood cell
J, K—Single nucleus ring forms
L—Double nucleus ring form
M—Mature macrogametocyte

Figure 11-7. Plasmodium falciparum in Thick Film. Giemsa's stain. ×2,000.

c. **Sources of Confusion or Error.** The inexperienced microscopist may be confused in the examination of thick films by bacteria or dirt from the skin; by dust particles on the slide; by vegetable spores, yeast cells, or fungi from the air; and by bacteria, molds, protozoa, or other contaminants from the distilled water used in staining. Probably the chief source of dirt on the thick film is the improperly cleaned skin. If rigid precautions are taken for the cleanliness of glassware and reagents, and the smear is made from a clean area and properly protected while drying, these disturbing factors will be entirely eliminated or reduced to a negligible minimum. Artifacts that may deceive the inexperienced will be found frequently to lie above the blood plane, or they may be refractile and focus out of the microscopic field unevenly. Sometimes red dots appear in the preparation without any visible cytoplasm. It is possible that some of these are at times remnants of malaria parasites, but one should never call a slide positive on these dots alone. If the dots are evenly distributed throughout the smear, they are more likely to be associated with the cells than if they are found in clumps or only in a part of the smear. If a red-stained coccus from the skin or a small nuclear remnant lies adjacent to a blue-stained particle of cellular substance or fibrin, there may be a resemblance to a parasite. This rarely occurs more than once or twice on a single slide, and numbers of free cocci will usually be found also, whereas free chromatin dots are rarely found in well-stained smears. As one becomes more familiar with the thick film, one is less likely to mistake a foreign body for a parasite. Thick films should not be diagnosed as positive on what appears to be only one parasite. The parasite should be unmistakable, or search should be continued until others are found. If this is impossible, later smears should be made.

11-10. Laboratory Reports:

a. To institute proper therapy, the physician needs a prompt, accurate report of the species of malarial parasite present. Unidentifiable species should be reported with the species report made as soon as possible. This is usually possible even though the species cannot be identified. The species diagnosis can still be made during the first few hours after therapy has been started. This identification has a definite bearing on the prognosis of the case: only infections with *P. falciparum* are likely to cause death, and only the infections with the other species are likely to cause relapses. Therefore, the laboratory report should first show the presence or absence of parasites; this should be followed by species identification, or subsequent specimens should be obtained to give a species diagnosis whenever possible.

b. Two additional reports are sometimes of importance—the forms of the parasites present and the relative or definite total number. The forms of the asexual cycle that are found usually have little bearing; often the clinical course will suggest the forms to be found. The presence of older forms of *P. falciparum* has a definite significance in the acute case. The reporting of gametocytes may have a bearing upon the control of malaria transmission, especially in *P. falciparum*. These forms are found in a significant number of cases when post-treatment or release examinations are made.

c. A simple method of enumeration of parasites, which may not be accurate but which will give a rough estimation of the number of parasites without any special equipment, is as follows: a thick smear of the blood is made at the same time that a white blood count is made. One hundred (or multiples of 100) white cells are counted on the thick film. In the same microscopic fields with these cells the malaria parasites are counted also. Then the parasites per cu mm are calculated by the following formula:

$$\frac{\text{X (Number of parasites per cu mm)}}{\text{White cell count per cubic mm}} = \frac{\text{Number of parasites counted in the same fields with 100 white cells}}{\text{Number of white cells counted (100 in this case)}}$$

Example:

$$\frac{X}{4{,}000} = \frac{1{,}200}{100}$$

$$100\,X = 4{,}800{,}000$$

$$X = 48{,}000/\text{cu mm}$$

11-11. Postmortem Demonstration. It is sometimes necessary to make postmortem examinations in order to find the parasites of *P.*

falciparum. In rare cases of death from *P. vivax* or *P. malariae* infections, parasites can be recovered from the peripheral blood or heart's blood. When death from *P. falciparum* infection is suspected—in all areas where this species is endemic or where the deceased has lived in such an endemic area within the last 6 months—the following procedure should be carried out:

a. **Impression Smears.** Smears or impressions should be taken of the spleen, bone marrow, liver, and gray matter of the brain. A clean slide is pressed tightly against the cut surface of spleen or liver, and the pulp is made to extrude by light pressure on the pieces of tissue held between the fingers. Bone marrow impressions are easily obtained by pressing a sectioned rib between the blades of bone forceps. Smears from the brain are obtained by crushing a small bit of the gray matter of the cortex between two slides and dragging the slides apart as in the making of a thin blood smear. When the smears and thin impressions have been made, they should be fixed and stained, using the same methods as for thin blood films.

b. **Fixation of Tissues.** Zenker's fixative is recommended for most tissues to be examined for protozoa. Formalin may also be used if the precipitate, which may be mistaken for malarial pigment, is first removed from the fixed tissue with alcoholic picric acid. Only the Zenker's fixation method is presented in this manual.

(1) *Zenker's Fixative.*

Potassium bichromate	2.5 g
Bichloride of mercury (corrosive sublimate)	5.0 g
Distilled water	100.0 ml
Glacial acetic acid	5.0 ml

(a) Dissolve bichromate and bichloride in water. Heat to dissolve completely.

(b) Do not mix the acetic acid with the bichromate-bichloride solution until the fixing fluid is to be used. It will produce changes in the chrome salt if added when prepared and allowed to stand.

(2) *Procedure For Fixation.*

(a) Mix the acetic acid with the bichromate-bichloride solution just before placing the tissues in the solution.

(b) Remove the tissues from the Zenker's fixative within 24 hours. Overnight is usually adequate.

(c) After fixation, wash the tissues in running tap water for 24 hours.

(d) Transfer the tissues to 70% ethyl alcohol tinted yellow with iodine. Tissues can be kept in the alcohol indefinitely.

c. **Staining Tissue Sections For Malaria Parasites.** Only glass or neutral plastic, never metal, containers should be used throughout the procedure. Slides may be carried through staining in (glass) racks, but it is recommended that they be handled individually during differentiation, for best result. The following steps are recommended for paraffin-imbedded sections:

(1) Place in xylene for 2 to 5 minutes; then change to fresh xylene for 2 to 5 minutes.

(2) Place in absolute ethanol for 2 minutes; then change to fresh ethanol for 2 minutes.

(3) Transfer to 95% ethanol for 1 minute. **NOTE:** For Zenker-fixed tissues, remove mercury precipitate with iodine, then clear in 70% ethanol.

(4) Hydrate in each dilution of ethanol, 80%, 70%, 50%, for 1 minute.

(5) Rinse in distilled water for 15 seconds.

(6) Neutralize in distilled, deionized, phosphate-buffered water (pH 7.2) from paragraph 10-2b(1)(a)*2* for 30 minutes.

(7) Stain sections in working Giemsa solution (made with 3.0 ml Giemsa stock [paragraph 10-2b(1)(a)*3*] in 100 ml of distilled, deionized, buffered water (pH 7.2) from paragraph 10-2b(1)(a)*2* for 8 hours or overnight.

(8) Rinse quickly in buffered water to remove excess stain. **NOTE:** Total time for differentiation (steps 9 and 10) should be 90 seconds.

(9) Differentiate in 0.2% acetic acid (0.2 ml of glacial acetic acid in 100 ml of distilled water) for 60 seconds.

(10) Transfer immediately to absolute ethanol for 15 seconds; then quickly change to a second absolute ethanol for 15 seconds.

(11) Rinse immediately in xylene first for 1 minute, moving constantly, then transfer to second xylene and allow to stand for 2 to 3 minutes.

(12) Mount in Permount or other neutral mounting medium.

Chapter 12
TRYPANOSOME AND LEISHMANIA PARASITES

12-1. Introduction. The trypanosomes and leishmanias belong to the large family, Trypanosomatidae. Members of that family are simple flagellates, which have only a single anteriorly directed flagellum. The members of the family differ widely in their parasitism; however, those that infect humans parasitize blood and tissues and utilize invertebrates (arthropods) as intermediate hosts. Those infecting humans belong to two genera: *Trypanosoma* and *Leishmania*. Four species of the genus *Trypanosoma* infect humans (table 12-1); two (*T. gambiense* and *T. rhodesiense*) are found in Africa and the other two (*T. cruzi* and *T. rangeli*) are found in South America. The *Leishmania* parasites, *L. donovani*, *L. tropica*, and *L. braziliensis* (table 12-2), are more widespread and are found in many of the tropical, subtropical, arid, and semiarid areas of the world.

12-2. Morphology. Four morphological stages may be found during the life cycles of blood and tissue flagellates. The stages vary with species. The morphological stages found in the vertebrate hosts are different from the morphological stages found in the invertebrate hosts. Also, some forms are found within cells and others are found outside of cells. The different stages are illustrated in figure 12-1.

a. **Amastigote (Leishmania-Form).** This is the usual intracellular stage. The body is round or ovoid and has no flagellum or undulating membrane. It is 3-4 microns long by 2 to 3 microns wide.

b. **Promastigote (Leptomonas-Form).** The body is elongated and somewhat flattened. The flagellum arises from the anterior tip of the body and there is no undulating membrane. This stage is usually found in the invertebrate host or in cultures. It can measure from 8 to 35 microns in length.

c. **Epimastigote (Crithidia-Form).** At this stage an undulating membrane and a flagellum are present. The kinetoplast (term for the blepharoplast and the parabasal body together) is just anterior to the nucleus. This stage is also usually found in the invertebrate host or in cultures. It can measure from 15 to 20 microns in length.

d. **Trypomastigote (Trypanosome-Form).** This stage has an undulating membrane and flagellum as does the epimastigote but the kinetoplast is posterior to the nucleus; therefore the flagellum originates from a position that is posterior to the nucleus. This is the form that is usually found in blood smears from patients infected with one of the *Trypanosoma* sp. parasites.

12-3. Trypanosoma Gambiense and Trypanosoma Rhodesiense. These parasites produce a disease in humans commonly called African sleeping sickness or trypanosomiasis. Since the two species are very similar, they are considered together. *Trypanosoma gambiense* is found in West Africa and *T. rhodesiense* in East Africa.

a. **Morphology.** The unstained trypomastigote appears as a colorless, slender body, 15 to 30 microns long. It is actively motile in fresh blood, but it makes little headway; the undulating membrane and flagellum can be visible. Stained with Wright's or Giemsa's stain, the nucleus is red and the cytoplasm pale blue; the kinetoplast stains purple. The relative position of these two bodies is that of a typical trypomastigote (figure 12-1). The flagellum appears as a fine red thread along the edge of the undulating membrane, free at the anterior end. These trypomastigotes are polymorphic, some showing altered shapes and having no free flagellum. The two species are indistinguishable as seen in human blood.

b. **Laboratory Diagnosis.** The trypanosomes can be recovered from lymph glands, blood, or spinal fluid. Early in the disease, best results are obtained by examination of material obtained from an enlarged lymph gland. Later the parasites can be readily recovered from the blood and occasionally from the spinal fluid. Cultures and animal inoculation can be employed when direct examination fails to confirm the diagnosis. However, cultures of *Trypanosoma gambiense* and *T. rhodesiense* are less satisfactory than of the other hemoflagellates.

12-4. Trypanosoma Cruzi. This parasite causes an illness known as Chagas' disease. It is most prevalent in South America with cases also reported from parts of Mexico and Central America. A few questionable cases have been reported from Texas. Man is only one of many mammals that are susceptible to the parasite. Many domestic and wild animals are reservoirs for this organism. *Trypanosoma cruzi* ordinarily requires a vector for transmission. However, infants can acquire the parasite by prenatal infection, and the organism is occasionally transmitted by blood transfusion.

a. **Morphology.** *T. cruzi* is found in the amastigote stage and the trypomastigote stage in vertebrate hosts. The amastigotes are found inside the hosts cells, especially visceral reticuloendothelial cells, myocardial tissue, and endocrine gland tissue. The trypomastigotes are found in the circulating blood. *T. cruzi* in the trypomastigote form is morphologically similar to the other trypanosomes, but it is different enough for you to identify. Sometimes the trypomastigote is a delicately spindle-shaped organism, about 20 microns long with only two or three curves in the narrow undulating membrane and a short, free flagellum. Other times, the organism appears as a considerably shorter, broader, more or less C-shaped organism, with or without a free flagellum. Regardless of the morphology of the trypomastigotes present they can be identified as *T. cruzi* by the large posterior kinetoplast (figure 12-2) that appears even larger in diameter than the posterior end of the parasite and the fact that no dividing forms are seen as may be seen with other trypomastigotes. The posterior end of the parasite appears swollen around the kinetoplast. In its intracellular phase, *T. cruzi* is a typical amastigote oval in shape, 1.5 to 5 microns in length, with large nucleus and a deeply staining parabasal body (figure 12-3). In reticuloendothelial cells, you cannot distinguish *T. cruzi* from *Leishmania*. A distinguishing feature to remember is that *T. cruzi* invades heart muscle cells and nerve tissue cells as an amastigote. *Leishmania* does not invade these tissues.

Table 12-1. Comparative Table of Trypanosomes.

	Common name of disease produced	Geographical distribution	Location of organisms in vertebrate hosts	Laboratory diagnosis
Trypanosoma gambiense	African sleeping sickness or more specifically Gambian sleeping sickness.	Wide area in tropical West Africa from Senegal to Luanda extending inland along the large rivers, particularly the Congo and Niger. Newer areas include Southern Sudan, Uganda, and the Great Lakes region.	Scarce in blood, usually more abundant in juice of enlarged lymph glands. Also occur in spleen which is often enlarged. In late stage of the disease they may be found in the cerebrospinal fluid and within tissues of central nervous system.	Fresh specimen, thin or thick-stained smear, hanging drop, centrifugalization, animal inoculation, culture, spinal fluid examination, or biopsy of lymphatics.
Trypanosoma rhodesiense	African sleeping sickness or more specifically Rhodesian sleeping sickness.	Eastern Africa from Kenya to southern Rhodesia and northern Mozambique, and inland across Tanganyika to Uganda and eastern Congo.	Scarce in blood, usually more abundant in juice of enlarged lymph glands. Also occur in spleen which is often enlarged. In late stage of the disease they may be found in the cerebrospinal fluid and within tissues of central nervous system.	Same as for T. gambiense, the method of choice usually depending upon the stage of the disease.
Trypanosoma cruzi	Chagas' disease.	Extends from pampas of Argentina to southwestern United States, few cases occur in Mexico and Central America, common in many parts of Venezuela, French Guiana, Brazil, Bolivia, Chile, Paraguay, and Uruguay.	Organism invades cells of the heart, voluntary muscles, various glands. Organisms usually scarce in the circulating blood.	Same as for T. gambiense plus xenodiagnosis. The method of choice depends upon the stage of the disease.
Trypanosoma rangeli	No known disease.	Much the same as T. cruzi.	Found only in blood.	Fresh specimen, thin or thick blood smear, hanging drop, culture and xenodiagnosis.

Table 12-2. Comparative Table of the Leishmania.

Parasites	Common name of disease produced	Geographical distribution	Localization of the parasites	Laboratory diagnosis
Leishmania donovani	Kala-azar, or visceral leishmaniasis.	In the old world it is common in India, North China, and the Sudan; around the Mediterranean and in western and middle Asia infantile type of disease occurs. It is also widespread in South America (parts of Brazil, Argentina, Paraguay, Bolivia, and Venezuela).	Leishman-Donovan bodies are widely distributed within the body, but special habitat seems to be large endothelial cells of the blood vessels and lymphatics. Abundant in spleen, liver, bone marrow, and sometimes found in circulating blood within the monocytes.	Liver or spleen puncture, biopsy of lymph glands, with culture of material recovered, culture of blood, and direct examination of stained blood smears. Animal inoculation with either type of specimen.
Leishmania tropica	Oriental sore, or cutaneous leishmaniasis.	Common in tropical cities of eastern Mediterranean.	Parasites are found in the dermal tissues of infected sores where increased numbers of large monocytes and other reticuloendothelial cells are literally packed with them.	Culture and direct examination of material from sores or biopsy specimens from edge of sores.
Leishmania braziliensis	Espundia, or South American muco-cutaneous leishmaniasis.	Occurs over large area of tropical America from Yucatan in Mexico through Central America into northern Argentina.	Initially parasites are localized in cutaneous sores as in the case of L. tropica. As disease advances they localize in mucous membranes of nose and pharynx.	Intradermal test, direct examination of exudates or biopsy from lesions, and cultures.

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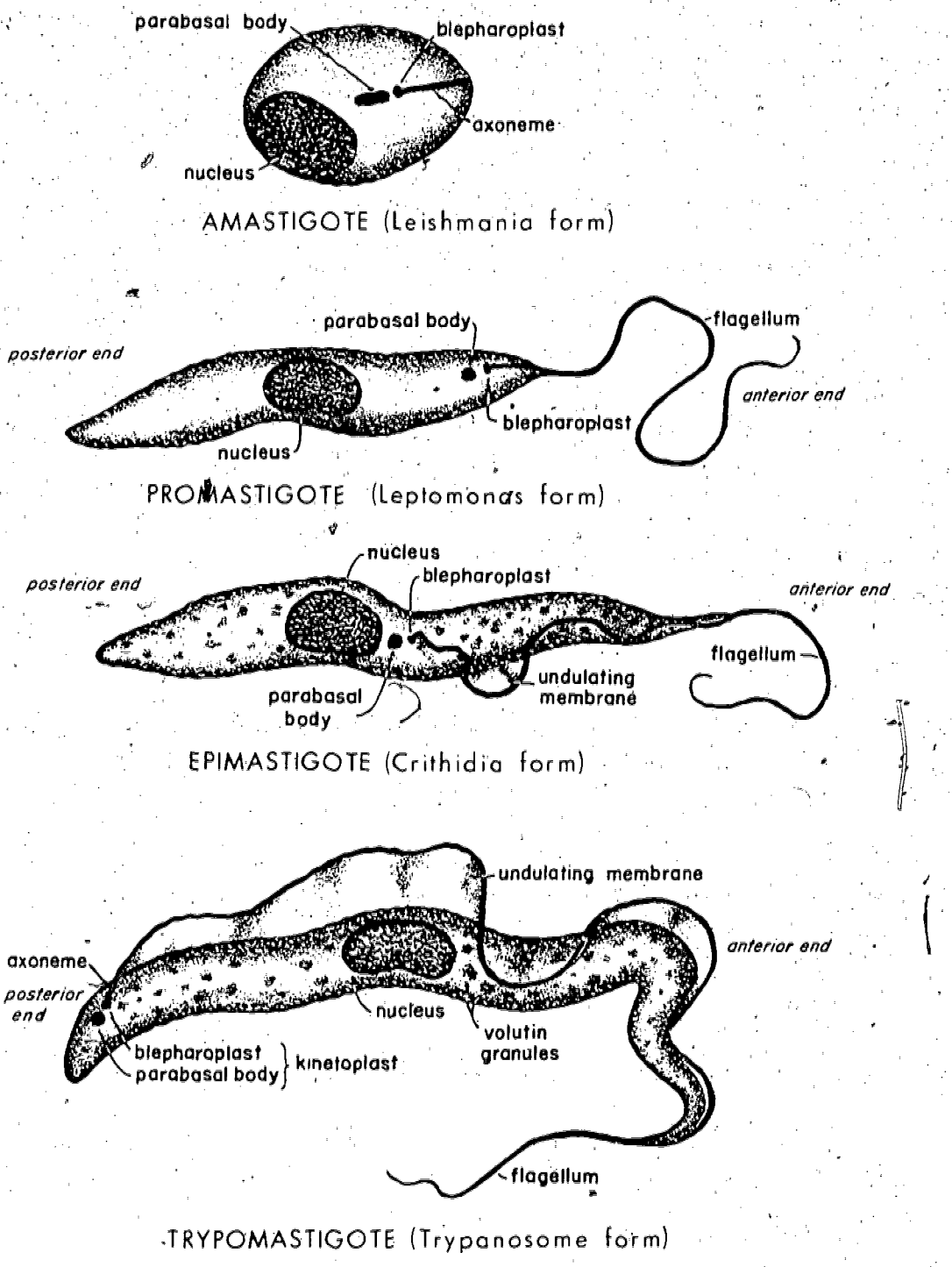

Figure 12-1. Morphological Forms of Hemoflagellates. Giemsa's stain. Greatly enlarged.

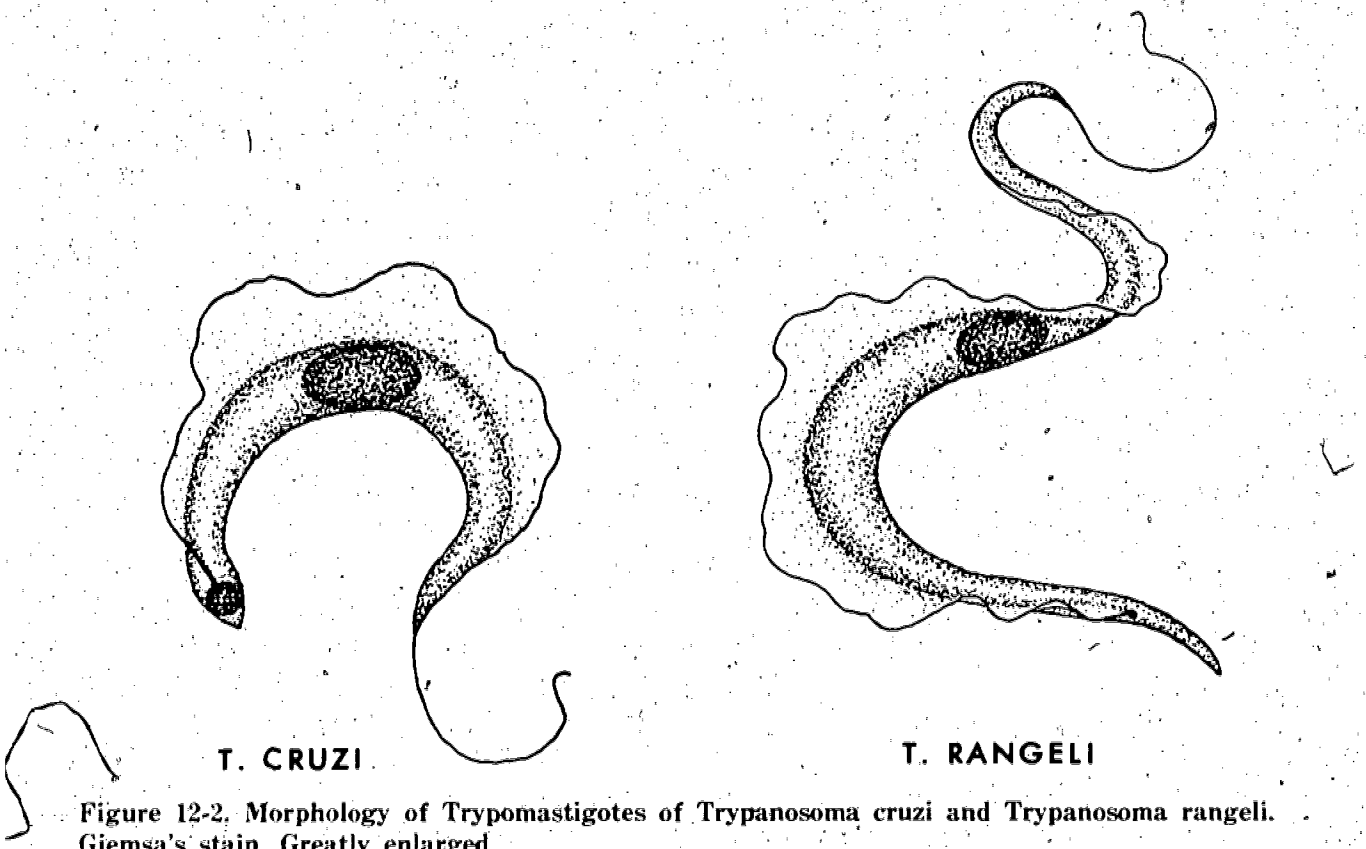

Figure 12-2. Morphology of Trypomastigotes of Trypanosoma cruzi and Trypanosoma rangeli. Giemsa's stain. Greatly enlarged.

b. **Laboratory Diagnosis.** Laboratory diagnosis is made by finding the typical trypomastigotes of *T. cruzi* in blood films during febrile periods. Aspirates of spleen, lymph node, liver, or bone marrow are the materials of choice for finding the intracellular amastigotes in living patients. Inoculate NNN media at the same time the smears are made. Quite often you can culture the organisms in this manner even if there are too few to find on a stained smear. Complement fixation is sometimes a satisfactory method of diagnosis when dealing with chronic cases. In endemic areas, xenodiagnosis is frequently employed. Animal inoculation procedures will pick up a large number of positives, but they are probably no more effective than cultures on NNN medium. Examine histological sections of postmortem heart muscle in suspected cases as that is the most likely source of the intracellular amastigotes.

12-5. **Trypanosoma rangeli.** *Trypanosoma rangeli* is not known to be pathogenic; however, it is found in some of the same areas as is *T. cruzi*. Therefore, it is important that the two be differentiated. *T. rangeli* is found in many of the same areas of northern South America and Central America as *T. cruzi*. *T. rangeli* is also similar to *T. cruzi* in that it employs many mammals in addition to humans as hosts.

a. **Morphology.** In human blood films the parasite is a trypomastigote (figure 12-2). It averages about 31 microns long, and it is quite slender. The extremities gradually taper to fine points. Unlike *T. cruzi* the kinetoplast is small and some distance from the posterior end. The nucleus is in the anterior half of the organism. The undulating membrane is broad and its margin usually has numerous undulations.

b. **Laboratory Diagnosis.** The trypomastigote is the only form that has been found in human infections. The parasite is difficult to find on direct blood smears because there are never very many in the circulating blood. The best way to find *T. rangeli* is to culture the

blood of suspected patients on NNN medium. Xenodiagnosis is another effective method of isolating the organism.

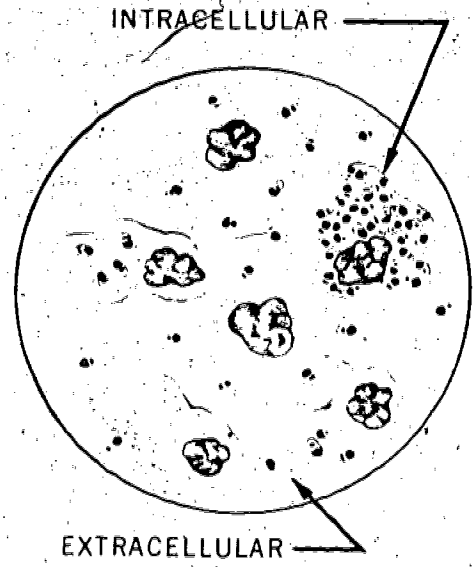

Figure 12-3. Intracellular and Extracellular Amastigotes. Giemsa's stain. ×2,000.

12-6. Leishmania donovani. The disease known as kala-azar or visceral leishmaniasis is caused by *Leishmania donovani*. It is more widely distributed than the other species of *Leishmania*. The parasite is found in many regions of Asia, Africa, Europe, South America, and Central America. *L. donovani* is found in many mammals in addition to humans.

a. **Morphology.** *L. donovani* exists in humans and other mammals only as intracellular amastigotes (figure 12-3). On rare occasions the parasites are present in small numbers in the blood, but they are usually found in the internal organs in reticuloendothelial cells. They have to be stained to be recognized. When stained with Wright's or Giemsa's stain, they have a large red nucleus and a purple rod-shaped kinetoplast that is perpendicular to the nucleus. The *Leishmania* amastigote measures 2-5 microns by 1.5-2.5 microns and has no flagellum.

b. **Laboratory Diagnosis.** The most reliable means of laboratory diagnosis is the actual demonstration of the organism. The specimen of choice is a Giemsa-stained bone marrow specimen. The organisms can also be found in spleen, liver, and lymph node biopsy impression smears or tissue sections, but these procedures pose a greater danger to the patient and yield no better results. The organisms are rarely observed in peripheral blood smears; however, if no other specimen can be obtained, prepare a Giemsa-stained smear from the buffy coat. The organisms can be cultured in NNN medium when inoculated with tissue homogenates of the above organs or occasionally from blood. However, the culture procedure is of limited diagnostic value because of the long time required to grow the organisms. Hamsters are very susceptible to the organism, and can be infected by intraperitoneal inoculation with the same materials used to inoculate the NNN culture medium. Infecting hamsters has the limitation that their use takes too long. Napier's aldehyde test of serum can be used as a presumptive screening test, but do not use it alone to rule out infection in suspected cases. *Leishmania* and *Trypanosoma* amastigotes can be confused with *Toxoplasma gondii* and *Histoplasma capsulatum* because all of them will be intracellular at times and are of similar size. Special stains are available to aid in the differentiation of *H. capsulatum*. Also, *Toxoplasma* does not have the kinetoplast that characterizes the *Trypanosoma* and *Leishmania* organisms and the *Histoplasma* organisms do not demonstrate a nucleus; they have a central dark-staining mass surrounded by a clear zone, but there is no true nucleus.

12-7. Leishmania tropica. The disease caused by *Leishmania tropica* is "Oriental Sore" or cutaneous leishmaniasis. It is frequently encountered in man, dogs, and rodents in the Middle East, and it is endemic in Mexico, Africa, India, and other parts of Asia.

a. **Morphology.** *Leishmania tropica* is morphologically identical to *L. donovani* and *L. braziliensis* as well as the amastigote of *T. cruzi*. However, it does produce a characteristic skin lesion that begins as a small, red papule, which enlarges to an inch or more in diameter. The lesion persists for several months to a year or more.

b. **Laboratory Diagnosis.** Parasites of *L. tropica* are found in macrophages around the margins of the necrotic lesions. Prepare Giemsa-stained smears from material scraped from the

inner margin of the sore or from material obtained by injecting and withdrawing a small amount of saline from the induration surrounding the lesion. Such specimens should be collected only by a medical officer. If the initial smears are negative, the only other thing to do is to collect another specimen and culture it on NNN medium.

12-8. Leishmania braziliensis. The mutilating disease known as South American mucocutaneous leishmaniasis, or espundia, is caused by *Leishmania braziliensis*. Initially the parasites produce localized cutaneous sores, as does *L. tropica*. As the disease progresses, the organisms localize in mucous membranes of the nose and pharynx. In the later stages of the disease, secondary foci occur in the nasal cavities, mouth, and pharynx. The disease can last for several years and can be fatal. *Leishmania braziliensis* occurs in large areas of tropical America from Yucatan in Mexico through Central America into northern Argentina. The amastigotes of *L. braziliensis* appear morphologically identical to those of the other *Leishmania* and *Trypanosoma*. The parasites can be found in Giemsa-stained preparations of exudates or impression smears of biopsies from involved areas. Also, cultures in NNN medium should be set up using material from the edge of the lesion for the inoculum. Montenegro's skin test provokes an allergic skin reaction in persons infected with *L. braziliensis*. The reaction can be used to differentiate *L. braziliensis* lesions from similar fungal lesions, such as those seen in sporotrichosis. The antigen for the test has to be prepared from cultured *L. braziliensis* organisms, and it is difficult to obtain except in endemic areas. This parasite, like *L. tropica* is very rarely found in circulating blood.

12-9. Laboratory Procedures:

a. **Direct Observation:**

(1) Place a small drop of the material to be examined on a glass slide. Any fresh material that is liquid, such as blood, spinal fluid, peritoneal fluid, or exudates from various sources is suitable.

(2) Cover the material with a coverslip. The amount of specimen should be just enough to barely fill the area under the coverslip.

(3) Examine the preparation systematically using the high-dry magnification and reduced light. When blood is examined, the preparation should be approximately two red blood cells thick. Thicker preparations make it difficult to see the parasites, and thinner preparations reduce the chance of finding the parasites in light infections.

b. **Stained Films.** Films can be stained with either Wright's stain or Giemsa's stain. However, just as in the case of malaria, the best results are obtained with the standard long method of Giemsa's staining. Blood, bone marrow, tissue, cerebrospinal fluid, peritoneal fluid, and almost any exudate can be used to prepare films for staining. The intracellular amastigotes can be as small as 2 microns; therefore, it is necessary to use oil-immersion magnification to study its morphology.

(1) *Blood Films.* Two types of blood films can be prepared: a thin film consisting of a single layer of cells, and a thick film consisting of many layers of cells. The thin film is most useful for a study of the morphology of the parasite and also for identification; it is useful for the detection of parasites only when they are relatively numerous. The thick film contains many more parasites per unit area making them the procedure of choice to detect the organisms much more readily in blood. And in some cases the parasites can be identified as to species from stained thick films. Detailed procedures for the preparation of stained blood films are outlined in paragraph 11-5.

(2) *Bone Marrow Films.* Stained thin films prepared from bone marrow are the specimens of choice in *Leishmania donovani* infections. They are also useful in moderate to heavy infections of trypanosomes. This type of thin film is prepared and stained in the same manner as given for blood in paragraph 11-5. Stained thick films of bone marrow are unsatisfactory because the density of nucleated cells and the high fat content frequently cause such films to come off the slides.

(3) *Tissue Impression Smear.* The tissues used to prepare impression smears can be biopsy, surgical, or autopsy specimens. The method provides a rapid method of diagnosing infections caused by many of the protozoa that invade tissues. Frequently the parasites are

more easily identified in such smears than in histological sections.

(a) To prepare the smears, touch a freshly cut surface of the tissue to a glass slide and then lift the tissue off; repeat that step until an area of 2 or 3 square cm has been covered with a thin film of cells. When properly done, a thin layer of cells adheres each time the tissue is touched to the slide. Avoid streaking the tissue along the slide; cells and parasites are distorted when the specimen is streaked.

(b) Allow the smear to dry at room temperature or warm slightly in a 37C incubator.

(c) Stain the smear by the Giesma method as outlined in paragraph 11-5 for thin blood films.

(4) *Cerebrospinal, Peritoneal, and Other Fluid Films.* When only a small amount of fluid is available, use it as is without centrifugation. However, when sufficient fluid is obtained, concentrate the cellular elements by centrifuging the fluid at 1,500 rpm for 15 minutes. Remove the supernatant fluid with a sterile pipet and make thin films with the sediment. Allow the film to thoroughly air-dry or warm slightly in a 37C incubator. Stain the films in the same manner as given for thin blood films in paragraph 11-5.

(5) *Exudate Films.* Films of exudates are prepared and stained in much the manner as thick and thin blood films. The procedures are outlined in paragraph 11-5. Examine the smear as if it were a blood film.

c. **Cultures.** All of the species of *Leishmania* and *Trypanosoma* that infect humans can be grown on culture media. *Trypanosoma cruzi*, *T. rangeli*, and all of *Leishmania* grow and reproduce very well on NNN medium. The specialized medium of Weinman gives the best results. However, the probability of obtaining a positive culture depends on the density of the parasites, the type of specimen, the type of culture medium employed, the species involved, and the freedom from contaminants such as bacteria and fungi. With the possibility that the parasites will not grow, do not rely on culture alone. Always perform other procedures such as the examination of fresh or stained specimens as well as serological tests and animal inoculation where available.

(1) *NNN Medium (Novy and MacNeal).*

Bacto agar	14 g
Sodium chloride	6 g
Distilled water	900 ml
Rabbit blood (sterile, defibrinated)	qs.

(a) Mix the agar and sodium chloride with the distilled water and heat to boiling.

(b) Dispense 5 ml quantities to large test tubes.

(c) Sterilize at 15 pounds pressure (121C) for 15 minutes in an autoclave.

(d) Cool to 45-50C, and add 2 ml of sterile defibrinated rabbit blood.

(e) Mix the blood well with the medium and place in a slanted position to cool.

(f) Incubate overnight at 37C, and check for sterility. Store under refrigeration.

(2) *Weinman's Medium.*

Nutrient agar, 1.5%, pH 7.3	31.0 g
Agar	5.0 g
Distilled water	1000.0 ml
Inactivated human plasma (sterile)	qs.
Human red blood cells (sterile)	qs

(a) Mix the nutrient agar, agar, and the distilled water and heat to boiling.

(b) Dispense in 6 ml amounts in large screw-capped test tubes.

(c) Sterilize at 15 pounds pressure (121C) for 15 minutes.

(d) The blood required for the plasma and red cells can be uncontaminated, expired, blood-bank blood or freshly drawn, citrated whole blood. Approximately 2 ml per tube is needed. The plasma of some individuals inhibits the growth of the trypanosomes. To reduce the possibility of this occurring, the plasma has to be inactivated and the cells thoroughly washed.

(e) Separate the cells and plasma and inactivate the plasma by heating for 30 minutes at 56C. Maintain sterile conditions throughout.

(f) Wash the red cells, as aseptically as possible, three times in three or more volumes of sterile 0.9 percent saline.

(g) Reconstitute the blood by mixing equal volumes of inactivated plasma and washed red cells.

(h) Cool the tubed medium to 45-50C, and add 2 ml of the reconstituted blood.

(i) Mix the blood well with the medium and place in a slanted position to cool.

(j) Incubate overnight at 37C, and check for sterility. Store under refrigeration.

(3) *Procedure for Liquid Specimens:*

(a) Introduce 1-2 ml of specimen into the culture tube maintaining sterility.

(b) Flame the mouth of the tube and replace the cap.

(c) Set up at least three tubes when there is sufficient specimen.

(d) Incubate the cultures at room temperature (approximately 24C).

(e) Check the culture at weekly intervals. Use aseptic precautions to remove a loopful of culture fluid after mixing by tapping tube. Place the material on a glass slide and add a coverslip. Examine the entire preparation systematically with the high-dry magnification of the microscope. Look for motile flagellates, which can occur singly or in small to large clumps or rosettes. In heavy to moderate infections the parasites can be found within 2 weeks; whereas, in light infections it might be as long as 2 months before any organisms can be found. Contamination with bacteria or fungi will inhibit growth of the parasites and is the greatest cause of failure with these cultures.

(4) *Procedure for Tissue Specimens:*

(a) Transfer 1-2 ml of sterile Locke's solution [paragraph 6-2a(2)(a)] to the culture tube with a sterile pipet. Employ aseptic technique during every step of the culture procedure.

(b) Transfer uncontaminated tissue specimens directly to the Locke's solution overlay.

(c) If the material to be cultured has been contaminated, add 20 units of penicillin and 40 units of streptomycin to each milliliter of Locke's solution used; then add the specimen to be cultured.

(d) Incubate and examine the culture as directed for liquid specimens in paragraphs 12-9c(3)(d) and 12-9c(3)(e).

d. **Animal Inoculation.** Animal inoculation is an excellent tool for isolating several organisms when such facilities are available, but the technique varies with the species of parasite suspected. However, it does take days or sometimes even weeks for the parasites to grow and reproduce in the animals to the point that a sufficient number is present for a diagnosis to be made.

(1) *Collection and Preparation of Specimens:*

(a) Employ the best technique possible when collecting specimens to avoid contamination with bacteria and fungi. Also, wear gloves throughout the procedure.

(b) Use sterile citrate or heparin tubes when collecting liquid specimens to prevent clotting.

(c) Prepare fine suspension of any tissue or coagulated specimen that is to be used for animal inoculation.

1. Snip larger tissue specimens into pieces no larger than 3 mm with sterile scissors.

2. Place tissue in sterile tissue grinder or mortar and add sufficient sterile 0.9 percent saline to make a mixture of approximately 1 part of tissue to 9 parts of saline.

3. Grind the mixture until it is a suspension of such fine texture that it will pass through a hypodermic needle.

(2) *Inoculation Procedure:*

(a) Cleanse the abdominal skin of each animal to be inoculated.

(b) To make mice more susceptible to the organisms, start with young males (not adults) and inject each one with 5 mg of cortisone subcutaneously.

(c) Inoculate 1.0 ml of the specimen intraperitoneally into a 15 g mouse. Inoculate correspondingly larger inocula in rats, hamsters, guinea pigs, etc.

(d) Sterilize all instruments and utensils used for preparing and inoculating the specimens. Sterilize them before washing.

(3) *Special Considerations for Each Hemoflagellate:*

(a) *Leishmania donorani. L. donorani* can be isolated by inoculating bone marrow, blood, spinal fluid, spleen, liver, and lymph node tissue into young hamsters. Bone marrow is the specimen of choice. Usually animal inoculation is not used to make the primary diagnosis, but it is used to confirm the diagnosis. The reason is that it can take 2 months or longer before the animals show signs of the disease. At that time the amastigotes can be found in the liver, spleen, bone marrow, and other specimens by

preparing bone marrow smears or tissue impression smears of the other tissues.

(b) *Trypanosoma cruzi and T. rangeli.* *T. cruzi* can be isolated from blood, heart muscle, bone marrow, spleen, liver, lymph node, and occasionally other tissues. Blood is most successfully used during the acute phase and during febrile episodes. Bone marrow, spleen, and lymph node tissues are frequently positive when the blood is negative. Heart muscle is the specimen of choice for postmortem examinations. Kittens, puppies, hamsters, guinea pigs, rats, and mice are susceptible to the organism. Inasmuch as mice are readily available and as susceptible to infection as the other animals, they are recommended. *T. rangeli* is usually present in small numbers and can be recovered from blood more regularly than other specimens. Most laboratory animals that are susceptible to *T. cruzi* are also susceptible to *T. rangeli.*

(c) *Trypanosoma gambiense and T. rhodesiense.* Both of the African trypanosomes can be isolated by inoculating blood (during early stages of infection), or lymph node tissue or spinal fluid (during later stages of infection) into guinea pigs, rats, and mice. Animals that are going to become positive usually do so within about 2 weeks. Those that are still negative after 4 weeks rarely become positive.

(d) *Leishmania tropica and L. braziliensis.* *L. tropica* and *L. braziliensis* produce skin lesions and mucocutaneous lesions in humans. The amount of material that is obtained for examination is very small and is usually best utilized for direct examination and culture on NNN medium.

(4) *Examination of Inoculated Animals:*

(a) *Leishmania donovani.* When animal inoculation is used to isolate *L. donovani*, sacrifice the hamsters after 3 months if they show no signs of disease. Sacrifice them sooner when they show symptoms of leishmaniasis. Prepare impression smears of a cut surface of the spleen, liver, and lymph nodes. Prepare a thin smear of a specimen of bone marrow and prepare stained histological sections of the same organs. Examine the smears and sections for the typical intracellular amastigotes.

(b) *Trypanosoma cruzi.* After 4 days check the mice every 2 days by clipping the tip of the tail and removing a small drop of blood to a microscope slide. Coverslip the blood and examine the entire area at high-dry magnification. If none of the mice become positive within 3 weeks, sacrifice them by withdrawing as much blood as possible from the heart, using a sterile 24 gage needle and 1 ml tuberculin syringe, and culture on NNN medium [paragraph 12-9c(1)]. Check the cultures as outlined in paragraph 12-9c(3)(c). Prepare stained histological sections of the heart muscles and examine for intracellular amastigotes.

(c) *Trypansoma rangeli.* Examine the blood of the mice as outlined for *T. cruzi.* If they are still negative after 3 weeks, withdraw their blood as for *T. cruzi* and inoculate NNN medium [paragraph 12-9c(1)]. It is not necessary to prepare histological sections when looking for *T. rangeli* because they are not known to invade tissues; however, *T. rangeli* and *T. cruzi* are found in the same areas so follow the case up just as for *T. cruzi* to prevent its being overlooked.

(d) *Trypanosoma gambiense* and *T. rhodesiense.* Clip the ear or tail of the animals to obtain a small drop of blood. Coverslip the drop of blood on a microscope slide and examine the entire area at high-dry magnification for motile trypanosomes. If none of the animals become positive in 4 weeks, sacrifice them by withdrawing as much blood as possible from the heart. Inoculate several tubes of Weinman's medium [paragraph 12-9c(2)]. Examine and follow the culture as outlined in paragraph 12-9c(3).

e. **Xenodiagnosis:**

(1) Xenodiagnosis is used extensively in areas where *Trypanosoma cruzi* and *Trypanosoma rangeli* are endemic. It is similar to the culture method except that the flagellates multiply in the insect vector (triatomid bug) instead of in a test tube. The procedure offers the advantages of no contamination with bacteria or fungi, no refrigeration required, and reduced chance of accidental infection.

(2) Clean triatomid bugs raised in the laboratory are allowed to take a blood meal from the patient. The bite of the bugs causes no pain because they secrete an anesthetic in their saliva. Place six or more hungry nymphs in a gauze-covered container. Then tape the contain-

er to the inner surface of the forearm with the gauze against the skin. Allow the bugs to feed until they take a full meal. Keep the bugs at room temperature. After 10 to 14 days and at weekly intervals afterward for 2 months or longer examine the fecal material of the bugs for flagellates. Larger nymphs can ingest up to 1 ml of blood at a feeding, and light infections can frequently be detected by this method when other methods fail.

f. **Aldehyde Test (Napier):**

(1) *Procedure:*

(a) To a test tube containing 1 ml of patient's serum, add 1 drop of 40% formaldehyde. Shake well.

(b) Allow to stand at room temperature.

(2) *Interpretation:*

(a) A positive test is indicated if the serum becomes opaque and gels within 3-30 minutes.

(b) A negative test is indicated when no reaction occurs within 24 hours.

(c) A doubtful reaction is indicated by the appearance of opaque serum which does not gel. This can occur in early infections.

(3) *Discussion:*

(a) The aldehyde reaction is a nonspecific test for increased serum globulin. The test is positive in about 85% of the infections with *Leishmania donovani*.

(b) False positive tests occur in any condition in which serum globulin is elevated. That includes schistosomiasis. Every attempt should be made to confirm the results of the aldehyde test before a final diagnosis is made.

Chapter 13
MISCELLANEOUS EXTRAINTESTINAL PARASITES OF HUMANS

SECTION A—PROTOZOA

13-1. Toxoplasma Gondii:

a. **Life History.** *Toxoplasma* is an intestinal coccidian of cats. It is spread through fecal contamination by oocysts resembling those of *Isospora*. Unlike typical coccidia, *Toxoplasma* multiplies extensively in other tissues, in addition to the intestine, in many species of mammals including humans. However, only cats are known to excrete oocysts in feces. Cysts or trophozoites in tissues cause infection when they are ingested with raw and undercooked meat, making carnivorism an excellent means of transmission. Also, congenital transmission plays a small but medically important role. Regardless of mode of transmission, mammals and birds other than cats can be regarded as facultative intermediate hosts. The oocysts of *Toxoplasma* become infective a few days after they are passed and remain infective for several months in water or moist soil.

b. **Morphology.** The oocysts passed in cat feces are ovoid, clear, thin-walled structures measuring about 10 by 12 microns. They are almost spherical and contain a central granular mass. These oocysts can be induced to sporulate by the same procedure outlined for *Isospora* and *Eimeria* in paragraph 6-5. After sporulation they contain two oval sporocysts similar to *Isospora*. Each sporocyst contains four sporozoites. In human tissues and exudates, trophozoites (figure 13-1) can be found singly or in groups that range from a few to many. The trophozoites are intracellular parasites, but they can be found outside of cells occasionally. They are ovoid in shape with one end attenuated and the other more rounded. They measure 2 to 4 microns in width and 4 to 7 microns in length. Stained organisms have an oval nucleus near the rounded end. The trophozoites of *Toxoplasma* resemble the organisms of *Leishmania*, but they have no kinetoplast. In some specimens there is a chromatin clot between the nucleus and the attenuated end. Trophozoites multiply by endodyogeny, and cells can become packed with organisms (figure 13-1). The individual organisms are sometimes crowded to the point that they lose their characteristic shape. Such cells are known as pseudocysts. At the proper time the process of endodyogeny leads to the production of true cysts containing many naked merozoites (figure 13-1), mainly in brain and muscle. Cells which become cysts are destroyed beyond recognition, and the parasites develop a highly elastic, tough membrane that distinctly separates the cysts from any host cells. Cysts contain few to many merozoites, but they usually have many. Giemsa's stain has been used to stain the parasites in tissue, but some find this unsatisfactory.

Figure 13-1. Toxoplasma gondii: Wright's stain. ×2,000.

c. **Laboratory Diagnosis:**

(1) *Direct Demonstration.* The trophozoites, including pseudocysts, can best be demonstrated by using many of the same laboratory procedures used to demonstrate *Leishmania donovani*; Giemsa-stained smears of tissue impressions [paragraph 12-9b(3)]; Giemsa-stained films of exudates and aspirates of spinal fluid or tissues [paragraph 12-9b(4) and 12-9b(5)]; histological preparations of suspected tissues; and rarely with stained blood films [paragraph 12-9b(1)]. In acute cases the specimens of choice for the demonstration of trophozoites are biopsies or aspirations of spleen, liver, lymph node, lung, brain, and other tissues. In chronic or latent cases, cysts are found in the brain, occasionally in muscle tissue, and rarely elsewhere. Trophozoites are rarely found in chronic or latent cases.

(2) *Animal Inoculation.* Mice are recommended for animal inoculation studies in cases of suspected toxoplasmosis. They are susceptible to *Toxoplasma gondii*, and they are relatively inexpensive. The same specimens that are recommended in the previous paragraph are recommended for animal inoculation. Collect, prepare, and inoculate the specimens as outlined in paragraphs 12-9d(1) and 12-9d(2).

(a) At the end of one week use a sterile needle and syringe to withdraw any peritoneal fluid present from one or more mice that have been anesthetized in an ether jar.

(b) Check the material microscopically for toxoplasmas after smearing and staining as for thin blood films in paragraph 11-5.

(c) If the peritoneal fluid is negative, sacrifice one mouse and prepare a suspension of the brain and other organs by the directions in paragraph 12-9d(1) as above. Inoculate that material into another group of mice. Treat the second group in the same manner as the first.

(d) The organisms are usually found in the first group of mice in positive cases. However, when organisms are not found, carry the test through three passages in mice. Sacrifice the mice at the end of 1 month and examine hematoxylin-eosin stained sections of the brains for the presence of cysts.

(3) *Immunologic Tests.* Immunologic tests can be an aid in diagnosing toxoplasmosis. The procedures are not recommended for the average laboratory, but they are generally available at the larger reference centers. The Sabin-Feldman dye test has long been the most reliable procedure available. More recently the immunofluorescent antibody and hemagglutination tests have come into use. Either one or both of these tests should be used in conjunction with the procedures to demonstrate the organisms in order to make a diagnosis.

13-2. Sarcocystis lindemanni. *S. lindemanni* is an incidental parasite of humans that causes no known disease. The organisms are found in elongated cylindrical, tubelike bodies in the muscle fibers of the diaphragm, tongue, chest, abdomen, and myocardia. The tubes range in size from microscopic up to 5 cm long. They are hyaline cysts that are surrounded by an outer envelope. The cyst is divided into compartments which contain many sporelike organisms (figure 13-2), each with a nucleus. The nucleus is located near one end of the organism, which is rounded. Their shape is similar to that of *Toxoplasma*, but they are about twice as large, 4 to 9 microns by 12 to 16 microns. *S. lindemanni* is rarely encountered in humans, but it can be confused with *Toxoplasma* and *Trypanosoma cruzi* or *Leishmania* if it is not considered when performing microscopic examinations of stained films and tissue sections.

13-3. Pneumocystis Carinii:

a. *Pneumocystis carinii* is found in the lungs of many mammals including humans. It is frequently observed in cases which demonstrate no symptoms of disease. However, the organisms are associated with interstitial, plasma-cell pneumonia in premature and young infants and older patients with immunologic deficiencies, especially in patients who have some disease for which they receive immunosuppressive chemotherapy as part of their treatment. This indicates that the parasite is an opportunistic invader of hosts with impaired defense mechanisms.

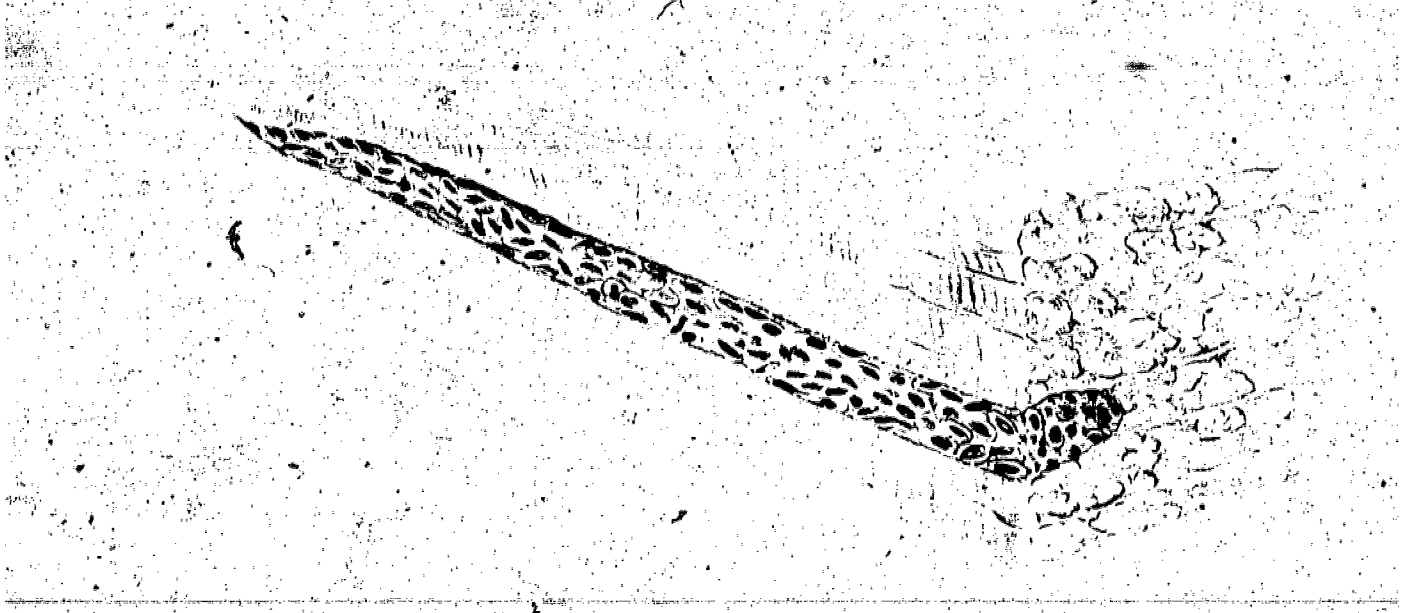

Figure 13-2. Sarcocystis lindermanni.

b. The parasites appear in tissue sections and tissue-impression smears as small, round or oval, cystlike structures that contain from one to eight uninucleated bodies (figure 13-3). The "cysts" are 5 to 8 microns in diameter. The uninucleate daughter cells are at first spherical; later, they become crescent-shaped structures that measure 1.5 to 2.5 by 0.5 microns.

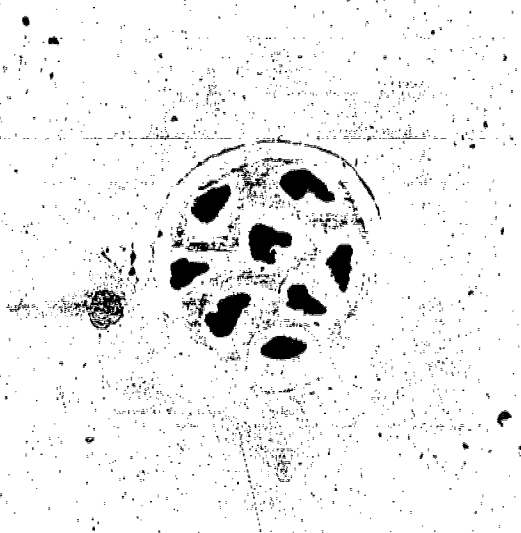

Figure 13-3. Pneumocystis carinii. Gram stain. Greatly enlarged.

c. The only presently available way to make a positive diagnosis is to demonstrate *Pneumocystis carinii* in lung tissue. The parasite can be identified in lung tissue-sections or lung tissue impression smears. The cut surface of the lung tissue appears more solid than normal with gray to gray-yellow patches. Upon microscopic examination the alveolar spaces are characteristically filled with a fine, foamy eosinophilic material. Large masses of the parasites are found in the foamy substance that fills the alveoli. Giemsa's stain of the tissue or impression smear will reveal the individual organisms within the "cysts." The individual organisms are Gram-negative, but the specific morphology is difficult to determine when Gram-stained. With both Giemsa's and Gram's stain the "cyst" wall does not stain but appears as a clear zone around the organisms. The "cyst" wall stains black with methenamine-silver stain making it stand out very clearly.

13-4. Entamoeba Histolytica:

a. The ameba, *E. histolytica*, has been found invading almost every organ and soft tissue of the human body. The invasion of the tissues is by a lytic process that causes necrosis which frequently leads to the formation of an abscess. The invasion of such extraintestinal tissues almost always occurs after the parasites have invaded the wall of the large intestine. The liver is the organ most frequently involved.

b. Suspected amebic abscess aspirate is frequently submitted to the laboratory for the demonstration of organisms. The trophozoites are located chiefly in the peripheral area of the abscess. Materials from the central portion of the abscess are less likely to reveal organisms. To insure that the two portions are collected separately, have the material removed in at least two aliquots. The first, usually yellowish-white, seldom contains amebae. The next, or subsequent portions, are reddish in color, and are more likely to contain organisms; the last part containing material from near the abscess wall, is most likely to contain organisms. The material is processed for examination and culture as outlined in chapters 5 and 6.

c. Tissues are examined for the demonstration of the amebae in suspected cases of extraintestinal amebiasis. The organisms are not easy to identify in routine sections stained by hematoxylin and eosin. To perform a useful screening procedure, fix the tissues in formalin and stain them with PAS (periodic acid Schiff) reagent. The amebae usually stand out as bright red bodies. The exact morphology is rarely clear, but the presence of such bodies is sufficient to warrant careful search of sections treated with stains that are better suited to the demonstration of morphology: iron hematoxylin, trichrome, or chlorazol black E [paragraph 7-3e(6)].

d. Antibodies are produced irregularly, or not at all, in intestinal amebiasis; however, in invasive amebiasis antibodies are regularly produced in detectable quantities. There are also serological procedures available that are highly specific for amebic antibodies, but the various procedures do vary in sensitivity. Since antibodies persist for unknown periods of time after termination of infection, a positive serological result is not in itself sufficient for the diagnosis of active amebic infection. A negative result by the more sensitive methods will help the physician rule out appreciable tissue invasion. The indirect hemagglutination test is available at many of the reference laboratories. The old complement fixation test and some other procedures are also in use; however, they do not regularly yield as reliable results as the indirect hemagglutination test.

13-5. Naegleria (Cause of Primary Amebic Meningoencephalitis):

a. The free-living ameba, *Naegleria*, can cause *meningoencephalitis* in humans. It has been recovered from such cases in many parts of the world and appears to have a worldwide distribution. Most of the patients from whom the organism has been recovered have had a history of swimming in fresh or brackish water a few days prior to onset of illness. *Naegleria* causes the same type of disease in laboratory animals when the organisms are introduced into the nasal passages. The organisms invade the nasal mucosa and penetrate into the brain. That is the probable mode of infection in human patients with the organisms gaining access to the nasal and inner ear passages while the victim is swimming. In addition to invading the central nervous system, the ameba can get into the circulatory system and spread to all organs of the body.

b. *Naegleria* is capable of transforming from amebae into highly motile flagellates. The organisms have a single nucleus that contains a large dark karyosome and a delicate nuclear rim. Living amebae, in cerebrospinal fluid actively extend blunt pseudopodia in different directions at almost the same time producing a "bubbling" appearance. Hyperactive, eruptive, spasmodic ameboid activity is characteristic of *Naegleria*. The ability of the ameboid forms to transform in vitro into actively motile flagellates is also an important characteristic of *Naegleria* that must be recognized to make a proper identification. The flagellate is elliptical or pear-shaped, with 2 to 4 flagella originating from the anterior end. The cysts are uninucleated. The cyst is spherical and has a thick wall with a smooth wavy capsule. The cyst wall has several apertures which, at times, are difficult to observe. The ameboid trophozoite measures 8-18 microns by 10-36 microns while the flagellated stage measures about 8 microns by 18 microns. The cysts are approximately 12 to 14 microns in diameter.

c. Laboratory diagnosis is made by demonstrating the amebae in spinal fluid. The amebae can be observed by examining direct wet preparations, and stained smears. They can also be cultured on trypticase soy medium seeded with *Enterobacter* species bacteria. Good

growth is usually obtained in 18 to 24 hours. *Naegleria* can be induced to encyst by allowing the culture plates to become cool and partially dry. To obtain flagellated forms, add warm fresh water to the culture medium on which the organisms are growing. Incubate again at 37C for 2 to 12 hours. Many of the ameboid forms will transform into flagellated forms. Flagellated forms have not been found in clinical material. Section and stain tissues from suspected cases as outlined for *E. histolytica* in paragraph 13-4c.

d. Prepare the following medium and culture spinal fluid or other material as outlined.

(1) *Neomycin Sulfate.* Prepare a 0.56 percent solution of neomycin sulfate from the dry powdered antibiotic in sterile, distilled water.

(2) *Nystatin.* Make a suspension of nystatin in sterile, distilled water that contains 1500 units of antibiotic per ml of water. Use dry, powdered nystatin to prepare the suspension.

(3) *Trypticase Soy Agar.* Prepare the trypticase soy agar from a commercial preparation. Prepare it at twice the strength recommended by the manufacturer.

(4) *Killed Enterobacter Aerogenes.* Remove colonies of *Enterobacter* from the surface of a 16 to 20 hour culture on trypticase soy agar. Suspend the organisms in trypticase soy broth. Bring the suspension to the proper density by adding organisms until 40% transmittance is reached when read in a spectrophotometer against an uninoculated trypticase soy broth tube. Place the suspension in a tightly capped tube and immerse in a 65C water bath for 30 minutes. The suspension can be stored indefinitely in a refrigerator.

(5) *Medium for Amebae Culture.* To 100 ml of sterilized trypticase soy agar that has been cooled to about 56C, add 5 ml of each of the antibiotic solutions plus 90 ml of sterile distilled water that is also about 56C. Pour the medium, in about 8 ml quantities, into 100 mm petri plates. Allow moisture to evaporate from the surface of the agar after it solidifies. Place approximately 0.05 ml of the killed *Enterobacter* suspension in the center of the plate. Spread the suspension over a central area of about 25 to 40 mm in diameter.

(6) *Inoculation.* Place a few small drops of a liquid specimen, such as spinal fluid, or small pieces of tissue near the center of the plate. Incubate the plates at 37C. Check the plates daily for 4 or 5 days for the emergence of amebae growing from the edge of the inoculated area.

(7) *Examination.* To check the plate for growth, observe the agar surface with a microscope using low power. The amebae are about twice the size of white blood cells and tend to wander into the peripheral zone of the agar where they eventually encyst.

SECTION B—HELMINTHS

13-6. Trichinella spiralis. *Trichinella* is actually an intestinal parasite, but it is considered in this section because it is usually diagnosed by finding the larvae in muscle tissue. Humans become infected when they ingest raw or insufficiently cooked flesh, usually pork, that contains infective larvae of the parasite. As expected, the parasite is most commonly found in pork-eating populations. *Trichinella* has traditionally been a serious problem in parts of Europe and the United States until recent decades. It is still widely distributed in Germany, Poland, Spain, Hungary, and the lower Danube countries. There are still reports of small epidemic outbreaks occurring in the United States and Latin America. In the last few years there have been several outbreaks reported from Thailand.

a. **Life Cycle.** A working knowledge of the life cycle of *Trichinella* is important to the technician. It tells him when and where certain stages of the parasite are most likely to be found. The cycle in man begins when he ingests meat, usually pork, that contains infective larvae. In the small intestine, the cysts that surround the larvae are digested, and the larvae enter the intestinal crypts. The larvae mature very rapidly. The adults (figure 13-4) are small white worms, just visible to the unaided eye, the male being 1.5 mm long and the female about 3.9 mm long. The male dies after mating and is passed from the intestine in a very short time. Upon fertilization, the female burrows more deeply into the intestinal mucosa and by the 5th to 7th day begins to deposit larvae directly into

the mucosa. A single female gives birth to several hundred larvae over a period of 4 to 16 weeks or more. These little larvae measure 100 microns long and 6 microns in diameter. They reach the mesenteric venules and lymphatics and become distributed to all parts of the body. The young larvae leave the capillaries and invade voluntary (striated) muscle. This is the *only* tissue in which the larvae are able to develop and grow. The muscles most frequently affected are in the diaphragm, larynx, and tongue, and the biceps, gastrocnemius, and deltoid muscles. One to two weeks after exposure, most of the larvae have reached striated muscle. About 3 weeks after exposure, the larvae in the muscles (figure 13-4) have grown to about 1 mm in length. At this stage they have become coiled and encapsulation has begun. The larvae are infective for another host when they reach the coiled stage. Calcification of the capsule begins after about 8 weeks. In 9 to 12 months most of the encapsulated larvae are completely calcified and dead, but some may live for several years.

b. **Laboratory Diagnosis.** A definitive diagnosis of *Trichinella* is very difficult to make in the early stages of the disease. During the very early stages, the physician has nothing to go on but the patient history. At that point the patient will have an eosinophilic leukocytosis. You can sometimes find *T. spiralis* larvae in the centrifuged sediment of hemolyzed blood during early phases of heavy infections or in the cerebrospinal fluid when CNS involvement is present. Larvae can usually be found in the suspected meat if it is still available. Use the compression procedure, or if large amounts of meat are available, use the digestion procedure to search for them. As the infection advances to the seventh to fourteenth day, larvae reach the muscles of the patient, and it becomes possible to recover them from muscle biopsies. The compression procedure is usually adequate to examine the entire biopsy in a short time because the amount of specimen is limited. On postmortem examinations obtain sufficient diaphragm muscle for digestion, compression, and histological sectioning. Diaphragm muscle usually gives the highest percentage of positives, but other striated muscles should also be examined. Skin-test antigens are available for *Trichinella*, and the physician will occasionally use them as an aid in diagnosing the disease. Reliable agglutination procedures are available for this infection. A bentonite flocculation test and an indirect hemagglutination test have been found to be sensitive and satisfactorily specific. The tests, when performed on acute and convalescent serum, will show a significant increase in titer.

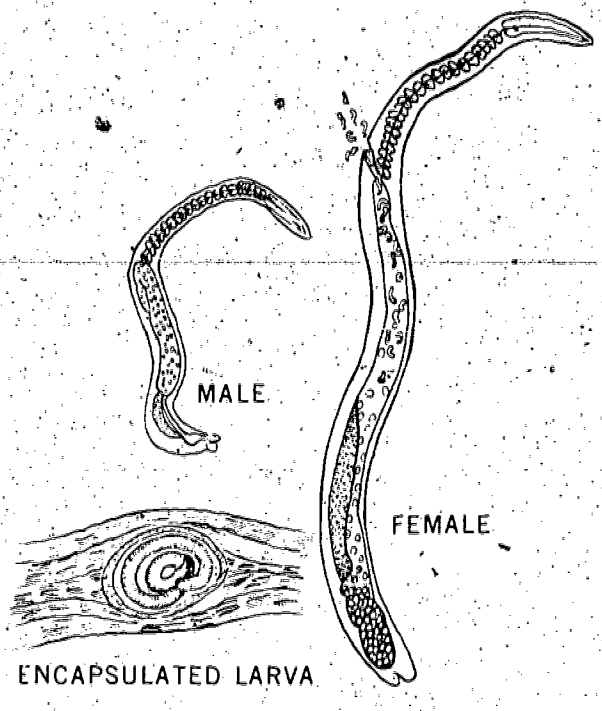

Figure 13-4. Trichinella spiralis. Adults ×50. Larva ×450.

c. **Laboratory Procedures:**

(1) *Compression Technique.* Press biopsy or autopsy specimens between glass slides before examining them. The tissues can be pressed thin enough to give clear visibility.

(a) Avoiding fat and connective tissues as much as possible, place a portion of the tissue, 10-20 cmm, depending upon its texture, on a 2 inch by 3 inch slide. Add a drop or two of saline.

(b) Tease fibrous samples into small strands with dissecting needles and cut nonfibrous samples into minute cubes.

(c) Spread the material along the middle third of the slide, cover it with a 1.5 inch by 3 inch slide. Compress the specimen as much as possible.

(d) Examine the preparation for larvae with the microscope under low power.

(2) *Concentration by Digestion.* Any nematode larvae can be concentrated by pepsin digestion and sedimentation. The digestion process is effective on all but bone, fat, and nerve tissues. To prepare the digestion fluid, dissolve 5.0 g of dry granular pepsin in 1 liter of normal saline and add 7.0 ml of concentrated hydrochloric acid to each liter of the solution.

(a) Grind or chop the tissue into bits not more than 2 or 3 mm thick.

(b) Transfer to a flask of suitable size to add at least 10 ml of digestion fluid for each gram of tissue.

(c) Incubate 6 to 12 hours at 37C, with occasional stirring.

(d) After stirring, fill one or two conical centrifuge tubes, immediately centrifuge at 2000 rpm for 1 minute, and decant.

(e) Resuspend the sediment in normal saline, recentrifuge, and decant.

(f) Add just enough saline to suspend the sediment. With a pipet transfer the sediment to a 1.5 inch by 3 inch slide.

(g) Coverslip and examine for motile larvae with the microscope under low power.

(h) If larvae are not found in the centrifuged portion of the sample, allow the remainder of the digested suspension to stand in the sedimentation flask for several more hours and reexamine.

13-7. Capillaria hepatica. *C. hepatica* is a delicate, threadlike parasite that lives in the parenchyma of the liver of mammals, especially rodents. The female worms pass eggs directly into the liver tissues, causing fibrosis. As the worms and eggs accumulate in the liver, considerable damage is done. To make a positive diagnosis, a liver biopsy must be performed. The accumulations of eggs appear as yellow splotches on the liver. Prepare a compression slide as in paragraph 12-6c(1). Search for worms and eggs. Morphologically the eggs (figure 9-15) are similar to those of *T. trichiura*. They measure 51 to 61 microns by 30 to 35 microns, which is slightly larger than those of *T. trichiura*. The eggs of *C. hepatica* are more barrel shaped, and the shells appear velvety, because they are finely pitted with minute pores.

13-8. Syngamus. Members of the genus *Syngamus* are small nematodes that live in the respiratory tract of birds and mammals. The males and females are permanently joined together for mating. Man is an incidental host for *S. laryngeus* and possibly *S. nasicola*. Human infections have been reported from Puerto Rico, Brazil, the West Indies, and the Philippines. The adult worms irritate the upper respiratory tract, causing coughing, sneezing, and an asthmalike condition. Diagnosis is made by finding the adult worms or eggs in sputum or feces. The eggs are oval, and the shell appears to be made up of a large number of prisms cemented together. Be on the lookout for these parasites, and do not confuse their eggs with pollen grains that are commonly observed in sputum and feces.

13-9. Thelazia. Two species of the genus *Thelazia* have been reported from the conjunctival sac of man. *Thelazia callipaeda* has been reported from several oriental countries. *Thelazia californiensis* has been reported from the western states of the United States. These parasites normally inhabit the conjunctival sac of dogs and other mammals. They are only incidental parasites of man. The worms measure about 4.5 mm by 0.25 to 0.85 mm. Females produce clear, thin-shelled, embryonated eggs which measure 57 by 35 microns. The worms have a rough cuticle which causes considerable damage to the tissues of the eye as they crawl back and forth across the front of the eye. Diagnosis depends upon the identification of the worm after it is removed from the anesthetized eye.

13-10. Dioctophyma renale. *Dioctophyma*, commonly known as the giant kidney worm, is widely distributed throughout the world. It is primarily a parasite of fish-eating carnivores, but it is occasionally found in horses, cattle, and humans. *Dioctophyma* adults are very large, reddish nematodes which inhabit and destroy the parenchyma of the kidney. Males measure from 150 to 400 mm long, and females measure up to 1,000 mm long. Females pass eggs that are unembryonated, dirty brown in color, have deep pittings in the shell except at the poles, and measure 64 to 68 microns by 40 to 44 microns.

Diagnosis is made by finding typical eggs in *urine*. At postmortem examination the adult worms will be found almost completely filling the capsule of a kidney.

13-11. Gongylonema pulchrum. *G. pulchrum* inhabits the mucosa of the esophagus of ruminants. It has also been found in pigs, bears, hedgehogs, monkeys, and occasionally in man. The parasite has been reported from most areas of the world. In man, the worm has been found in the mucosa and subdermal connective tissues in the vicinity of the mouth, not the esophagus. It produces local irritation and inflammation. The adult worms are threadlike, and the females are larger than the males. They may measure up to 145 mm long. The females lay fully embryonated eggs which are transparent, thickshelled, broadly ovoidal, and measure 50 to 70 microns by 25 to 37 microns.

13-12. Nematodes Unable to Develop Completely in Humans:

a. **Dirofilaria:**

(1) *Dirofilaria immitis. D. immitis,* the heartworm of dogs, is occasionally found encapsulated in the lungs of humans. Sexually mature worms have been found in people, but no microfilariae of the parasite have been found in their blood. The worms are usually found on radiographic examinations as *coin lesions* of unknown etiology which require surgery to rule out a malignancy. Histological sections reveal the worms in the lesions. Skin tests and serological tests that employ *Dirofilaria immitis* can be helpful in cases presenting such coin lesions.

(2) *Dirofilaria "conjunctivae."* A group of *Dirofilaria* sp. are found in tumors and abscesses of the subcutaneous tissues and tissues of the eyes of humans. The various species of *Dirofilaria* that cause these infections in humans are usually common parasites of the native animals of the particular area. They are referred to collectively as *Dirofilaria "conjunctivae."* Sexually mature worms are sometimes found in the excised tumors, but microfilariae are not found circulating in the blood of the patient.

b. **Angiostrongylus cantonensis.** *A. cantonensis* causes the disease "eosinophilic meningitis" in humans. It is normally a parasite of rats. It is enzootic in most areas of the Pacific. Snails, planarians, shrimp, and many other invertebrates serve as intermediate hosts. The larvae in the rat migrate from the intestine to the brain for a period of development before they go to the lungs of rats where they mature and reside. Humans also become infected by eating raw snails or shrimp, but the worms normally do not complete their migration to the lungs. They remain in the brain and spinal column, causing *eosinophilic meningitis.* The worms develop to the adult stage in humans, but they do not reach full maturity. Infections occur frequently in humans in many of the Pacific islands, Vietnam, and Thailand. People who are infected have very severe headaches and other neurological symptoms. The spinal fluid is usually loaded with eosinophils. Occasionally immature worms can be recovered from the spinal fluid. There are other helminths that sometimes cause a similar syndrome; but in the areas where *A. cantonensis* is common among rats, it is the most frequent cause of eosinophilic meningitis in man. A skin test utilizing antigen made from the worm may be helpful in making a diagnosis.

c. **Anisakis.** *Anisakis* is a member of the *Ascaris* group. It is normally a parasite of fish-eating mammals. Humans become infected by eating insufficiently cooked marine fish. The parasite is commonly found in Japan. It is occasionally found in Europe, and it can be expected in any area where raw marine fish are eaten. Marine fishes harbor the larvae of the parasite, which develop very little when ingested by humans. They burrow into the mucosa of the stomach or intestine causing lesions which are mistaken for peptic ulcers or malignant tumors. The immature parasites are found coiled in the raised tumors.

d. **Oesophagostomum.** *Oesophagostomum* is a parasite of monkeys and apes in Asia, Africa, and South America. The parasites are about the same size as hookworms, and they attach to the mucosa of the cecum and colon. They are differentiated from hookworms because their buccal capsule opens forward and is guarded by a crown of bristles. The larvae are ingested, and in humans they work their way deep into the mucosa of the abdomen or intestine. There they produce tumorlike masses and abscesses. Tu-

mors containing several parasites can be as much as 5 cm in diameter. Surgical removal of the tumorlike masses and direct examination of histological sections are the only means of diagnosis available.

e. **Lagocheilascaris.** *Lagocheilascaris minor* is normally a parasite of the intestinal tract of the cloudy leopard. In humans it inhabits the tissues of the neck near the jaw, the nasal passages, and the maxillary sinuses. *L. minor* does develop to maturity in humans and even passes eggs which closely resemble those of *Toxocara*, but they obviously develop atypically. Large abscesses usually develop around the worms, and both worms and eggs can be recovered from the abscess exudates. Human infections have been reported from Tobago, Trinidad, and Surinam.

f. **Gnathostoma spinigerum.** *G. spinigerum* is a parasite that lives in tumors within the walls of the stomachs of dogs, cats, and certain wild carnivores. The parasite is found in animals in Southeast Asia, Japan, China, India, Indonesia, parts of Europe, and Australia. Most human cases are reported from Thailand. When humans become infected, the larvae of the parasite migrates around the patient's tissues, producing a kind of creeping eruption and occasionally causing a serious problem by invading the eye. As in the case of most tissue-invading nematodes, there is an eosinophilia associated with the infection. The larvae are quite large (10 to 50 mm by 1 to 2.5 mm) and can be readily identified when removed from their channel in the tissues. They have a large head bulb (figure 13-5) that has eight rings of hooklets.

Figure 13-5. Gnathostoma spinigerum. ×2.

g. **Toxocara (Cause of Visceral Larva Migrans).** The genus *Toxocara* includes ascarids that infect dogs and cats. They are widely distributed over most of the world. Most birds and mammals, including humans, can become infected with the larvae of the parasites by ingesting infective eggs. *Toxocara* larvae will live for years in such infected animals. In humans the disease produced by these larvae is known as *visceral larva migrans*. Young children about 1 to 4 years old are most frequently infected. That is to be expected since they put everything, including dirt, in their mouths. *Toxocara* larvae migrate through the body and concentrate particularly in the liver in humans. The resulting disease may be very mild, with an eosinophilia of 20% to 80% the only symptom noted. Frequently the disease is severe with intermittent fever, cough, hepatomegaly, and hyperglobulinemia. It is sometimes fatal. Specific diagnosis requires identification of the larvae, which can be found in liver biopsies. Skin tests and serological procedures have shown some promise, but as yet they are not reliable.

h. **Ancylostoma (Cause of Cutaneous Larva Migrans).** Some species of the genus *Ancylostoma* cause a disease known as *cutaneous larva migrans*, also called "creeping eruption." The parasite most frequently implicated as the cause of the disease in humans is *Ancylostoma braziliense*, a hookworm of dogs and cats. The disease is most commonly encountered in sandy coastal areas of the United States all along the Atlantic and Gulf Coast from New Jersey to Texas. It is also found in many other subtropical and tropical coastal regions of the world. The infective larvae of the parasites penetrate the skin and migrate around unable to find their way to the intestine. They cause an intensely itching tissue irritation. As the larva migrates, it leaves a wandering discolored path. The

larvae sometimes continue to migrate around for weeks or months, resulting in extensive skin involvement. A diagnosis of cutaneous larva migrans is made by the physician on the appearance of the lesions as the larvae cannot be recovered for identification.

13-13. Tapeworm Larvae in Humans:

a. **Taenia solium (Cysticercosis).** Humans become infected with the larvae by ingesting eggs of *Taenia solium*. The larval stage of *Taenia* is called a *cysticercus*, or *bladder worm*. It has one invaginated scolex and one bladder. After the egg is ingested, the onchosphere hatches, penetrates the intestinal wall, and enters the blood stream. It can lodge in any tissue, but it is most frequently found in the brain, eye, and voluntary muscle. The larvae mature in about 2 months. The cysticercus (figure 13-6) is semitransparent, white in color, oval in shape, and it is about 1 cm in diameter. The bladder is filled with a clear fluid. A dense knoblike structure is located on one side; it is the invaginated scolex. Living larvae lie relatively free in the tissue causing little trouble unless they become lodged in some vital area such as the eye or brain. They begin to die and degenerate after 3 to 5 years. The degenerating larvae cause an inflammatory reaction which leads to fibrosis and calcification in muscle tissue, but in the brain or spinal cord they rarely calcify. There the tissue reactions lead to neurological symptoms.

b. **Echinococcus (Echinococcosis or Hydatid Disease).** *Hydatid cysts* are the larvae *Echinococcus granulosus* and *E. multilocularis*. Dogs and other canines are hosts for the adult worms of both species. Herbivorus animals are the usual intermediate hosts of *E. granulosus* while rodents are the intermediate hosts for *E. multilocularis*. Hydatid disease in humans, caused by the larvae of *E. granulosus*, is most often found in the sheep-raising areas of the world. The disease caused by *E. multilocularis* is found most often in Arctic regions and Central Europe. *E. granulosus* larvae are known as unilocular cysts in soft tissues and osseous when in bone. *E. multilocularis* larvae are known as multilocular cysts.

(1) *Echinococcus granulosus: Hydatids.* Of the two types of cysts produced by larvae of *E. granulosus* the unilocular is the most frequently encountered. The unilocular cyst (figure 13-6) has a cavity that is filled with fluid. The cyst wall surrounds a thin germinative layer. The wall is made up of a thick, laminated, noncellular, membrane. *E. granulosus* embryos can lodge in any organ or tissue, but are most often found in the liver and lungs. In these tissues they develop slowly into a hydatid cyst. They steadily enlarge and after 10 to 20 years they can be more than 15 cm in diameter and contain more than a liter of fluid. The fluid usually contains many scoleces (hydatid sand) and daughter hydatids. Larvae that become lodged in bony tissues develop completely differently because there is no limiting membrane formed. The hydatid develops freely in the bony canal and continues to spread as the bone is eroded.

(2) *Echinococcus multilocularis: Hydatids.* The multilocular or alveolar hydatid of *E. multilocularis* is usually found in the liver and rarely in the lungs. Humans appear to be poor intermediate hosts for this tapeworm because few, if any, scoleces are produced in hydatids in humans. The multilocular hydatids develop probing, minute cavities surrounded by a hyaline membrane. Frequently there is no fibrous capsule present, allowing the organism to grow without confinement. This hydatid can destroy much of the surrounding host tissues. As the hydatid grows, necrosis and cavitation occur. On rare occasions the larvae can metastasize to other areas of the body.

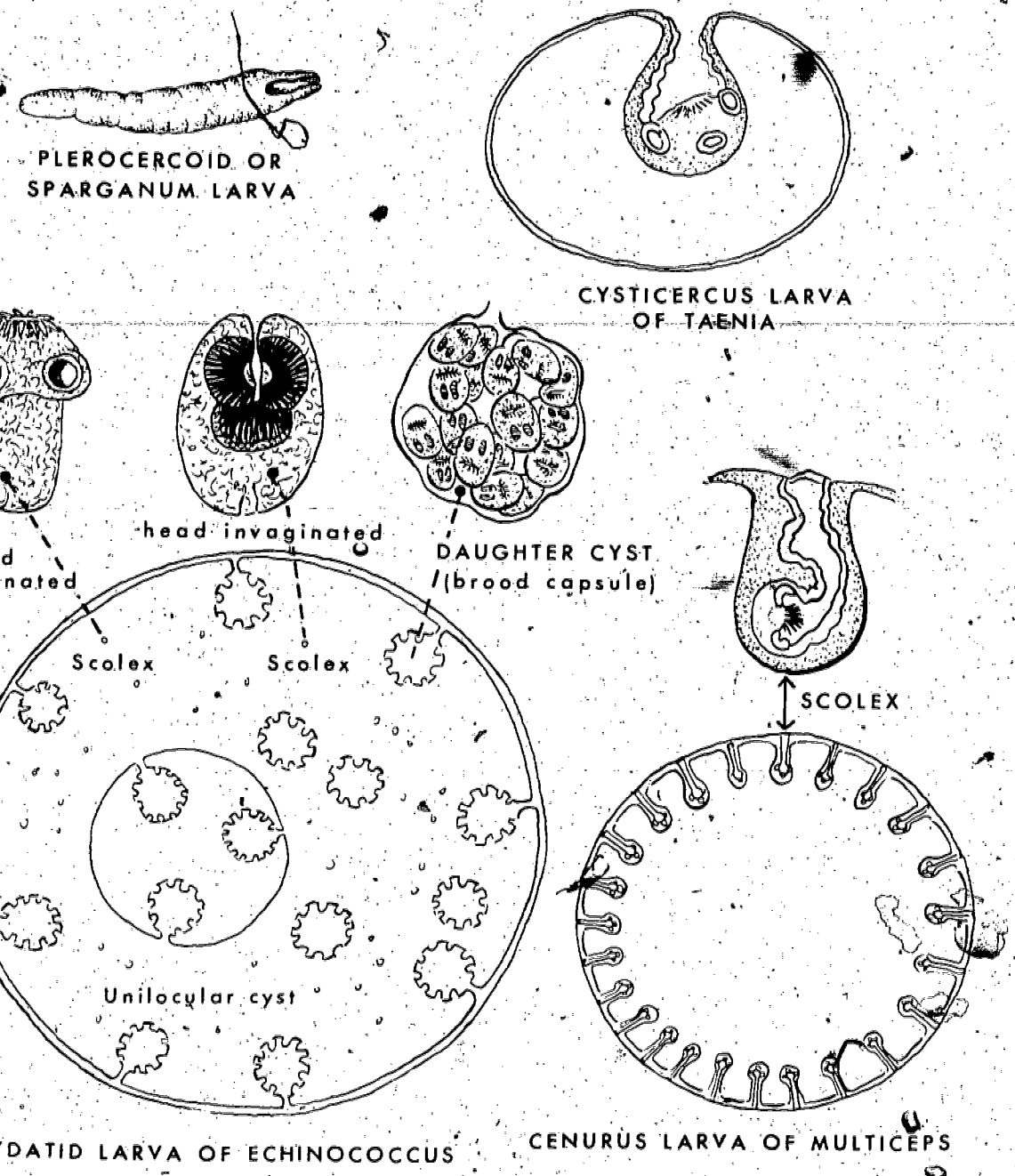

Figure 13-6. Tapeworm Larvae in Humans. Various magnifications.

c. **Multiceps (Cenurus Disease).** Several species of the genus *Multiceps* have been found to cause *cenurosis* in humans. Dogs and their wild relatives are most commonly the natural hosts of the adult worms, and herbivorus animals are usually the intermediate hosts that become infected with the larva, known as a *cenurus*. Man may also become infected with these larvae. The larvae reach their extraintestinal sites through the blood stream. They can lodge and develop in any soft tissues, but most cases have been found in the brain and spinal cord. In the tissues the embryos transform into the cenurus type of larva (figure 13-6), which has many scoleces developing from the germinative membrane lining the cavity. The cenurus has the appearance of a bladder that is filled with a clear fluid. It is transparent, white in color, and on the inside wall the attached scoleces appear as dense, white knobs.

d. **Spirometra (Sparganosis).** Sparganosis in humans is an infection caused by the plerocercoid larvae of pseudophyllidean tapeworms belonging to the genus *Spirometra*. Most of the human infections with unbranched spargana occur in the China Sea area. A few cases have been found in other parts of the world. A branching type, *Sparganum proliferum*, has been found in Japan and the United States. Humans acquire the infection by ingesting uncooked intermediate hosts that are "carrying" the parasite. The ingested larva penetrates the gut wall and migrates through the tissues as it grows into a sparganum larva. Human infections also take place through the practice of placing poultices of frog or snake flesh on open wounds or other lesions, especially of the eyes. The animal flesh can contain sparganum larvae which actively penetrate into the covered lesion. Sparganosis of the eye is frequently obtained in that way in parts of the Orient. The larvae of *S. proliferum* occur almost anywhere in the body, and the branched proliferating larvae break up into segments, which then develop into more spargana. The sparganum (figure 13-6), is a wrinkled, whitish, ribbonlike object, a few millimeters in width and up to several centimeters in length. The anterior end can invaginate, and there is evidence of the grooves that are present on the mature scolex. The majority of the larvae found in humans are from the subcutaneous tissues.

e. **Laboratory Diagnosis of Larval Tapeworm Infections.** Serological and allied tests offer the most practical method of diagnosis. However, the reactions are of a group character making it necessary for test results to be carefully correlated with clinical observations. Occasionally, negative test results do not exclude infection. The most specific test for echinococcus is the intradermal or *Casoni* reaction. Standard serological tests are also available but are of less value except in old or complicated cases. Similar techniques are available for the diagnosis of cysticercosis and sparganosis, but the results are less reliable. A diagnosis of echinococcosis can be made by identifying free scoleces and scattered hooklets from hydatid cyst fluid. Cysticercus larvae can be identified after surgical removal or at autopsy by the presence of a miniature scolex of *Taenia solium*. Sparganum larvae can be identified grossly, or they can be flattened, stained, and identified microscopically by their typical morphology.

13-14. Trematodes:

a. **Cercariae That Produce Dermatitis.** The disease known as *schistosome dermatitis* is caused by cercariae of certain nonhuman schistosomes penetrating the skin of humans thus invoking a sensitization reaction which becomes stronger on repeated contact. Humans come in contact with these cercariae by swimming in cercariae-infected fresh or salt water. The disease is found in many of the tropical, subtropical, and temperate regions of the world including many areas of the United States. The responsible cercariae can sometimes be recovered from the water and identified, but there is no practical method for removing them from the skin. The cercariae cause a prickling sensation and local or general urticaria shortly after penetrating the skin. The initial response soon subsides but leaves a raised spot on the skin. After a few more hours the lesions begin to itch intensely, and the raised spots change into papules. The reaction reaches its maximum in 2 to 3 days and then gradually subsides. It does not persist as long as cutaneous larva

migrans and does not leave the migrating trails in the skin as do the hookworm larvae.

b. **Paragonimus westermani in Unusual Locations:**

(1) In *Paragonimus westermani* infection the parasites can encyst in the intestinal wall, peritoneum, diaphragm, or pleura, failing to complete their migration to the lung. In these cases the eggs do not have access to the respiratory tract, and diagnosis can only be established by biopsy. Occasionally fistulous tracts are established between abdominal cysts and the intestine or skin. In these cases it is possible to recover eggs in the stool or skin discharge. In the absence of communication with a surface, diagnosis depends on removal of one of the bluish cysts from the intestine, peritoneum, diaphragm or pleura and identification of the adults and eggs by teased preparations and microscopic sections.

(2) Usually in pulmonary paragonimiasis the eggs are present in the sputum. If the disease is suspected and eggs are not demonstrable in the sputum, needle aspiration of one of the subpleural cysts can reveal the typical eggs. Lymph node, skin, or brain involvement sometimes occurs as the result of ectopic migration in paragonimiasis. Cysts or abscesses formed in these sites are examined for eggs and adult worms by smears, teased preparations, and microscopic sections.

c. **Schistosome Infections.** In schistosomiasis infections biopsy procedures are very helpful when eggs are not found in the stool or urine. The purpose of biopsy is to demonstrate the characteristic eggs; the adults will usually not be obtained. When biopsy *is* rewarded by the finding of adults, the most reliable criterion for the identification (exclusive of the characteristics of the eggs) is the appearance of the integument on cross section. *S. mansoni* shows coarse tubercles; *S. haematobium*, fine tubercles; and *S. japonicum*, a smooth nontuberculate integument. Biopsies intended to recover the diagnostic eggs should be selected from granular, eroded, or polypoid areas in the mucosa of the rectum (via proctoscopy) and bladder (via cystoscopy). The tissue obtained may be divided, one part being processed for tissue sections and the other part cut into fine pieces with scissors, macerated in saline, and unstained slides prepared for identification of the characteristic eggs of one of the three species. Needle biopsy of the liver may reveal pseudotubercles containing eggs in the portal areas. Schistosome eggs are also occasionally found in the lung and brain.

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SUMMARY OF REVISED, DELETED, OR ADDED MATERIAL

This manual revises and updates parasitologic isolation, differentiation and identification techniques; discussess host/parasite/vector relationships; and presents the text in a completely reorganized format.

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GLOSSARY OF TERMS

Amastigote. A "leishmania-form" organism that is the usual intracellular stage of *Leishmania* and *Trypanosoma* parasites.

Arostyle. Rod-like organelles which function as internal structural support in certain protozoans, characteristically in the intestinal and vaginal flagellates.

Blepharoplast. A minute oval or round granule forming a part of the complex known as the kinetoplast in blood and tissue flagellates belonging to the genera *Trypanosoma* and *Leishmania.*

Cestode. A common name applied to the tapeworms as a group.

Charcot-Leyden Crystals. Perfectly formed, six-sided crystals that are attenuated in the longitudinal axis terminating in needlelike points at the poles.

Chromatin. The more readily stainable portion of a cell nucleus which displays a characteristic pattern useful in species identification.

Chromatoid. Variously shaped aggregations of material which stain deeply with hematoxylin, contained within the precystic and cystic stages of certain species of parasitic amebae.

Cilia. Minute hairlike structures which serve as organelles of locomotion in protozoans belonging to the class *Ciliatea.*

Ciliates. A general term applied to protozoans of the class *Ciliatea,* characterized by the presence of numerous fine hairlike fibrils on the surface of the body which serve as organelles of locomotion.

Cyst. A structure, the outer covering of which consists of a protective layer which envelops the protoplasm of protozoans and enables them to survive under adverse environmental conditions.

Cysticercosis. The term applied to a disease in which the larval stage of a *Taenia* sp. invades body tissue.

Cysticercus. A larval form of *Taenia* sp. in which a single scolex is enclosed in a bladderlike cyst.

Cytoplasm. The protoplasm of the cell other than that of the cell nucleus.

Cytostome. An opening within the outer wall of certain species of highly developed protozoans which serves as a primitive mouth through which solid food or waste material passes in or out of the cell.

Diurnal. Activity occurring during the day.

Echinococcosis. The term applied to the disease which results from infection with the *Echinococcus* sp.

Ectoplasm. The outer clear zone of cytoplasm on the immediate margin of the cell.

Epismastigote. A stage of hemoflagellate ("crithidia-form") that is usually found in the invertebrate host or in cultures. It has an undulating membrane and a flagellum. The kinetoplast is just anterior to the nucleus.

Fibrils. Minute filaments which serve as organelles of locomotion in certain species of protozoans.

Filariform. The infective-stage larvae of hookworms and *Strongyloides.*

Flagellate. A general term applied to protozoans of the class Zoomastigophorea characterized by the presence of whiplike flagella which serve as organelles of locomotion.

Gamete. The sexual cell which is the end product of gametogeny in the life cycle of the malaria parasite. In a process comparable to that of fertilization in higher forms, the macrogamete and microgamete combine to produce the zygote within the body of the mosquito.

Glycogen. The chief carbohydrate form in which food is stored in tha animal body. In protozoans glycogen stains very poorly with hematoxylin, and in stained preparations the poorly stained aggregations of glycogen are termed "glycogen vacuoles."

Helminth. A general term applicable to the various species of worms which may be parasitic in man.

Hemozoin. The pigment found within malaria parasites. Also the pigment deposited in body tissue as a result of the rupture of infected red cells at the completion of the schizogenous cycle of the malaria parasite.

Hermaphroditic. Possessing male and female reproductive organs in the same individual.

Hexacanth. The six-hooked embryo of certain species of tapeworms which is liberated from the egg at the time it hatches.

Hyaline. Glassy and transparent, or nearly so.

Hydatid. The cyst stage of a tapeworm larva in which the cyst contains daughter cysts, each of which contains many scoleces.

Karyosome. One of the spherical masses of chromatin in the nucleus of a cell, generally situated at or near the center of the nucleus.

Leishmaniasis. Any of the three diseases caused by members of the genus *Leishmania.*

Leishman-Donovan Body. The intracellular amastigote of *Leishmania donovani.*

Merozoite. Asexual forms in the developmental cycle of the malaria parasite which are liberated into the blood stream when the schizont reaches maturity.

Metacercaria. The encysted resting stage of a trematode either within the tissues of a crustacean or fish, or upon the surface of aquatic or semiaquatic vegetation.

Miracidium. The free-swimming larva liberated into the water from the egg of a fluke at the time the egg hatches.

Nematode. A general term applicable to all species of roundworms.

Nocturnal. Activity limited to the hours of darkness.

Nucleus. A spherical body within a cell which forms the essential and vital part that controls the cell's activities. It is distinguished from the remainder of the cell by its denser structure and consistent organization of chromatin material.

Onchocerciasis. A term applied to the disease which results from infection with the parasite *Onchocerca volvulus.*

Oocyst. The swollen saclike structure which develops in the stomach wall of the mosquito as a result of invasion by the zygote. When mature, it gives rise to the malarial sporozoites. Also, a stage in the development of coccidia.

Operculum. The cap which covers the opening through which embryo of certain species of flukes and tapeworms escape from the eggs at the time of hatching.

Paragonimiasis. The term applied to the disease which results from infection with the parasite *Paragonimus westermani.*

Proglottids. Individual divisions of the chain of segmentlike structures which make up the body of tapeworms, exclusive of the head and neck.

Promastigote. A stage of hemoflagellates that is "leptomonas-form" in shape, usually found in the invertebrate host or in cultures. Its body is elongated and somewhat flattened. A flagellum arises from the anterior end, and it has no undulating membrane.

Protozoans. Single-celled animals characterized by the fact that the body is composed of one or more nuclei surrounded by cytoplasm and contained within a limiting cell membrane.

Pseudopod. A temporary protrusion of the outer margin of the cell wall of an ameba serving for purposes of locomotion and feeding.

Rhabditiform. Nematode larvae that have a muscular, bulbed esophagus.

Rostellum. A process at the anterior end of certain parasitic intestinal worms that can be either armed with hoods or unarmed according to species.

Schistosome. The general term applied to the blood flukes.

Schistosomiasis. A term designating the disease produced by infection with any of the three species of blood flukes which parasitize humans.

Schizogony. The asexual cycle of sporozoa.

Scolex. The attachment end of a tapeworm consisting of the head and neck.

Sparganosis. The term applied to the disease produced by the migration within body tissues of pseudophyllidean tapeworm larvae.

Sporocyst. a. A case or cyst secreted by some sporozoans preliminary to sporogony; also the resulting encysted sporozoan.

b. A saccular structure that is the first reproductive form of a digenetic trematode in the molluscan host. It buds off cells from its inner surface which develop into rediae within the sporocyst.

Sporozoite. The infective stage of the malarial parasite which migrates to the salivary gland of the mosquito.

Strobila. The entire adult tapeworm including the head, neck, and chain of proglottids.

Trematode. A common name applied to the flukes as a group.

Trichinosis. A disease caused by infection with the "pork-worm," *Trichinella spiralis.*

Trophozoite. The active vegetative feeding motile stage of a protozoan.

Trypanosome. A blood and tissue flagellate of the genus, *Trypanosoma.*

Trypomastigote. A stage of hemoflagellates that has an undulating membrane and flagellum which originates from a kinetoplast that is situated posterior to the nucleus. It has the typical "trypanosome-form" shape.

Vacuole. A term applied to various small aggregations of material (generally food) which float about in the cytoplasm within protozoan cells.

Vector. A carrier, especially the animal (usually an arthropod) which transfers an infective agent from one host to another.

Viable. In living conditions.

Volutin. Chromatin-like bodies found within the cytoplasm of trypanosomes.

Xenodiagnosis. Diagnosis accomplished by allowing a known natural uninfected vector to feed upon a suspected infected individual for the purpose of attempting to recover the organism from the vector.

Zygote. The cell resulting from the fusion of the two gametes.

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