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LABORATORY PROCEDURES FOR THE DIAGNOSIS OF INTESTINAL PARASITES

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
Centers for Disease Control

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LABORATORY PROCEDURES FOR THE DIAGNOSIS OF INTESTINAL PARASITES

Dorothy M. Melvin, Ph.D.
and
Marion M. Brooke, Sc.D.

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U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
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LABORATORY TRAINING AND CONSULTATION DIVISION
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PREFACE

This manual is based on material developed for courses in the "Laboratory Diagnosis of Parasitic Diseases," which have been presented by the Centers for Disease Control (CDC) since 1945. The authors have been associated with these courses since their beginning and have evaluated a great deal of material in preparing laboratory manuals and brochures for student use. This manual contains information on practical procedures for performing parasitologic examinations in public health and medical laboratories. It includes information on collecting and processing specimens, methods of examination, and establishing reliable routine laboratory regimens for different types of laboratories. Information on collecting and shipping specimens for serologic testing is also included, although immunodiagnostic procedures are not discussed. The diagnostic stages of intestinal helminths and protozoa have been described by the authors in another publication: *Morphology of the Diagnostic Stages of Intestinal Parasites of Man* (Brooke and Melvin, 1978), and are not included here.

Like previous editions this third edition of the manual is divided into two sections: (1) a text section in which the available diagnostic procedures and methods are described and evaluated, and (2) a techniques section which gives step-by-step directions for diagnostic procedures, formulas for solutions, and methods for preparing and examining specimens. The techniques section also contains information on microscopy, the calibration of ocular micrometers, quality control, and safety precautions.

Some of the material in this manual has been taken from *Amebiasis: Methods in Laboratory Diagnosis* by Marion M. Brooke (out of print) and the self-instructional lesson prepared by CDC, *Amebiasis: Laboratory Diagnosis: Part III: Laboratory Procedures* (see Reference Materials in QUALITY CONTROL for source).

The authors acknowledge the contributions of numerous associates over the years and thank the various people who have assisted in the preparation of the manual, particularly Miss Edwina B. Davis, Chief, and Mr. Philip Thompson, Publication Activities, Center for Infectious Diseases, who edited the material, Dr. John Krickel, Director of the Laboratory Training and Consultation Division, who helped in developing the format of the original manual, Mrs. China Christian who typed the manuscript, and Mr. Ed Biel, artist, Graphics

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Section, Publication Management, who prepared most of the illustrations.

Dorothy M. Melvin, Ph.D.
Marion M. Brooke, Sc.D.

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INTRODUCTION

Accurate clinical diagnosis of intestinal parasite diseases is difficult and laboratory confirmation is usually necessary. Demonstrating the diagnostic stage or stages of the parasite by directly examining specimens is the most reliable method of establishing a diagnosis of most parasitic infections, although indirect (serologic) methods are available for a few in which the organism is not readily demonstrated, including trichinosis, echinococcosis, extraintestinal amebiasis, and chronic schistosomiasis.

This manual is concerned with direct laboratory procedures used in recovery and identification of intestinal parasites and related species (atrial, bladder, and lung parasites) and with the collection of satisfactory specimens. Competent laboratory work is dependent on several factors: 1) personnel trained in examining specimens and accurately identifying organisms, 2) adequate laboratory facilities, including a good microscope, and 3) satisfactory specimens. Any laboratory procedure used for directly demonstrating parasites is only as reliable as the microscopist who examines the specimen; therefore, all persons attempting to make parasitologic examinations should have intensive training and supervised experience.

The primary purpose of this manual is to present selected methods which can be used routinely in laboratories performing parasitologic examinations and which, when performed properly, are reliable and thorough.

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SPECIMEN COLLECTION AND HANDLING

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SPECIMEN COLLECTION AND HANDLING

The usual diagnostic stages of intestinal parasites are helminth eggs and larvae and protozoan trophozoites and cysts. Specimens obtained for diagnosis should be collected and handled so that these stages, when present, will be identifiable when the specimens reach the laboratory. In fact, reliable diagnoses of parasitic diseases begin with satisfactory materials, and the importance of properly collected specimens cannot be overemphasized. Inadequate samples and old or poorly preserved materials are usually of little or no value in establishing a diagnosis and may lead to erroneous conclusions.

The most common type of body material submitted for parasitologic examination is feces, but other materials such as sputum, urine, aspirates, tissue scrapings, and specimens obtained by biopsy may be submitted in certain cases.

FECAL SPECIMENS

•COLLECTION

NORMALLY PASSED SPECIMENS

To obtain satisfactory material for examination, adequate instructions and proper containers for collecting fecal specimens must be provided. Persons who have little or no knowledge of the proper procedures for submitting samples to the laboratory often make the collections; therefore, complete and explicit directions should be given to the person responsible for obtaining the specimen. Printed instructions with line drawings to illustrate certain steps are often included in the collection kits the laboratory supplies. In hospitals or clinics, brief but specific directions should be supplied to ward or nursing stations.

Specimens for diagnosis may be divided into three categories: 1) those received directly from hospitalized patients; 2) those submitted by outpatients; and 3) those shipped or mailed to the laboratory. In hospital or clinic laboratories, most patient specimens fall into the first two categories, although proficiency testing specimens may be received by mail. Public health and reference laboratories

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most often receive shipped specimens. The criteria for satisfactory material are essentially the same for all three categories, but the method of submission, described in the section on Transportation and Preservation, will vary.

In collecting fecal specimens, several factors should be considered: type of container, size and age of the specimen, and substances that might interfere with examinations. The container should be clean, dry and sufficiently large to accommodate a sample of adequate size. It should also be leakproof, with a tight-fitting lid to prevent spills. A half-pint waxed cardboard carton with an operlapping lid is recommended for routine collection (fig. 1). This type of carton can be defecated into directly, does not leak, and permits labeling directly on the box. Plastic cups of similar size with snap-in lids are also satisfactory. Larger sizes of both types of containers are available.

Feces should be passed directly into the container or on clean paper and transferred to the container for transporting to the laboratory. In hospitals, specimens may also be collected in bedpans if the feces are not contaminated with urine.

Information identifying the patient and the date and hour of passage should be recorded on the carton or should accompany the specimen. The time of passage is especially important, since trophozoites soon die outside the body and other stages may undergo changes that would hamper identification. Ideally, unpreserved specimens should arrive in the laboratory within 1 to 2 hours after passage. Older specimens, especially if they contain trophozoites, may be erroneously reported as negative. A suggested identification label for specimen containers is as follows:

Name _____

_____

Age _____ **Male ( )**

Female ( )

Date _____ **and**

hour _____ **AM / PM stool passed**

To permit adequate macroscopic and microscopic examination, the entire fecal passage should be submitted to the laboratory. This not only provides sufficient material for several techniques, but

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allows the technologist to select a particular portion of the stool for examining. For example, soft or mucoid material is preferred for preparing smears for staining and more formed portions are used for concentration. Since organisms inhabiting the lower colon may often be present in the outer layer of the fecal bolus, having the entire stool will allow the technologist to scrape material from the surface for examining. Surface material is often useful in diagnosing *Schistosoma mansoni* and *Entamoeba histolytica* infections.

If the entire fecal specimen cannot be submitted, a quantity of about 20 to 30 grams (the size of a medium-size walnut, if formed, or 2 to 3 tablespoonsful, if loose or watery) should be provided to the laboratory.

Several substances, generally referred to as "interfering substances," will make the specimen unsatisfactory for parasitologic examinations. Urine and water will destroy trophozoites, and feces contaminated with either are not suitable for examination. Specimens collected in bed pans should be checked to be sure they are free of urine. Occasionally, feces are dipped out of toilet bowls and thus mixed with water. Not only does water destroy trophozoites, but there is a danger of coprozoic free-living organisms contaminating the feces and making diagnosis difficult. In addition, feces deposited on soil (as may happen with outpatients) are not satisfactory, since free-living larvae and other contaminants from the soil may cause errors in diagnosis.

Certain drugs and compounds will also render the stool specimens unsatisfactory for examination or for detection of organisms. Among these are antidiarrheal compounds, antibiotics, antacids, bismuth, and barium (Juniper, 1962). Specimens for parasitologic diagnosis should be obtained before these compounds are used, otherwise collection must be delayed until the effects have passed. Specimens should not be collected for 7 to 10 days after barium or bismuth have been given, since crystals and particles of these compounds in the feces will interfere with examination and may destroy trophozoites by their abrasive action. Antibiotics often cause a temporary decrease or absence of organisms in the stools and reliable diagnoses may not be possible for 2 to 3 weeks or more. In addition, stools should not be collected from patients receiving gallbladder dye until about 3 weeks afterwards (McQuay, 1969).

Specimens collected in very cold regions should not be allowed to freeze, since freezing and thawing may destroy protozoan cysts and trophozoites (Goldman and Johnson, 1950; Chang, 1955).

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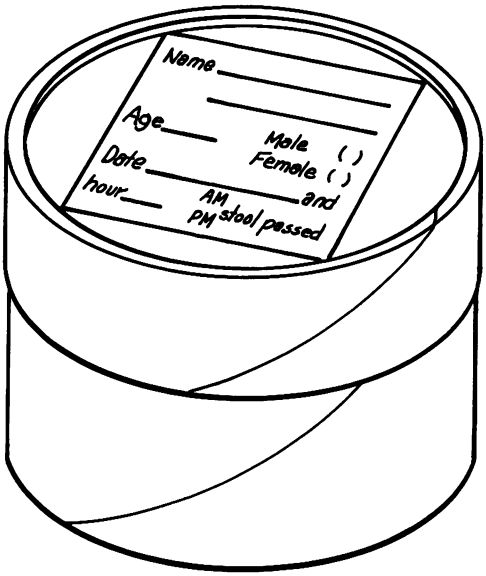
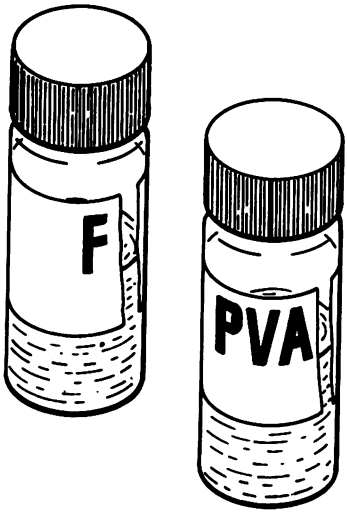
Figure 1.
Containers for Collection of Fecal Specimens



Carton for Unpreserved Specimens Submitted Immediately to the Laboratory



Two Vials Containing Formalin and PVA—Fixative Solutions for Preserving Specimens which ARE NOT Sent Immediately to the Laboratory

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MULTIPLE SPECIMENS

Because of the intermittent passage of certain parasites from the host and limitations of available diagnostic techniques, the possibility of finding organisms is increased by examining multiple specimens (Andrews, 1934; Sawitz and Faust, 1942; Wykoff and Ritchie, 1952; Swartzwelder, 1952; Alicna and Fadell, 1959). A single, normally passed specimen reveals only about one-third to one-half of the species present. Thus, a physician receiving a report of "no parasites found" on a single specimen should realize that this does not exclude the possibility of the patient being infected. In general, nematode species such as *Ascaris lumbricoides,* hookworm and *Trichuris trichiura* shed eggs more or less constantly and may be detected daily in feces. Other parasite species, however, especially the protozoa, are passed irregularly and possibly for only a few days at a time (Marsden, 1946). The number of cysts of *Entamoeba histolytica* passed in the stools fluctuates from day to day,with cyclical peaks occurring 7 to 10 days apart (Tsuchiya, 1932; Lincicome, 1942; Kershaw, 1946; Stamm, 1957). *Giardia lamblia* cysts are also passed intermittently at intervals ranging from 2 to 3 days to 7 or 8 or more (Rendtorff, 1954; Danciger and Lopez, 1975). Egg production in certain of the helminth infections, particularly the schistosomes and *Diphyllobothrium latum,* is also irregular. Proglottids of *Taenia* species may be passed at intervals. Thus collecting specimens at 2- to 3-day intervals seems preferable to obtaining them on successive days.

Workers do not agree on the number of specimens that should be examined to rule out infections. If time permits, however, at least 2 or 3 normally passed stool specimens, preferably spaced 2 to 3 days apart, should be examined before one resorts to catharsis or sigmoidoscopy. Normally passed specimens are more likely to contain cysts which can be identified with greater reliability then trophozoites (Bijlmer, 1948; Brooke *et al.*, 1953; Stamm, 1957; and Goldman, 1964). Since organisms may be rare in passages following purgation for a week or more, normally passed specimens should be collected before the purge is given.

PURGED SPECIMENS

In some cases, purged specimens (obtained following administration of a laxative) may be desirable or necessary. Several workers have shown that purgation increases the possibility of finding organ-

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isms in a single specimen (Andrews, 1934; Alicna and Fadell, 1959). In cases of protozoan infections, however, trophozoites predominate in purged specimens, and species identification of trophozoites, as already stated, is usually less reliable than identification based on cysts. Saline laxatives such as sodium sulfate or buffered phosphosoda should be used for purgation. Mineral oil, bismuth, or magnesia compounds are unsatisfactory. Oil globules interfere with examination, and crystalline debris from bismuth and magnesia compounds may obscure the organisms or affect the appearance of trophozoites.

The patient receiving catharsis should be directed to collect all bowel movements in separate boxes numbered in series and to deliver them immediately to the laboratory. Postcathartic specimens should be examined for eggs, larvae, and cysts as well as for trophozoites. If the examination may be delayed or if it is necessary to ship the specimen to the laboratory, a portion of the material should be mixed with polyvinyl alcohol (PVA)-fixative, preferably upon collection or as soon afterwards as possible (figs. 1 and 2). (See the paragraph on Preserved Specimens for additional information on PVA-fixatives.) This practice may be advisable even in clinics and hospitals where, theoretically, the specimens are taken directly to the laboratory and examined, but where considerable delay may actually occur.

ENEMA COLLECTION

Specimens may also be obtained by using a saline solution enema. Since intestinal parasites seldom locate in the lower colon (Hegner *et al.*, 1938), enemas tend to be less efficient than purges for bringing down organisms. However, saline enema material can be used for parasitologic examination in lieu of other types of specimens. It should be handled as suggested for purge specimens.

•TRANSPORTATION AND PRESERVATION

As stated earlier, the method of collecting and submitting fecal specimens will vary with the location of the patient in relation to the laboratory. Either unpreserved or preserved specimens may be submitted.

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UNPRESERVED SPECIMENS

Specimens from hospital or clinic patients are usually unpreserved and should reach the laboratory within 1 to 2 hours after passage. Since protozoan trophozoites do not multiply or encyst outside of the body, they degenerate and die soon after passage. Therefore, it is essential that unpreserved feces be brought to the laboratory without delay. If the patient is having diarrheic stools, specimens should be received immediately after passage, within ½ to 1 hour. Because diarrheic specimens are more apt to contain trophozoites than cysts there is a greater urgency in getting these to the laboratory.

Outpatients also usually submit unpreserved specimens, but in many cases, unavoidable delays occur which may result in morphologic alterations in parasites, particularly in protozoa. Ideally, therefore, preservatives should be used to keep the organisms in good condition until the material can be examined. If specimens can be brought to the laboratory within 12 to 24 hours, the patient should be provided with a carton and a vial (20- to 30-ml capacity) of PVA-fixative (Brooke and Goldman, 1949). He/she should be instructed to pass the stool into the container and to mix a portion with the fixative. Both the unpreserved and the PVA-fixed specimens should be submitted to the laboratory.

More information on the use of PVA-fixative will be found under Preserved Specimens.

If outpatient specimens will be delayed for longer than 24 hours, unpreserved feces may be unsatisfactory for reliable diagnoses. In these cases, specimens should be preserved as described in the following section.

PRESERVED SPECIMENS

Many diagnostic laboratories receive specimens through the mails. If these are delayed in reaching the laboratory and are not preserved properly, any organisms present (particularly protozoa) may be unrecognizable or completely disintegrated by the time the specimen is examined. Adequate and proper preservation is, therefore, essential. In addition, the shipping containers must meet postal requirements for mailing laboratory materials. These are described in the paragraph entitled Packing Specimens for Mailing.

Helminth eggs and larvae and protozoan cysts can be preserved in 5 or 10 percent Formalin in a 1:3 proportion of stool and fixative. The 5 percent solution is preferred because cysts, eggs of *Hymeno-*

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lepis nana and *Hymenolepis diminuta*, and *Stronygloides* larvae may become distorted in 10 percent Formalin. Formalin does not satisfactorily preserve trophozoites and is not recommended for this stage.

For preserving trophozoites (and cysts), PVA-fixative should be used, in a 1:3 proportion of stool and fixative. After preservation in PVA-fixative, trophozoites present will remain suitable for staining and identification for several months. In the CDC laboratory, specimens are generally usable for 1 year. Different investigators have demonstrated the effectiveness of PVA-fixative in preserving the intestinal protozoa, particularly the trophozoite stage (Goldman and Brooke, 1953; Myers *et al.*, 1959; Melvin and Brooke, 1962; Markell and Quinn, 1977).

The PVA-fixative currently in use is Schaudinn's fixative to which PVA powder, a synthetic resin, has been added. Since Schaudinn's solution contains mercury, an environmental pollutant, workers are investigating the use of nonmercuric solutions in fixing protozoa for permanent staining. Thus, PVA-fixative solutions which do not contain mercuric compounds may be available in the future as substitutes for the present PVA-Schaudinn's fixative. In this manual, however, the term "PVA-fixative" refers to PVA-Schaudinn's fixative unless otherwise indicated.

The formula for preparing PVA-fixative, directions for use, and commercial sources for both PVA powder and fixative solutions are described in the section on Preservation of Specimens.

For specimens that must be shipped to the laboratory, a two-vial method which enables the laboratory to recover all diagnostic stages of the intestinal parasites (fig. 2) is recommended (Brooke and Goldman, 1949). One vial of the kit contains PVA-fixative and the other contains 5 or 10 percent Formalin. Screw-cap vials of 20- to 30-ml capacity are recommended. In the United States, a number of State public health laboratories have provided such shipment kits with good results (Kelly, 1952; Harper *et al.*, 1957). PVA-fixative is stable and may be kept for 6 months or longer in tightly stoppered bottles, but when it is dispensed in small vials, it may harden or jell after a relatively short time and become unsuitable for use. Therefore, to be sure that the fixative in the collection kit is in good condition, an expiration date should be indicated on the vial. Two months is a practical time limit for routine purposes.

Another satisfactory preservative for stool specimens is the merthiolate-iodine-formaldehyde (MIF) solution (Sapero and Lawless,

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1953) which preserves trophozoites as well as cysts, eggs, and larvae. MIF solution has been recommended for survey work when only one procedure, the MIF concentration (Blagg *et al.*, 1955) is performed. (See section on Preservation of Specimens.) This solution can be substituted for Formalin in the two-vial collection method. In using MIF as a preservative, the iodine solution must be added to the merthiolate-formaldehyde solution just before the specimen is collected. Commercial MIF collection kits have simplified this procedure and eliminated many of the problems in using MIF preservative. Most of the cysts and some trophozoites can be identified in wet-mount preparations (MIF and others), but identification of trophozoites should be confirmed in permanently stained films. Therefore, a vial of PVA-fixative should be included in the collection kit so that stained slides can be prepared.

Several commercial collection kits are available for parasitologic specimens. Most of these are satisfactory, but kits that contain vials of PVA-fixative as well as vials of Formalin or MIF solution are recommended for general or routine stool collections.

Regardless of the preservative used, proper proportions of feces and solution and thorough mixing are essential for good preservation of the organisms. The collector should be given complete and easy-to-follow directions such as shown in figure 2. The patient should be instructed to pass the specimen into a dry container or onto a paper, then to transfer a portion of the stool to the vials of preservative and mix thoroughly. Mixing should be emphasized. Applicator sticks and/or tongue depressors should be provided for this purpose. To get the proper proportions of feces and preservative, draw a line on the vial or put the label on the bottle so that the line or label top is at an appropriate level. The collector is instructed to add feces until the fluid comes to the designated mark. The collector should also legibly record all pertinent information (patient's name, and the time and date of passage) on the bottle label or on a piece of paper attached to the bottle.

HELMINTHS

Adult worms or proglottids of tapeworms are passed with or without fecal material. They can be picked out of feces and placed in a bottle of saline or water or in 70 percent alcohol or 10 percent Formalin. If they are to be sent immediately to the laboratory they should not be preserved, since preservation without prior relaxation may cause the organism to contract or may distort it.

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CULTURE MEDIUM FOR SHIPPING

An egg-extract culture medium has been developed for shipping *E. histolytica* trophozoites in fecal specimens or sigmoidoscopic material from the field to the laboratory (Gleason *et al.*, 1960). Screw-cap tubes are used to facilitate mailing. In this medium at room temperature, trophozoites may remain viable up to 4 days, enabling the laboratory to obtain cultures of living trophozoites. For best results, freshly passed feces should be inoculated into the medium. Details of preparation and use are given in the section on Cultivation.

PACKING SPECIMENS FOR MAILING

Broken or damaged specimens are of little or no value to the recipient laboratory. Therefore, persons shipping specimens to diagnostic or reference laboratories or elsewhere should be especially careful to pack the specimens with adequate "cushioning" or protection. Furthermore, the outer container should be clearly addressed to insure its arrival in the proper laboratory. A second label should be placed on the inside in case the outer label is defaced during transit. Before packing, the sender should make certain that each specimen is properly and completely labelled and tightly capped or sealed and that all essential information accompanies it.

Parasitologic specimens sent through the mails are usually preserved and thus are nonviable and noninfectious. Nevertheless, postal regulations require that laboratory and clinical specimens be packed in special containers in such a manner that materials will not leak during transit. Extracts from Title 39, Parts 21, 123, and 124 of the Code of Federal Regulations governing the shipment of harmful matter will be found on pages 21-25.

Other Federal Regulations (for example, see 124.1(b)) may be applicable, depending upon the specific contents of the package and the method of shipment. The cited regulations are subject to amendment from time to time, and the shipper bears full responsibility for compliance with the latest regulations.

Styrofoam containers which fulfill these requirements are available (figure 3). In addition, double mailing containers consisting of a heavy cardboard outer cylinder and a metal inner cylinder may be used (figure 3). Several sizes of these are available, but perhaps the most useful for parasitologic specimens is the one with the outer cylinder of 6-5/8 inches by 2-3/4 inches. The vials of preserved feces

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or the culture tubes should be sealed with adhesive tape around the top to prevent leakage, wrapped in suitable absorbent packing material (cotton or ordinary toilet tissue are satisfactory), and placed in the inner cylinder. Identifying information can be wrapped around the inner cylinder which is inserted into the outer container.

Prepared slides, such as stained fecal smears or mounts of helminths, do not have to meet those postal requirements and can be shipped in cardboard or plastic holders, slide boxes, styrofoam mailers, or other suitable containers if they are packed carefully to prevent damage or breakage. Slides can be put in plastic slide holders or wrapped in facial tissue, toilet tissue, or other soft paper. Toilet tissue makes an excellent wrapping if the following method is used. Place a slide on the tissue and fold the paper over once or twice to surround the slide. Place a second slide on top of the wrapped slide and roll the whole packet over again (figure 4). In this way, the slides are protected during transit and can be easily unrolled from the packing by the receiver.

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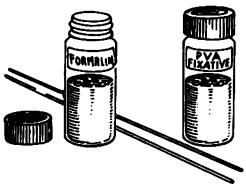
Figure 2.
Use of PVA-Fixative and Formalin for Submitting Stool Specimens to be Examined for Parasites

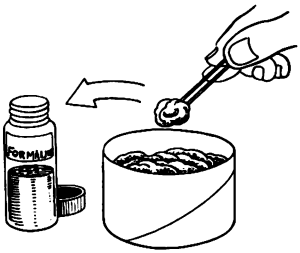
ADAPTED FROM BROOKE AND GOLDMAN, 1949

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

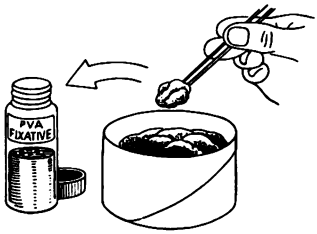


1 THE KIT CONSISTS OF TWO GLASS VIALS (ONE WITH 5% FORMALIN AND ONE WITH PVA-FIXATIVE) AND TWO APPLICATOR STICKS.

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

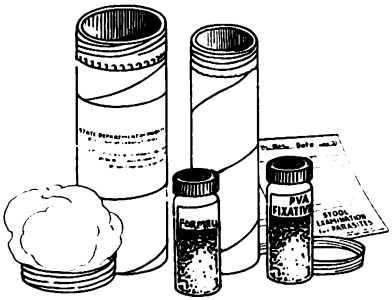


3 USING APPLICATOR STICKS, PLACE A QUANTITY OF THE STOOL (ABOUT THE DIAMETER OF A QUARTER) INTO THE VIAL CONTAINING FORMALIN, SCREW CAP ON TIGHTLY.



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.

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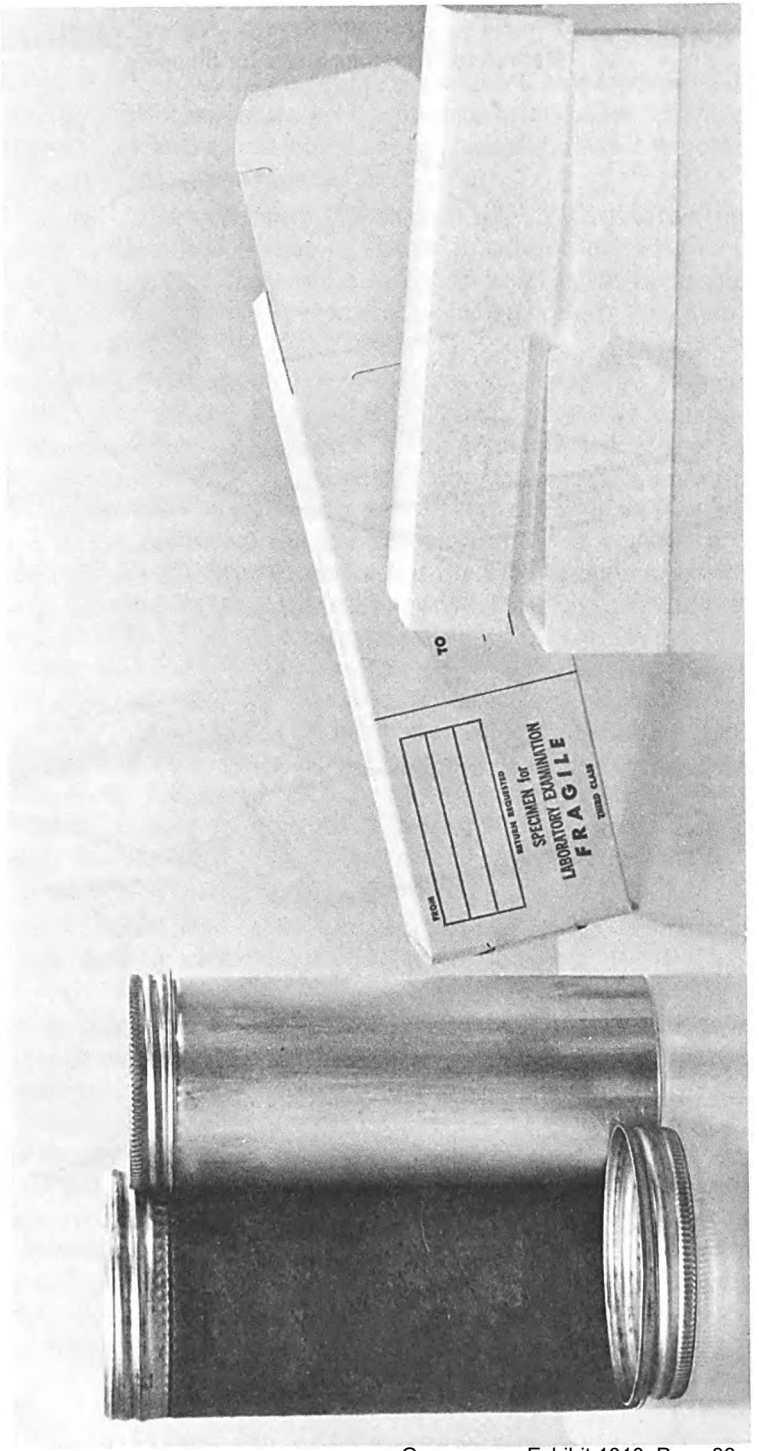

Figure 3.
Containers for Shipping Fecal Specimens

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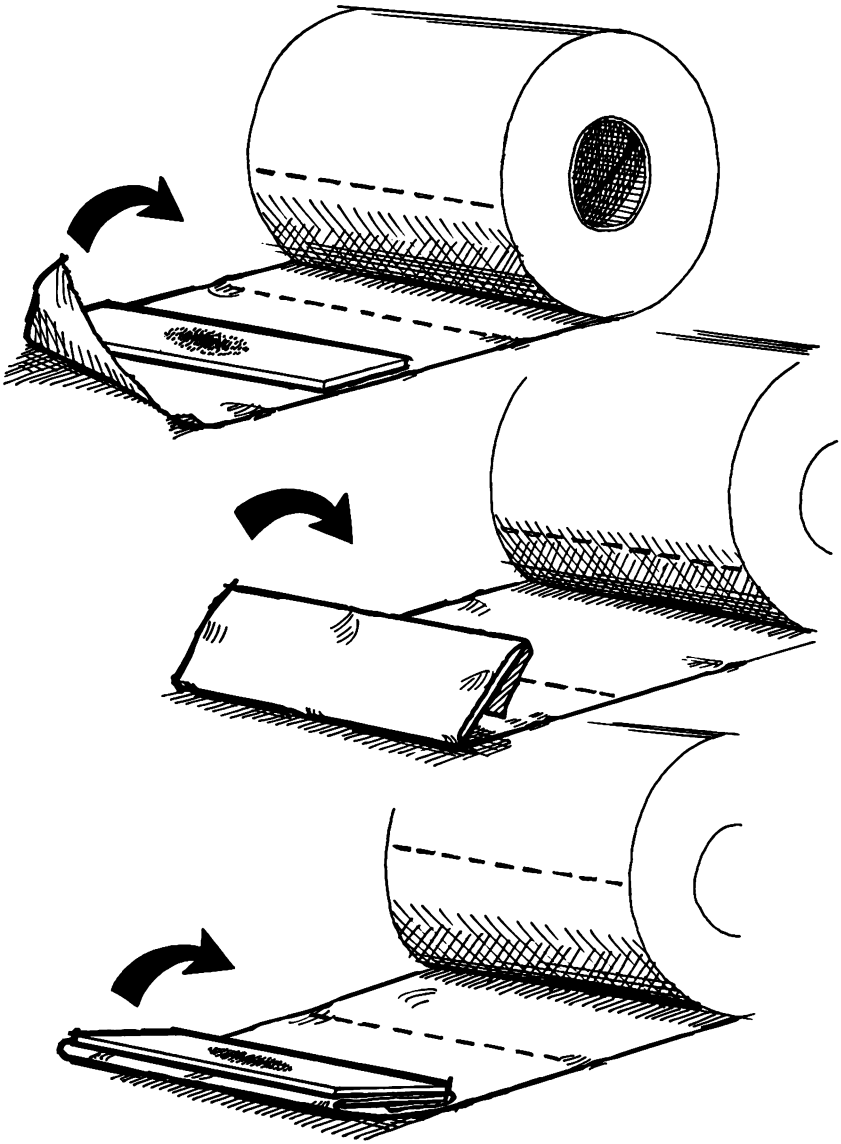
Figure 4.
Method for Wrapping Slides for Shipping



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•STORAGE AND HANDLING OF FECAL SPECIMENS

Not only must specimens be properly collected and transported to the laboratory, but they must be handled properly after arriving in the laboratory. Good specimens can be easily ruined by neglect, resulting in unreliable diagnoses.

Preserved specimens (those in Formalin, MIF, PVA-fixative) may be examined at the convenience of the microscopist and may be left on the table or counter. There is no need to refrigerate these specimens; in fact, if PVA-fixed specimens are refrigerated, they harden and become useless.

Unpreserved specimens, on the other hand, should be examined immediately upon receipt, or stored in such a way that organisms will be maintained in an identifiable condition. If feasible, the feces should be examined while warm, especially if protozoan infections are suspected and the specimen is soft or diarrheic. If examination must be delayed, however, the specimen should not be kept artifically warm. Elevated temperatures hasten the death of the organisms, particularly amebae trophozoites (Tsuchiya, 1945; Gurevitch and Delightish, 1947). Although cysts will survive longer than trophozoites (Chang and Fair, 1941; Simitch *et al.*, 1954), they too will degenerate fairly quickly at elevated temperatures. Helminth eggs and larvae are more resistant to environmental changes than protozoan trophozoites and cysts, but development or hatching, which could complicate diagnosis, may occur. In general, therefore, if a fresh specimen cannot be examined immediately, keep it at room temperature for the next 2 to 3 hours or in the refrigerator (4°C) if longer storage is necessary.

Specimens should not be stored in a freezer, since freezing and thawing will destroy protozoa (Goldman & Johnson, 1950; Chang, 1955).

Unpreserved specimens occasionally arrive in the laboratory when no one is available to examine them. These can be handled in several ways, but they must be taken care of at the time they are received. If feasible, preparations for permanent stains can be made and put into fixative (smears may remain in fixative for 2 or 3 days without harm) and the remainder of the specimen can be put in the refrigerator. In this way, trophozoites, if present, will be fixed while in the best possible condition. A second method is to preserve portions of the specimen in 5 percent Formalin or MIF solution and in PVA-fixative. If possible, the material should be preserved as soon as passed. If neither of these methods is practical, specimens can be put into the

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refrigerator. Trophozoites may not be as typical after a weekend at 4°C as if they had been fixed shortly after passage, but they generally are identifiable in stained preparations. Refrigeration for a day or two would not affect the appearance of cysts, eggs, or larvae.

It should be emphasized that regardless of the handling procedure used, specimens must be taken care of **as soon as possible after passage.** They should reach the laboratory within 1 to 2 hours and, once in the laboratory, must be properly stored or preserved to maintain organisms in good condition for diagnosis.

OTHER SPECIMENS

Certain of the intestinal parasites pass diagnostic stages in material other than feces and occasionally intestinal parasites, such as *E. histolytica*, may be located in the liver, lungs, intestinal mucosa, or other tissue. These specimens are usually examined in local laboratories, but if they are to be mailed, they must be packed according to postal regulations as described for fecal specimens.

•SPUTUM

Sputum specimens should be collected in suspected cases of paragonimiasis. Although *Paragonimus westermani* eggs are commonly found in sputum, in many cases they may be present in the stools (Komiya and Yokogawa, 1953), and for this reason, fecal specimens as well as sputum specimens should be examined. Pulmonary amebiasis and echinococcosis possibly may also be detected by examining sputum specimens. In cases of suspected amebiasis, sputum should be examined immediately for trophozoites or preserved in PVA-fixative for staining later. Trophozoites of *E. histolytica* must be differentiated from those of *Entamoeba gingivalis*, a commensal parasite of the mouth that might be present. If examination must be delayed, sputum for diagnosis of paragonimiasis or echinococcosis may be preserved in 10 percent Formalin.

•URINE

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

The optimal urine specimen for revealing eggs of *S. haematobium* has been shown to be the one passed about or shortly after noon

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rather than the previously recommended early morning specimen (Stimmel and Scott, 1956; Pugh, 1979). Furthermore, eggs are more frequently present in the last few drops of the specimen than the first portion voided and more likely to be found in urine containing blood or pus (Belding, 1965). It has been shown that exercise, such as running for 10 minutes, increases the chances of finding eggs. Since the passage of schistosome eggs fluctuates, repeated daily examinations for 3 consecutive days must often be performed to demonstrate the eggs.

For detecting *T. vaginalis*, fresh urine specimens, preferably the first portion of voided urine (Roth, 1944), should be used. Old specimens are not satisfactory, since the flagellates may be dead or immotile or the urine may become contaminated with a species of *Bodo*, a nonparasitic flagellate, which might be confused with *T. vaginalis.*

•ANAL SWABS

Anal swabs are the usual means of detecting *Enterobius vermicularis* infections, since the female worm ordinarily deposits eggs on the perianal region rather than within the intestinal tract. Two types of preparations are commonly in use: the cellulose-tape slide preparation (fig. 25, Miscellaneous Procedures) (Graham, 1941; Brooke *et al.*, 1949) and the Vaseline-paraffin swab (Markey, 1950). Because of the peculiar migratory habits of the female, these swabs are best collected either between the hours of 9 p.m. and midnight or early in the morning before defecation or bathing.

The responsibility for taking the swab usually rests with the patient, or more commonly with the parents of the patient since children are the chief victims of this parasite. Explicit instructions on the proper techniques of collecting, handling, and transporting the swabs should be provided. An illustration of the cellulose-tape method may be useful. The person collecting the specimens should also be instructed to wash the hands thoroughly after each collection to avoid cross-contamination of swabs from several patients. He or she should understand that several daily collections may be necessary.

Since migration of the females may not occur every day, examination of a single swab will miss a significant percentage of the pinworm infections; therefore, repeated collections should be made. Sadun and Melvin (1956) demonstrated that a single cellulose-tape examination revealed only 55 percent of the infections and that six

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consecutive daily collections were necessary to pick up essentially all of the infections. For practical purposes, three consecutive daily collections might be routinely examined, since these authors found that this number revealed 90 percent of the cases. Either type of swab may be stored in the refrigerator, without appreciable damage, for as long as 2 months before it is examined.

Anal swabs are used for collecting *Taenia* eggs, especially in *T. saginata* infections where proglottids may migrate out of the anus (Rijpstra *et al.*, 1961). They can be collected with a cellulose-tape swab like that for *E. vermicularis* eggs.

Other helminth eggs, especially those of *Trichuris* and *Ascaris* may also be found on anal swabs (Jeffery, 1950). However, anal swabs are not recommended for use in the diagnosis of these infections.

•VAGINAL AND URETHRAL SWABS

Vaginal swabs or scrapings and urethral swabs may be collected for diagnosis of *T. vaginalis* infections. In female patients, a sterile cotton tipped applicator or a sterile, nonlubricated dry speculum should be used, and the patient should not douche for 3 to 4 days before the specimen is collected (Perl *et al.*, 1956). In male patients, urethral discharge and prostatic secretions are usually obtained. Sterile cotton swabs may be used to collect specimens from the urethral orifice.

Specimens should be examined immediately after collection before the organisms lose their motility. If the material cannot be examined in the collecting room, it should be placed in a tube containing a small amount of sterile saline and sent immediately to the laboratory for examination. In addition, portions of the exudate may be inoculated into culture media, and smears for staining may be prepared and placed immediately in Schaudinn's fixative. If the examination will be delayed or if the specimen must be transported to a distant laboratory, some of the materials should be put into PVA-fixative for future staining.

•DUODENAL DRAINAGE MATERIAL

In infections with *Strongyloides stercoralis* and *Giardia lamblia*, duodenal drainage often reveals organisms when the stool specimens are negative (Jones and Abadie, 1954; Gondard, 1956; Kamath and Murugasu, 1974); therefore, when diagnosis cannot be established by fecal examinations, duodenal drainage material should be collected.

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In addition to drainage, duodenal material may be collected using a nylon thread coiled in a weighted pharmaceutical capsule which the patient swallows (Beal *et al.*, 1970; Bezjak, 1972; Thomas *et al.*, 1974). One end of the thread is held as the capsule is swallowed. As the capsule moves along the digestive tract, the thread plays out and becomes coated with mucus from the duodenal area. After 3 or 4 hours, the thread is withdrawn (the capsule separates), and the mucus is scraped off and examined.

•SIGMOIDOSCOPIC MATERIAL

When, in suspected cases of amebiasis, stool specimens are negative, material may be obtained from the intestine by sigmoidoscopy (Brooke *et al.*, 1959). If a patient has had a recent bowel movement when he reports to the clinic, sigmoidoscopy may be performed immediately. If a spontaneous bowel movement does not occur, a laxative may be given. In this case, sigmoidoscopy must be delayed 2 or 3 hours after purgation. A cleansing enema should not be given, since it may remove organisms from the area which can be viewed through the sigmoidoscope.

A serologic pipette (1 ml) with a rubber bulb should be used to aspirate material from visible lesions and the mucosa. This method is preferable to obtaining material with a cotton swab. The pipette is quite satisfactory for moist lesions, but it is less satisfactory for drier ulcers that are not covered by readily removable exudate (Wilmot, 1962). For these, and moist lesions as well, a curette or Volkman spoon may be used to gently scrape pathologic areas or the mucosal wall (Manson-Bahr, 1944). Bronchial biopsy forceps have also been used successfully (Wilmot, 1962).

•ASPIRATED MATERIAL

Liver abscesses suspected of being caused by *E. histolytica* are sometimes drained, and the aspirate is examined for trophozoites. Organisms are located chiefly in the peripheral area of the abscess and aspirates from the center portion are less likely to reveal trophozoites. The Amoebiasis Research Unit, Durban, South Africa (Freedman *et al.*, 1958; Elsdon-Dew, 1960) recommended the following procedure. At least 2 portions of exudate should be removed and numbered. The first portion, usually yellowish-white, seldom contains amebae. Later portions, reddish in color, are more likely to contain organisms, and the portion containing material from near the wall is most likely to be positive.

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•BIOPSIED MATERIAL

Biopsied material is obtained for diagnosis of parasitic infections such as trichinosis, schistosomiasis, amebiasis, and, occasionally, larva migrans. Trichinosis infections are more commonly diagnosed by serologic methods, but in some cases tissue may be examined directly for the presence of larvae. Small portions of skeletal muscle, usually the deltoid or gastrocnemius, may be removed and examined. Tissue from the rectum, colon, and bladder can be obtained for diagnosis of schistosomiasis (Ottolina and Atencio, 1943; Erfan and Talaat, 1947; Gelfand, 1948; Lichtenberg and Valladares, 1955), and punch biopsies of rectal tissue (Juniper *et al.*, 1958; Manson-Bahr and Muggleton, 1957) or liver biopsy tissue (Nelson *et al.*, 1955; Doxiades *et al.*, 1961; Wilmot, 1962) may be used in the diagnosis of amebiasis. In suspected cases of larva migrans, liver biopsy may be resorted to for diagnoses, although the chances of detecting larvae in small liver biopsies are somewhat remote. Duodenal biopsy has been used for diagnosing giardiasis (Kamath and Murugasu, 1974) but stools and duodenal aspirates are more commonly examined.

Biopsy is not recommended for echinococcosis because of the danger of complications resulting from spilling or leaking hydatid fluid into the surrounding tissue.

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POSTAL SERVICE MAILING REGULATIONS

EXCERPTS FROM TITLE 39 – POSTAL SERVICE

Postal Union Mail
PART 21–CONDITIONS APPLICABLE TO ALL CASES

Sec.
21.1 Preparing and addressing.
21.2 Postage.
21.3 Prohibitions and restrictions.
21.4 Documentation.

AUTHORITY: The provisions of this Part 21 issued under 39 U.S.C. 401, 407.

SOURCE: 37 F.R. 22578, Oct. 20, 1972, unless otherwise noted.

§ 21.1 Preparing and addressing.

(a) *Preparing*–(1) *General responsibility.* It is the responsibility of the sender to prepare articles securely and to address them clearly and correctly. Use strong envelopes or other wrappings. Take into consideration the nature of the articles being mailed and the distance they must travel to reach the country of address.

(2) *Envelopes.* Use envelopes of light color (but not brilliant colors) that do not interfere with the legibility of the address and postmark. Envelopes must be rectangular in shape, and must not measure less than 3 inches in width (height) and 4¼ inches in length. (Effective October 1, 1973, envelopes must measure at least 3½ inches in width and 5½ inches in length.) The use of envelopes measuring over 9 inches by 12 inches is not recommended. Window envelopes meeting the conditions in § 141.1(a) (4), with the exception of open-panel envelopes and envelopes with more than one panel, are acceptable in international mail. Open-panel envelopes are accepted only in ordinary (unregistered) mail to Canada.

(3) *Letter mail (LC).* Letters and packages prepaid at the letter rate of postage (letter-packages) may be placed in envelopes or prepared in package form. Sealing of letters and letter-packages is at the option of the sender, except that registered letters and letter-packages must be sealed. See § 42.2(b)(1).

(4) *Other articles*(AO). (i) Printed matter, matter for the blind, and small packets must be prepared in such a way that the contents are protected but so as not to hinder quick and easy inspection of the contents. The articles must be placed either in wrappers, on rollers, between cardboard or in bags, boxes, envelopes, or containers that are unsealed but closed in such a way as to permit easy opening and reclosing. Fasteners that offer no danger may be used or the items may be tied with string or twine in a manner that will permit them to be easily untied. AO mail must not be sealed, even if registered. The manner of pre-

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paration must be such that other pieces of mail do not run the risk of being trapped.

(ii) As an exception to subdivision (i) above, articles which would become spoiled if packed according to the general rules, and items in a transparent packing permitting a check of the contents are admitted in a hermetically sealed packing. The same applies to industrial and vegetable products in packing sealed by the manufacturer or by an examining authority in the country of origin. In such cases the sender or the addressee may be required to assist in a check of the contents, either by opening certain of the items or in some other satisfactory manner.

(iii) Articles mailed under "Other Articles" classifications must be marked to identify the class of mail, as provided in § § 22.3(e) (2), 22.4(e), and 22.5(e).

(b) *Packing requirements for certain articles*–(1) *Glass*. Articles of glass or other fragile materials must be securely packed in boxes of metal, wood, or strong corrugated fiberboard filled with paper, wood shavings, or other protective material that prevent the articles from moving about or coming in contact with each other or with the sides of the box in course of transmission.

§ 21.3 Prohibitions and restrictions.

(5) *Perishable biological materials*. Perishable biological materials, including those of pathogenic nature, when sent in the postal union mail are accepted in airmail "letter packages." The following conditions apply:

(i) *Mailing restrictions*. If a country prohibits perishable biological materials this is shown under "Prohibitions" in the country item in Postal Service Publication 42. The packages must be packed as prescribed in subdivision (iii) below and must bear distinctive violet labels by which they can be readily recognized and receive careful handling and prompt delivery.

(ii) *Qualification of mailers. (a)* Only officially recognized laboratories may send or receive letter packages containing perishable biological materials. Laboratories of the following categories are so designated:

Laboratories of local, State, and Federal government agencies.

Laboratories of federally licensed manufacturers of biologic substances derived from bacteria and viruses.

Laboratories affiliated with or operated by hospitals, universities, research facilities, and other teaching institutions.

Private laboratories licensed, certified, recognized, or approved by a public authority.

(b) A laboratory desiring to mail letter packages containing materials of this kind shall make written application on its letterhead stationery to the Mail Classification Division, Finance Department, U.S. Postal Service, Washington, D.C. 20260, explaining its qualifications and those of the prospective addressee to send and receive such materials, and stating how many packages are to be

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mailed. On approval, the mailer will receive a sufficient number of the violet labels for the contemplated shipments.

(iii) *Packaging. (a)* Perishable biological material not of a pathogenic nature must be packed in a nonporous container surrounded by sufficient absorbent material to take up all the liquid and must be placed in an outer protective container where it should fit tightly to avoid any shifting.

(b) Perishable biological material of a pathogenic nature must be packed in a tightly closed bottle or tube or heavy glass wrapped in thick absorbent material rolled several times around the bottle or tube and tied at the ends, sufficient in quantity to absorb all the liquid; the wrapped container must be placed in a strong well-closed metal box constructed to prevent any contamination outside of it. This metal box must be wrapped in cushioning material and placed in an outer protective box where it should fit tightly to avoid shifting. The outer container must consist of a hollow block of strong wood, metal, or other equally strong material with a tight lid so fitted that it cannot open during transportation.

(c) In addition to the requirements in *(a)* and *(b)*, packages must comply with the regulations governing the transmission of such materials in the domestic mail.

(d) The mailer must place on each package one of the violet labels mentioned in subdivisions (i) and (ii)*(b)* of this paragraph.

PART 123—NONMAILABLE MATTER

§ 123.1 Introduction.

(a) *Description.* Nonmailable matter includes all matter which is by law, regulation, or treaty stipulation, prohibited from being sent in the mail or which cannot be forwarded to its destination because of illegible, incorrect, or insufficient address.

(b) *Applicability.* The harmful or objectionable items identified in this part are some of the matter which may not be sent through the mail, as a matter of absolute prohibition. See Part 124 for matter mailable only under special rules or conditions. Notwithstanding any statement contained in Part 123, the burden rests with the mailer to assure that he has complied with the law. In addition to the nonmailable items mentioned in this part, certain other articles are prohibited in the mail to military post offices overseas (part 126).

(c) *Penalties for violation.* Servere penalties, by fine or imprisonment, or both, are provided for persons who knowingly mail or cause to be mailed, any matter which has been declared nonmailable under law.

(d) *Nonconformity with postal regulations.* Regardless of its nature, matter may not be mailed in any form if done in violation of postal regulations for such reasons as failure to pay postage, improper size or weight, improper permits, improper addresses, etc.

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(e) *Responsibility of mailer.* When mailers are in doubt as to whether any matter is properly mailable, they should ask the postmaster. Even though the Postal Service has not expressly declared any matter to be nonmailable, the mailer of such matter will be held fully liable for violation of law if he does actually send nonmailable matter through the mail.

§ 123.2 Harmful matter.

(a) *General provision of law.* Any articles, compositions, or materials, which may kill or injure another or injure the mail or other property, are nonmailable. This includes but is not limited to:

(1) All kinds of poison or matter containing poison.

(2) All poisonous animals, except scorpions (see § 124.3(3), all poisonous insects, all poisonous reptiles, and all kinds of snakes.

(3) All disease germs or scabs.

(4) All explosives, flammable material, infernal machines, and mechanical, chemical, or other devices of compositions which may ignite or explode.

PART 124—MATTER MAILABLE UNDER SPECIAL RULES

§ 124.1 Legal restrictions.

(a) *Harmful matter.* (1) Certain items barred from the mail, as set forth in Part 123, may be mailed if prepared and packaged in accordance with this part. These are items not outwardly or of their own force dangerous or injurious to life, health, or property.

(2) This part covers generally some of the more common situations; however, the burden rests with the mailer to assure that he has complied with the law and that anything shipped by him has been properly prepared and packaged. The ordinary test of adequate preparation and packaging is whether the contents of a parcel are safely preserved under ordinary hazards of mail handling and transportation.

(3) Products, materials, and devices are created or modified with such frequency that the Postal Service is unable to issue general rulings in advance to govern adequate preparation and packaging. Any mailer may, however, request the Postal Service in advance for a specific ruling as to mailability of his item. The request should be addressed to the local postmaster, who will forward it to the Classification Division, Office of Rates and Classification, Finance Department, U.S. Postal Service, Washington, D.C. 20260.

(b) *Applicability of other laws.* (i) Although not unmailable, as defined in part 123, certain other items may be mailed only if they comply with applicable Federal laws and regulations.

(2) Any special conditions or limitations placed on transportation or

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movement of certain things shall govern admissability to the U.S. mail, when imposed under law by the U.S. Department of the Treasury; U.S. Department of Agriculture; U.S. Department of Commerce; U.S. Department of Health, Education and Welfare; U.S. Department of Transportation; or any other Federal department or agency having legal jurisdiction.

(c) *Penalties.* Failure to comply with the regulations of the Postmaster General, as prescribed in this part, as to matter otherwise nonmailable constitutes a violation of law.

§ 124.2 Conditions for mailing.

(a) *General nature of precautions required.* (1) The restrictions against mailing of harmful matter, from which relief is granted by this part, are intended to prevent damage or harm to postal and transportation personnel, to prevent damage or destruction of other mail and property, to avoid obnoxious odors, and to prevent the spread of disease and infection.

(2) Basic precautions, covered generally in this section relate to the inner containers holding the harmful matter, internal cushioning and protection, and exterior packaging and marking.

(b) *Liquids (nonflammable) and powders.* (1) Precautions must be taken in the case of liquids, pastes, salves, ink powders, pepper, snuff, or other pulverized materials against damage to mail and property from leakage and against caustic, irritant, toxic, or soiling effect on mailhandling personnel.

(2) Containers must meet any applicable Federal specifications. Closures must effectively seal the contents against leakage. Friction tops must be fastened with solder, clips, or otherwise so that they will not come off under impact.

(3) Glass or other breakable containers of liquid must be placed to withstand handling en route. The container must be cushioned inside the carton to absorb shock and impact. Where feasible, absorbent material must be used to take up all the liquid in case of breakage.

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METHODS OF EXAMINATION

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METHODS OF EXAMINATION

FECAL SPECIMENS

• MACROSCOPIC

When unpreserved stool specimens are received in the laboratory, they should be examined macroscopically to determine the consistency of the specimen and the presence or absence of proglottids, whole worms, blood, mucus, and other abnormal conditions. Four terms are generally sufficient to designate the consistency of the specimens: watery(W), loose(L), soft(S), and formed(F). Since (if specimens are recently passed) the more fluid specimens have the greater likelihood of containing protozoan trophozoites, they should be examined in the order listed above. Figure 5 demonstrates the categories of consistency and the distribution of cysts and trophozoites. Distribution of eggs and larvae is less affected by the consistency of the feces.

The age of the specimen should also be noted. Since trophozoites are more or less fragile structures, they die fairly soon after passage from the body, but cysts, which are more resistant, survive for longer periods. Thus, a report of "no parasites found" on a "watery" specimen 24 hours old may be meaningless with regard to the intestinal protozoa; on the other hand, a similar report on a "formed" specimen 24 hours old would have greater significance. Recording the consistency and age of the stool specimen in the laboratory record and in the report will permit the laboratory director and the physician to better evaluate results of the examination.

In some cases, macroscopic examination for helminths may reveal organisms which might be overlooked if only a microscopic examination were made. Small helminths, proglottids, or scoleces may be demonstrated by comminuting a small amount of specimen with water and slowly straining the suspension in small portions through a piece of screen wire, a sieve, or a layer of gauze. A suggested method for examining feces for helminths is described in the section on Miscellaneous Procedures.

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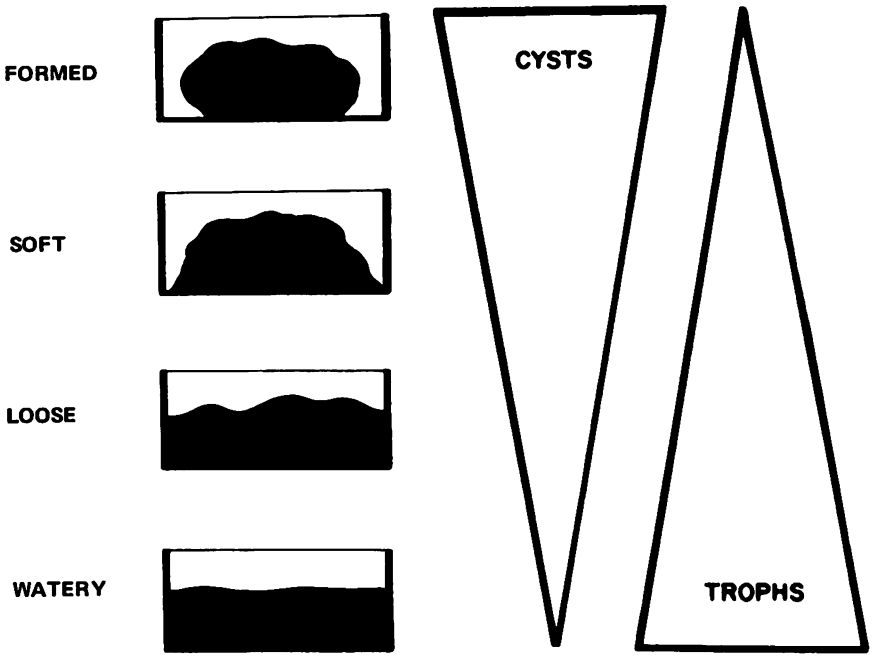
Figure 5.
Consistency of Feces and Distribution of Cysts and Trophozoites



•MICROSCOPIC

The microscopic examination of feces may reveal helminth eggs, larvae, and small adults; protozoan trophozoites and cysts; discharged body cells, such as macrophages, indicative of bacterial infection, epithelial cells, and red blood cells sometimes indicative of ulceration or of hemorrhagic or vascular lesions; various inanimate objects, such as Charcot-Leyden crystals which occasionally are correlated with amebiasis and other parasitic infections; *Blastocystis hominis,* recently identified as a protozoan (Zierdt *et al.*, 1967;

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Zierdt, 1973, 1978); fungi; plant cells, including stone cells, parenchymal cells, starch granules, and pollens, which may be confused with cysts or eggs; and plant fibers and plant or animal hairs which may be confused with nematode larvae.

Since the differentiation of intestinal parasites depends on observing the morphologic characteristics of the diagnostic stages, having a good binocular microscope with proper illumination is essential. The make of microscope is not important so long as it meets the standards described in the section on Microscopy.

The observation of structural details, particularly in wet mount fecal suspensions, is facilitated by reducing the light or regulating it until elements in the specimen are clearly visible. A too-bright light will obscure many structures. Begin with a lower intensity and increase it until satisfactory illumination is obtained. Best results are achieved by leaving the substage condenser in the optimal position and regulating the light intensity by means of the iris diaphragm. Improper focusing of the condenser and improper illumination may cause difficulties in examining specimens. Attention to these two factors will increase the observer's efficiency. See the section on Microscopy.

Size of the organism is an important criterion in identifying many parasite species, especially helminth eggs. A calibrated ocular micrometer disc, placed inside the ocular of the microscope, should be used to accurately measure the size of organisms. The technique for calibrating the micrometer is described in the section on Microscopy. In addition, a sound-slide program, designed as a self-instructional lesson, on calibration of the micrometer is available on loan from most State Laboratories.

The procedures used for microscopic examinations vary, depending on many factors such as the consistency of the specimen, the organism suspected, the type of equipment available, and the time that can be spent. No one technique is 100 percent effective in revealing organisms. The technologist should be familiar with several methods and their advantages and disadvantages. Microscopic techniques include direct wet mount examinations, concentration, permanent staining, and cultivation procedures, and special methods for certain infections. Details of specific procedures including preparation of solutions and step-by-step performance are in the Technique portion of this manual.

The diagnostic stages that may be recovered by each method are presented in Table 1 on page 41.

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WET MOUNTS

Wet mounts made directly from stool specimens require the least equipment of any of the available examination methods and are the simplest to prepare. By using various solutions, all types and stages of organisms encountered can often be identified. For wet mounts of fresh or unpreserved feces, saline and iodine mounts are routinely prepared. If preserved feces are used, the Formalin preservative serves as a diluent and additional saline is usually not necessary for the unstained mounts. In some cases, special temporary stains may be used to demonstrate parasite structures. See the section on Direct Wet Mounts for the technique of preparing and examining mounts.

Saline Mounts—Physiological saline constitutes the most normal environment for all types and stages of organisms which may be encountered in the stool specimens. Eggs and larvae can be readily detected and identified in saline mounts. Both the trophozoites and cysts of intestinal protozoa appear highly refractile and the trophozoite (if living) may exhibit characteristic locomotion. Because of these properties, protozoa can be more easily spotted in saline than in any other type of preparation, even with the low-power objective. Some structures, such as the chromatoid bodies in cysts and red blood cells in trophozoites, are more easily seen in saline than in stained temporary mounts, although others, such as nuclei, may be invisible.

Temporary Stains—Although helminth eggs and larvae can be identified more easily in unstained mounts, the use of various temporary stains will aid in identifying protozoa.

Iodine solutions are used primarily for staining cysts to determine the number and structure of the nuclei. The solutions will also stain glycogen masses and thus help to identify *Iodamoeba bütschlii* cysts. The iodine solution must be of the right concentration, preferably about 1 percent strength, to insure good results (fig. 13, Direct Wet Mounts). Dobell's or D'Antoni's iodine solutions are recommended. Lugol's iodine can be used but must be diluted 1:5 for fecal preparations. Undiluted, it is too strong and will cause the feces to clump, perhaps obscuring or entangling organisms. A too-weak iodine solution, such as Gram's, will not stain the organisms properly.

Iodine solutions fade rapidly and, for optimum staining, should be prepared fresh every 10 to 14 days.

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The nuclei of amebae trophozoites, like those of cysts, are indistinct or invisible in saline preparations. However, iodine is unsatisfactory for the trophozoite stage, since it may distort the entire organism. Therefore, various staining solutions are recommended to assist in identifying amebic trophozoites.

Quensel's stain (Svensson, 1935) stains the nuclei and helps to differentiate 3 genera of ameba – *Entamoeba, Iodamoeba* and *Endolimax.* Unfortunately, it may not be adequate for differentiating trophozoites of *E. histolytica* and *E. coli,* a common problem in many laboratories. Quensel's stain either does not stain flagellates or stains them poorly, including *Dientamoeba fragilis,* now classified as a flagellate rather than an ameba (Camp *et al.*, 1974; Honigberg, 1974). Furthermore, it will not stain living or unpreserved cysts and thus, helps the microscopist to distinguish rounded-up trophozoites from cysts.

Merthiolate-iodine-formaldehyde (MIF) solution stains well both the cysts and trophozoites of all the amebae (Sapero and Lawless, 1953). Like Quensels, however, it does not stain *D. fragilis* very well.

Nair (1953) showed that the visibility of the nucleus of the *E. histolytica* trophozoite depends on the pH of the suspending solutions and has suggested acetate or phthalate buffers in the lower pH ranges for bringing out nuclear details. Thus, one need not have the more complicated staining solutions. Methylene blue, pyronin, and other available stains in a buffered solution of the desired pH will serve the purpose. Buffered methylene blue solutions have been found satisfactory for routine use.

CONCENTRATION

When organisms are rare or not found in direct mounts, the feces may be concentrated. Many reliable techniques have been developed and this type of examination has become a part of the laboratory parasitologic routine because it increases the chances of detecting organisms and insures a more thorough examination. The concentration procedures are applicable to helminth eggs and larvae and protozoan cysts, but not to protozoan trophozoites. The latter are usually distorted or destroyed during the process.

Concentration techniques are generally divided into flotation procedures and sedimentation procedures. The specific techniques used will depend in part on the organism to be searched for and the type of material to be examined. To be successful, the solutions

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used in these methods must have certain characteristics: for flotation techniques, the solution must float the majority and preferably all of the eggs, larvae, and cysts in the specimen; for sedimentation methods, the solution must permit the settling of all of these elements.

Flotation– This type of concentration was introduced for the recovery of hookworm eggs (Bass, 1910). The procedures make use of a solution which has a higher specific gravity than the organisms to be floated so that the organisms rise to the top and the debris sinks to the bottom. Eggs and cysts usually have a specific gravity ranging between 1.05 and 1.15, although a few eggs, such as *Clonorchis, Opisthorchis,* and related species, have specific gravities over 1.20 and cannot be concentrated by the usual flotation techniques.

The most common techniques used are the zinc sulfate centrifugal flotation (Faust *et al.*, 1938) and the brine flotation (Willis, 1921). The zinc sulfate solution is approximately a 33 percent aqueous solution of specific gravity 1.18 or 1.20. The former density is commonly used with fresh specimens and the latter with formalinized specimens. The density should be checked for accuracy with a hydrometer, since, because of impurities, a 33 percent solution may not give the required specific gravity. The sodium chloride or brine is a saturated aqueous solution prepared from crude salt and has a specific gravity of 1.20. These solutions may be stored in tightly stoppered bottles, but, if used over long periods, the specific gravity should be rechecked monthly.

The zinc sulfate technique is an effective procedure revealing both protozoan cysts and helminth eggs and larvae in unpreserved specimens (Shrivastav, 1954; Guinn, 1959). (See section on Concentration Procedures.) With various slight modifications, it has been used successfully in numerous laboratories. It is particularly useful for concentrating *Giardia lamblia* cysts and *Hymenolepis nana* eggs; however, operculated eggs and those of the schistosomes are not recovered. Furthermore, in some instances distortion of protozoan cysts may occur. Harper (1964) has modified this method by preparing an initial Formalin feces suspension. The tube of material is centrifuged, the supernatant fluid decanted, and the sediment used for the usual concentration technique. Preparation of a formalinized fecal suspension permits delayed examinations of the specimen without degeneration or deterioration of the organisms. The Formalin also reduces distortion in the cysts. The use of the zinc sulfate flo-

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tation method on formalinized specimens has been evaluated by Bartlett *et al.*, 1978.

The brine flotation is particularly recommended for recovering hookworm eggs, but it is also useful in recovering other helminth eggs especially those of *Ascaris, Trichuris, Taenia,* and *Hymenolepis nana.* (See section on Concentration Procedures.) Operculated eggs, schistosome eggs, and protozoan cysts will not be recovered with this method. Therefore, because of its restricted use, this technique should not be used as a primary routine diagnostic procedure.

Sedimentation—In these techniques, the organisms are sedimented either by gravity or centrifugation. Feces may be suspended in tap water alone, or various solutions can be used to clarify the suspension, free the organisms from the debris, and aid the settling of these elements. The sedimentation procedures are useful in concentrating protozoan cysts and helminth eggs and larvae, including the schistosome and operculated eggs. By substituting saline for the tap water, protozoan trophozoites may occasionally be recovered.

The simple gravity sedimentation is time consuming and somewhat less effective than other methods but does possess certain advantages. (See section on Concentration Procedures.) By using saline, viable eggs of the schistosomes may be freed from the fecal debris and recovered in the sediment. If examinations of the sediment fail to reveal suspected schistosome infections, the material can be subjected to the egg hatching technique. (See section on Miscellaneous Procedures.) The gravity sedimentation method is probably one of the most practical for obtaining immature, mature, and degenerate eggs of the schistosomes (Maldonado and Acosta-Matienzo, 1953). The time involved may be shortened by centrifuging the fecal suspension.

Various sedimentation techniques use chemical solutions, such as hydrochloric acid, acetic acid, or sodium sulfate, to clarify the material and liberate the organisms from the fecal debris. Telemann (1908) devised the first of these techniques, an acid-ether method which is particularly useful in recovering schistosome eggs. Many modifications of this method have been developed, but none are satisfactory for recovering protozoa. Alicna and Fadell (1959) reported that acid-ether techniques were more effective than either zinc sulfate or Formalin-ether methods in recovering helminth eggs and larvae.

The substitution of Formalin for the acid or sodium sulfate made

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possible an effective sedimentation technique for revealing both protozoa and helminths (Ritchie, 1948). This Formalin-ether (FE) sedimentation technique is satisfactory for both unpreserved and Formalin-preserved specimens and has the added advantage of recovering operculated eggs and those of schistosomes as well as other helminths. A modification of this method (Maldonado *et al.*, 1954) that uses Triton NE (a nonionic detergent) in the Formalin increased the effectiveness of the FE technique in recovering helminth eggs. Ritchie *et al.*, (1960) found that the pH of the Formalin affected recovery of cysts and eggs. *Ascaris* and *Schistosoma japonicum* eggs were recovered best at pH 10.0, but hookworm egg recovery was best at pH 4.0 and pH 7.0. Recovery of *Trichuris* eggs and protozoan cysts was not particularly affected by changes in pH, but pH 7.0 seemed to give the best results. These data suggest that Formalin adjusted to a neutral pH might be more satisfactory than unbuffered solutions in recovering organisms.

The FE method, like the zinc sulfate technique, is especially useful as a routine diagnostic procedure. In comparative studies with the zinc sulfate flotation procedure, the FE sedimentation technique has been considered more efficient (Ritchie *et al.*, 1952, 1953; Vinayak *et al.*, 1967). In an unpublished study conducted by CDC, the FE technique gave slightly better results than the zinc sulfate on both unpreserved and preserved specimens. In general the cysts are more normal in appearance and are more easily identified in the preparations from FE concentrations. Since Formalin fixes the organisms, the nuclei and chromatoid bodies of cysts are more distinct than in the zinc sulfate method. Furthermore, less distortion of small cysts, such as *Endolimax nana* or *Entamoeba hartmanni*, occurs. Occasionally, with zinc sulfate flotations, cysts, especially those of *E. nana*, will appear "caved-in" on one side. Moreover, if the flotation stands for some time, many organisms will sink to the bottom of the tube and examining the surface film may cause infections to be overlooked. The FE sedimentation not only recovers a wider range of organisms but also permits keeping the tubes longer without affecting the results of the examination. After pouring off the supernatant, the tube can be stoppered to prevent drying and the sediment can be examined later. If the quantity of fluid with the sediment is minute, a drop or two of saline or Formalin should be added. Tubes left for several hours or overnight should be placed in the refrigerator to prevent drying. The FE technique, however, is not as efficient as the zinc sulfate method for detecting *G. lamblia* cysts, *H. nana* eggs or *I. bütschlii* cysts.

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Unfortunately, because of its flammability, ether has been banned in some laboratories. Recently, CDC has found that ethyl acetate is an acceptable substitute for ether in the FE procedure. It is effective in concentrating eggs, larvae, and cysts and is less hazardous to use than ether (Young *et al.*, 1979). Ethyl acetate effectively concentrates both formalinized and unpreserved feces, but is less effective than ether with fatty specimens.

The MIF-ether concentration (MIFC) technique (Blagg *et al.*, 1955) is essentially the FE technique with MIF solution substituted for Formalin. However, the alcohol and acetone in the tincture of merthiolate apparently reduce the specific gravity, and this reduction may increase the concentration of eggs. The MIFC technique can be performed on either fresh specimens or those preserved in the MIF solution.

Regardless of which technique is used, the mounts should be carefully and systematically examined.

Technical Errors—The efficiency of any concentration procedure is dependent to a great extent on the capability of the technologist performing the technique. Numerous technical errors which would make the results invalid can occur. For example, the sample of specimen used may be too small for adequate examination or too large to be properly concentrated. The suspensions may not be thoroughly comminuted or the supernatant may be carelessly decanted, resulting in loss of material. In the flotation methods, the specific gravity of the solutions may be too low to float the organsims, resulting in poor recovery, or too high, resulting in distortion of cysts and other stages. Carelessness in handling flotation specimens may disrupt the surface film and force some of the organisms into lower fluid levels.

PERMANENT STAINS

Stained fecal smears are usually prepared for the identification of protozoa. Helminth eggs and larvae may be found on such smears, but, often, they are distorted or the stain obscures their differential characteristics. Occasionally, however, special stains or clearing procedures are used in identifying the helminths.

Protozoa—Various authorities have emphasized the importance of using permanent stained fecal preparations for demonstrating and

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accurately identifying intestinal protozoa (Brooke *et al.*, 1963a, 1963b; Goldman, 1964; Healy, 1964; Healy *et al.*, 1969; Gleason *et al.*, 1970; and others). In temporary wet mounts, unless indisputably typical characteristics of *E. histolytica* or other protozoa are observed (preferably in the cystic state), identification should be considered only tentative. Confirmation in permanent stained slides, especially of amebae trophozoites, is often essential for accurate diagnostic work. Since the procedure need not be time consuming, all specimens found to contain amebae trophozoites or questionable organisms should be stained. Furthermore, permanent stained fecal preparations may reveal organisms, especially small protozoa, not found by other techniques (Brooke, 1960; Brooke *et al.*, 1962, 1963a; Melvin and Brooke, 1962; and others).

Stained fecal smears afford the most satisfactory method for keeping positive evidence of the intestinal protozoa identified in a laboratory. In view of the existing doubt about the reliability of the available diagnostic characteristics, persons responsible for identifying intestinal protozoa should maintain evidence of their positive findings. It is generally recommended that permanent stained preparations be kept for 1 to 2 years.

Permanently stained preparations also make it possible to obtain consultation when needed. Even the authorities in the field welcome the opinions of others on certain specimens, and permanent stained preparations enable an individual to obtain assistance more easily. If the personnel in a clinical laboratory cannot prepare permanent stained slides, specimens preserved in vials of PVA-fixative or dried PVA-fixed fecal smears can be sent to a reference diagnostic laboratory for processing.

For several decades, the Heidenhain's iron hematoxylin stain technique was the most widely used procedure for staining fecal smears. The original classic method required 1 to 2 days to complete, but many workers consider the results worth the time spent, since Heidenhain's method produces perhaps the most critically stained organisms of any available procedure. The time needed to complete the technique can be shortened by heating certain of the solutions without noticeable differences in stain reactions. Since this method requires a degree of technical skill and can be quite tedious, however, it is not commonly used in diagnostic laboratories. A number of simpler staining techniques which can be completed in less than an hour have been developed: for example, the iron-hematoxylin-phosphotungstic acid stain (Tompkins and Miller, 1947) and the trichrome stain (Wheatley, 1951) techniques. A comparative study

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(Markell, 1956) showed that the trichrome method is satisfactory for diagnostic purposes and many laboratories, including CDC, currently use it for routine staining.

Kohn (1960) developed a staining procedure using chlorazol black in which fixation and staining take place in a single solution. Destaining is not necessary, which simplifies the procedure to some extent. A modification of this technique (Gleason and Healy, 1965) has been found satisfactory for staining protozoa in fecal smears or tissues. Although unpreserved feces stain well with this stain, PVA-fixed specimens do not. (PVA-Schaudinn's fixative contains mercuric chloride which must be removed for satisfactory stains and the Kohn procedure does not have an iodine-alcohol wash to do this.) The chlorazol black stain could be of particular value in hospital or clinic laboratories, where unpreserved specimens are usually obtained, since there are fewer steps in this technique than in other procedures and they require less attention.

Specimens fixed in Formalin are unsatisfactory for staining because the material will not adhere properly to the slide and the organisms, especially the chromatin portion, stain poorly. Unfortunately, the formalinized feces cannot be mixed with PVA-fixative to overcome the lack of adhesion because the two solutions are not compatible and a cloudy precipitate forms. A method for staining cysts recovered by the Formalin-ether sedimentation technique has been developed (Ruff *et al.*, 1970), but the results are variable and staining of Formalin-fixed material is not recommended for routine use in clinical or public health laboratories.

Arensburger and Markell (1960) described a procedure for concentrating cysts, mixing the concentrate with fresh feces, and staining smears by the usual procedures for permanent preparations. (See section on Staining Procedures.) To date no satisfactory procedure for concentrating trophozoites in fecal material for subsequent staining is available. However, a procedure for washing and centrifuging feces in Schaudinn's fixative, which increases the number of trophozoites and cysts in the stained preparation, has been described (Scholten, 1972).

In preparing permanent stained slides, fecal material may be stained in bulk in test tubes, but the results vary and the degree of staining or differentiation is more difficult to regulate than with smears made directly on the slide. Practice and experience are usually necessary to achieve good preparations.

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Helminths—In most instances, helminth adults can be identified macroscopically, or microscopically in temporary wet mounts without staining. Observation of small, living organisms in saline often provides valuable information. Helminths should always be handled as gently as possible, especially in the living condition, to prevent rupture and distortion. If small enough, they can be sucked up in a wide-bore pipette and placed in a dish of saline. Small tapeworms can be handled either with camel's hair brushes, blunted needles, or applicator sticks with edges cut in the shape of thin, flat spatulas. Larger organisms, of course, are not as fragile and if care is taken not to damage them, they can be handled with any suitable blunt instrument. Whole mounts of the worms or proglottids are best examined under a low-power magnification. If adult helminths are received frequently, a 3.5X objective on the compound microscope is desirable. A dissecting microscope such as that commonly used in entomology studies or even a good hand lens also may be used.

Special stain procedures are available for helminth adults but are not used routinely to identify the common human intestinal species. In determining nematode morphology, clearing the specimen will usually result in a satisfactory preparation for differentiating structures. The clearing agents commonly used are carbol-xylene (1:3), lacto-phenol (1:1), beechwood creosote, xylene or glycerin. The specimen should be turned and rolled so that it can be observed at various positions. Cestode proglottids, especially those of the *Taenia* species, may also be cleared for identification. The cleared specimens can be mounted in balsam or synthetic media such as Permount, for permanent preparations. A PVA-lacto-phenol medium which combines the clearing and mounting processes has been advocated for small nematodes (Rubin, 1951). Modifications of this method have been used successfully in the CDC laboratories to clear and mount both nematodes and cestode proglottids. (See section on Staining Procedures.)

Hematoxylin or carmine stains are widely used for trematodes or cestodes where species differentiation is a problem. Either Delafield's or Ehrlich's hematoxylin, usually available in most laboratories, will give excellent results. Both are aqueous stains and best results are achieved when very dilute solutions are used. This permits slower penetration, and the specimen can be watched and removed from the solution when the structures become stained. Furthermore, dilute stain solutions acting over a long period (1 to 2 days) will not require a great deal of destaining. Semichon's acetic

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carmine is also a good stain for routine purposes, especially for tapeworm proglottids.

Directions for clearing and staining procedures will be found in the section on Staining Procedures. Additional information is available in various texts such as Guyer, *Animal Micrology;* Pearce, *Histochemistry;* and Lillie, *Histopathologic Technique and Practical Histochemistry.* (See the list of references for publishers.)

CULTIVATION

Like staining techniques, cultivation procedures are used primarily in the diagnosis of intestinal protozoa, although certain special techniques have been developed for cultivating helminth larvae.

Protozoa– Cultivation has two chief advantages: to increase the number of organisms available for observation and to revive trophozoites that may be degenerating. It is a useful tool for very fresh specimens in which organisms are rare or are not found by other methods (Norman and Brooke, 1955a).

In the diagnosis of protozoan infections, cultivation can be used to advantage with fresh specimens if there are adequate facilities, time, and interest, but it is not a recommended procedure for laboratories which receive specimens by mail. All of the intestinal protozoa except coccidia can be cultured on artificial media, although *Balantidium coli* and *Giardia lamblia* require special media. *Giardia* can be grown in tissue cultures (Karapetyan, 1960) and in artificial media (Karapetyan, 1962; Meyer and Pope, 1965; Danciger and Meyer, 1971; Meyer, 1970, 1976, 1979; Visvesvara, 1980) although with difficulty. Axenic cultivation of *Giardia* trophozoites and excystation of cysts in vitro have also been accomplished (Meyer, 1970, 1976; Bingham *et al.*, 1979; Bingham and Meyer, 1979;Lakhonina, 1978).

Several media are available for the cultivation of intestinal protozoa: liver extract (Cleveland and Collier, 1930); modified Boeck and Drbohlav's or LER (Boeck and Drbohlav, 1925; Reardon and Rees, 1939); egg yolk infusion (Balamuth, 1946); alcohol egg extract (Nelson, 1947), charcoal medium (McQuay, 1956). Charcoal media and *"Entamoeba"* media, which is basically the Cleveland and Collier medium, can be purchased in dehydrated form from Difco. Selecting a single medium and becoming proficient in its use is generally better, however, than using several media.

Small quantities of penicillin and streptomycin should be added to

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most media before inoculation to suppress bacterial multiplication (Springarn and Edelman, 1952; Norman and Brooke, 1955a). At least 2 tubes of media should be inoculated with different quantities of the specimen. The sediments in the tubes are examined at the end of 24 hours of incubation at 37°C, and if organisms are not found in the first examination, again after 48 hours. In addition, subcultures into fresh tubes of media may be made at the end of 24 hours. The subcultures should also be examined after 24 and 48 hours' incubation.

Although *E. histolytica* tends to culture more easily than the other species, no satisfactory differential medium that will isolate this pathogen alone from stool specimens is available. For this reason, after the amebae have been established in culture, the necessity of differentiating the species by morphologic characteristics remains. Generally speaking, if the trophozoites demonstrate rapidly progressive locomotion and have an *Entamoeba*-type nucleus, they can usually be identified as *E. histolytica.* The other species, particularly *E. coli,* do occasionally exhibit the same activity. In examining the cultures, the technologist should prepare stained and unstained wet mounts from the sediment in the tubes for identification of species. Mixing the sediments with PVA-fixative will facilitate the preparation of stained smears, since the PVA acts as an adhesive (Norman and Brooke, 1955b).

Cultivation may be of assistance in diagnosing trophozoites in the process of degeneration. If the organisms have not deteriorated too much, they may revive in culture and produce progeny with more typical characteristics. Furthermore, if encystation can be accomplished, perhaps by incubating for 72 hours, cultivation may be a method of obtaining the more diagnostically reliable cystic stages.

Although as previously stated, coccidia cannot be cultured, a type of "cultivation" or "maturation" procedure may be used in some cases where immature oocysts are found in feces and identification of genus is doubtful. Immature oocysts of *Eimeria* (animal species) and of *Isospora* are difficult to distinguish and, since *Eimeria* oocysts are sometimes accidentally ingested and passed in human feces, generic identification may be important. The simplest method of doing this is to allow the immature oocysts to mature and to determine genus from the mature stage. The procedure is described in the section on Miscellaneous Procedures.

Helminths—Cultivation of helminth larvae is used chiefly in differentiating hookworm and *Strongyloides* larvae. Occasionally,

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larvae found in feces cannot be specifically identified, or infection with both species may be suspected. In these cases, cultivation to obtain filariform larvae may be advantageous.

Sand or charcoal cultures are often used, and the larvae are collected by the Baermann apparatus (Baermann, 1917). (See fig 24, Cultivation.) In this way, larvae free of feces and culture material are obtained. Several modifications of the Baermann apparatus have been devised for recovering hookworm larvae (Beaver, 1953; Watson and Al-Hafidh, 1957) and *Strongyloides* larvae (Ferriolle, 1959). This type of apparatus and procedure can also be used for isolating or recovering various nematode larvae in soil samples, but free-living larvae must be distinguished from parasitic species. Free-living larvae can be killed by a dilute solution of hydrochloric acid; parasitic species are unharmed (Shorb, 1937). (See section on Cultivation.)

Feces can be cultured on filter paper strips in a test tube, a procedure that is easier and simpler to perform than charcoal or sand cultivation (Sasa *et al.*, 1958). (See fig. 25, Cultivation.) Larvae of *Strongyloides,* hookworm, and *Trichostrongylus* are recovered by this method. Cultivation, especially the filter paper method can also be used as a diagnostic procedure when hookworm or *Strongyloides* infections are suspected but eggs or larvae cannot be found by the usual methods.

Directions for culturing larvae and using the Baermann apparatus are included in the section on Cultivation.

TABLE 1
DIAGNOSTIC STAGES RECOVERED BY DIFFERENT METHODS

METHOD	STAGE			
	PROTOZOA		HELMINTHS	
	Trophs	Cysts	Eggs	Larvae
Direct Wet Mount	+	+	+	+
Concentration	–	+	+	+
Permanent Stain	+	+	–	–
Cultivation	+	–	–	+

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OTHER PROCEDURES

Cellophane Thick Smear—A cellophane thick smear for the detection of helminth eggs has been developed (Kato and Miura, 1954; Komiya and Kobayashi, 1966). This technique uses a specially treated cellophane covering in place of a coverslip. Feces are placed on a slide, covered with the cellophane strip and pressed with a rubber stopper into a more or less uniform layer. The preparation is then allowed to clear for 1 hour at room temperature before it is examined. The method is considered effective for fairly large eggs such as hookworm and *Ascaris*, but it is not satisfactory for small eggs and for protozoa. Its limitations make it generally unsuitable for routine use in clinical and public health laboratories. The technique is described in the section on Miscellaneous Procedures.

Egg Hatching Technique—This procedure is used in diagnosing schistosome infections, particularly *S. mansoni*, and is considered to be sensitive and reliable in detecting schistosomiasis. Feces are suspended in chlorine-free water in a narrow-mouthed flask and allowed to stand overnight. Viable eggs will hatch, and the miracidia will be found swimming in the upper layers of the water. The details of the technique are described in the section on Miscellaneous Procedures.

Filtration Procedure—Filtering urine samples through an 8-μm Nucleopore filter has been used to recover *S. haematobium* eggs (Peters *et al.*, 1976 a,b). By using a measured aliquot of urine, the procedure can be quantitative. Filtering saline suspensions of fecal material through Nucleopore filters may also be used to recover *S. mansoni* and *S. japonicum* eggs (Peters *et al.*, 1976a).

Estimation of Worm Burden—The physician can often use some indication of the intensity of helminth infections and, for this purpose, quantitative egg counting procedures have been developed for some helminth infections. Egg counts provide an estimate only of the worm burden and should not be considered an accurate worm count. Nevertheless, counts do enable one to classify the infection as light, moderate, or heavy. Fluctuation in numbers of eggs shed, the amount of feces passed, distribution of eggs in feces, and errors in sampling introduce variations in the counts. If performed properly, however, they provide an index of infection which is of value in determining the degree of infection. The counts are used to estimate

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the worm burdens in population groups when mass therapy or community control measures are being considered. In individual patients, egg counts may be useful both in indicating the intensity of infection and in following the efficiency of a therapeutic regimen.

Ascaris, Trichuris, and Hookworm—Egg counts have been used for many years to determine worm burdens in *Ascaris, Trichuris* and hookworm infections. This information is especially useful in distinguishing between "hookworm infection" and "hookworm disease." In these three nematode species, fairly reliable egg counts can be made because the egg production is more or less constant, the approximate daily output per female worm is known and (as with other helminths) the worms do not multiply in the body. Whenever possible, egg counts should be performed on unpreserved fecal specimens in which hookworm, *Ascaris,* or *Trichuris* eggs are found.

A number of egg counting procedures are available. Two widely used and reliable techniques are the dilution egg count technique (Stoll, 1923) and the direct egg count (standardized smear) technique (Beaver, 1949a, 1950). Comparison of these two methods indicates that they are equally satisfactory for routine egg counts (Melvin *et al.*, 1956). The direct smear, however, is perhaps easier for technologists to perform than the dilution method and requires less time. In addition to these procedures, the Kato cellophane thick smear technique has been adapted for quantitative use in nematode infections (Martin and Beaver, 1968). These methods are described in the section on Miscellaneous Procedures.

Other Infections—The quantitative procedures are less often applied to other helminths, since either the shedding of eggs is irregular or the eggs are not ordinarily passed in the feces. Counts can be performed, of course, but in most cases they should be done with the understanding that only rough estimates of worm burdens may be obtained.

Egg count procedures have been devised for estimating density of infections with *Opisthorchis* (Sadun, 1955), *S. haematobium* (Stimmel and Scott, 1956; Scott, 1957; Bradley, 1964; Peters *et al.*, 1976a, 1976b); *S. mansoni* (Bell, 1963; Martin and Beaver, 1968); and *S. japonicum* (Yogore *et al.*, 1978). Quantitative methods are particularly useful in categorizing schistosome infections as light, moderate, or heavy intensity. The egg output has been correlated with clinical level and course of infection (Cheever, 1968; McCul-

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lough and Bradley, 1973; Hiatt, 1976; Lehman *et al.*, 1976; Smith *et al.*, 1979; Warren *et al.*, 1979a, 1979b; Yogore *et al.*, 1978 and other investigators).

A coefficient of infection has been used as an index to the intensity of *Enterobius* infections in different population groups, and, within limits, can be applied to individual patients (Sadun *et al.*, 1956).

Several quantitative procedures are described in the section on Miscellaneous Procedures.

OTHER SPECIMENS

•SPUTUM AND URINE

The methods of examining feces are also applicable to urine and sputum. Usually, simple centrifugation of urine and subsequent examination of the sediment is sufficient to reveal any protozoa or helminths present. Sputum specimens may be satisfactorily concentrated by either acid-ether or Formalin-ether techniques or by mixing the material with 3 percent sodium hydroxide and centrifuging.

•ANAL SWABS

Anal swabs collected for diagnosis of pinworm infections are handled according to the type of preparation used. They should be examined as soon as possible after collection, but if a delay is necessary, they should be stored in the refrigerator. Cellulose tape slides may be examined directly or cleared by adding a drop of toluene or xylene under the tape (Beaver, 1949b), thus making the debris more or less transparent and the eggs more easily visible. Vaseline-paraffin swabs are handled according to the directions in the section on Miscellaneous Procedures. These two techniques are about equally efficient, although when collections are made during the day, the Vaseline-paraffin swab reveals a few more infections than the cellulose tape slide.

Cellulose tape swabs are examined for *Taenia* eggs in the same way as for *Enterobius* eggs.

•VAGINAL AND URETHRAL MATERIAL

Vaginal swabs or scrapings or urethral swabs are most often obtained for detection of *T. vaginalis* infections. Examining the speci-

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men by wet saline mount is the simplest and easiest method; the mounts should be prepared and examined immediately after the material is collected. If present, trichomonads are usually readily detected and recognized.

Vaginal or urethral exudates may also be cultured. Cultivation is considered the most sensitive and best diagnostic procedure for *T. vaginalis*, especially in light infections, and should be used routinely when organisms are not detected in direct wet mounts. The combination of cultivation and direct wet mount examinations substantially increases the number of infections detected (Perl, 1972; McCann, 1974; Honigberg, 1978) and in studies at CDC, cultivation has been found to double the number of positive cases detected (Fouts and Kraus, 1980). Furthermore, culturing may be more convenient, since the tubes of media can be inoculated at the time of collection and examined later. Scrapings, swabs, or material obtained with a speculum are introduced into the tube of medium and incubated at 37°C. Readings are made at 48- or 96-hour intervals. If a sterile cotton swab is used to collect material, the entire swab is placed in the tube. Several media are available including trypticase-liver-serum medium (Kupferberg *et al.*, 1948; Burch *et al.*, 1959), Feinberg's medium (Feinberg and Whittington, 1957), Modified Diamond's Medium (Fouts and Kraus, 1980) and others. Specific information on culturing *T. vaginalis* is in the section on Cultivation.

Stained smears of vaginal and urethral material may be made but are not recommended for the diagnosis of trichomoniasis. The trichomonads are usually distorted and difficult to recognize in dried preparations. A recent study showed that Papanicolaou smears, which are frequently used to detect *T. vaginalis* infections, are unreliable and lead to a significant number of erroneous diagnoses (Perl, 1972). Gram's, Wright's, and Giemsa stains of dried preparations have similar drawbacks. If stained smears must be made, better results are obtained by fixing the wet smears immediately in Schaudinn's or by putting the material into PVA-fixative and then staining it by the methods used for feces. Although distortion is less by the latter method, organisms in stained preparations are often atypical and are difficult to identify. Therefore, wet examinations, either direct or preparations from cultures, are recommended.

•DUODENAL DRAINAGE MATERIAL

Material obtained by duodenal drainage for diagnosis of strongyloidiasis and giardiasis can be examined directly in wet-mount prepa-

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rations. Gondard (1956) has stated that *Giardia* trophozoites are lysed in bile and the specimen should be preserved in 5% Formalin unless examined immediately. Material obtained by the nylon thread method (Enterotest) is also examined in wet-mount preparations. Mucus adhering to the thread is scraped off, emulsified in saline, and examined directly for the presence of trophozoites or larvae.

•SIGMOIDOSCOPIC MATERIAL

The amount of material obtained is usually very small, especially if the lesions have been scraped rather than aspirated. Direct saline mounts should be made immediately and examined for amebae trophozoites. The material is obtained from the lesion and mixed at once with a drop of saline. Macrophages and epithelial cells will probably be present in the material also and must be differentiated from amebae trophozoites. If organisms or suspected organisms are found, a drop of Quensel's stain or buffered methylene blue solution can be added to stain the internal structures. In addition, a permanent stain can be made and, indeed, may be necessary for positive identification. Since the quantity of specimen is limited, two or three drops of PVA-fixative can be added to the saline mount, mixed thoroughly, and spread into a smear for staining. The smears are dried and stained as for fecal smears. The lesion material often seems to coagulate in the fixative, however, and good smears are difficult to obtain. Juniper (personal communication) has recommended that the material be pressed between two slides, "squashed" as it were, to remove the lumps. In the CDC Laboratory, this procedure has been found satisfactory for obtaining even, uniform preparations. Sigmoidoscopic material can be cultured, but the minute quantity available may cause difficulties in obtaining positive cultures. Generally, if trophozoites are present, they can be detected in wet mounts.

When sigmoidoscopy is performed away from laboratory facilities, the material can be placed in a tube containing a small quantity of sterile saline and taken at once to the laboratory. It should be kept warm during transit. If too much saline has been added, the material can be gently centrifuged (about 250 or 300g or 1,000 r/min, for 1 minute) and wet mounts made from the sediment. If the specimen cannot be sent to the laboratory immediately, the material may be put into PVA-fixative for subsequent staining. Material can also be inoculated into a shipping culture medium for transportation to the laboratory.

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•ASPIRATED MATERIAL

Material aspirated from abscesses or other lesions should be examined as soon as possible. Such specimens are received more frequently for diagnosis of extraintestinal amebiasis than for other infections and should be expected to contain only the trophozoite stage of the ameba. Since this stage rapidly deteriorates, delays in examining the material may result in unreliable diagnoses. Demonstrating amebae in the thick pus removed from the abscesses is difficult for several reasons: 1) most of the organisms are in the peripheral area of the abscess and often relatively few are free in the abscess contents; 2) the organisms may be partially immobilized by coagulum; 3) since the fluid is sterile, amebae will not grow in culture when the material is inoculated in the manner routinely used for stool cultures. Streptodornase and Streptokinase, proteolytic enzymes, have been used in freeing the amebae from aspirates of liver abscesses (Freedman *et al.*, 1958; Wilmot, 1962) A suggested procedure for treating aspirated material is given in the section on Cultivation.

Aspirated specimens can be examined by the routine procedures used to demonstrate or recover trophozoites from feces. They may be examined in wet saline mounts and, if trophozoites are found, temporary stains such as Quensel's or buffered methylene blue may be used to confirm the findings. Permanent stains also can be made by any of the usual techniques for fecal preparations. If the aspirated material is liquid, it should be mixed with PVA-fixative which acts as an adhesive as well as a fixative. Difficulties in obtaining satisfactory smears may be encountered, however. Aspirated material can be cultured, if the medium is conditioned with *Clostridium perfringens*. (See section on Cultivation.)

•BIOPSIED MATERIAL

Biopsied material may be examined by several methods: sectioning and staining, compression, teasing, and digestion. The method used will depend to some extent on the type of tissue and the organisms suspected.

In the diagnosis of trichinosis, muscle tissue may be examined by any of the techniques listed (Gould, 1945). The simplest and quickest methods, of course, are compression and teasing. Digestion of the entire biopsy, however, may reveal larvae when the infection is too light to be detected by direct examination. The digestion and compression techniques are given in the section on Miscellaneous Procedures.

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Tissue to be examined for schistosome eggs may be compressed between two slides (Lichtenberg and Valladares, 1955) or cleared in lacto-phenol (1:1) and examined directly. The usual histologic methods of sectioning and staining can also be used.

Tissue for examination for *E. histolytica* should be sectioned and stained. Juniper *et al.*(1958) have described an improved technique for handling punch biopsies from suspected amebiasis cases. It is described in the section on Miscellaneous Procedures.

On occasion, liver biopsy may be done for the diagnosis of larva migrans infections. Histologic methods are most often used in preparing the specimen for examination. Serial sections should be examined.

Duodenal biopsies have been successful in diagnosing giardiasis when examinations of feces or duodenal contents have not revealed organisms. Both sections of the tissue and impression or "touch" preparations from mucus adherent to the biopsy tissue have been used (Kamath and Murugasu, 1974). The tissue and mucus preparations may be stained with Giemsa, trichrome or other staining procedures suggested for intestinal protozoa.

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SELECTION OF PROCEDURES FOR LABORATORY REGIMENS

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SELECTION OF PROCEDURES FOR LABORATORY REGIMENS

Since many techniques and procedures have been advocated for the laboratory confirmation of intestinal parasites, some selection has to be made in establishing a laboratory regimen. Each type of examination has advantages and limitations, and all are subject to technical errors. In general, the more types of examination included in the laboratory routine, the greater the number of infections discovered. From a practical standpoint, however, the laboratory personnel must decide if the increase in positive findings warrants the increased time and expense of performing the added techniques. In practice it would seem advisable to select certain procedures for routine use and to reserve others as supplementary techniques for special occasions. For routine purposes, the regimen should include methods which will permit the recovery and identification of both trophozoites and cysts of intestinal protozoa and eggs and larvae of helminths. The accessibility of the patient and whether specimens are received fresh or through the mail will also influence the choice of techniques.

PROCEDURES FOR REFERENCE DIAGNOSTIC OR PUBLIC HEALTH LABORATORIES

•FECAL SPECIMENS

Laboratories which are not associated with clinics and hospitals generally do not have the opportunity to examine fresh specimens. Reference diagnostic laboratories, for example, those of State health departments, usually have to depend upon specimens delivered by carrier or by mail. These laboratories, therefore, should provide physicians using their services with shipping kits for properly submitting fecal specimens for parasitologic examinations.

As discussed in the section on Collection, a two-vial kit (one vial containing 5 or 10 percent Formalin or MIF solution and the other, PVA-fixative) has been found satisfactory. Portions of the specimen should be put into the two vials immediately after passage and thor-

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oughly mixed with the solutions (fig. 2). See the sections on Specimen Collection and Handling and Preservation of Specimens.

When the preserved specimens arrive in the laboratory, direct wet-mount examinations and a concentration method (preferably the Formalin-ether or Formalin-ethyl acetate sedimentation technique) should be performed on the portion preserved in Formalin. Direct wet-mount examinations are recommended since concentration techniques do not always reveal all species of organisms present. Permanent stained smears should be prepared from the portion preserved in PVA-fixative. The PVA-fixed material can be satisfactorily examined only as permanent stain preparations; wet mounts of this material are of no value. Table 2 lists recommended methods for preserved specimens.

The wet mounts, either direct or concentrated, should be examined for cysts, eggs and larvae; the stained smears are searched for trophozoites and cysts. Preparations should be examined in the manner described in the paragraph "Examination" in the section on Direct Wet Mounts. Figure 6 suggests an appropriate procedure for preserved specimens.

•OTHER SPECIMENS

Occasionally, anal swabs, vaginal or urethral exudates, aspirates, sputum and other materials are received by the laboratory for parasitologic examinations. Suggested procedures for handling and examining these specimens are discussed in the next section.

PROCEDURES FOR HOSPITALS AND CLINIC LABORATORIES

Laboratories in clinics and hospitals have advantages in the diagnosis of parasitic diseases over those which must depend on the mails for delivery of specimens. Since fresh unpreserved specimens can be obtained in clinic and hospital laboratories, direct wet-mount examinations, concentrations, and permanent stained smears should be used (fig. 6). In some cases cultivation may be of value in diagnosing protozoan infections (Norman and Brooke, 1955a), although this is not recommended for routine purposes. Furthermore, additional or purged specimens can frequently be obtained in the clinic or hospital, and the patient is available for sigmoidoscopy, if necessary.

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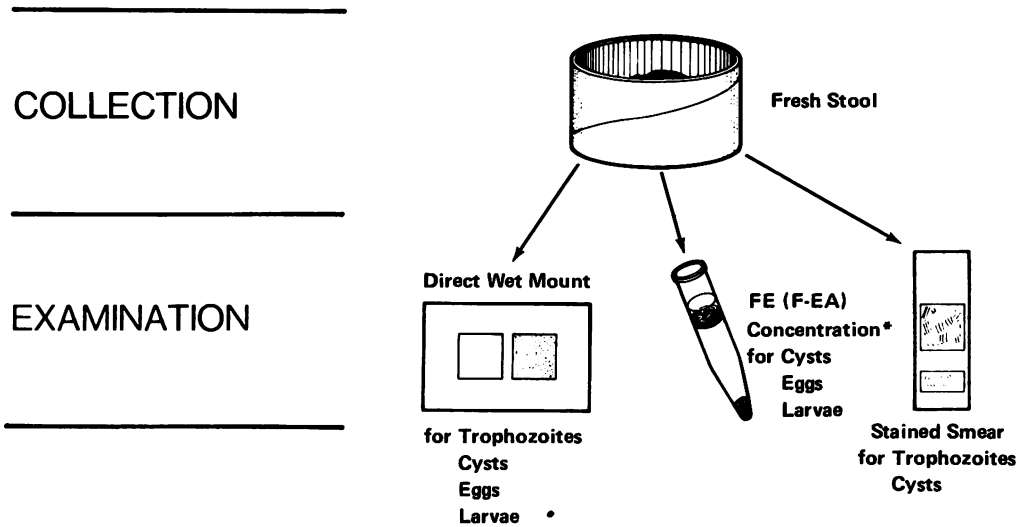
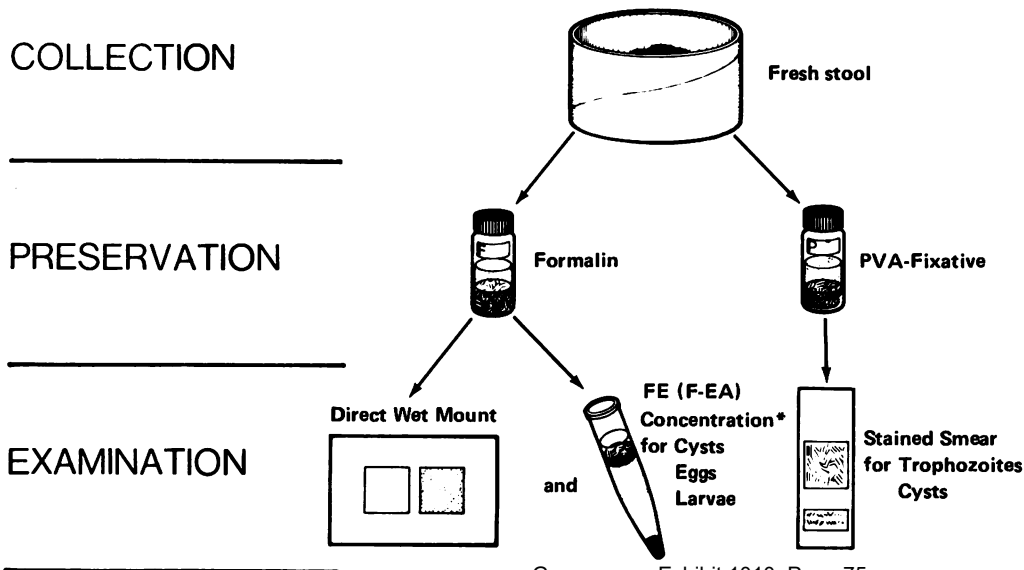
Figure 6.
Parasitologic Examination of Stool Specimens

UNPRESERVED SPECIMEN



NOTE: Concentration procedures may not be needed for diarrheic specimens. Stained smears may not be needed for all formed specimens.

PRESERVED SPECIMENS



*Zinc sulfate centrifugal flotation may be substituted for FE sedimentation.

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•FECES AND SIGMOIDOSCOPIC MATERIAL

NORMALLY PASSED SPECIMENS

Normally passed specimens should be delivered to the laboratory immediately. As soon as the specimen is received, the technologist should examine it macroscopically, noting the consistency of the feces and any abnormalities present. If the specimen is soft, loose, or watery, it should be examined microscopically at once. Formed specimens can be left for a few hours without undue deterioration, but for best results they should be examined as soon as possible. Fresh unpreserved specimens should be examined routinely by direct wet-mounts, concentration methods (except loose and watery specimens), and permanent stained preparations (except as noted in paragraph 4 below). Table 2 lists the methods recommended for unpreserved specimens received from inpatients and for specimens from outpatients.

The following methods and sequence of examination are suggested as a routine procedure for unpreserved parasitologic specimens.

1. Immediately upon receipt of the specimen, prepare two smears and place them in fixative (Schaudinn's or other fixatives suitable for permanent stains) or, if preferred, emulsify a portion of the feces in PVA-fixative. Thus, smears for permanent staining will be available if and when they are needed. If necessary, smears can be left in Schaudinn's fixative for a day or two or even longer, without harm. If chlorazol black E stain is used, put the smears directly into the solution since it is both fixative and stain combined.

2. Examine direct wet-mounts, both unstained and stained. If mucus is present, include these portions in the feces selected for the preparations.

3. Perform a concentration technique, either a FE or FEA (Formalin-ether or Formalin-ethyl acetate) sedimentation or a zinc sulfate flotation, on all except loose and watery specimens which would be expected to contain trophozoites but not cysts. In suspected cases of helminthiasis, however, concentrations should be performed on all types of specimens, because eggs and larvae may be present in watery as well as formed feces. The laboratorian may select another concentration procedure, but whatever method is used should efficiently recover both helminth eggs and larvae and protozoan cysts.

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4. Routinely stain and examine at least one of the fixed smears for protozoa if the specimen is soft, loose, or watery. Permanent stains should also be examined if organisms suggestive of *E. histolytica* or if questionable objects are found in wet mounts (regardless of consistency), or if the specimen reveals any abnormalities (blood, mucus, or other material). If the specimen is formed and no organisms or suspicious structures are seen in direct wet-mounts or concentrates, permanent stained preparations need not be examined. (Ideally, permanent stains should be done routinely on all specimens, but time may not permit this.) The trichrome and chlorazol black stain techniques are especially useful for hospital and clinic laboratories.

   As stated previously, cultivation may be advantageous, especially in suspected cases of amebiasis but is not recommended as a routine procedure.

Outpatient specimens are examined by the same methods as inpatient specimens, except permanent stains should be made from the PVA-fixed portion, if available.

TABLE 2
LABORATORY ROUTINE FOR PARASITOLOGIC EXAMINATIONS

TYPE OF SPECIMEN	METHODS		
	Direct Wet Mount	Concentration	Permanent Stain
I. Unpreserved feces			
a. Formed	+	+	(+)*
b. Soft	+	+	+
c. Loose	+	–	+
d. Watery	+	–	+
II. Unpreserved and	+	+ (Unless diarrheic)	
PVA-fixed			+
III. Formalin and	+	+	
PVA-fixed			+

*if unidentified organisms or objects are found in the wet-mounts.

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If no organisms or only unidentified organisms are found in a specimen from a patient suspected of having a parasitic infection, especially amebiasis or giardiasis, additional specimens should be obtained.

PURGED SPECIMENS

All of the passages following a purge should be examined, but if this is not feasible, at least three, including the first, should be examined. If formed material is present in the first passage, it should be concentrated by either the Formalin-ether or Formalin-ethyl acetate sedimentation or the zinc sulfate flotation method. All specimens should be examined by temporary wet mounts and permanent stained smears should be prepared from at least one of the soft specimens. Since organisms may sometimes be found in mucus more easily than in nonmucoid material, flecks of mucus should be examined either as wet mounts or stained slides. If desired, one or more of the specimens can be cultured. After purgation, any protozoan infection which may be present will require several days to develop to a demonstrable level. Hence, there is little advantage in collecting additional stools, with or without the use of laxatives, until a week has elapsed.

SIGMOIDOSCOPIC MATERIALS

Materials collected by sigmoidoscopy for diagnosis of amebiasis should be examined immediately, in the same or an adjacent room in which the sigmoidoscopy is performed. Material which is aspirated or scraped from the mucosa should be mixed with saline and mounted. It is not necessary to use a warm stage, but ideally the room should be warm. After completing the direct examinations, the coverslip can be removed, the material mixed with three or more drops of PVA-fixative and stained (Brooke *et al.*, 1959).

COMBINATION OF SPECIMENS COLLECTED BY DIFFERENT METHODS

If there is a sufficient time and clinical justification, examining several specimens collected by different methods from a patient suspected of having intestinal parasites will increase the possibility of detecting infections, particularly amebiasis. For example, laboratory personnel might establish the following routine: 1) examination of three normally passed stool specimens collected 2 to 3 days apart,

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2) purgation with examination of three or more passages and 3) sigmoidoscopy with examination of aspirated materials if amebiasis is suspected.

Since sigmoidoscopy reveals only a few inches of the colon and permits recovery of such small amounts of material, it should seldom be the sole method used.

•OTHER TYPES OF SPECIMENS

For diagnosis of infections caused by certain species of intestinal parasites, materials other than feces will be received for examination. These materials should be examined as described under the heading "Other Specimens" in the section Methods of Examinations and in the following paragraphs.

ASPIRATED MATERIAL

Material aspirated from lesions is more often obtained in suspected cases of amebiasis than in other parasitic infections. It should be examined for the presence of trophozoites directly in wet mounts, using saline or temporary stains such as Quensel's or buffered methylene blue, or cultured (as previously described). If trophozoites or questionable structures are found, permanent stained slides of either fresh or PVA-fixed material should also be prepared.

ANAL SWABS

For the diagnosis of enterobiasis, anal swabs are received, usually cellulose-tape slide preparations or Vaseline-paraffin swabs. Refer to the section on Miscellaneous Procedures.

URINE, VAGINAL SWABS, DUODENAL DRAINAGE MATERIAL, AND SPUTUM

The handling of urine, vaginal swabs, duodenal drainage material and sputum has been discussed in the previous section and depends in part on the organism to be searched for. Selecting a technique for these body materials is not so much a problem as collecting and handling the specimen before examination. In all cases, the specimens should be examined as soon as possible, put into tubes of culture media, or preserved in appropriate solutions: Formalin for urine or sputum to be examined for helminth eggs, or PVA-fixative for urine, sputum, vaginal material or urethral exudate to be examined

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for protozoa. Preservatives may also be used for duodenal drainage or aspirates to be examined for *Giardia*.

As stated previously, material obtained for the diagnosis of *Trichomonas vaginalis* should be examined by wet mount, cultivation, or both, rather than by stained smear.

PROCEDURES FOR POSTTREATMENT SPECIMENS

Disappearance of symptoms in a patient with a parasitic infection is not sufficient evidence of a "cure." Posttreatment specimens should be examined to determine the effectiveness of therapy.

With most drugs used to treat patients with protozoan infections, organisms may not be found shortly after therapy even though the infection has not been eradicated. Thus, unless symptoms reappear earlier, the first posttreatment specimen should be collected approximately 4 weeks after therapy. Additional examinations after 3 and 6 months are advisable to detect later relapses.

In helminth infections, posttreatment examinations may be made after 1 to 2 weeks. Persons who have been treated for *Taenia* infections should be checked 2 to 3 months later.

The specimens should be collected in the same manner as for the initial diagnosis, that is, three normally passed specimens should be collected at 2- to 3-day intervals. In some cases, the specimens may be more conveniently and economically collected at the patient's home and shipped to the laboratory in Formalin (or MIF) and PVA-fixative. The patient can be given three collection kits and instructed to submit specimens on specified dates.

The specimens should be examined as described previously for normally passed feces.

Posttreatment follow-up for enterobiasis should be made by examining anal swabs. Some workers recommend that the patient be examined for 7 days following completion of therapy and then periodically for 1 month. For practical purposes, three consecutive daily swabs might be examined 7 to 10 days after therapy and at weekly intervals for 3 weeks. If swabs become positive after being negative for a month, the patient has probably reacquired the parasite.

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REPORTING RESULTS

All parasites found, both pathogenic and nonpathogenic species, should be reported by their scientific names. All helminths are considered pathogenic or potentially so, but certain intestinal protozoa are commensals or nonpathogens. Although commensals have no clinical significance, they do indicate that the patient has ingested material contaminated with feces. In persons harboring amebae, multiple infections have been found more frequently than can be accounted for by chance association (Boughton and Byrd, 1938; Eyles *et al.*, 1953). Finding a nonpathogenic species, therefore, increases the likelihood that the individual may be infected with a pathogen. Additional specimens should be examined.

In certain situations it might be advisable to identify the protozoa reported as pathogenic or nonpathogenic species.

Occasionally protozoa, especially amebae, cannot be specifically identified. In these cases, an equivocal result should not be reported lest it be misinterpreted. A report of "organisms resembling *E. histolytica*," for example, may be interpreted as final identification of this pathogen and may lead to needless treatment of the patient. Vague reports of "amebae found" may also be accepted as indicating the presence of *E. histolytica*. If species identification cannot be made, "unidentified amebae found," "unidentified flagellates found," or "unidentified nematode (or trematode, cestode or helminth) egg (or larva) found" should be reported. Additional specimens should be examined.

Although stages of helminths (eggs or larvae) are usually reported, stages of protozoa need not be reported. If cysts are found in the stool, trophozoites must be present in the intestinal tract.

If no organisms are found, the specimen should be reported as "no parasites found (NPF)" and not as "negative." If additional laboratory procedures had been used or if some other person had examined the specimens, organisms might possibly have been found.

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PRESERVATION OF SPECIMENS

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PRESERVATION OF SPECIMENS

Feces and other body materials, unless examined very soon after passage, must be preserved if organisms are to remain in a satisfactory, recognizable condition.

A single solution is not available for preserving all of the diagnostic forms (helminth eggs and larvae and protozoan cysts and trophozoites) and for providing material that can be both concentrated and permanently stained. Therefore, more than one preservative solution may be needed to permit reliable diagnoses.

Some fixatives such as merthiolate-iodine-formaldehyde (MIF) preserve all four stages, but permanent stains cannot be prepared from these specimens. Since MIF solution involves temporary stains for wet-mounts, it is discussed in the section dealing with wet-mounts.

FORMALIN

While Formalin is not the ideal preservative for protozoan cysts, it is satisfactory for cellecting specimens to be examined for eggs, larvae, and cysts. In addition, it is simple to prepare, is stable, and does not require any "last minute" mixing. Two concentrations are recommended: 5% for preserving specimens containing protozoa and 10% for specimens containing helminths. For routine purposes where both helminths and protozoa are sought, 5% is preferred. *Ascaris* eggs, and sometimes *Trichuris* eggs, will continue to develop in 5% concentrations, but this does not interfere with identification of these species. Neutral Formalin is more satisfactory for the preservation of cysts than unbuffered Formalin, especially for long-term preservation of teaching or reference specimens. Thus, it is recommended that the Formalin be buffered with sodium phosphate buffers.

•PREPARATION

A. UNBUFFERED FORMALIN

1. **Formalin, 10%**

Formalin (commercial formaldehyde) . . . . . 10 ml
Water (tap or distilled) . . . . . 90 ml

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

2. **Formalin, 5%**

Formalin (commercial formaldehyde)	5 ml
Water (tap or distilled)	95 ml

Mix thoroughly.

B. BUFFERED NEUTRAL FORMALIN SOLUTIONS

1. **Buffered Neutral Formalin, 10%**

Na_2HPO_4	6.10 gm
NaH_2PO_4	0.15 gm
Formalin (commercial formaldehyde)	800.0 ml
Water	7,200.0 ml

Mix the formaldehyde with the water. Add the buffer salts and mix thoroughly. This makes 8 liters of neutral 10% Formalin.

2. **Buffered Neutral Formalin, 5%**

Na_2HPO_4	6.10gm
NaH_2PO_4	0.15gm
Formalin (commercial formaldehyde)	400.0 ml
Water	7,600.0 ml

Prepare as for 10% buffered Formalin.

Although Formalin solutions keep indefinitely, the laboratory may wish to prepare a quantity smaller than 8 liters. The following method is suggested:

Because of the small amount of acid buffer used, it is advisable to weigh the quantities of each buffer salt as listed above. Mix the two thoroughly and store the mixture in a tightly closed bottle. Prepare 1 liter of either 10% or 5% Formalin and add 0.8 gm of the buffer salt mixture.

•TECHNIQUE FOR USING

SPECIMENS FOR ROUTINE DIAGNOSIS

Mix 1 portion of feces in about 3 volumes of Formalin. Since Formalin is chiefly a preservative for cysts, eggs, and larvae, the more formed portions should be selected, and the softer parts saved for PVA fixation.

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TEACHING OR REFERENCE SPECIMENS

If specimens are preserved for reference or teaching purposes, the following procedure is suggested.

1. Thoroughly comminute the fecal specimen with saline or water and strain through gauze or screens to remove coarse debris. Beakers or other suitable containers may be used, but a conical sedimentation flask is preferred.
2. Allow the specimen to settle for at least an hour.
3. Carefully pour off the supernatant fluid and preserve the sediment in 2 to 3 volumes of either 10% or 5% buffered neutral Formalin. For protozoan cysts, helminth larvae, and certain eggs such as *Hymenolepis diminuta* and *Hymenolepis nana*, 5% Formalin is best. For other organisms, use 10%. At least 3 parts of Formalin to 1 of fecal material should be used to insure adequate preservation.
4. If material is stored for future study, it should be checked frequently for evaporation of the Formalin. Protozoan cysts and helminth larvae can be maintained in better condition if the preservative is changed at 6-month intervals. Addition of fresh neutral Formalin retards degeneration of the organisms.
5. Certain protozoa and helminths are more difficult to preserve than others and require greater care in handling. Among these are *Entamoeba histolytica* cysts, *H. nana* eggs, and rhabditiform larvae of *Strongyloides*. The other protozoa and helminth eggs preserve somewhat better and can be kept in good condition for longer periods.

PVA-FIXATIVE

The polyvinyl alcohol (PVA)-fixative solution developed by Brooke and Goldman (1949)—a mixture of Schaudinn's fixative and the water-soluble resin—provides a means of preserving trophozoites (and cysts) for subsequent examination and is particularly useful for shipping specimens to reference laboratories. Fresh specimens, feces or other materials, suspected of containing trophozoites are mixed with the PVA-fixative. Immediately, or months later, permanent stained films can be prepared from the preserved material and trophozoites and cysts, if originally present, can be demonstrated by microscopic examination. A distinct advantage of PVA-fixative is that it makes possible successful staining of organisms in fluid speci-

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mens. The solution serves as an adhesive as well as preservative and prevents loss of organisms during the staining procedure. PVA-fixed specimens and culture sediments can be kept for several months to a year and, thus, can be used as reference or teaching material.

As discussed in the section Specimen Collection and Handling: Preserved Specimens, the currently used PVA-fixative (prepared with Schaudinn's solution) contains mercuric chloride and disposal of the material may contribute to mercury contamination of the environment. Fixatives that do not contain mercuric chloride are being investigated and, in the future, mercury-free PVA-fixative solutions may be available as substitutes for the PVA- Schaudinn's fixative. In this manual, however, PVA-fixative refers to PVA- Schaudinn's fixative.

PVA powder is stable and will remain satisfactory for years. As mentioned previously (Specimen Collection and Handling: Preserved Specimens), PVA-fixative can be kept for 6 months to 1 year in tightly closed bottles. The small quantities dispensed into vials for specimen collection, however, may jell or harden after a time. Therefore, a time limit for satisfactory use (2 months is practical) should be indicated on the collection vials. Vials should be labelled: "Do Not Use After _____," with a specific date given and "POISON" because of the mercuric chloride in the fixative.

There are many grades of PVA powder produced by several companies, but the grades of high hydrolysis and low or medium viscosity are more satisfactory for preparing fixative solution for protozoa. PVA powders produced by J.T. Baker Co. and Eastman Chemical Co. have been found satisfactory for preparing PVA-fixative. The powders are available through retail outlets selling Baker and Eastman products. Neither of these companies produces PVA-fixative, but the fixative solution can be purchased from several commercial sources including Medical Chemical Corporation (Medi-Chem), Box 445, Santa Monica, California 90404; Marion Scientific Corporation, 9233 Ward Parkway, Kansas City, Missouri 64114; and Meridian Diagnostics, Inc., P.O. Box 44216, Cincinnati, Ohio 45244. PVA-fixative is also included in some commercial kits.

•PREPARATION

SATURATED AQUEOUS SOLUTION OF MERCURIC CHLORIDE

Mercuric chloride crystals	75 to 80 gm
Distilled water	1,000 ml

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Dissolve mercuric chloride in water by heating. Let cool. Excess mercuric chloride will crystallize out. Decant the clear solution or filter. Store in a glass-stoppered bottle. The exact amount of mercuric chloride needed to produce a saturated solution may vary but 75 to 80 gm usually will be sufficient.

MODIFIED SCHAUDINN'S FIXATIVE

Glacial acetic acid	5.0 ml
Glycerin	1.5 ml
Schaudinn's fixative	93.5 ml

(2 parts saturated aqueous solution of mercuric chloride and 1 part of 95% ethyl alcohol)

Add the acid and glycerin to the fixative and mix thoroughly.

PVA-FIXATIVE SOLUTION

1. Slowly add 5 grams of PVA powder to 100 ml of modified Schaudinn's fixative at room temperature, stirring constantly.
2. Heat to about 75°C or higher until powder dissolves and suspension clears. Do not boil. A magnetic stirrer with a heating element (stirrer-hot plate) will facilitate preparation.
3. Cool to room temperature. The solution should be clear and free of lumps. Solution prepared with some lots of PVA may remain turbid and exhibit some precipitate upon cooling. Unless these conditions are excessive, they will not interfere with satisfactory use of the solution. PVA-fixative remains satisfactory for several months and can be used either at room temperature or heated to 50°C.

Burrows (1967) recommended the following improved method of preparing PVA-fixative:

MODIFIED SCHAUDINN'S FIXATIVE

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

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

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PVA MIXTURE

Glycerin	1.5 ml
PVA powder	5.0 gm
Distilled water	62.5 ml

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

PVA-FIXATIVE SOLUTION

1. Heat a water bath (or large beaker of water) to 70° to 75°C. Adjust heat to maintain this temperature range. A hot plate-magnetic stirrer can be substituted for the water bath.
2. Place the loosely stoppered flask containing the PVA mixture in the bath for about 10 minutes, swirling frequently. If the hot plate-stirrer is used, put a stirring magnet in the flask and adjust the speed to give a slow to moderate stirring action.
3. When the PVA powder appears to be mostly dissolved, pour in the fixative solution, restopper, and swirl to mix.
4. Continue to swirl mixture in the bath (or on the hot plate) for 2 to 3 minutes to dissolve the remainder of the PVA, to allow bubbles to escape and to clear the solution.
5. Remove the flask from the water bath or hot plate and let cool. Store the PVA-fixative in a screw-cap or glass stoppered bottle.

PVA-fixative prepared by this method is "reliably adhesive, does not thicken with age" (Burrows, 1967), and fixes amebae and other protozoa as well as the solutions prepared by the original method. In the CDC laboratory, most of the PVA-fixative is prepared by this method. Any laboratory experiencing difficulty in preparing satisfactory PVA-fixative solutions should try this method.

•TECHNIQUE FOR USING

To obtain the full advantage of PVA-fixative as a preservative, specimens should be thoroughly mixed with the solution immediately after passage, before the organisms lose their characteristic

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morphology. Use approximately 3 parts of fixative to 1 part of feces or other material. Feces or other materials may be mixed with PVA-fixative on slides or in vials. Fixing material directly on the slide is appropriate when the quantity is small and/or when trophozoites have been found in direct saline mounts and require permanent staining for identification. Slide fixation is especially useful with the small amounts of material obtained by sigmoidoscopy or by aspiration. Such material can be examined directly in saline, the coverslip removed, and PVA-fixative added to the saline suspension and spread into a film for future staining.

Preserving specimens in vials is more common, especially when collecting routine specimens, and, if sufficient material is available, fixation in vials is preferable. Vials of PVA-fixed material are especially useful if a number of slides are needed for teaching or reference purposes.

On Microscope Slides—A drop of dysenteric stool or other material is placed on a microscope slide and mixed with 3 drops of PVA-fixative. With an applicator stick, the mixture is then spread (not smeared like a blood film) over approximately one-third of the glass surface as described in the section on Staining Procedures: Preparation of Smears, and as shown in figure 18. Dried films remain satisfactory for staining for a few months. For best results, however, they should be stained within 2 months after preparation.

In Vials—A quantity of specimen is thoroughly mixed in a vial containing 3 or more parts of PVA-fixative. Although films for staining can be prepared immediately, better fixation is obtained after 1 hour. Smears can also be made weeks or months later; in the CDC laboratory, good preparations can be obtained up to approximately 1 year. (See section on Staining Procedures: Preparation of Smears and figure 18). If the specimen in the vial jells, it can be liquefied by heating in a water bath before making the films. Unfortunately, this can be done only 1 to 2 times before the smears become unsatisfactory. If specimens re-jell after the second liquefication, they should be discarded.

Staining—PVA films are permeable to all commonly employed staining reagents. The films are therefore handled in the same way as smears fixed in the conventional manner with Schaudinn's fixative. (See section on Staining Procedures.)

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MICROSCOPY

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MICROSCOPY

A good microscope and light source are essential for examining parasitologic specimens, since the identification of organisms depends on the observation of morphologic details. Some audiovisual aids are available on the use of the microscope and the calibration of the micrometer. See the section on Quality Control and Training Aids for further information.

EQUIPMENT

•CRITERIA FOR MICROSCOPE

The make of microscope is not important so long as it is equipped with the following:

1. Objectives: low-power (10X, 16mm)
   high-dry (43-45X, 4mm)
   oil-immersion (97-100X, 1.8 mm)
   A 3.5 or 4X low-power objective is desirable but not essential. Also, 20X objectives may be used satisfactorily.

2. Oculars: 10X and 5X (optional, but desirable)
   Special oculars such as 10X or 12.5X wide-field or compensating oculars may also be used.

3. Calibrated ocular micrometer. The disc should be placed in one of the oculars, preferably 10X, and left there. It should not be removed between uses because this often results in a dirty ocular and a dirty micrometer. Since organisms are constantly being measured, the micrometer should always be readily available. The technique for calibration is described below.

4. Adjustable mirror (if illuminator is not built in).

5. Adjustable substage condenser with regulatory iris diaphragm.

6. Adequate illumination, either a built-in illuminator or a separate lamp.

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•CRITERIA FOR ILLUMINATOR

1. **Built-in illuminators**
   a. Voltage regulator
   b. Filters: blue clear glass and white ground glass or the equivalent.
2. **Separate illuminator**
   a. Preferably equipped with condensing system, regulatory iris diaphragm, and the maneuverability to be raised or lowered and focused at different levels.
   b. 100-watt bulb (75-watt bulb may be used in some lamps).
   c. Removable filters: blue clear glass and white ground glass.

The need for blue and white filters cannot be overemphasized, since the bright yellow light obtained without these can cause difficulties in seeing the minute details necessary for species identification. Filters other than the blue and white types described are not as effective in producing the kind of lighting needed.

Certain other types of lamps that take a 75-watt or 100-watt bulb and either have, or can be fitted with, filters are satisfactory. However, the small box-type lamp often used as a substage illuminator and similar lamps may not give sufficient light to adequately observe details in specimens.

The proper use and care of the equipment is equally important. The following paragraphs on these subjects should be given special attention.

ILLUMINATION AND ALIGNMENT

The microscopes and illuminators should be properly adjusted and aligned. To do this, place the lamp about 6 to 8 inches away and directly in front of the microscope. If the filters are removable, take these out of the holder and direct the beam of light onto the center of the microscope mirror. The flat side rather than the concave side of the mirror should be turned towards the light. The reason for using the flat side is demonstrated in Diagrams A and B of figure 7. After the microscope and mirror have been aligned, the mirror is adjusted so that the object or area to be examined appears illuminated to the naked eye. The microscope field should be filled with light.

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For optimal use the substage condenser should be in the upper range of the rack. Generally, with high-dry or oil-immersion objectives, the condenser is in the top position. (See Diagrams A and C, figure 7.) Occasionally, with some microscopes, it may be necessary to lower the condenser slightly to fill the field with light when using the low-power objective. The optimal position can be determined by removing the ocular and, with the substage iris diaphragm fully open, adjusting the condenser so that the back lens of the objective is filled with light. Light intensity should be regulated with the iris diaphragm, *not* by raising or lowering the substage condenser. When the optimal position is determined, the condenser should be left in that position.

CARE OF THE MICROSCOPE

The microscope should be kept clean and free from dust, and the surface lenses of both objectives and oculars should be cleaned frequently with a good grade of lens paper. Dust particles may interfere with the observation of the structural details of organisms. Oil should be removed from the oil immersion objective as soon as the examination is finished. Oil left on the lens may form a film that is difficult to remove or seep into the objective causing a cloudy or hazy appearance. A good quality, low-viscosity immersion oil, such as Cargille's grade A, low viscosity, is recommended. Cedar, mineral, or similar oils may damage both the slide preparations and the microscope. Cedar oil tends to produce a hardened film over the lens, and, despite cleaning efforts, mineral oil frequently seeps into the objective. Excessive use of xylene to clean oculars or objectives should also be avoided.

The microscope should be dusted regularly to remove surface dust and should be kept covered when not in use.

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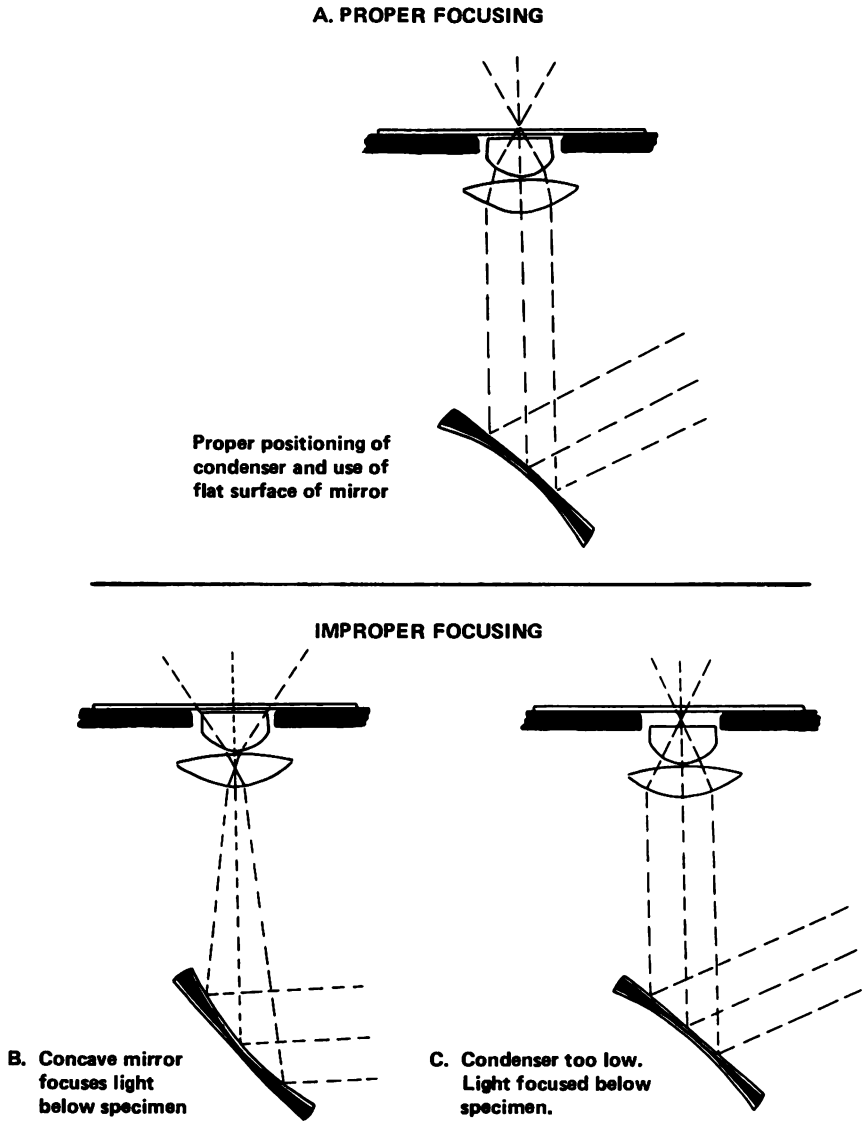
Figure 7.
Use of Microscope Mirror and Condenser



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CALIBRATION OF OCULAR MICROMETER

Size is one of the most important criteria used in identifying parasites or their diagnostic stages, especially helminth eggs. The unit of measurement is the micromillimeter (μm), sometimes referred to as a micron (μ). A rough estimation can be obtained by comparing the parasite with the size of human red blood cells (7.5 to 8μ). However, if an organism is to be accurately measured, a calibrated micrometer disc placed within the ocular of the microscope should be used. The type suggested is a disc with a line scale divided into 50 spaces or ocular micrometer units. Ocular micrometers are relatively inexpensive and are available through several scientific supply companies.

Inasmuch as the units on the ocular micrometer are arbitrary and the exact value of each varies with different objectives and microscopes, the value must be calculated with 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. This may be done by superimposing the image of the unknown ocular scale on the known scale of a stage micrometer (slide with a carefully etched and calibrated scale of 0.1-mm and 0.01-mm divisions) as demonstrated in figure 9. Stage micrometers are considerably more expensive than ocular micrometers and unless microscopes are calibrated frequently, a stage micrometer might be borrowed rather than purchased. Several State Laboratories have stage micrometers for loan; CDC also has some available for 10-day loan periods. (Contact the Parasitoloby Training Branch, Laboratory Training and Consultation Division.)

The procedure for calibration is as follows:

1. Remove the 10X ocular from the microscope and unscrew the top or bottom lens, depending on the model of microscope. Place the micrometer disc on the diaphragm within the ocular or on top of the bottom lens in newer models so that the engraved side is underneath (fig. 8). Screw back the lens and insert the ocular in the microscope. Be careful to keep both micrometer and lens clean and free of dust which might interfere with observations.
2. Place the stage micrometer on the stage and focus on some portion of the scale with the 10X objective.
3. Looking through the microscope, examine the ruling of the stage micrometer so that you can distinguish between the large (0.1-mm) and the small (0.01-mm) divisions.

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4. Adjust the field so that the 0 line on the ocular micrometer is exactly superimposed upon the 0 line on the stage micrometer. If the two scales are placed so the lines intermesh but the scale numbers are apart, readings are easier.

5. Without moving the stage micrometer, find another point at the extreme right where two other lines are exactly superimposed. The 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.

6. Knowing that each large division of the stage micrometer equals 0.1 mm and each small division equals 0.01 mm, determine the total distance (in millimeters) between the two points of superimposition and the number of small ocular units neccessary to cover the same distance (fig. 9).

   For example: Suppose 49 ocular units equal 8 large stage units or 0.8 mm.

7. Calculate the number of millimeters that is measured by one small ocular unit.

   For example: 49 ocular units = 0.8 mm

   $$1 \text{ ocular unit} = \frac{0.8 \text{ mm}}{49} \text{. or } 0.0163 \text{ mm}$$

8. Because measurements of small organisms or structures are usually given in micromillimeters (μm), or microns (μ), rather than millimeters, the above determination must be converted to micromillimeters. Since 1 mm equals 1,000 μm, the millimeter determination multiplied by 1,000 will give the number of micromillimeters measured by 1 small ocular unit.

   For example: 0.0163 mm X 1,000 = 16.3μm

9. Record the calibrations of the ocular micrometer obtained with the 10X oculars combined with each of the three objectives. The lines of the stage micrometer will increase in magnification while those of the ocular micrometer will remain the same. With high and oil magnifications, the thinner ocular micrometer line must be centered on the broader stage micrometer line for more accurate measurement.

10. The size of any microscopic object can be quickly determined by measuring it with the ocular micrometer, noting the particular lens combination, and referring to the record of the ocular micrometer calibrations. For ready reference, the values for 1 to 50 units can be recorded in a chart such as the one following.

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OCULAR MICROMETER CALIBRATIONS

Ocular magnification 10X

Low-Power Obj. 16 mm		High-Dry Obj. 4 mm.		Oil-Immersion Obj. 1.8 mm.	
Units	Micromillimeters	Units	Micromillimeters	Units	Micromillimeters
1	16.3	1	3.5	1	1.7
2	32.6	2	7.0	2	3.4
3	48.9	3	10.5	3	5.1
4		4		4	
5		5		5	
6		6		6	
7		7		7	
8		8		8	
9		9		9	
10		10		10	

Figure 8.
Insertion of Micrometer Disc into Ocular of Two Types of Microscopes

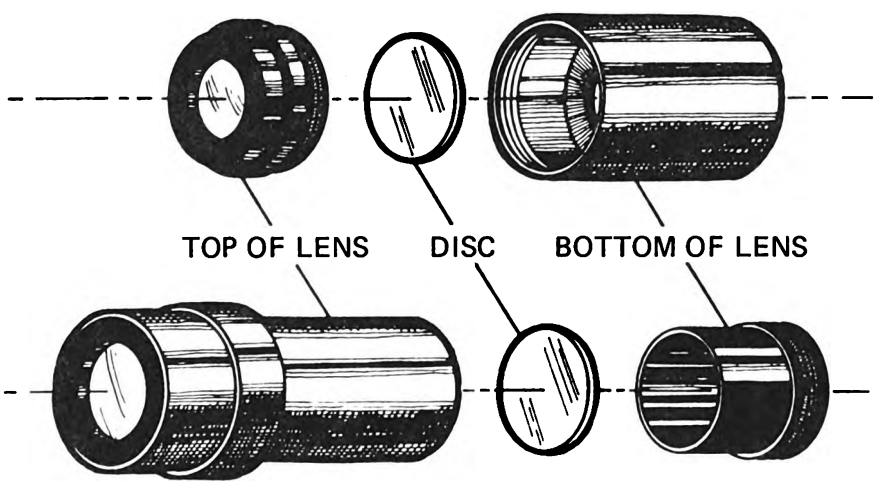

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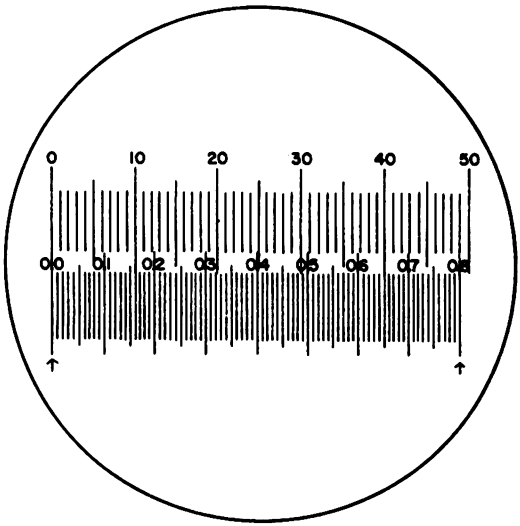
Figure 9.
Calibration of Ocular Micrometer



Ocular Micrometer — Top Scale
Stage Micrometer — Bottom Scale

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DIRECT WET MOUNTS

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DIRECT WET MOUNTS

In addition to having good equipment, the microscopist should be proficient in preparing suitable mounts and in examining these preparations. An audiovisual aid demonstrating proper preparation and examination of smears is available. See section on Training Aids for details.

PREPARATION OF MOUNTS

For direct wet mounts, as well as those from concentrated or cultured material, 3x2-inch slides instead of 3x1-inch should be used. The wide slide affords more space for preparation, permits the mount to be sealed around the edges (fig. 11), and prevents contamination of the microscope or the hands of the observer as might occur if the preparations "ran-over" on narrow slides. Ordinarily, a 22-mm-square coverslip of #1 thickness is used. For oil-immersion examination, #2 thickness is too thick for focusing. Density of the mount is also important. Fecal preparations should be thick enough that fine print can just be read through them (fig 10d). If the mount is too thick, focusing will be difficult and the organisms may be obscured. If the mount is too thin, locating organisms takes longer and may be missed entirely. Mounts should be prepared as follows:

•UNPRESERVED FECES

Place a drop of physiological saline (0.85%) on one end of the slide and a drop of iodine (approximately 1%) on the other end (fig. 10a). Using an applicator stick or toothpick, pick up a very small portion of feces and emulsify it in each solution (fig. 10b). Criteria for proper density should be kept in mind. Large particles should be avoided if possible, since the coverslip will not lie evenly on the slide. Cover the preparations, seal as described below, and examine. If too little fluid has been used and the material does not fill up the coverslip area, additional saline or iodine may be run under the edge. The feces will not be evenly mixed with the diluent, of course, but the preparation can be more easily examined than if vacant spots are present. On the other hand, the coverslip should

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not float on the fecal suspension. In this case, a piece of facial tissue or absorbent towel can be used to remove the excess fluid before sealing. Touch the tissue to the edges of the coverslip, but be careful not to remove too much fluid. Iodine preparations will fade rather quickly if exposed to light, so these mounts cannot be left indefinitely before examining. If several preparations are made at one time, a slide folder may be used to hold them.

•SEALING PREPARATION

Sealing the mounts with a mixture of Vaseline-paraffin (vaspar) (approximately 1:1) is recommended as a routine procedure to prevent drying, thus permitting reexamination or examination at a later time. Such preparations may last as long as 2 to 3 days. Sealed mounts prevent the "flow" of the material and can be more easily examined with oil-immersion (as may be necessary to identify small protozoa) than unsealed preparations. Secure the coverslip by first placing a drop of hot vaspar on the four corners of the mount (fig 11). This will anchor the coverslip in place. A cotton swab or camel's hair brush can be used to apply the vaspar. Next, spread a thin layer around the edges of the preparation (fig. 11). The layer of vaspar should cover the edge of the coverslip, sealing it to the slide, but should not be very thick. If the layer is thick enough to form a ridge, the microscope objectives may become coated with the mixture as they are swung into position. Too much of the coverslip should not be covered with vaspar, since a maximum surface of the preparation should be available for examination.

•FORMALIN PRESERVED FECES

If the quantity of Formalin is more than 2 or 3 times that of the feces, let the feces settle and discard the excess before resuspending the material. Place a small drop of iodine near one end of the slide. In preserved specimens, the Formalin replaces the saline used in mounts of fresh feces, and the suspension can be used directly. Thoroughly mix the specimen before taking a sample, since eggs, larvae, and cysts settle to the bottom of the bottle. A drop of material from the top layers of an unmixed positive specimen may not contain organisms. Using 2 applicator sticks or toothpicks held together, remove a drop of the suspension to the slide (fig 10c).

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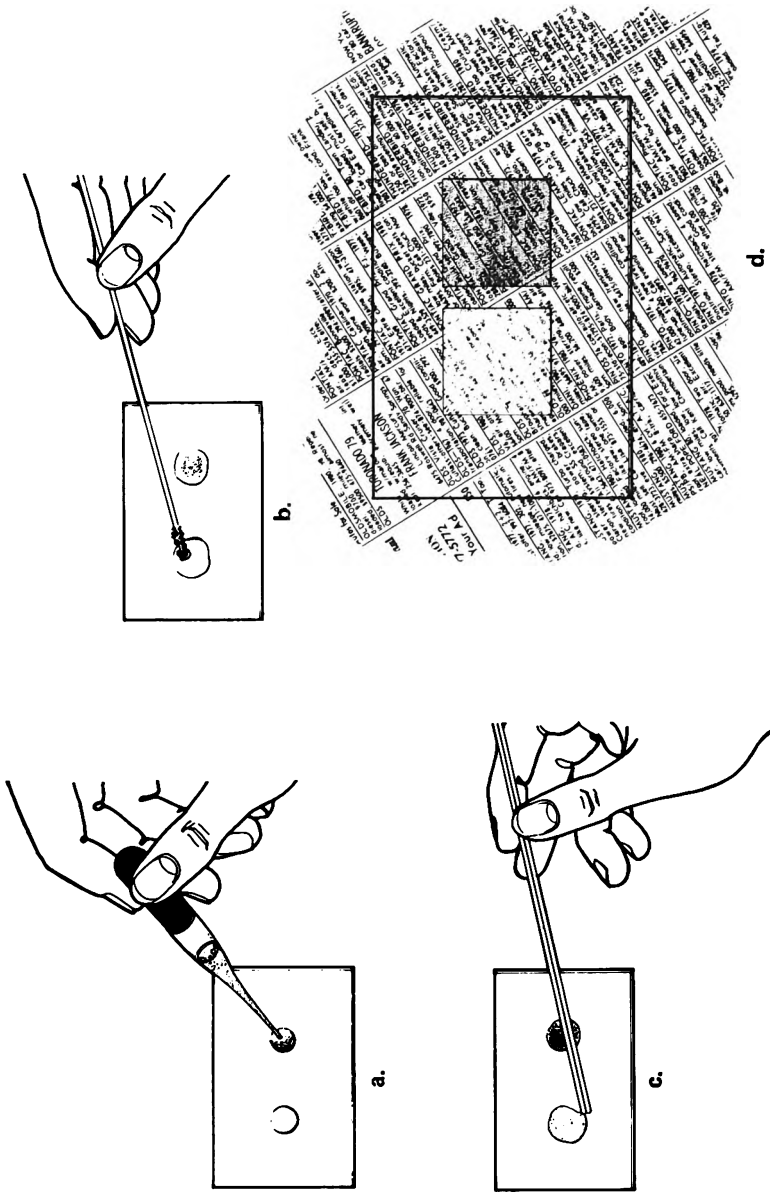
Figure 10.
Preparing Wet Mounts of Fecal Material



a. Drops of saline and iodine are placed on a 3 x 2-inch slide.
b. Emulsify portions of unpreserved feces in diluents.
c. Use 2 applicator sticks to remove drop preserved fecal suspension to slide.
d. Correct density of mounts.

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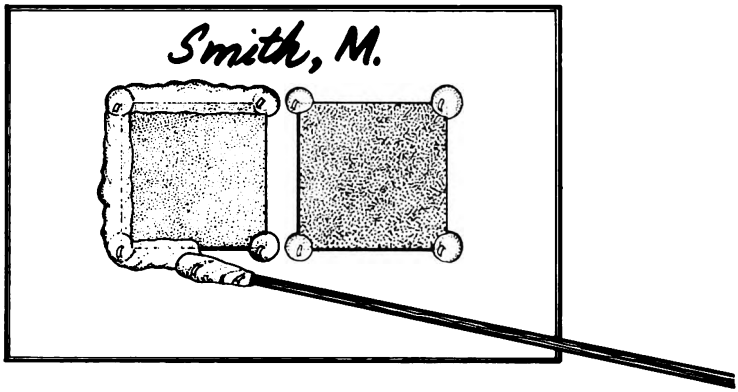
Figure 11.
Sealing Mounts with Vaspar



If the suspension is thick, it may be necessary to add a small drop of saline to dilute it to a satisfactory density. Add a second portion to the iodine drop and mix. Cover both preparations and seal as described above. The same techniques used for fresh fecal mounts can be used to add additional fluid (saline can be added to the unstained mount) or to remove it. The preparation must be of the correct density for adequate examination.

In making mounts from preserved specimens, a small drop of iodine must be used so that the preparation will not be too thick. Since the iodine will be diluted by the Formalin in the specimen, a slightly stronger concentration than usually used may be needed to obtain adequately stained structures. The need for a more concentrated iodine can be determined by trial. A too-strong solution, however, should be avoided. In the CDC laboratory, Dobell's iodine is used routinely, and a darker, stronger solution is made up for use with formalinized specimens by adding extra iodine crystals to the potassium iodide solution.

•CONCENTRATED SPECIMENS

Preparations from concentrated specimens are made in the same fashion as that described for Formalin preserved material. Pipettes

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or loops, depending on the technique employed, are used to transfer material to the slides. Whether or not additional saline must be used will depend on how much fluid the sample contains. Often concentrated material, especially that from sedimentation procedures, will contain grit or large particles. Avoid getting these in the mount if possible, since, if this occurs, the coverslip will not lie evenly on the slide. If particles do get into the preparation, sometimes they can be pushed to one side with the corner of the coverslip or with an applicator stick. (If you hold the applicator stick by the ends and break it, you can usually get a slanted or pointed edge which can be more easily used than the blunt end.) Occasionally, particles can be flattened by pressing gently on the coverslip. If the particle is grit, however, the coverslip may break. When this happens, remove the broken pieces, add another small drop of thoroughly mixed suspension to the material left on the slide, and try again. If the preparation becomes "messy" around the edges of the coverslip, a piece of tissue can be used to clean it. Unless excessive, however, the "messy" edges can simply be covered with the vaspar; there is little, if any, danger of contaminating the vaspar by doing this. If despite everything, the preparation contains particles which cause the coverslip to "rock," add saline or iodine to fill up the space and seal. The density of the preparation will be uneven and may be too thick in spots, but this is preferable to gaps in the mount.

•OTHER TYPES OF MATERIAL

Wet mounts of urine to be examined for *Trichomonas vaginalis* or *Schistosoma haematobium* eggs are usually prepared from the sediment of a centrifuged specimen. (See p. 44) No special stains are needed to demonstrate either organism. Preparations may be sealed or left unsealed if they are examined within a few minutes.

Vaginal and urethral material may be mixed with a small drop of saline and examined directly for *Trichomonas vaginalis*. Neither temporary methylene blue stain nor iodine should be used.

Trichomonas trophozoites will lose their characteristic motion within a short time and begin to round up. Also, specimens should not be chilled because this affects the motility, but warm stages are not necessary, since the heat from the microscope lamp will be sufficient. For best results mounts prepared for this parasite should be examined as soon as possible.

Wet mounts of aspirated lesion material to be examined for *E.*

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histolytica require special treatment, as described on p. 154. Temporary stains such as Quensel's or buffered methylene blue (Nair's solution) may be used to stain the trophozoites. Preparations should be sealed as described above.

Duodenal drainage can be examined directly with or without saline. Duodenal aspirates and material obtained by nylon thread should be mixed with a small drop of saline. Temporary stains are of no value with these mounts.

Sigmoidoscopic material may be mixed with a drop of saline and examined for amebae trophozoites. Additional preparations with temporary stains such as Quensel's or buffered methylene blue may be useful. Preparations should be sealed as described above.

Sputum specimens are also examined as wet mounts. A small portion of the specimen can be mixed with saline, or if the material is thick and viscous, it can be diluted with several volumes of saline in a large tube and shaken vigorously for 30 to 45 seconds. If the specimen is being examined for *P. westermani* eggs, dilute sodium hydroxide (about 3% to 5%) may also be used to break up the thick portions. Sputum is only rarely examined for amebae trophozoites, but in these cases it must be handled more gently. Mounts with saline and with temporary stains, such as Quensel's or buffered methylene blue, may be made.

EXAMINATION

Helminths eggs and larvae are large enough to be seen under low-power magnification (10X objective), although for study of structural details, high-dry magnification (43 or 44X objective) may be needed. Oil-immersion is not necessary and, in fact, may tend to confuse the observer. Cysts and trophozoites of protozoa can be detected with the low-power objective but higher magnifications are necessary for identifying species. Large protozoa, such as *Entamoeba coli* and *Balantidium coli*, and species like *Giardia lamblia* may be easily identified with high-dry magnification, but others, especially small cysts like *Endolimax nana* or *Entamoeba hartmanni* may require oil-immersion magnification for accurate identification. Diagnosis of *E. histolytica* cysts is also more reliable with oil-immersion examination.

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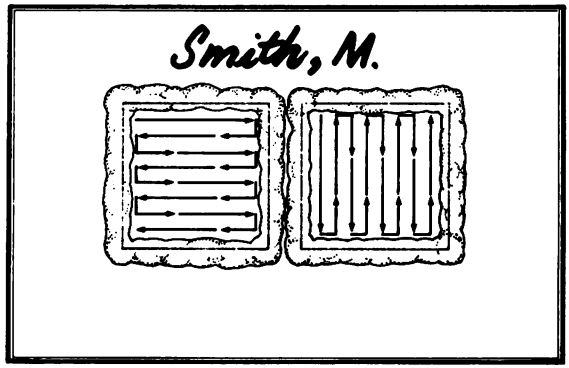
The light should be reduced for low-power observations, since most organisms will be overlooked with bright light. Illumination should be regulated so that cellular elements in the feces are readily visible.

For routine examinations, a systematic scanning, beginning with one corner of the mount and covering the entire preparation, is recommended (fig. 12). This reduces the chances of missing scarce organisms that might be present. Scanning is done with the 10X objective. When organisms are found, the high-dry and oil-immersion objectives can be used for more detailed study. At least one complete saline (or unstained) mount should be searched carefully. An experienced microscopist usually requires from 10 to 15 minutes to do this.

The saline or unstained mounts should be examined first, since eggs, larvae, trophozoites (if living), and cysts can be more readily detected in this type of mount than in the iodine preparation. If structures resembling cysts are seen, the iodine preparation should be examined. If no cysts or suggestive structures are found, the iodine mount need not be examined unless the microscopist wishes to do so. In certain instances, final species identification of protozoa can be made; in others, only presumptive diagnoses will be possible. Permanent stained preparations or additional specimens may be needed. Except in rare cases, stained preparations are essential for identifying species of amebae trophozoites.

Figure 12.
Method of Examining Wet Mount



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In temporary wet mounts, eggs or larvae may sometimes need to be turned over or reoriented so that the structural details may be seen. This can be done by gently tapping the coverslip with an applicator stick. Occasionally an egg may be lying at an angle or in a different plane and appear unfamiliar. This is particularly true with *Trichuris*, which may be oriented so that the viewer gets an optical cross-section or oblique view that may be unrecognizable as the familiar *Trichuris* egg. *Schistosoma mansoni* eggs also sometimes lie so that the spine does not show, and they too may need to be rolled over to be recognized.

Wet mounts of other types of material should be examined in the same manner as described for fecal specimens.

SOLUTIONS

•IODINE SOLUTIONS

Several iodine solutions can be used to stain protozoan cysts satisfactorily. The two described below have been widely used and are simply prepared. The one recommended by Dobell and O'Connor (1921) is a 1% iodine solution that should be prepared fresh about every 10 to 14 days for best results. Lugol's iodine, which must be diluted 1:5 for use, should be prepared fresh about every 3 weeks; the 1:5 dilution should be prepared every 10 to 14 days.

PREPARATION

1. DOBELL AND O'CONNOR'S IODINE SOLUTION:

Iodine (powdered crystals)	1 gm
Potassium iodide	2 gm
Distilled water	100 ml

Dissolve potassium iodide in the water. Add the iodine crystals and shake thoroughly. The crystals may not dissolve completely, but the solution should be a definite reddish-brown or the color of medium-strong tea. See fig. 13, page 84, for correct color. Filter or decant into a dropping bottle or other type of dispenser. Replace after 10 to 14 days.

Most clinical laboratories do not use 100 ml of iodine solution in a 10- to 14-day period and preparing this amount of stain is wasteful. For practical purposes, the following procedure is suggested.

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1. Prepare 500 ml of 2% potassium iodide (10 gm dissolved in 500 ml distilled water).

2. Store this stock potassium iodide solution in a glass-stoppered bottle. A brown glass bottle is preferred. The solution will keep for several months, although it may become slightly yellow with age. This does not interfere with its use.

   Pour a small amount (20 or 30 ml) into a small beaker or Erlenmeyer flask. Add some iodine crystals (about 0.2 or 0.3 gm; the exact amount is not important) and shake until the solution is the proper iodine "color" or strength (fig 13). Replace after 10 to 14 days.

2. LUGOL'S IODINE SOLUTION:

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

Dissolve the potassium iodide in the distilled water. Slowly add the iodine crystals and shake until dissolved. The solution will be a strong reddish-brown. Filter. Store in a tightly stoppered, brown bottle away from the light. Dilute a portion 1:5 with distilled water for use in staining protozoan cysts.

TECHNIQUE

A small portion of feces or fecal suspension is comminuted in a drop of the iodine solution, mounted with a coverslip, and sealed with heated vaspar. The preparation should be of the correct density for microscopic examination.

STAIN CHARACTERISTICS

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 may 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.

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Figure 13.
Dobell's Iodine – Correct Color

•MERTHIOLATE-IODINE-FORMALDEHYDE (MIF) SOLUTION

The MIF solution can be used for staining protozoan cysts and trophozoites in wet-mount preparations and as a preservative for these stages and helminth eggs and larvae (Sapero and Lawless, 1953). Slightly different formulas are recommended for these two uses.

PREPARATION

1. Wet-Mount Preparations

Lugol's solution (see below)	0.10 ml
Formaldehyde (USP)	0.15 ml
Tincture merthiolate, No. 99, 1:1,000 (Lilly)*	0.75 ml

One ml of stain is sufficient for 25 to 30 fecal preparations. The stain is prepared fresh each day. The amounts of the 3 ingredients may be varied for different purposes. The greater

*Tincture of Merthiolate, No. 99, 1:1,000 (Lilly) (Eli Lilly & Co., Indianapolis, Indiana) must be used, since this contains eosin, the stain ingredient in the second stage.

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the iodine strength, the more immediate the staining reaction; however, the cysts and trophozoites are more easily detected at a lower iodine strength. Cytoplasmic detail is more distinct at a greater formaldehyde strength.

2. **Preservation of Specimens**

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 solutions and store in brown bottle.

B. Lugol's Iodine Solution (Prepared Fresh Every 3 Weeks)

Iodine crystals (powdered)	5 gm
Potassium iodide	10 gm
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.

Mix solutions A and B together as indicated in item 2 below to prepare the MIF preservative.

TECHNIQUE

1. **Wet Mounts**
   a. Place 1 drop of distilled water on a slide using a standard medicine dropper.
   b. Add 1 drop of MIF stain (prepared for wet mounts).
   c. Comminute a portion of the fecal specimen in this mixture. Have the suspension of the correct density for a satisfactory mount.
   d. Mount with coverslip, seal, and examine. The preparation may be examined several hours later without loss of staining or details.
2. **Preservation of Specimens**
   a. Add 2.35 ml MF stock to 0.15 ml Lugol's solution *immediately before use* because prior addition of the iodine causes a dense precipitate to be formed and the iodine fails to act satisfactorily on the protozoa.

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b. Thoroughly mix a portion of the feces about the size of a pea in the MIF solution (approximately 1 part of feces to 2 or 3 parts of preservative).

   Do not use too much feces. For larger samples, use increased amounts of the MIF stain in the same proportions of MF stock and iodine solutions.

c. To examine, take a drop of fluid from the top layer of the sedimented feces and place on a slide. A preparation roughly comparable in density to a direct smear should be obtained.

STAIN CHARACTERISTICS

The staining reaction in both direct smears and preserved specimens is divided into two phases: 1) an iodine phase in which cysts and trophozoites stain yellow-green or yellow-brown and 2) an eosin phase which replaces the iodine phase and is permanent.

In the iodine phase; nuclei stain dark brown and in the eosin phase, dark red to jet black. Cytoplasm of the trophozoites and cysts changes from yellow-green or brown in iodine to an eosin (pink or red) color in the second stage. Occasionally, refractory mature cysts are encountered which fail to stain in the eosin phase. Nuclear elements of all species except *Dientamoeba* are fairly well defined. Glycogen appears as a dark brown area in the iodine stage and as a colorless area in the eosin stage. Chromatoid bodies are characteristic in appearance. Flagella may be detected on flagellate trophozoites. Helminth eggs also stain and retain their normal characteristics. Fecal debris stains darker brown than parasites or blood and tissue cells. No distortion occurs, but for good contrast, a blue filter should be used in the illumination system.

•QUENSEL'S STAIN SOLUTION

The nuclei of amebae trophozoites are indistinct or entirely invisible in saline preparations. Iodine solution is unsatisfactory for staining this stage, since it may distort the entire organism. Quensel's solution is recommended for the study of trophic amebae in temporary preparations (Svensson, 1935) and may be used with unpreserved feces or sediment from cultures.

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PREPARATION

A. Stock Solutions

1. *Sudan III Saturated Alcoholic Solution*

   Sudan III powder ..... 1.6 gm
   80% ethyl alcohol ..... 100 ml

   Add the stain to the alcohol. Shake thoroughly. Let stand for a few hours or overnight to be sure the solution will be saturated. If all of the stain dissolves, add more powder until no more will go into solution. Filter or decant to remove the excess powder and store in a screw-cap or glass-stoppered bottle.

2. *Methylene Blue, Saturated Aqueous Solution*

   Methylene blue powder, medicinal ..... 3.5 gm
   Distilled water..... 100 ml

   Add the stain to the water and shake thoroughly. Let stand for a few hours, shake at intervals. If all of the stain goes into solution, add more. Filter. Store in a screw-cap or glass-stoppered bottle.

3. *Cadmium Chloride Solution*

   Cadmium chloride (c.p.) ..... 10 gm
   Distilled water..... 100 ml

   Dissolve the cadmium chloride crystals in the water. Store in a screw-cap or glass-stoppered bottle.

B. Quensel's Stain Solution

Sudan III, saturated alcoholic solution ..... 20 ml
Methylene blue (medicinal) saturated aqueous solution..... 30 ml
Cadmium chloride, 10% aqueous solution..... 50 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 now and then 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.

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5. Remove all excess liquid from precipitate by placing the filter paper with the precipitate upon another filter paper. Leave overnight.
6. Transfer precipitate to a fresh filter and rapidly pour through 25 to 30 ml of distilled water.
7. Dissolve the washed precipitate in 250 ml of distilled water.
8. Filter in a few days if fine crystals of cadmium chloride precipitate.

TECHNIQUE

A small amount of the fecal sample or culture sediment is picked up with an applicator stick, toothpick, or pipette (culture) and thoroughly comminuted in a drop of Quensel's solution. The mixture is covered with a coverslip and sealed.

STAIN CHARACTERISTICS

After about 10 to 20 minutes, the amebae trophozoites are stained a pale blue with their nuclei a deeper blue shade. The stained nuclei present the same morphologic characteristics that they do in a permanently stained hematoxylin preparation. Food inclusions within the cytoplasm are also stained. After ½ to 1 hour, the organisms become overstained and can no longer be identified. Occasionally, organisms fail to stain. The nuclei of *Dientamoeba fragilis* do not stain well, but the presence of 1 or 2 nuclei may be discernible. The use of a warm stage may aid in the staining of *Dientamoeba*. *Blastocystis hominis* stains beautifully, but ciliates, flagellates, and living cysts do not stain. Cysts preserved in Formalin will stain, however, but details are not always easily seen.

•BUFFERED METHYLENE BLUE SOLUTION

The pH of the stain solution has been found the deciding factor in bringing out the morphologic details of the nuclei of protozoan trophozoites in wet mounts. Satisfactory results can be obtained by dissolving biological dyes, such as methylene blue, in an appropriately buffered solution (Nair, 1953). The buffered methylene blue stain can be substituted for Quensel's. Staining results are the same and the buffered methylene blue solution is easier to prepare than Quensel's stain. For staining trophozoites of *E. histolytica*, the exact

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pH is not critical and the optimum range may vary with the buffer system and dyes employed. Directions for preparing acetate buffers with the pH range of 3.6 to 4.8 are given below, or if desired, commercially available buffer tablets can be used. Phthalate buffers with a pH range of 2.0 to 2.5 are also satisfactory.

PREPARATION OF SOLUTIONS

A. Stock Solutions

1. *Solution A, 0.2M Acetic Acid:*

Glacial acetic acid ..... 11.55 ml
Distilled water..... 988.45 ml

Mix the acid with the water to give a 0.2M solution. Store in a glass-stoppered bottle.

2. *Solution B, 0.2M Sodium Acetate:*

Sodium acetate ($NaC_2H_3O_2$) ..... 16.4 gm

–OR–

Sodium acetate, crystalline ($NaC_2H_3O_2\ 3H_2O$) .... 27.2 gm
Distilled water to make..... 1,000.0 ml

Dissolve sodium acetate in 300 to 400 ml of water in a 1-liter volumetric flask. Add water to the l-liter level. Mix thoroughly. Store in a glass-stoppered bottle.

B. Acetate Buffer Solution

For specific pH, mix the indicated portions of Solutions A and B and dilute with distilled water to a total volume of 100 ml.

pH	A	B
3.6	46.3	3.7
3.8	44.0	6.0
4.0	41.0	9.0
4.2	36.8	13.2
4.4	30.5	19.5
4.6	25.5	24.5
4.8	20.0	30.0

C. Temporary Stain

Biological dyes such as methylene blue or pyronin may be used. The quantity of dye that should be dissolved in the buffer solutions can be determined by a little experience. The amount will

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depend upon the solubility of the dye in the buffer at different pH levels. In practice, a small quantity of methylene blue (0.06%) in acetate buffer at pH 3.6 has given satisfactory results.

TECHNIQUE

A small amount of the fecal sample or culture sediment is picked up with an applicator stick, toothpick or pipette and thoroughly comminuted in a drop of the buffered methylene blue solution. The mixture is covered with a coverslip and sealed.

STAIN CHARACTERISTICS

After a few minutes the amebae trophozoites are stained a pale blue with their nuclei a deeper blue shade. The stained nuclei present the same morphologic characteristics that they do in a permanently stained hematoxylin preparation. Food inclusions within the cytoplasm are also stained. The nuclei of *D. fragilis* stain to a degree with this solution and the presence of 1 or 2 nuclei may be discernible. Occasionally, some organisms fail to stain.

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CONCENTRATION PROCEDURES

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CONCENTRATION PROCEDURES

CALCULATION OF CENTRIFUGAL SPEED

Since the models and sizes of centrifuges used for concentrating specimens vary considerably, the use of g (gravity) forces instead of r/min is suggested. In the procedures given in this section, the r/min indicated are the speeds obtained on International table model, horizontal head centrifuges. The same r/min on a different model may produce a different g force. Therefore, the estimated g force is listed in parenthesis after the r/min. Unless a comparable table model centrifuge is used, the laboratory should calculate the speed (r/min) necessary to achieve the appropriate g force with the particular model being used.

A nomograph for calculating centrifuge speeds follows. The rotating radius of the centrifuge head is the basis for the calculation and must be carefully determined. As indicated in the footnote on the nomograph, this is done by measuring the distance from the center of the drive shaft to the middle of the column of material being centrifuged. The tube must be in the fully extended position when the distance is measured.

G forces have not usually been given in reports of either the original or subsequent studies on the technical procedures for parasitologic specimens. Those given in this Section are the ones obtained in the CDC laboratory, and they are estimated g forces for routine work. Apparently, the g force can vary, within limits, without interfering with the effectiveness of the procedure. The g forces found most effective in the CDC laboratory range between 500 and 700 g for the faster, stronger centrifugations and between 300 and 450 g for the slower centrifugations.

If the g force used does not seem to be effective, the technologist should alter it. When material and organisms appear to concentrate poorly, the g force may need to be increased slightly. Collapsed organisms, if caused by centrifugation and not the density of the solution being used, indicate that the g force should be decreased.

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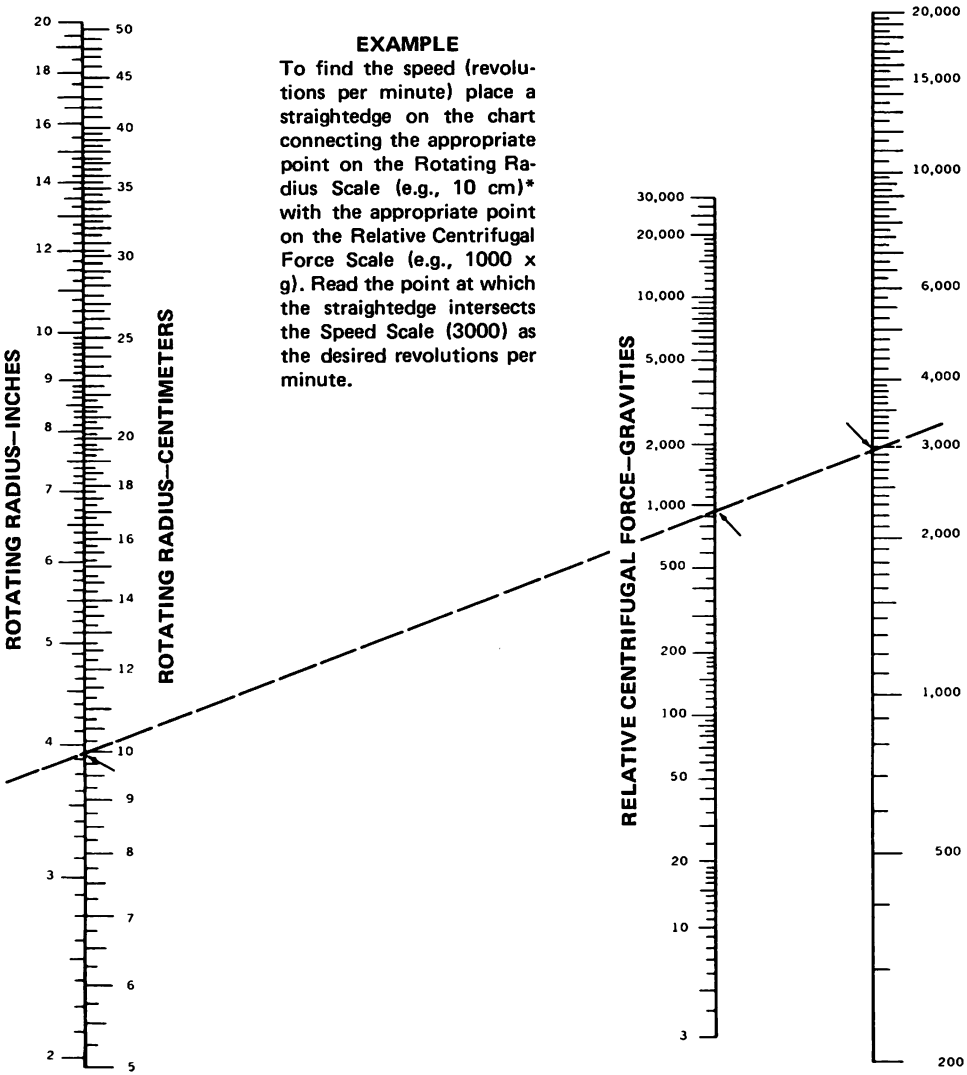
Nomograph for Calculating Centrifuge Speed



EXAMPLE

To find the speed (revolutions per minute) place a straightedge on the chart connecting the appropriate point on the Rotating Radius Scale (e.g., 10 cm)* with the appropriate point on the Relative Centrifugal Force Scale (e.g., 1000 x g). Read the point at which the straightedge intersects the Speed Scale (3000) as the desired revolutions per minute.

*To determine the rotating radius of the centrifuge head, measure—with the tube in the extended position—from the center of the drive shaft to the middle of the liquid column you are centrifuging.

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FLOTATION PROCEDURES

•MODIFIED ZINC SULFATE CENTRIFUGAL FLOTATION TECHNIQUE

The zinc sulfate centrifugal flotation procedure was the first concentration technique developed for the recovery of both helminth eggs and larvae and protozoan cysts. Operculated eggs and those of the schistosomes are not recovered, however. The use of water for preparing the fecal suspension will destroy *Blastocystis* and remove some of the debris usually present. The original method developed by Faust *et al.* (1938) included 2 steps which are omitted in the modified procedure described below. These were 1) preparing a fecal-water suspension in a beaker and 2) straining a portion of the suspension through gauze into a small tube. In the modified method, the suspension is prepared directly in the tube. In addition, unless the specimen is very oily, the number of washings by centrifugation is reduced to one.

Organisms which rise to the surface of the solution will begin to sink after 1 hour. Therefore, for optimum recovery, slide mounts should be prepared as soon as the technique is completed. Since prolonged exposure to zinc sulfate may cause small cysts to distort and become difficult to recognize, mounts should not be allowed to stand for several hours before being examined.

PREPARATION OF SOLUTION

1. In a volumetric flask, dissolve 330 gm of zinc sulfate (USP)* in about 600 ml of warm distilled water. After the zinc sulfate is thoroughly dissolved, add water to make 1 liter of solution. A technical grade of zinc sulfate can also be used if the insoluble salts are removed from the solution by filtering before use.
2. Check the specific gravity with a hydrometer. It should read 1.18. If the reading is not correct, adjust the specific gravity by adding water or zinc sulfate as needed. Usually 330 grams of zinc sulfate will give a solution of the correct specific gravity, but this may vary with the water used; therefore, the specific gravity should be carefully checked.

*These quantities are for dry zinc sulfate crystals. Crystals with obvious moisture will give a lower specific gravity.

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3. Some workers prefer a specific gravity of 1.20. Dissolve 330 gm of zinc sulfate in 500 ml of warm distilled water. After the zinc sulfate has dissolved, add water to make 700 ml of solution. Check the specific gravity with a hydrometer. If it does not read 1.20, adjust by adding water or zinc sulfate as needed.

   This solution of higher specific gravity should be used when a formalinized fecal specimen is to be examined.

TECHNIQUE

A. Unpreserved Fecal Specimens

1. Using 2 applicators, comminute a fecal sample about the size of a small pea in a 100x13-mm tube half-filled with tap water. Make certain that all obvious particles are broken up and that a suspension is formed.
2. Add additional tap water until the tube is two-thirds full.
3. Centrifuge this preparation for 1 minute at approximately 2,500 r/min (650 g).
4. Pour off the supernatant fluid into a container holding a disinfectant, for example cresol or amphyll.
5. Repeat this washing only if the stool is very oily or contains excessive debris (supernatant fluid will be very cloudy).
6. Add sufficient zinc sulfate solution to fill the tube half full.
7. Using an applicator, break up the packed sediment thoroughly and suspend it in the solution.
8. Add additional zinc sulfate solution to fill the tube to within ½ inch (1 cm) of the top.
9. Centrifuge this suspension for 1 minute at 2,500 r/min (650g).
10. Without shaking or spilling, carefully place the tube in a rack. The openings in the rack should be just large enough to accommodate the tube and keep it in an upright position. Racks with large openings will allow the tube to lean and the surface level will be slanted. This may cause reduced recovery of organisms.
11. The material in the surface layers can be removed in either of two ways: a) by raising the fluid level to form a meniscus on top or b) by removing a portion of the surface film with a wire loop (preferred) or a capillary pipette. (See fig. 14).

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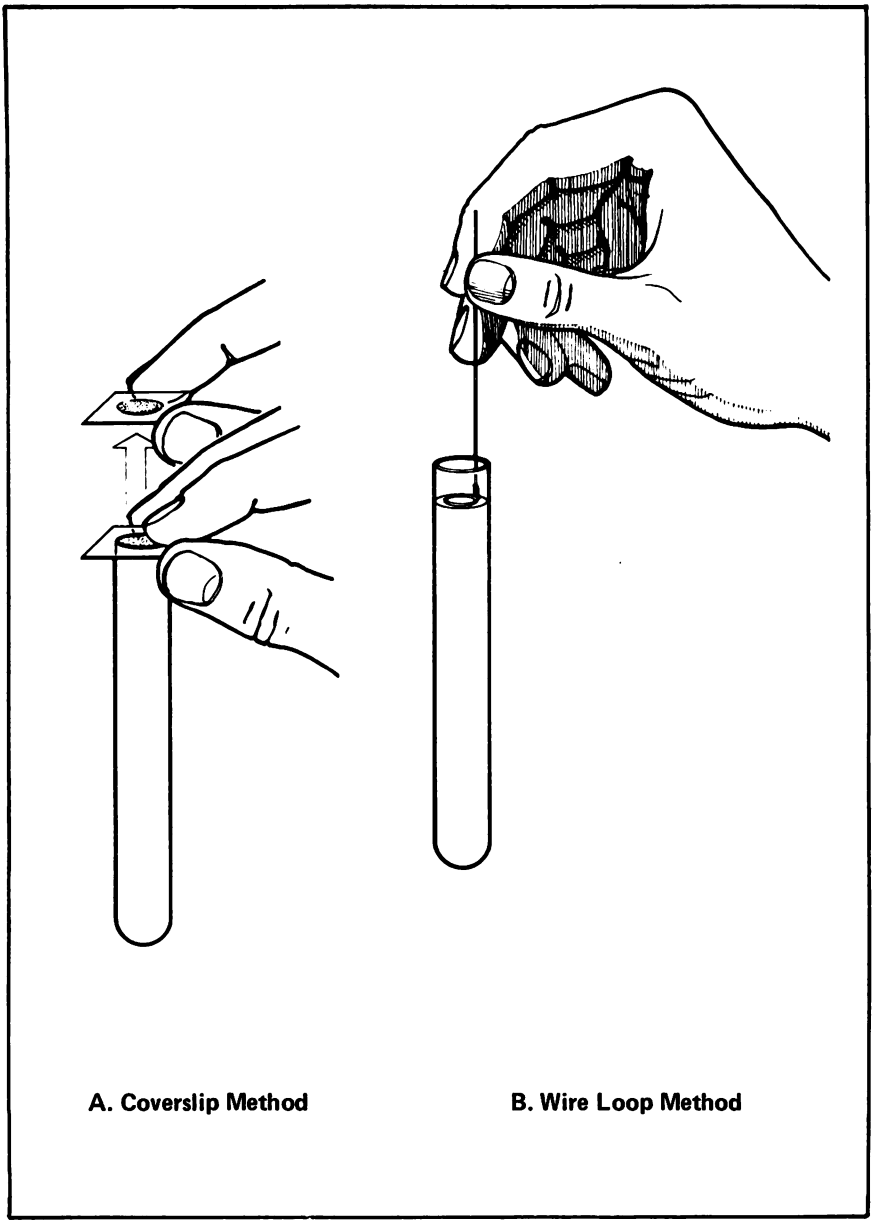
Figure 14.
Zinc Sulfate Centrifugal Flotation
Method of Obtaining Sample for Examination



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a. **Raising Fluid Level**

1) Slowly fill the tube to the brim with zinc sulfate without allowing any runover. Using a pipette or dropper, add the solution down the side of the tube to prevent disturbing the surface film.

2) Place a clean, grease-free #1 coverslip (22x22-mm) on top of the tube so that the under surface touches the meniscus. Leave undisturbed for about 10 minutes. No bubbles of air should be present.

3) Deftly remove the coverslip with a straight, upward motion (fig. 14a). A drop containing eggs, larvae, and cysts (if present) will adhere to the underside of the coverslip.

4) Lower this onto a drop of iodine stain placed on a clean 3x2-inch slide. Seal the preparation.

b. **Removing Surface Film**

1) Make a small wire loop so that the loop is at right angles to the stem.

2) Slide the loop gently under the surface film (fig. 14b) and remove 2 or 3 loopfuls of material to a 3x2-inch slide.

3) A capillary pipette may also be used to transfer a portion of this film to a slide; be sure the tip of the pipette is not below the surface.

4) Add a drop of iodine, cover with a coverslip, and seal the preparation.

12. Examine for eggs, larvae, and cysts.

B. Formalin-Preserved Fecal Specimens

Formalinized fecal specimens require a higher specific gravity to float eggs, larvae, and cysts than fresh specimens, so a zinc sulfate solution with specific gravity of 1.20 should be used. The following procedure is adapted from Bartlett *et al.* (1978).

1. The Formalin-feces suspension should be approximately 1 part of feces and 2 to 3 parts of Formalin. If the suspension is too thick, dilute with 10% Formalin to approximately these proportions.

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2. Strain enough of the Formalin-feces suspension through *wet* gauze to fill a 100x13-mm tube about two-thirds to three-fourths full.

   (Note: Tap water may be added to the tube if necessary).

3. Follow steps 3-12 as described for fresh fecal specimens, except use zinc sulfate with specific gravity 1.20. After the first centrifugation (Step 3) there should be about ½-inch of sediment in the tube.

•CONCENTRATED BRINE FLOTATION TECHNIQUE

This technique (Willis, 1921) is simple and efficient for the recovery of helminth eggs other than operculate and schistosome eggs. It is especially recommended for recovery of hookworm eggs. Protozoan cysts, however, will be unrecognizably shrunken.

PREPARATION OF SOLUTION

Prepare the brine solution by stirring sodium chloride (table salt is satisfactory) into hot or boiling tap water until the excess added will not go into the solution. This saturated solution should be checked with a hydrometer after cooling to insure that the specific gravity is 1.20. The solution should be filtered before using.

TECHNIQUE

1. Place a sample of feces about the size of a small marble (approximately 1 or 2 grams) into a vial about 1 inch in diameter and 2 to 2½ inches high, with a capacity of approximately 20 ml (fig. 15).
2. Fill this one-fourth full with the saturated salt solution.
3. Using 2 applicators, mix thoroughly, suspending the feces as a fine silt.
4. Fill the vial to the brim with salt solution.
5. Carefully superimpose a grease-free 3x2-inch slide. The slide must contact the surface of the suspension completely without any airpockets being formed and without overflow.
6. Allow the preparation to stand for 10 minutes to 1 hour. The optimum time is 10 to 15 minutes.
7. Lift the slide straight up without tipping. A drop containing the eggs will adhere to the undersurface of the slide.

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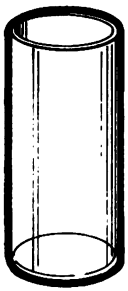
8. Invert the slide without allowing the drop to slide off.
9. Examine for eggs immediately, before the preparation begins to dry. A coverslip is not necessary.
10. Inverting the slide without spilling the drop is awkward and difficult for some people. A coverslip can be used in place of the slide, if the vial is not too wide. After the appropriate time, the coverslip is lifted straight up and placed drop side down on a slide. These preparations usually do not need to be sealed with vaspar if they are examined immediately.

Figure 15.
Vial for Brine Flotation



SEDIMENTATION PROCEDURES

Most of the sedimentation techniques require the fecal suspension to be strained. The gauze or cheesecloth used should not be too thick, lest organisms get trapped. Two layers of a wide-mesh flimsy type material or one layer of medium-mesh material should be used. Finely woven material should be avoided. The cheesecloth or gauze should be wet before use.

•GRAVITY SEDIMENTATION TECHNIQUE

One of the simplest techniques applicable to the recovery of all eggs, larvae, and cysts is a combination of straining and sedimentation. This method is especially suitable for operculate and schistosome

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eggs. The use of a large fecal sample in contrast to the smaller ones used in other techniques is of help, especially when only small numbers of eggs are present. Since the technique is essentially a washing process, concentration is often not marked, however, and the preparation usually contains much confusing debris. Faust and Ingalls (1946) recommend the use of a 0.5% solution of glycerin in tap water as a substitute for plain tap water as an aid in reducing surface tension, thereby increasing the yield of organisms.

TECHNIQUE

1. Place a sample of feces about the size of a walnut (approximately 5 gm) in a 250-ml beaker, fill it one-fourth full with tap water and mix thoroughly, using a tongue depressor or 2 applicators.
2. Add sufficient tap water to fill the beaker three-fourths full.
3. Strain this suspension through a funnel containing 1 or 2 layers of *wet* cheesecloth into a sedimentation flask (fig. 16) or cone-shaped graduate flask (250-ml or 500-ml size).
4. Allow the suspension to settle for 1 hour.
5. Carefully pour off two-thirds of the supernatant fluid without allowing any of the sediment to escape.
6. Add fresh tap water near the top of the flask so that the sediment becomes uniformly resuspended; let settle for another hour.
7. This washing procedure may be repeated until the supernatant fluid is relatively clear. Generally one washing will suffice. Schistosome eggs hatch within a short time after the feces are diluted with water. Therefore, if schistosome eggs are being sought, allow the suspension to settle only once; do not wash. Saline can be substituted for the water to retard or prevent hatching.
8. Carefully pour off the last wash water without losing any of the sediment.
9. Insert the tip of a long capillary pipette with rubber bulb attached into the *top* layer of the sediment and remove a portion to a 3x2-inch slide. If the preparation is too thick, dilute with a drop of saline. Iodine preparations should also be made.
10. Cover with a coverslip, seal, and examine.

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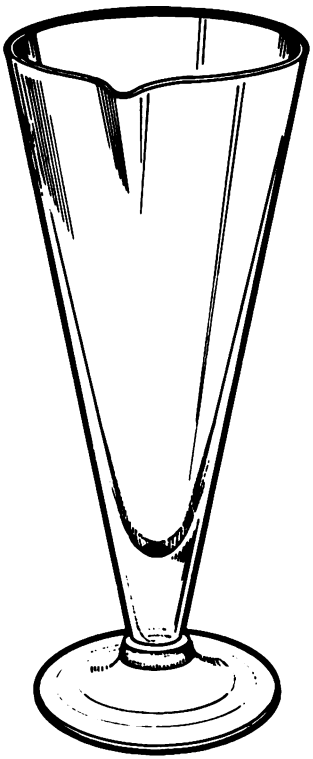
11. Take additional samplings from the middle and bottom layers of sediment.

Figure 16.
Sedimentation Flask



•CENTRIFUGAL SEDIMENTATION TECHNIQUE

This technique is essentially the same process as the gravity sedimentation, except that centrifugation concentrates the material more quickly. The technique has been found to be moderately effective for recovering eggs (particularly of *Schistosoma japonicum* and *S. mansoni)*, larvae and cysts. It is rapid, reveals the organisms in a living condition, does not require expensive reagents to perform, and samples a large quantity of feces. Like the gravity sedimentation, however, the effective concentration is not marked and confusing

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debris is usually present. Centrifugation is the recommended procedure for concentrating urine for the recovery of *Schistosoma haematobium* eggs.

FECES

1. Place a portion of feces about the size of a walnut (4 to 5 gm) in a 250-ml beaker. Fill the beaker one-fourth full with tap water and mix thoroughly with a tongue depressor to obtain an even fecal suspension. Break up any lumps or fecal masses.

   The fecal suspension can be prepared in a tube instead of a beaker if it is more convenient. Place the feces in a large test tube (25x150-mm), fill about one-half with water and mix thoroughly. If the feces are soft, they can be mixed with the water by stoppering the tube and shaking it for at least 1 minute.

   Physiological saline may be substituted for water to prevent hatching of schistosome eggs that might be present.

2. Strain the suspension through a funnel containing 1 or 2 layers of *wet* cheese cloth or gauze into a 50-ml centrifuge tube.
3. Centrifuge at 1,000 to 1,500 r/min (300 to 400 g) for 1 minute. Do not exceed 1,500 r/min since some types of eggs may collapse at higher speeds.
4. Carefully pour off the supernatant into a suitable disinfectant solution, without disturbing the sediment.
5. Using a long capillary pipette with a rubber bulb attached, remove a portion of the *top* layer of sediment to a 3x2-inch slide. If the preparation is too thick, dilute with a small drop of saline. Iodine preparations should also be made. Cover the samples with a 22-mm square coverslip, seal, and examine as described for wet mounts.
6. Remove a sample from the *bottom* layer and handle in the same manner.

URINE

1. Fill a 15-ml centrifuge tube three-fourths full with the urine specimen.
2. Centrifuge at 1,500 r/min (400 g) for 1 minute.
3. Carefully pour off the supernatant fluid.

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4. Using a long pipette with rubber bulb attached, remove a portion of sediment to a 3x2-inch slide, cover with a coverslip, and seal.
5. Examine for eggs under low power; confirm under high-dry, if necessary. *T. vaginalis* trophozoites may also be recovered by this method.

• ACID-ETHER SEDIMENTATION TECHNIQUE

This method is useful for concentrating most helminth eggs, especially for those of *S. mansoni* and *S. japonicum*. It is not satisfactory for the diagnosis of protozoan cysts.

The procedure, originally developed by Telemann (1908), relies upon the use of an acid, for example HCl, to dissolve albuminous matter, such as casein, soaps, mucin, phosphates, and various calcium salts. Ether is then added to dissolve neutral fats and free fatty acids. These actions plus mechnical agitation result in the liberation of eggs from the debris in which they are buried.

The disadvantage of the technique is that the embryos in the eggs recovered are dead. Thus, in attempting to evaluate treatment for schistosomiasis, one cannot tell whether live or dead eggs are being passed. In addition, ether is a hazardous and flammable compound and may present problems in laboratory use.

PREPARATION OF HYDROCHLORIC ACID SOLUTION

Concentrated hydrochloric acid ..... 40 ml
Distilled water ..... 60 ml

This results in a 15% concentration of hydrochloric acid.

TECHNIQUE

1. Place a sample of feces about the size of a marble in a test tube or beaker containing 5 to 10 ml of the 15% hydrochloric acid.
2. Comminute throughly with applicator sticks to form a homogeneous suspension.
3. Strain 5 ml of the suspension through wet cheesecloth or gauze into a 15-ml conical centrifuge tube, preferably graduated.
4. Add an equal quantity of ether; insert a rubber stopper and shake thoroughly for about 1 minute, keeping the thumb on the stopper. Loosen the stopper at least once to release the pressure.

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5. Remove the stopper, and centrifuge at 2,000 r/min (450-500 g) for 1 minute.
6. Four layers will appear in the tube: 1) a top ether layer, 2) a plug of fecal debris, 3) an acid layer, and 4) a bottom sediment containing eggs and larvae, if present.
7. Loosen the plug of debris by holding the tube horizontally and inserting an applicator stick between the plug and the wall of the tube in a ringing motion.
8. Quickly but carefully pour off the ether, plug and acid layers leaving the sediment undisturbed.
9. Wipe the inside of the tube with a cotton swab to remove debris adhering to the glass; this prevents fatty material from flowing down into the sediment.
10. Use a capillary pipette with a rubber bulb attached to remove a portion of the sediment to a 3x2-inch slide. The sediment should be stirred before taking the sample.
11. Place a coverslip on the drop and examine under low power. If necessary, confirm findings under high-dry.

Occasionally eggs are trapped in the layer of debris and consequently, are not recovered in the sediment. To reduce the adhesive forces and increase settling of the eggs. a small quantity of Triton (a nonionic detergent) can be added to the acid-feces mixture (Weller and Dammin, 1945). Add 0.1 to 0.2 ml of a 10% aqueous solution of Triton X-100* to the mixture in Step 1.

•FORMALIN-ETHER SEDIMENTATION TECHNIQUE

This concentration procedure is efficient in recovering protozoan cysts and helminth eggs and larvae, including operculated and schistosome eggs (Ritchie, 1948). Less distortion of cysts occurs with this technique than with the zinc sulfate method, and it is more effective in concentrating formalinized specimens. *H. nana* eggs may be missed, however, and concentration of *Giardia lamblia* cysts and *Iodamoeba bütschlii* cysts may not be very good.

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*Triton X-100 is available through several scientific supply companies. A 10% aqueous solution is prepared by adding 10 ml of stock Triton X-100 to 90 ml of distilled water.

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TECHNIQUE FOR UNPRESERVED SPECIMENS

1. Comminute a portion of stool about the size of a small walnut (about 5 gm) in sufficient saline so that 10 ml of suspension will yield about 1 ml of sediment upon centrifugation. The suspension can be prepared in the carton in which it is submitted (if portions for permanent staining are removed first) or in a beaker or a flat-bottom paper cup. Water may be used in place of saline.

2. Strain about 10 ml of the suspension through a small funnel, containing *wet* gauze or cheesecloth into a 15-ml conical centrifuge tube. To conserve glassware, a cone-shaped paper cup (about a 4-ounce size) with the point cut off can be substituted for the funnel.

3. Centrifuge at 2,500 r/min (650 g) for 2 minutes. Decant supernatant. About 1 ml of sediment should be present (fig. 17A). If the amount is much larger or smaller, adjust to the proper quantity in the following manner.

   a) Amount too large

   Resuspend the sediment in saline (or water) and pour out a portion. For example, if the amount is twice the desired quantity, pour out about half (or slightly less) of the suspension. Add saline (or water) to bring the fluid level to about 10 ml and centrifuge again.

   b) Amount too small

   Pour off the supernatant and strain a second portion of the original fecal suspension into the tube. The amount to be strained can be determined from the amount of sediment; that is, if about half of the quantity necessary is obtained with the first centrifugation, strain another 10 ml into the tube. Centrifuge again.

   It is not necessary to have an *exact* quantity of sediment in the tube, but the quantity should approximate the amount indicated above. Too much or too little sediment will result in an ineffective concentration.

4. If the supernatant is very cloudy, resuspend the sediment in fresh saline (or water), centrifuge and decant as before. Usually 2 washes are sufficient to eliminate material that can be removed by water.

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5. To the sediment, add 10% Formalin (preferably buffered to a neutral pH) to bring the level of material to 9 ml. (See p. 60 for preparation of buffered Formalin.) Mix the Formalin and sediment thoroughly and allow to stand for 5 minutes or longer. At this point, the Formalin-feces mixture may be stoppered and saved until a later time.

   Note: Plastic squeeze bottles for saline and Formalin will facilitate dispensing these solutions into the tubes.

6. Add 4 ml of ether, stopper the tube, and shake vigorously in an inverted position for at least 30 seconds. Remove the stopper with care.
7. Centrifuge at 2,000 r/min (450-500g) for 2 minutes. Four layers should result: 1) layer of ether, 2) plug of debris, 3) layer of Formalin, and 4) sediment (fig. 17C).
8. Free the plug of debris from the sides of the tube by ringing with an applicator stick and carefully decant the top 3 layers. Use a cotton swab to clean debris from the walls of the tube and prevent it from settling down into the sediment (fig. 17D).
9. With a pipette, mix the remaining sediment with the small amount of fluid that drains back from the sides of the tube and prepare iodine and unstained mounts in the usual manner for microscopic examination (fig. 17E). If not enough fluid is left in the tube, a drop of saline can be added to the sediment.
10. If examination of the specimen is delayed, add 1 or 2 ml of 10% Formalin to the sediment and stopper the tube. Formalinized sediments may be kept for some time if they do not dry. Remove the excess Formalin before making the mounts, otherwise the specimen may be too thin for satisfactory preparations.

TECHNIQUE FOR FORMALIN-PRESERVED SPECIMENS

1. Thoroughly stir the formalinized specimen.
2. Depending on the size and density of the specimen, strain a sufficient quantity through *wet* gauze into a conical 15-ml centrifuge tube to give the desired amount of sediment indicated below. Usually 4 to 5 ml is sufficient unless the fecal suspension is thin.

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3. Add tap water to make 10 ml of suspension, mix thoroughly, and centrifuge at 2,000 to 2,500 r/min (500-650g) for 2 minutes.
4. Decant supernatant and, if desired, wash again with tap water. The amount of sediment should be about ½ ml (fig. 17B). If too much or too little is present, adjust the quantity by the same methods described in step 3 for fresh material. In Formalin-preserved specimens, the Formalin has already clarified the feces to some extent and further clarification is caused primarily by the ether. Therefore, the reduction of the sample (or sediment) is not as great as with fresh feces, and the initial quantity must be less.
5. Add 9 ml of 10% Formalin (preferably buffered) to the sediment and mix thoroughly. (See p. 60 for formula for buffered Formalin.)
6. Add 4 ml of ether, stopper the tube, and shake vigorously in an inverted position for at least 30 seconds. Remove the stopper with care.
7. Centrifuge at 2,000 r/min (450-500 g) for 2 minutes. Four layers should result, as stated above (Step 7).
8. Free the plug of debris from the sides of the tube by ringing with an applicator stick, and carefully decant the top 3 layers. Use a cotton swab to remove debris adhering to the glass.
9. Mix the remaining sediment with the small amount of fluid that drains back from the side of the tube (or add a drop of saline, if necessary) and prepare iodine and unstained mounts in the usual manner for microscopic examination. After decanting the upper 3 layers (Step 8), a small amount of Formalin may be added to the sediment, the tube stoppered, and mounts prepared later.

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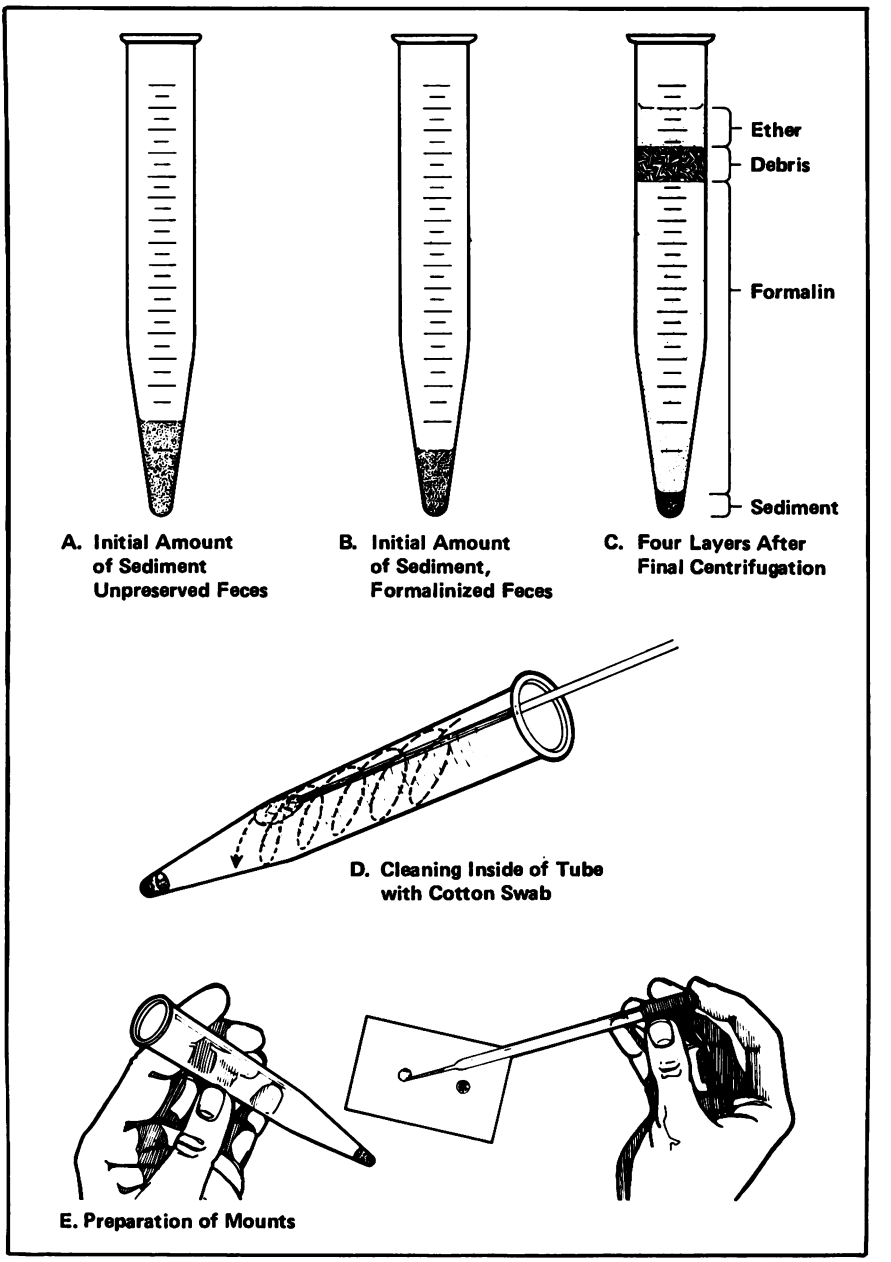
Figure 17.
Formalin-ether Sedimentation Technique



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•FORMALIN-ETHYL ACETATE SEDIMENTATION TECHNIQUE

In laboratories where ether is banned, ethyl acetate may be substituted as a solvent. Use the same quantity of ethyl acetate as ether and follow the same procedure. Ethyl acetate is equivalent to ether in recovering organisms from formalinized specimens (numbers of *Giardia* cysts and *H. nana* eggs are often increased), but it is less effective than ether on fresh unpreserved feces, especially if the feces are fatty or contain a lot of mucoid material. If the entire inner surface of the tube (to just above the fluid-feces level) is cleaned with a cotton swab, most of the fatty globules are removed and mounts are cleaner and easier to examine.

•MERTHIOLATE-IODINE-FORMALDEHYDE CONCENTRATION TECHNIQUE (MIFC)

Specimens preserved in MIF solution may be concentrated by either gravity sedimentation or the MIFC sedimentation technique. The latter procedure is similar to the Formalin-ether concentration in that it recovers both protozoan cysts and helminth eggs and larvae (Blagg *et al.*, 1955). The technique is the same as for the FE except that the Formalin is replaced by MIF solution. (See directions for the use of MIF as a preservative.)

1. GRAVITY SEDIMENTATION

   A certain amount of concentration will occur if the specimen is allowed to settle normally. Since eggs, cysts, and larvae tend to collect in the upper layers of the sediment, mounts for examination should be prepared from this area.

   Sedimentation can be speeded up by centrifuging the specimen (or a portion of it) at about 2,000 to 2,500 r/min (500-650g) for 1 minute.

2. MIFC CENTRIFUGAL METHOD

   a. Thoroughly mix the MIF fecal suspension and strain into a 15-ml centrifuge tube. If the quantity of specimen is insufficent, add fresh MIF preservative to make 10 ml.

   b. Add 3 ml of ether. Stopper with a rubber stopper and shake thoroughly for at least 1 minute. Remove the stopper with care.

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c. Centrifuge at 2,000 r/min (450-500 g) for 1 minute. Four layers will result: 1) ether, 2) plug of debris, 3) MIF solution, and 4) sediment.

d. With the tube in a horizontal position, loosen the plug with an applicator and pour off everything except the sediment. Use a cotton swab to remove debris adhering to the glass.

e. With a capillary pipette, remove a portion of sediment to a slide, cover with a coverslip, seal and examine.

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STAINING PROCEDURES

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STAINING PROCEDURES

FECES

Permanent stained preparations are primarily used for identification of trophozoites, occasionally cysts, and for confirmation of species. They also afford a permanent record of the organism encountered and permit consultation and reference diagnosis where needed. In addition, small organisms, missed by other examinations may be found on stained films. The classic staining technique is the long Heidenhain's iron-hematoxylin method, but, for routine diagnosis, shorter procedures such as the trichrome method (Wheatley, 1951), the phosphotungstic acid technique (Tompkins and Miller, 1947), and the chlorazol black stain technique (Kohn, 1960; Gleason and Healy, 1965) have been developed. The solutions and schedules for these methods are described below.

•PREPARATION OF SMEARS

For permanent stain preparations use 3x1-inch slides. They should be clean and grease-free. Frosted end slides are recommended, since the identifying information can be written on the frosted area with an ordinary lead pencil. If nonfrosted end slides are used, etch the identification into the glass with a diamond-point pencil. China- or glass-marking pencil marks will wash off during staining.

UNPRESERVED SPECIMENS

Using an applicator stick, make a thin, even smear of the fecal sample by streaking the material back and forth on the slide (fig. 18a). The technique is similar to streaking an agar plate. If necessary, dilute feces with physiological saline (0.85%). A thin, even smear of feces is essential for good preparations (fig 19a). An absolutely uniform density cannot be obtained, but the feces should be as evenly distributed on the slide as possible. Smears that are too thick stain poorly and will be too dense to examine adequately. Thin smears destain too rapidly, and unless organisms are numerous, they may be missed or require prolonged searching to be found. Immerse slide into Schaudinn's fixative ***immediately*** after preparation. ***The smear must not be permitted to dry from the time it is made until it is mounted. This is very important.*** Slides may be left in the fixative for several hours or days, if necessary. If the staining schedule for an iron-hematoxylin procedure must be interrupted before or after

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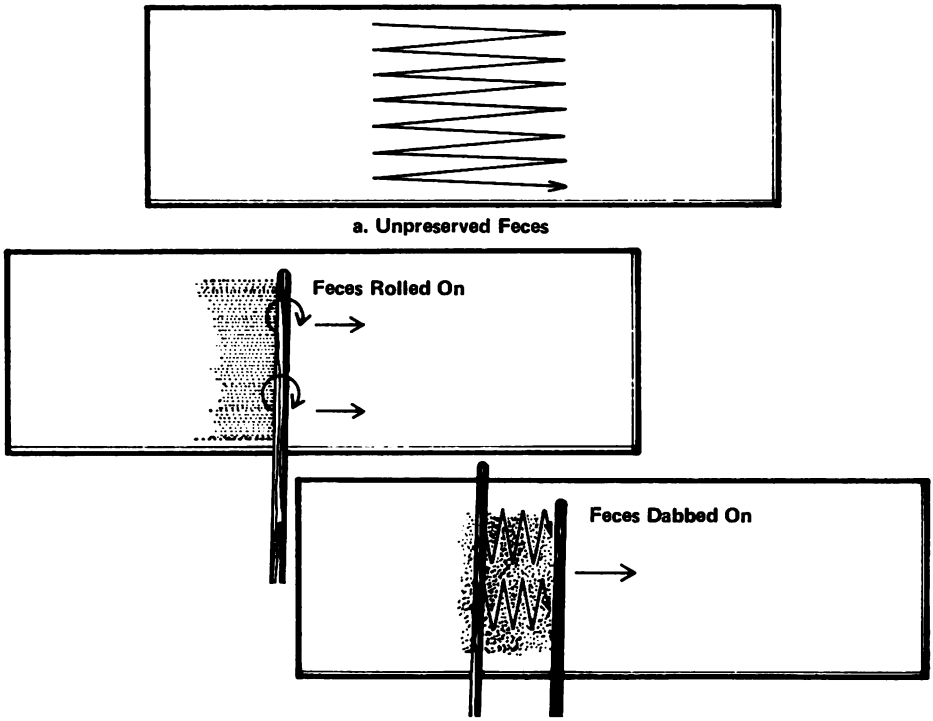
the mordanting-staining process, slides may be stored for long periods in 70% alcohol without subsequently changing the regular schedule. If the schedule must be interrupted between the mordant and stain, store slides in 70% alcohol but repeat the mordanting step when ready to stain. In the trichrome procedure, the slides may be left in the second 70% alcohol (step 4).

Figure 18. Preparation of Smears for Staining



a. Unpreserved Feces

b. PVA-fixed Feces

Arensburger and Markell (1960) have developed a procedure for concentrating cysts, mixing the concentrate with fresh feces, and making stained preparations of this mixture. The procedure is as follows:

1. Mix about 1 ml or 1 gm of feces (a portion about the size of a marble) in 10 to 15 ml of tap water.
2. Strain approximately 10 ml of the suspension through 2 layers of *wet* gauze (1 layer of fine-mesh gauze is sufficient) into a 15-ml centrifuge tube.

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3. Add 1 to 2 ml of ether. Stopper and shake thoroughly.
4. If necessary, add water to fill the tube to a level approximately 1 cm below the top.
5. Centrifuge at 2,500 r/min (650 g) for 45 seconds.
6. Loosen the plug which forms at the top, and decant the supernatant fluid.
7. Add 2 to 3 ml of physiological saline to the sediment and mix thoroughly.
8. Fill the tube to 1 cm from the top with saline.
9. Centrifuge at 2,500 r/min (650 g) for 45 seconds and decant the supernatant.
10. With an applicator stick, add a small quantity of the original fecal specimen to the sediment in the tube. Mix thoroughly to form a homogeneous suspension of feces and sediment.
11. Transfer the mixture, or a portion of it, to a 3x1-inch slide and prepare a smear as described above for fresh specimens.
12. Place the smear immediately in Schaudinn's fixative.

Smears prepared from this material may be stained by the usual staining procedures. The use of water to prepare the initial suspension eliminates *Blastocystis* and some of the debris. The saline, used in the successive steps, decreases the osmotic effect that might damage any remaining trophozoites, if present in the original feces, when the concentrate and feces are mixed.

PVA-FIXED SPECIMENS

With an applicator stick, spread (not smear) 2 or 3 drops of the preserved specimen evenly and smoothly over approximately one-third of the surface of a microscope slide. A rolling motion or an up and down dabbing motion can be used to apply the specimen to the slide (fig. 18B). To prevent peeling of the film during staining, be sure the material extends to the top and bottom edges of the slide (fig. 19B). The films are allowed to dry thoroughly for several hours or overnight either in a 37°C incubator or at room temperature. The films should *dry thoroughly* to prevent the material from washing off the slide during staining. Slides may be left unstained up to 2 months.

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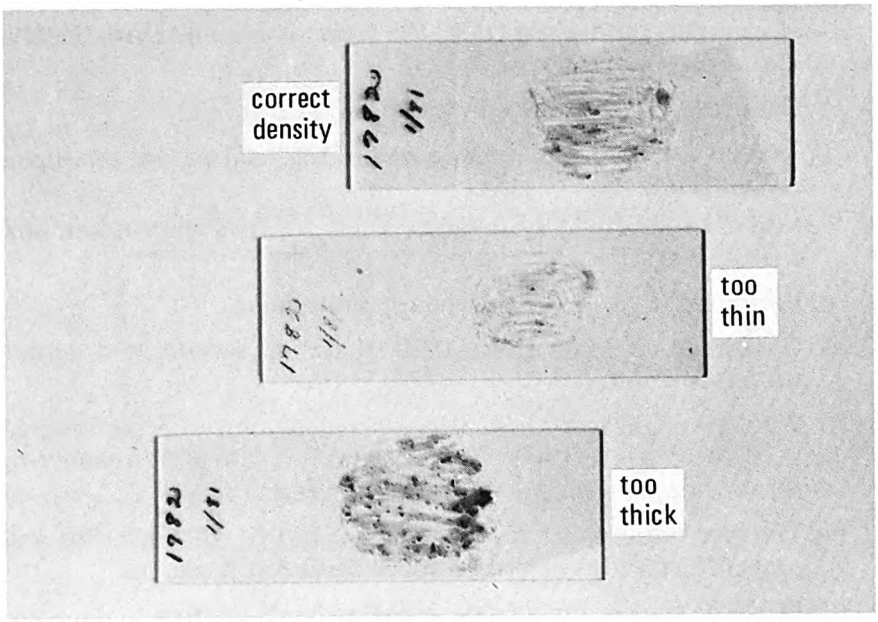
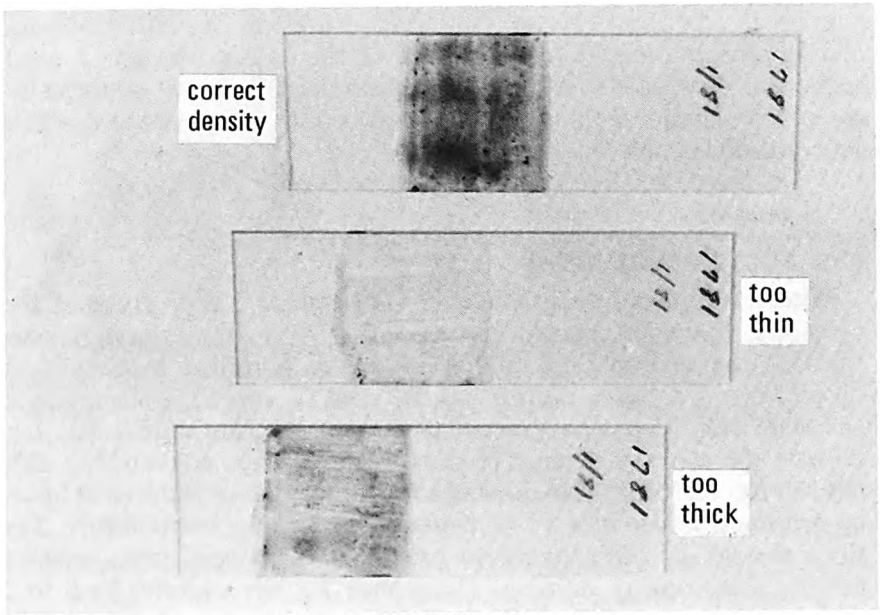
Figure 19. Smears for Staining



a. Unpreserved Feces



b. PVA-fixed Feces

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A more rapid procedure for preparing PVA-fixed fecal smears has been developed by the California State Health Department Laboratory (1965). Thoroughly stir the PVA-fixed specimen and pour 2 to 4 ml into a small tube (85x15-mm). Centrifuge for 5 minutes at 2,500 r/min (650 g) and decant the PVA fluid. Remove the PVA solution adhering to the glass by swabbing the inside of the tube with a cotton swab. Using an applicator stick, remove a portion of the sediment to a piece of facial tissue to absorb excess PVA. The sediment will have to be "raked out" of the tube and should be piled up, not spread out on the tissue. With an applicator stick, smear the partially dry fecal material on a slide in the manner described for fresh fecal specimens. If the specimen is mucoid, fatty. or watery, coating the slide with a *very thin* film of clear human serum before making the smear will cause the fecal material to adhere better. The serum is applied by touching a cotton swab dipped in serum to the slide and wiping off the excess. Too much serum will produce a poor stain. The preparation can be stained immediately without drying. The procedure for fresh fecal smears can be used instead of the longer schedule usually recommended for PVA smears.

Slides of PVA-fixed material may be stored in 70% alcohol, as described for fresh specimens, if the staining schedule must be interrupted.

•EXAMINATION

Permanent stained smears should be examined with the oil-immersion objective. Although cysts and trophozoites of *E. coli* and trophozoites (and occasionally cysts) of *E. histolytica* can be detected under low or high-dry magnification, structural details cannot be seen clearly except with oil-immersion magnification. Also, organisms may be overlooked in stained smears if low power is used to locate them, so for practical purposes, stained preparations need to be examined only with oil-immersion objectives. The use of a 43X-, 44X-, or 50X-oil immersion objective will speed up the examination, however. Some people find that a 5X- or 6X-ocular in combination with the usual oil-immersion objective (97X-100X) facilitates examination.

Focus on the smear with the low power (10X) and locate an area that appears to be neither thin nor too thick. Switch to oil-immersion for examination. In examining the smears, however, both light and dark (thin and thick) areas should be looked at. Usually organsims are more easily located in thin areas, but in some preparations

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these portions may be poorly stained or the organisms poorly differentiated. In these cases, the thicker portions would be of more diagnostic value. Organisms are not uniformly mixed with feces, so the examiner should examine several areas of the mount; for example, top, center, and bottom or left, center, and right areas. For a reliable report, a competent microscopist should examine the smear for at least 15 to 20 minutes, especially if organisms are not found.

•QUALITY CONTROL

Quality control in staining procedures is very important. Refer to the section on QUALITY CONTROL for details concerning changing solutions and evaluation of finished preparations.

•PREPARATION OF SOLUTIONS

1. FIXATIVE (SCHAUDINN'S SOLUTION)

Ethyl alcohol, 95% ..... 1 part
Saturated aqueous mercuric chloride..... 2 parts
(See page 62 for preparation.)

Mix the alcohol and mercuric chloride solution thoroughly. Before use, add 5 ml of glacial acetic acid per 100 ml of solution.

2. IODINE ALCOHOL

Prepare a stock solution by adding sufficient iodine crystals to 70% alcohol to make a dark, concentrated solution. Either ethyl or isopropyl alcohol may be used. For use, dilute some of the stock with 70% alcohol until a strong tea-colored solution is obtained. See figure 20 on page 117 for correct color. The exact concentration is not important, but the solution should not be too dark, since the iodine may stain the protozoa and interfere with subsequent hematoxylin or trichrome staining. If it is too light, the mercuric chloride in the fixative will not be removed and mercuric chloride residue in the finished preparation will interfere with examination.

3. MORDANT

Ferric ammonium sulfate (violet crystals)..... 4.0 gm
Distilled water..... 100.0 ml

Dissolve the ferric ammonium sulfate crystals in the distilled water. The crystals are often in large lumps. Crush these so they will dissolve more quickly. A regular household-type hammer

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works fine, but crush the material before weighing. This solution should be prepared fresh just before use.

Note: Ferric ammonium sulfate crystals should be stored in the refrigerator.

Figure 20.
Iodine-Alcohol -- Correct Color

4. STAINS

a. Stock Hematoxylin Stain (10%)

Hematoxylin (powder or crystals) 10gm
95% or 100% ethyl alcohol to make . . . . . 100 ml

Dissolve hematoxylin in a small quantity of alcohol. Add alcohol to make 100 ml. Let ripen for 6 weeks before diluting for use in staining. When ripe, the stain is a deep orange-brown, clear solution.

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b. Hematoxylin Staining Solution (0.5%)

Stock solution, ripened	5.0 ml
Distilled water	95.0 ml

Add stock hematoxylin to the distilled water and mix. This makes a 0.5% staining solution. The dilute aqueous solution is not stable and must be prepared fresh each day or when needed.

c. Trichrome Stain

Chromotrope 2R	6.0 gm
Light Green SF	1.5 gm
Fast Green FCF	1.5 gm
Phosphotungstic acid	7.0 gm
Acetic acid (glacial)	10.0 ml
Distilled water	1,000.0 ml

Put the dry stains into a clean flask. Add the glacial acetic acid, shake to mix, and allow the mixture to stand ("ripen") for 30 minutes. Add the distilled water. Shake to mix thoroughly. The stain is stable and is used without diluting. Good stain is deep purple, almost black.

d. Chlorazol Black E Stain

(1) Basic Solution

90% ethyl alcohol	170 ml
Methyl alcohol	160 ml
Glacial acetic acid	20 ml
Liquid phenol*	20 ml
1% phosphotungstic acid**	12 ml
Distilled water	618 ml

Add alcohols and acids to distilled water and mix thoroughly.

Since 1,000 ml of Basic Solution are needed to prepare the stock stain and additional Basic Solution is needed for the stain dilution, 2 liters should be made. It is best to make these separately. Preparing a single 2,000-ml quantity sometimes does not produce as effective a solution as a 1,000-ml amount.

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*Liquefy phenol crystals by heating in a water bath. Loosen cap!

**1 gm of phosphotungstic acid crystals dissolved in 100 ml of distilled water.

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(2) *Stock Stain Solution*

Chlorazol black E dye ..... 5 gm
Basic solution ..... 1,000 ml

Weigh out dye, put in mortar, and grind for at least 3 minutes. Add a small amount of basic solution and grind for 5 minutes. Allow particulate matter to settle a few minutes and pour off the liquid into a separate dry, clean container. Add more basic solution and continue the grinding, mixing process until all of the dye appears to be in solution. Add any remaining basic solution, bottle the stain, and put aside for 4 to 6 weeks to ripen. A black sediment settles out within a few days, leaving a black cherry-colored liquid which is the fixative-stain. Filter the stain through Whatman #12 filter paper before use. Keep the filtered stain in a stoppered bottle protected from moisture, dust, and dirt.

5. DESTAINING SOLUTIONS

a. Ferric Alum Solution (2% Solution)

Dilute mordant (solution #3) 1:1 with distilled water.

This solution is good for about 1 week but is better if prepared fresh each 2 days. Prepare only enough for 1 Coplin jar, about 40 ml.

b. Phosphotungstic Acid Solution

Phosphotungstic acid ..... 2.0 gm
Distilled water ..... 100.0 ml

Dissolved the acid in the water. Store in a glass-stoppered or screw-cap bottle. The solution is good indefinitely.

c. Saturated Aqueous Picric Acid Solution

Add approximately 2 gm of picric acid crystals to 100 ml of water. Shake thoroughly; let stand for several days, shaking at intervals. If all the crystals dissolve, add more. After 3 to 4 days, some undissolved picric acid should remain in the flask. Let settle. Use the clean solution from top of the bottle or filter. Store in a glass-stoppered bottle. The solution is good indefinitely. Unlike picric acid crystals, picric acid solution is stable and presents no problem in storage.

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d. **Acid Alcohol**

Acetic acid	4.5 ml
90% ethyl alcohol	995.5 ml

Add the acid to the alcohol and put in a glass-stoppered bottle. The solution is good indefinitely.

(Note: Do NOT use 95% alcohol.)

6. **CARBOL-XYLENE**

Add 1 volume of liquefied carbolic acid (phenol) to 3 volumes of xylene. Phenol crystals can be liquefied by heating the jar in a water bath. Loose the jar lid!

Pour the carbol-xylene solution into a glass-stoppered bottle. Label: Carbol-Xylene 1:3. The solution will keep for months or a year if it is kept tightly closed so moisture can not get in.

For chlorazol black stain, 1:2 carbol-xylene is preferred.

Note: Liquid Phenol solution which contains water cannot be used in carbol-xylene solutions.

7. **LITHIUM CARBONATE SATURATED AQUEOUS SOLUTION**

Dissolve lithium carbonate powder in distilled water until no more will go into solution. About 1 to 2 gm per 100 ml water is usually sufficient. Store in a screw-cap or glass-stoppered bottle. The solution is good indefinitely.

•TECHNIQUES

A. HEIDENHAIN'S IRON-HEMATOXYLIN STAIN

The Heidenhain's iron-hematoxylin technique, a procedure that has been used for a long time, is described in many textbooks. The "classical" long method gives excellent staining results if the procedure is carefully followed, especially with regard to the destaining of the organisms. By heating the fixative, mordant, and stain, the procedure can be shortened considerably, however, without loss of staining details. Both the long and short Heidenhain's techniques for fresh fecal specimens are given below. PVA-fixed material can be stained by either method, but better results will be obtained by using the procedure particularly adapted for PVA specimens. (This procedure is given separately.)

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Unpreserved Specimens

1. Schaudinn's fixative
   (Solution #1) ..... 5 minutes at 50°C, 1 hour at room temperature
   (Smears may be left in Schaudinn's fixative for 2 or 3 days.)
2. 70% alcohol plus iodine
   (Solution #2) ..... 5 minutes
3. 50% alcohol..... 3 minutes
4. Tap water ..... 3 minutes
5. 4% iron alum mordant
   (Solution #3)..... 10-20 minutes at 40-50°C, 12-24 hours at room temperature
6. Water (distilled or tap)
   two changes..... 3 minutes (total)
7. 0.5% hematoxylin
   (Solution #4b) ..... 5-10 minutes at 40-50°C, 12-24 hours at room temperature
8. Tap water, two changes..... 3 minutes (total)
9. 1%-2% iron alum destain
   (Solution #5a) ..... By observation under the microscope (usually requires 1-3 minutes or more)

   -or-

   Saturated aqueous picric acid
   (Solution #5c) ..... 5-10 minutes or more by observation under the microscope

   This is the critical step in this technique and the process must be checked carefully. At intervals of ½ minute, 1 minute, 2 or more minutes, depending on the destain solution used, the smear should be removed from the solution, rinsed thoroughly with water to slow down or stop the destaining process, and examined microscopically with low or high power. Using a 3x2-inch slide under the wet slide will facilitate examination and protect the stage of the microscope. Repeat the process until optimum results are obtained. The destaining is completed when the structural details in the organism, such as nuclei, are visible, or, if organisms cannot be located, until the background material is more gray than black. The time will vary widely

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depending on the smear, the organisms, and other factors. *Experience and practice are necessary to get the best results.* An experienced technician may destain visibly without microscopic observation. If time permits, several films may be prepared from one specimen and destained by varying the time, that is, for 1 minute, 2 minutes, etc. Usually at least one slide from a group is properly destained. This is time-consuming, however, and the destain process should be controlled by microscopic observations. Since picric acid destains more slowly than iron alum, it can be better controlled and is generally preferred.

10. Running tap water (or several changes) ..... At least 5-30 minutes (for permanence of stain)
11. 70% alcohol plus a few drops of saturated lithium carbonate (Solution #7) per stain jar ..... 3 minutes
12. 95% alcohol ..... 5 minutes
13. Carbol-xylene ..... 5 minutes
14. Xylene ..... 3 minutes
15. Mount with coverslip using Permount or other suitable mounting media.

PVA-Fixed Material

The staining of dried PVA films is essentially the same as for other types of fecal smears except for a variation in the time required for certain steps and the omission of the usual Step 1, or fixation in Schaudinn's solution. Use of PVA-fixative solution as a preservative makes further fixation unnecessary.

1. 70% alcohol plus iodine (Solution #2) ..... 20 minutes
2. 50% alcohol ..... 10 minutes
3. Tap water ..... 5 minutes
4. 4% iron alum mordant (Solution #3) ..... 8-12 hours
5. Water (distilled or tap), two changes ..... 3 minutes (total)
6. 0.5% hematoxylin (Solution #4b) ..... 8-12 hours (or overnight)

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7. Tap water, two changes..... 3 minutes (total)
8. Saturated picric acid destain
   (Solution #5c) ..... 15-20 minutes, or by observation under the microscope

   This is the critical step in this technique. See the note with step 9 of the standard procedure. The time may vary depending upon the smear, the organism, and other factors. Experience and practice are necessary to get the best results.
9. Running tap water, or
   several changes .....30 minutes (for permanence of stain)
10. 70% alcohol plus a few drops
    of saturated lithium carbonate
    solution per stain jar ..... 10 minutes
11. 95% alcohol..... 10 minutes
12. Carbol-xylene ..... 10 minutes
13. Xylene..... 10 minutes
14. Mount in Permount or other mounting media

Stain Reactions

Well-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. Background material usually stains blue-gray.

Protozoan cysts and trophozoites are generally not distorted, with the exception of trophozoites of *Chilomastix* and *Trichomonas*. The latter tend to round up and are often atypical in appearance. Nonstaining organisms may indicate poor fixation. With fresh stools, poor fixation can be largely overcome by heating the fixative to 56°C and fixing for 5 to 10 minutes. Cold fixation (room temperature) with fresh fixative, however, gives more critically stained organisms. With the exception of *E. coli*, 30 minutes' cold fixation is usually sufficient. For *E. coli*, 2 to 3 hours at room temperature or ½ hour at 56°C may be necessary to insure thorough fixation of cysts. Immature and young cysts fix more easily than old cysts, and trophozoites frequently are fixed in 2 to 3 minutes.

Mononuclear and polymorphonuclear leucocytes, *Blastocystis*, yeasts and molds may cause confusion in diagnosis unless the observer is trained in differential morphology of the protozoa.

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The organisms in PVA-fixed material stain as described above, although cysts such as *E. histolytica* and *E. coli* may be distorted in PVA preparations.

IRON-HEMATOXYLIN-PHOSPHOTUNGSTIC ACID STAIN

The phosphotungstic acid technique of Tompkins and Miller (1947) is a good short hematoxylin staining procedure for routine diagnostic purposes. The destaining is self-limiting and does not require control by microscopic observation, as does the classical technique. The stain reaction is similar to that described for Heidenhain's stain except that the slides are bluer in appearance. This procedure gives better results with fresh fecal smears than with preparations from PVA- fixed feces.

Technique

1. Schaudinn's fixative (Solution # 1) ..... 5 minutes at 50°C, 1 hour at room temperature
2. 70% alcohol plus iodine (Solution # 2) ..... 5 minutes
3. 50% alcohol ..... 3 minutes
4. Tap water ..... 3 minutes
5. 4% iron alum mordant (Solution # 3) ..... 3-5 minutes at room temperature
6. Distilled or tap water ..... 1 minute
7. 0.5% hematoxylin (Solution #4b) ..... 1 minute at room temperature
8. Distilled or tap water ..... 1 minute
9. 2% phosphotungstic acid destain (Solution #5b) ..... 2 minutes or more
10. Running tap water (or several changes) ..... 5 minutes (total)
11. 70% alcohol plus a few drops of saturated lithium carbonate solution per stain jar ..... 3 minutes

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12. 95% alcohol. . . . . 3 minutes

13. Carbol-xylene . . . . . 5 minutes

14. Xylene. . . . . 3 minutes

15. Mount with coverslip using Permount or other suitable mounting media.

Stain Reactions

The organisms stain as described for Heidenhain's hematoxylin stain, but the preparations are bluer than those obtained by the longer method.

TRICHROME STAIN

The trichrome technique of Wheatley (1951) is a rapid staining procedure which gives good results for routine purposes. The method is simple in that overstaining and differentiation are not necessary to bring out the morphologic details of the parasites, nor is it necessary to mordant before staining. However, for practical purposes, destaining the smears gives better differentiation of the organisms, and this step should be included. The suggested stain set-up is shown in figure 21. The stain solution is stable and may be used repeatedly, the lost volume being replaced by adding stock solution. Staining over 15 smears daily (in 50 ml of stain), however, tends to weaken the stain. If stain is allowed to evaporate in the open air for 3 to 8 hours, strength will return. This can be accomplished by leaving the cover of the staining dish off overnight. The staining of unpreserved and PVA-fixed material differs chiefly in the increased time required for the latter and in the omission of the fixative step, since the material in the PVA solution is already fixed. Both procedures are given below.

Note: Slides should ***always*** be drained between solutuions. When the slide is removed from a solution, touch the end to a paper towel for 2-4 seconds to remove excess fluid. Then put in the next solution. This should be done at each transfer. Otherwise, the solutions become contaminated with material from the previous jar and poor stains will be obtained.

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Technique for Unpreserved Specimens

1. Schaudinn's fixative (Solution # 1) ..... 5 minutes at 50°C, 1 hour at room temperature
2. 70% alcohol plus iodine (Solution# 2) ..... 1 minute
3. 70% alcohol (1) ..... 1 minute
4. 70% alcohol (2) ..... 1 minute
5. Stain (trichrome) (Solution #4c) ..... 2-8 minutes
6. 90% alcohol, acidified (Solution #5d) ..... 5-10 seconds, total Usually a brief dip (in and out) is sufficient.

   Since the acid alcohol continues to destain as long as it is in contact with the material, the time allowed *should include the few seconds between the time the slide is removed from the destain and the rinse in 95% alcohol* (step 7). For more effective removal of the acid destain, two 95% alcohol washes are suggested instead of one. These should be changed frequently to prevent them from becoming so acid that the destaining process will continue. Prolonged destaining in acid alcohol (over 10 seconds) may cause the organisms to be poorly differentiated, although larger trophozoites, particularly those of *E. coli*, may require slightly longer periods of decolorization.

   Note: If several slides are being stained simultaneously, they should be destained separately. Remove only one slide at a time from the stain, destain it, rinse in the 95% alcohols, and place it in the carbol-xylene (step 9).

7. 95% alcohol (1) ..... Rinse briefly
8. 95% alcohol (2) ..... Rinse twice
9. 100% alcohol or carbol-xylene ..... 1 minute
10. Xylene ..... 1-3 minutes
11. Mount with coverslip using Permount or other mounting media.

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Technique for PVA-Fixed Material

1. 70% alcohol plus iodine (Solution #2) ..... 10-20 minutes
2. 70% alcohol (1)..... 3-5 minutes
3. 70% alcohol (2)..... 3-5 minutes
4. Trichrome stain (Solution #4c) ..... 8 minutes
5. 90% alcohol, acidified (Solution #5d) .....5-10 seconds

   (See previous paragraph on destaining. If more than one slide is being destained, handle one at a time as described for fresh specimens; get slide to step 7 (95% alcohol #2) then go back and destain another slide.

6. 95% alcohol (1)..... Rinse to remove acid destain
7. 95% alcohol (2)..... 5 minutes
8. Carbol-xylene ..... 5-10 minutes
9. Xylene..... 10 minutes
10. Mount with coverslip using Permount or other mounting media.

Stain Reactions

The cytoplasm of thoroughly fixed and well-stained cysts and trophozoites is blue-green, tinged with purple. Occasionally, *E. coli* cysts may stain slightly 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 frequently occur in the color reaction of ingested particles. Background material usually stains green thus contrasting with the protozoa.

Nonstaining cysts and those staining predominately red are most frequently associated with incomplete fixation. Unsatisfactorily stained organisms obtained from specimens submitted in PVA-fixative usually indicate incomplete fixation associated with poor emulsification. Thorough emulsification of preferably *soft* stools will yield critically stained cysts and trophozoites. Degenerate forms stain pale green, although understained or overdestained organisms may also appear green.

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Eggs and larvae stain red and contrast strongly with the green background. Thin-shelled eggs often collapse when placed in mounting medium, although some diagnostic features may be retained, especially if the smear is examined immediately.

Mononuclear and polymorphonuclear leucocytes and *Blastocystis* present the same diagnostic problems that they present when stained with hematoxylin. Like the protozoa, the nuclei of pus cells and tissue cells stain red and the cytoplasm, green. However, the cytoplasm of these cells does stain more greenish than that of the protozoa.

D. CHLORAZOL BLACK E STAIN

Chlorazol black E stain technique (Kohn, 1960) is a simple procedure in which fixation and staining take place in a single solution. No destaining is necessary. The modified stain technique (Gleason and Healy, 1965) may be used for staining protozoa in fecal smears or in tissues. The stain gives good results with fresh fecal smears but is not satisfactory for PVA-fixed material. PVA-fixative contains mercuric chloride. Smears must be washed with iodine alcohol to remove this compound; otherwise, it will interfere with staining and subsequent examination. The chlorazol black E procedure does not have an iodine alcohol wash. (PVA-fixed specimens are better stained by iron-hematoxylin or trichrome stains, the procedures which do include this step.)

The optimum staining dilution and time for each liter, or "batch," of stain must be determined. The stock stain may be kept indefinitely, and stain dilutions may be used repeatedly. Repeated use "wears out" the stain dilution, however, and when slides appear visibly red rather than greenish-black at the end of the staining period, the dilution should be discarded. Approximately 20 slides can be stained satisfactorily in a 50-ml Coplin jar of diluted stain before deterioration makes change necessary. Deterioration of the stain is dependent on use (slides stained) and not on time, since satisfactory stains may be obtained with stain dilutions up to 30 days old if the number of slides stained has not been excessive. Slides that appear red can be restained in fresh stain.

Fecal Smears

1. Determination of Optimum Dilution and Staining Time

The optimum dilution and staining time must be determined for each liter of fixative-stain. The following series of dilutions and staining periods are recommended for this purpose.

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Stain Solution	*Basic Solution*	*Hours*
Undiluted	–	2-3
1	1	2-4 to overnight
2	1	2-4
1	2	2 to overnight
1	3	4 to overnight

A solution producing good overnight staining does not appear to overstain when left for several days.

Trial smears should be stained with each dilution according to the technique given below and the optimum dilution and time selected for routine staining with the "batch" of stain concerned. More than one dilution-time combination may be satisfactory and the choice of which to use will depend on the laboratory schedule and urgency of diagnosis. The range most commonly found satisfactory is a 1:2 dilution for 2 hours to overnight, although for best results, the exact combination should be determined.

2. Technique

1) Fixative-stain dilution
(for routine use, use dilution and time predetermined) . . . . 2 hours to overnight

2) 95% ethyl alcohol . . . . . 10-15 seconds

3) Carbol-xylene (1:2) . . . . . 5 minutes

—or—

100% ethyl alcohol . . . . . 5 minutes

4) Xylene . . . . . 5 minutes

5) Mount in Permount or other suitable media.

Protozoa in Tissue

Tissue slides are prepared following the usual histologic procedures for fixation, embedding, sectioning, and preparation of slides. The sections are treated as usual in xylene and alcohols to remove the paraffin and prepare them for staining. Good stains have been obtained on sections of 5 to 7 microns in thickness.

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1. *Determination of Optimum Dilution and Staining Time*

For staining protozoa in tissue, use the optimum stain dilution as determined for protozoa in feces, but substitute distilled water for the basic solution as the diluent.

Stain Solution	*Distilled Water*	*Hours*
Undiluted	–	4-6
1	1	4-8 to overnight
2	1	4-8
1	2	4 to overnight
1	3	8 to overnight

Note that the staining time is twice as long for tissues as for fecal smears. The most common satisfactory time is about 6 to 7 hours with the optimum dilution.

2. *Technique*

1) Fixative-stain ..... 4 hours to overnight (as determined)

2) 95% alcohol ..... 10-15 seconds

3) Carbol-xylene (1:2) ..... 5-10 minutes

4) Xylene ..... 5-10 minutes

5) Mount in Permount or other suitable media.

Stain Reactions

Protozoa in fresh fecal specimens stain green to gray-green; in older stools, organisms are gray to black. Nuclei, chromatoid bodies, karyosomes, and cell membranes stain dark green to black. Ingested red cells may vary from pink to black. *E. coli* cysts may stain pink or green, and rarely, *E. histolytica* cysts stain faintly pink.

In tissue, *E. histolytica* trophozoites have the same appearance as in feces.

OTHER MATERIALS

Permanent stain preparations to demonstrate ameba trophozoites may sometimes be prepared from material aspirated from a lesion or obtained with a sigmoidoscope. Thick, viscous, aspirated material may need special treatment to free organisms, as described on p.152; otherwise, the procedures are those used for fecal smears.

Stains of urethral or vaginal material are not recommended for the demonstration of *T. vaginalis.*

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ADULT HELMINTHS

Preparing stained helminths involves a procedure similar to that used for fecal smears, although before staining, the worms must be relaxed and carefully fixed. Some of the more common methods of relaxation, fixation, and staining are described below.

To secure good preparations, start with clean organisms. Remove mucus and adherent debris by gently shaking the helminth in saline, or by using fine needles or brushes. Be careful not to damage the organism in any way. Prior to relaxation and fixation, it should be kept in saline to prevent drying.

•COLLECTION OF SPECIMENS

FROM FECAL SPECIMENS

The worms most commonly submitted for diagnoses are those passed with the feces. If the specimen is watery or loose, organisms can be removed with camel's hair brushes, blunt needles, or applicator sticks. If the feces are mushy or formed, they can be mixed with water to facilitate detection and removal of worms. Ocassionally, *Taenia* proglottids will be on the surface of the feces, and can be easily collected. If worms are not readily observed, the feces can be strained or examined in small portions as described in the section on Miscellaneous Procedures. Organisms should be handled carefully and put immediately in physiological saline.

FROM TISSUE

Tissue containing larval parasites may occasionally be received by the diagnostic laboratory. Portions of muscle, liver, brain, or other tissue suspected of harboring *Trichinella* or other nematodes are usually handled by histologic methods. However, larger forms such as cycticerci can often be carefully dissected out with blunt instruments.

FROM ANIMAL HOSTS

Animals are generally good sources of parasites for teaching purposes and a variety of organisms may be collected from the intestinal tract and from tissue.

a. **Intestinal Tract**

If the host is relatively small, the entire gut can be tied off at the esophageal and rectal ends and removed to a dish of physiological saline.

Many organisms can be removed by washing the gut with physiological saline. A beaker or pipette, depending on the size

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of the intestinal tract, can be used to introduce the saline into one end of the gut. The entire washings should be collected in a dish and examined for parasites.

Parasites can also be obtained by slitting the gut. Starting at one end, a continuous lengthwise slit using fine shears is made to expose the lumen. For larger animals, individual sections of gut can be systematically opened and examined. Large organisms, e.g., tapeworms, may become immediately visible, but smaller ones may not be apparent without careful search. Gently shake the opened intestine in the fluid since this may dislodge some organisms or make them more visible. If possible, place the dish beneath a dissecting microscope for closer scrutiny. Some cestodes and trematodes may be firmly attached to the tissues by hooks or suckers. These can best be obtained without damage by allowing the organisms to free themselves over a period of time. Placing the dish in the refrigerator for a few hours will sometimes accomplish this. Careful dissection of the tissue around the attached end will hasten the process.

Freed organisms, if small enough, can be sucked up in a wide-bore pipette and placed in a dish of clean physiological saline. Small tapeworms can be handled either with camel's hair brushes, blunted needles inserted underneath them, or applicator sticks which can be cut to a thin, flat spatulate carrying edge. Larger organisms of course are not as fragile and can be handled with any suitable blunt instrument so long as they are not damaged.

b. From Tissue

Tissue forms, e.g., cysticerci, should be carefully dissected out with blunt instruments. Liver flukes can be obtained by cutting the organ open and applying pressure to squeeze the worms out of the tissue.

•PREPARATION OF SOLUTIONS

FIXATIVE

1. Formalin – 5%

Formalin	5 ml
Water	95 ml

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2. **Alcohol-Formalin-Acetic Acid (AFA)**

Commercial Formalin	10 parts
Ethyl alcohol, 95%	25 parts
Glacial acetic acid	5 parts
Glycerin	10 parts
Distilled water	50 parts

3. **Gilson's Fixative**

Nitric acid, conc.	15 ml
Glacial acetic acid	4 ml
Mercuric chloride	20 gm
Ethyl alcohol, 70%	100 ml
Distilled water	880 ml

Allow mixture to stand for 3 or 4 days, then filter.

4. **Bouin's Fixative**

Picric acid (saturated aqueous solution)	75 ml
Formalin	25 ml
Acetic acid	5 ml

STAINS

1. **Alum Cochineal**

Potassium alum	30 gm
Cochineal	30 gm
Distilled water	400 ml

Boil for 1 hour. Cool and filter. Keep filtrate. Boil the filter paper in 200 ml distilled water for 30 minutes. Filter. Add the second filtrate to first filtrate and boil 30 minutes. Filter and make up to 400 ml with distilled water. Store in glass-stoppered bottle. The solution will keep indefinitely.

2. **Delafield's Hematoxylin**

   a. *Prepare a saturated aqueous solution of ammonium alum as follows:*

   Add aluminum ammonium sulfate (ammonium alum) crystals to hot distilled water, stirring continuously, until no more crystals will go into solution. Let cool. Excess alum will crystallize out. Pour off the clear solution or filter. Store in a glass-stoppered bottle. The solution will keep indefinitely.

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b. Stain Solution

Hematoxylin crystals	4 gm
95% ethyl alcohol	25 ml
Ammonium alum, saturated aqueous solution	400 ml

Dissolve the stain crystals in the alcohol and add the ammonium alum solution. Place in loosely capped containers and keep in sunlight for 2 weeks.

Then add a mixture of:

Methyl alcohol (acetone free)	100 ml
Glycerin	100 ml

Place in a glass-stoppered bottle and expose to direct sunlight for at least a month. Filter before use.

Delafield's hematoxylin is good indefinitely if kept tightly closed and free of moisture.

3. Reynold's Stain

Alum cochineal	3 parts
Delafield's hematoxylin	1 part
Distilled water	25 parts

This mixture may be used immediately, but it is best to allow it to stand for a few hours after mixing. A fine precipitate will settle out but it can be removed by filtering. The mixture is then ready for use. The solution is stable.

4. Semichon's Acetic Carmine

Acetic acid (glacial)	1 part
Distilled water	1 part

Place in small flask; add excess carmine powder. Stopper and place flask in boiling water for 15 minutes. Cool, decant supernatant, and filter. Store in a glass-stoppered bottle. The stain is good indefinitely.

•STAINING TECHNIQUE

PREPARING WORMS

Mucus and adherent debris should be carefully removed from the worms by means of a soft brush or by fine needles in a dish of saline. Before fixation, worms should be relaxed by *one* of the following methods.

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1. Place trematodes in a bottle half-filled with physiological saline. Shake vigorously for 2 to 3 minutes to relax worms. Pour off saline, add solution consisting of 1 part fixative and 1 part saline and shake again for 2 to 3 minutes. Pour off solution and add full-strength fixative. Fix for the recommended time.

2. Put trematodes and cestodes (worms and proglottids) in physiological saline or water in the refrigerator for 1 to 2 hours to overnight. Pour off saline or water and rapidly add fixative. Some internal degeneration may sometimes result from this procedure as in the case of schistosomes, but it is satisfactory for most organisms. Fix for the recommended time.

3. Wrap tapeworms about a glass jar or piece of plate glass. Holding the two ends of the glass, rotate the organism in fixative until contractions cease. Fix for the recommended time.

4. Put small trematodes on a slide in small drop of saline. Put a coverslip on top of organisms so that *gentle* pressure is exerted. Run fixative under coverslip. Fix for the recommended time.

5. Strips of tapeworm proglottids can be cut from the body of the worm with a sharp scalpel. The scolex should be the first portion cut off so that future handling of the worm will not cause it to contract. Place the strips between large pieces of glass or between 3x2-inch slides which are then tied with string to flatten the organisms slightly. Do not bind too firmly or they will distort. Drop into fixative and allow to remain until contractions cease. Cut off string; it is best, however, not to remove organisms from between slides to prevent curling. Be sure that the fixative is in contact with proglottids. Fix for the recommended time.

6. For large tapeworms, cut off long strips of proglottids and place alongside one another on pieces of heavy paper toweling soaked in fixative. The strips may be gently flattened and extended to a normal length. Place another layer of fixative-soaked toweling on top and allow preparation to remain for 30 minutes. Then add sufficient fixative to cover but not float worms away and allow to remain for recommended time.

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FIXING WORMS

Fix worms by one of the following methods:

1. **Formalin Fixative**

   Organisms can be rapidly and simply fixed with hot (not boiling) 5% Formalin. When the organisms are relaxed or extended, pour off most of the saline or water, then rapidly pour the hot Formalin over them. Usually this procedure will fix the worms without allowing too much contraction to occur. After several hours, pour off the fixative and add fresh, unheated 5% Formalin. Fixed organisms can be kept indefinitely if the container is sealed to prevent evaporation. They may be left in Formalin or washed in 35% and 50% alcohols and stored in 70% alcohol. If desired, organisms can be stained immediately after fixing or later whenever time permits.

2. **AFA Fixative**

   Use solution cold. Mixture acts both as fixative and preservative and specimens may be left in it for months. Ordinarily, 1 or 2 days is sufficient for fixation.

3. **Gilson's Fixative**

   May be used cold or as a steaming solution. In hot solutions, the organism is killed quickly and tends to remain in a more natural and extended condition; however, delicate structures may be distorted. Organisms should be fixed for 15 minutes to 6 hours depending on size. Overfixation should be avoided. The mercuric chloride in the fixative should be washed out before the organisms are stained.

4. **Schaudinn's Fixative**

   May be used hot or cold. With hot solutions, worms are rapidly killed and tend to retain a natural condition, however, delicate structures may rupture or distort. Worms should fix for 10 to 24 hours depending on size. The mercuric chloride in the fixative must be removed before organisms are stained.

5. **Bouin's Fixative**

   Used cold. Parasites may be left for 6 to 24 hours or longer. For best results, the yellow picric acid must be washed out completely before the staining step. A pinch of sodium bicarbonate or a few drops of saturated aqueous lithium carbonate in the washing alcohols will usually bleach the organisms satisfactorily. Parasites can be stored in 70% alcohol if not stained immediately.

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STAINING PROCEDURE

Pre-Staining Preparations

Following fixation with Gilson's or Schaudinn's fixatives, it is necessary to remove the mercuric chloride from the tissues by washing in iodine alcohol (steps 1 and 2 of the staining technique).

If AFA fixative or Formalin is used, no washing in iodine is necessary; after fixation, start with Step 5 of the staining technique and continue.

If Bouin's is used, start with Step 3 of the staining technique after washing out the picric acid.

Technique for Staining

1. Transfer fixed organisms directly to iodine-alcohol (70% ethyl alcohol to which iodine has been added to give it a strong tea color). Allow to remain with occasional agitation for 12-24 hours depending on thickness of the organism.
2. Transfer to 70% ethyl alcohol for 2-3 hours. Organisms can be stored here indefinitely or stained immediately.
3. 50% alcohol – 1 hr.
4. Distilled water – 10 min.
5. *Stains.* The time required for staining depends on the stain used and the thickness of the organism.
   a. *Alum-cochineal Stain.* Staining usually requires 24 to 36 hours, depending on the size and density of the parasite. After staining, wash the organism in water for 15 to 20 minutes, then place it in 70% ethyl alcohol. With this stain, destaining (Step 7) is usually not necessary and may be omitted.
   b. *Delafield's Hematoxylin Stain.* Stain for 1 hour or longer, depending on the size of the organism. Organisms may be left in diluted stain for much longer periods and watched until staining has reached the desired point.
   c. *Reynold's Stain.* Stain 4 hours to overnight. Small objects may stain in 4 hours, but most organisms should stain overnight.
   d. *Semichon's Acetic Carmine Stain.* Stain 24 hours.
6. 70% ethyl alcohol . . . . . 2 minutes

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7. Acid alcohol (1 ml concentrated HCl to 99 ml of 70% ethyl alcohol) ..... until internal structures are visible by strong transmitted light
8. 70% alcohol..... 1-2 minutes
9. Neutralize in 70% alcohol to which lithium carbonate has been added. (Add several drops of a saturated aqueous solution of lithium carbonate to each 100 ml alcohol.) ..... 1 hour
10. 95% alcohol..... 1 hour
11. 100% alcohol (1)..... 30 minutes
12. 100% alcohol (2)..... 1 hour
13. 100% alcohol plus cedarwood oil (for clearing), equal parts..... ½-1 hour
14. Cedarwood oil..... 1 hour
15. Mount in Permount or other suitable media.

    Note: Do not allow the organism to dry at any time until it has been mounted.

•CLEARING HELMINTHS WITHOUT STAINING

Clearing helminths without staining reveals most structural details, and the species can ordinarily be identified more quickly by clearing than by using lengthy staining procedures. The cleared specimen should be rotated and oriented in the preparation for optimum observation of details. Tapeworm proglottids and small worms are often handled in this manner.

PREPARATION OF SOLUTIONS

1. **Carbol-Xylene**

   Carbolic acid (Phenol), liquefied..... 1 part
   Xylene..... 3 parts

   Phenol crystals can be liquefied by placing the jar in a hot water bath. Loosen the cap before heating!

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2. **Lacto-Phenol**

Phenol, liquefied	1 part
Lactic acid	1 part

Liquefy phenol crystals by heating the jar in a hot water bath. Loosen the cap!

3. **Lacto-Phenol-PVA**

Rubin (1951) advocated the following proportions:

PVA, 15% aqueous solution*	56 parts
Lactic acid	22 parts
Phenol	22 parts

In the CDC laboratory, less clearing and better detail is obtained with a medium of the following proportions:

Lacto-Phenol solution	30 ml
PVA, 15% aqueous solution*	70 ml

This medium may be made up as 20 - 80 ml, respectively, if clearing is too extensive with the above proportions. Good for small nematodes.

4. **Other Clearing Agents**

a. Beechwood creosote - undiluted
b. Glycerin - undiluted
c. Xylene - undiluted

These compounds are especially useful for clearing *Taenia* proglottids. Beechwood creosote is also used to clear arthropods and small nematodes.

TECHNIQUE

The following procedure is suggested.

1. Relax the organisms as previously described.
2. Fix in hot 70% alcohol or 5% Formalin for several hours. After fixation in Formalin, small nematodes may be mounted directly in lacto-phenol- PVA solution, which serves as both a clearing and mounting medium.
3. Put in one of the following clearing solutions. Clearing can be observed visually.

*Use same PVA powder as for PVA-fixative.

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a. **Carbol-xylene (1:3):** Clearing is very rapid. The uterine branches of proglottids can be seen easily by holding the segment between 2 slides towards the light, or with a hand lens.

b. **Lacto-phenol (1:1):** An equal volume of glycerin may be added. This solution is good for nematodes, but prolonged contact may clear the organism too much.

c. **Beechwood creosote:** Clearing is slower than with the above solutions, but organisms remain soft and pliable.

d. **Xylene:** Clearing occurs fairly quickly, but the specimen may become brittle. Tapeworm proglottids, however, are cleared effectively with xylene.

e. **Glycerin:** Fairly satisfactory with nematodes such as hookworm, but may occasionally cause shrivelling or wrinkling.

f. **Lacto-phenol-PVA:** This is a fairly good clearing and mounting medium for small nematodes. The relative proportions of PVA and lacto-phenol may be varied to give the degree of clearing desired.

•DEHYDRATION-CLEARING HELMINTHS FOR PERMANENT MOUNTS

Permanent mounts for reference or teaching purposes may be prepared by clearing specimens (without staining) in the same way as for diagnosis. However, better permanent preparations will be obtained if the fixed worms are first dehydrated and then cleared in xylene or carbol-xylene. Dehydration is accomplished by passing the organism up through a graded series of ethyl alcohols: 20%, 35%, 50%, 70%, 85%, 95%, and 100%. Leave the organism in each alcohol for 30 minutes to 1 hour.

The cleared specimen is mounted in Permount or other suitable mounting media. Small pieces of fine capillary glass tubing, broken coverslips, or similar materials can be arranged on either side of the organism to provide support for the coverslip (fig. 22). Without these, the coverslip will rock and the finished preparation will have a slanted surface.

Lacto-phenol-PVA mounts are satisfactory for permanent preparations of small nematodes. For best results and to keep the organism in the proper position while the mount is drying, support the coverslip as described in the previous paragraph.

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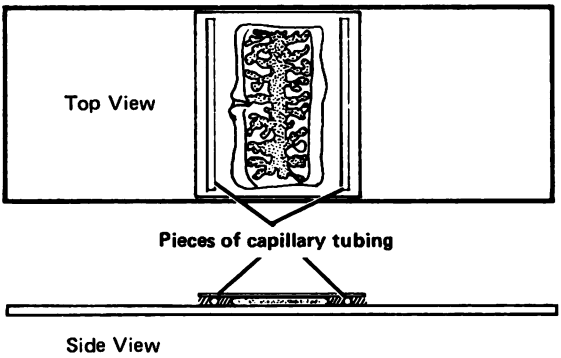
Figure 22.
Use of Supports for Whole Mounts of Helminths



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CULTIVATION PROCEDURES

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CULTIVATION PROCEDURES

INTESTINAL PROTOZOA

Various media have been used in cultivating amebae and intestinal or atrial flagellates. Since *B. coli* and *G. lamblia* require special media, the procedure for culturing these two organisms has not been included. Some of the commonly used media for amebae and flagellates are presented.

•MEDIA

MODIFIED BOECK AND DRBOHLAV'S MEDIUM

This medium consists of a solid egg base and an overlay of Locke's solution (Boeck and Drbohlav, 1925; Reardon and Rees, 1939). It supports growth of most of the intestinal protozoa and satisfactorily maintains them. Since it does not require fresh serum and uses only materials usually available in laboratories, it is recommended for routine purposes.

Glassware and material used in the preparation should be sterile because the length of time the medium is sterilized may not be adequate to kill large numbers of bacteria in the solid egg base. Experience with the efficiency of one's autoclave will determine whether aseptic precautions are necessary.

Modified Locke's Solution (Overlay)

Sodium chloride ($NaCl$)	8.0 gm
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.2 gm
Potassium chloride (KCl)	0.2 gm
Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	0.01 gm
Disodium hydrogen phosphate (Na_2HPO_4)	2.0 gm
Sodium bicarbonate ($NaHCO_3$)	0.4 gm
Potassium dihydrogen phosphate (KH_2PO_4)	0.3 gm
Distilled water	1,000.0 ml

Add the above chemicals in the order listed to the distilled water. Mix each until dissolved. Boil 10 minutes. A precipitate will form. Cool to room temperature and filter through paper. Sterilize in an autoclave for 15 minutes at 15 lbs (121°C).

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Preparation of Egg Slants

1. Wash 4 fresh eggs and carefully break them in a 1,000 ml flask containing a few glass beads or into a blender. It is usually easier to break 1 egg at a time into a beaker and then transfer it to the flask through a small funnel. Any eggs that do not appear fresh should be discarded. A "blood spot" in a fresh egg does not harm the medium.
2. Add 50 ml of sterile Locke's solution and emulsify by shaking the flask.
3. Filter the mixture through gauze into a dispenser.
4. Dispense into sterile test tubes (15x125-mm) in such a quantity that there will be a short butt and a 4 to 5 cm slant.
5. Inspissate. If an inspissator is not available, inspissate in the autoclave as follows:

   If the autoclave has a jacket, allow the pressure to reach 15 lbs. Place the tubes in the chamber in the desired slanted position. Close all exhaust valves and the door to the chamber. This procedure will entrap the air in the chamber. Allow the steam from the jacket to enter the chamber as fast as possible until the pressure gauge registers 15 lbs. Hold at this pressure for 15 minutes. Cut off the steam supply and with all doors and valves still tightly closed, allow the pressure to drop gradually.

   If the autoclave has no jacket, place the tubes in the chamber and close all exhaust valves and the door tightly. If the drain at the bottom of the autoclave has no valve, stopper it with a rubber stopper. Turn on the steam and allow the pressure to rise to 15 lbs. The temperature of the mixture of air and steam will not be above 85°C. Hold at this pressure for 15 minutes. Turn off the steam and allow autoclave to cool gradually. Sudden drops of pressure tend to disrupt the medium, causing bubbles and/or rough surfaces.
6. Allow the slants to cool and cover with Locke's solution so that the overlay forms a column 5 to 6 cm deep.
7. Sterilize in the autoclave under 15 lbs of pressure for 15 minutes (temperature 121°C). Allow the pressure to drop gradually.
8. Incubate at 37°C for 24 hours and check for sterility. If the fluid is clear, the medium can be stored in the refrigerator for future use. Fresh medium is more satisfactory than stored medium, but if necessary, media can be kept for a month.

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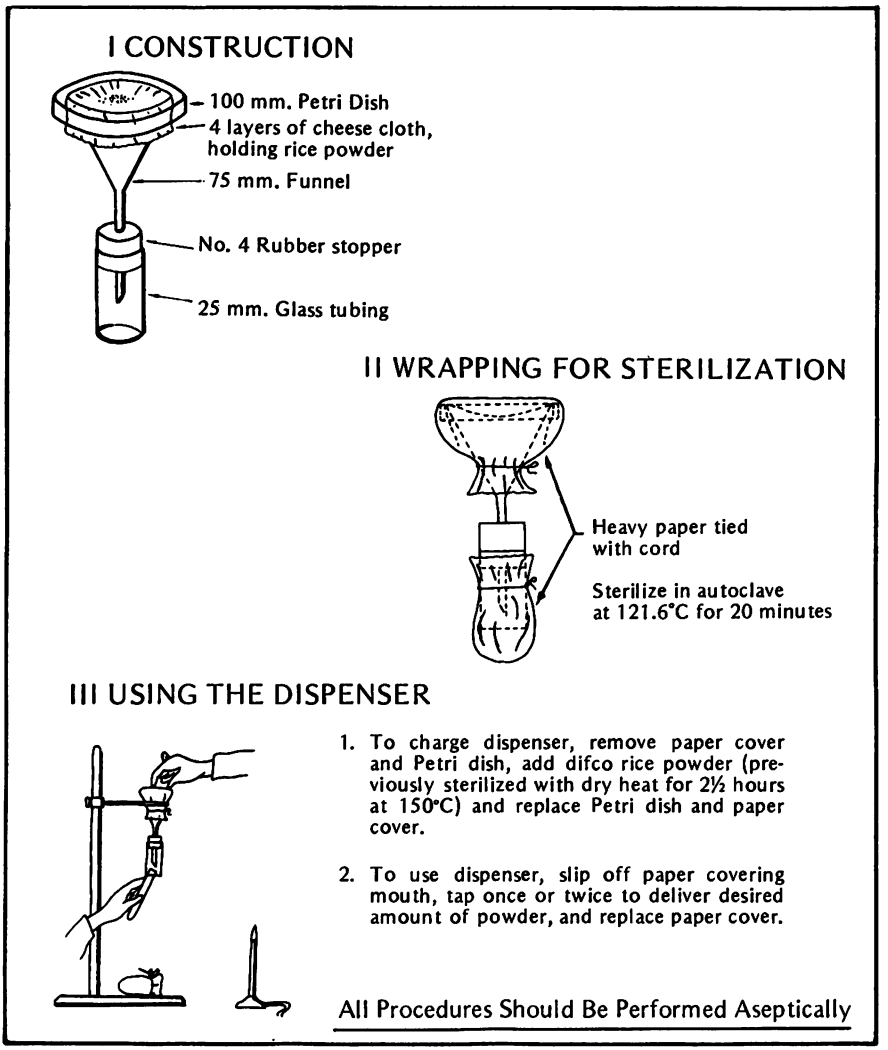
Sterile Rice Starch

Difco Rice starch (or its equivalent) is used. Spread a few grams evenly in a large tube and heat in a hot air oven for 1½ hours at 150°C. Keep the tube in a horizontal position during sterilization. If larger quantities are needed, use the dispenser, funnel-type container as shown in figure 23. Sterilize in the autoclave at 15 lbs (121°C) for 20 minutes.

Figure 23
Rice Powder Dispenser



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EGG YOLK-INFUSION MEDIUM

This is an all-liquid, egg yolk infusion medium to which liver has been added (Balamuth, 1946). It is good for isolating most intestinal protozoa, and, furthermore, it may be stored for several months without marked deterioration.

Liver Extract Solution

Wilson's Dry Extract of Liver (powder) ..... 5 gm
Distilled water ..... 100 ml

Dissolve the liver extract powder in the distilled water and heat to the boiling point. Filter through ordinary filter paper and autoclave at 15 lbs (121°C) for 20 minutes.

Buffer Solutions

A. Stock Buffers (1 M Solutions)

1. Dibasic Phosphate Solution

K_2HPO_4 ..... 87.09 gm
Distilled water to make ..... 500 ml

In a 500-ml volumetric flask, dissolve the K_2HPO_4 in part of the distilled water. Add water to make 500 ml of solution. Store in a glass-stoppered bottle.

2. Dihydrogen Phosphate Solution

KH_2PO_4 ..... 13.61 gm
Distilled water to make ..... 100 ml

In a 100-ml volumetric flask, dissolve the KH_2PO_4 in part of the distilled water. Add water to make 100 ml of solution. Store in a glass-stoppered bottle.

B. Buffer Solution (M/15 Solution)

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

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

Preparation of Medium

A. From Fresh Egg Yolks (approximately 200-250 ml amount);

1. Crumble the yolks of 4 hard boiled eggs and mix thoroughly with 125 ml of 0.85% NaCl. Use a rotary beater, electric mixer, or a blender.

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2. Heat the mixture in the top part of a covered double boiler for 20 minutes after it reaches 80°C.
3. Add 20 ml of distilled water to make up for evaporation.
4. Filter through a Büchner funnel under reduced pressure. Use several layers of #2 Whatman filter paper (or equivalent).
5. Add 0.85% NaCl to the filtrate to bring the volume back to 125 ml.
6. Autoclave filtrate at 15 lbs pressure for 20 minutes.
7. Cool to below 10°C (a yellowish sediment will form) and refilter through the Büchner funnel.
8. Add an equal volume of the M/15 buffer solution to the filtrate to complete the yolk infusion.
9. If liver extract is used, add 1 part of the liver extract to 9 parts of the yolk infusion.
10. Autoclave at 15 lbs pressure for 20 minutes.
11. The medium may be refrigerated in the flask or tubed immediately in 7 to 10-ml amounts and refrigerated.

B. From Dehydrated Egg Yolks (approximately 1,000-ml amount):

1. To 288 gm dehydrated egg yolk, add 288 ml of distilled water and 1,000 ml of 0.85% saline.
2. Mix thoroughly with an electric mixer or in a blender until emulsion is smooth.
3. Heat in the top of a double boiler, stirring constantly, until the egg yolk material coagulates (about 20 minutes).
4. Add 160 ml distilled water to make up for evaporation.
5. Filter the mixture through a muslin bag. The bag may be squeezed gently to obtain the maximum amount of filtrate. The pulpy product of dehydrated egg yolks is difficult to filter through a Büchner funnel and a muslin bag is preferred.
6. Measure the filtrate; add 0.85% saline to make 1,000 ml.
7. Dispense the filtrate into 2 one-liter Erlenmeyer flasks and autoclave for 20 minutes at 15 lbs pressure.
8. Refrigerate overnight or until solution is thoroughly chilled. A yellowish sediment will form.

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9. Filter while cold through a Büchner funnel, under reduced pressure, using several layers of # 2 Whatman filter paper (or equivalent). Pour the filtrate in small amounts into the funnel and change the filter papers frequently.
10. Measure the filtrate and add an equal volume of buffer solution.
11. If liver extract is used, add 5 ml of the extract per liter of medium.
12. The medium may be left in flasks or dispensed in 7- to 10-ml amounts in tubes. Autoclave at 15 lbs pressure for 20 minutes.
13. Store the tubes or flasks in the refrigerator. There may be an accumulation of sediment in the bottom of the flasks which can be removed by filtration. Re-autoclaving will not harm the medium.

LIVER INFUSION AGAR MEDIUM

This medium consists of a solid liver infusion agar slant with an overlay of physiological saline and fresh horse serum (Cleveland and Collier, 1930; Cleveland and Sanders, 1930). It is claimed to be more specific for *E. histolytica* than for the other protozoa which do not grow well on it.

Serum Saline Solution (Overlay)

Fresh horse serum	1 part
Physiological saline (0.8% NaCl in distilled water)	6 parts

Mix and sterilize by filtration.

Agar Solution

Dehydrated liver infusion agar (Difco)	30 gm
Disodium phosphate (Na_2HPO_4)	3 gm
Distilled water	1,000 ml

Dissolve the agar and disodium phosphate in the distilled water.

Agar Slants

1. Dispense the liquid agar into tubes in an amount sufficient to give an agar slant 4 to 5 cm long *without* a butt.
2. Sterilize at 15 lbs pressure for 20 minutes.
3. Add sufficient overlay to cover the slant.

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CHARCOAL MEDIUM

A charcoal medium originally prepared for culturing *Mycobacterium tuberculosis* (Hirsch, 1954) has been modified for culturing intestinal protozoa (McQuay, 1956). The modified medium may be either a diphasic medium, consisting of a charcoal agar slant with an overlay of buffered saline, or a liquid medium. Both are equally satisfactory for culturing protozoa, but the liquid medium is easier to prepare.

The charcoal medium is particularly good for isolating and maintaining *Entamoeba hartmanni* and *Dientamoeba fragilis,* although other species, including *Entamoeba histolytica*, will also grow on it. The modified Boeck and Drbohlav's medium, however, is better for isolating and maintaining *E. histolytica.*

Charcoal Agar Slant – Diphasic Medium

A. Buffered Saline Overlay

1. Solution A (M/15 KH_2PO_4)

Potassium dihydrogen phosphate (KH_2PO_4), anhydrous ..... 9.07 gm
Distilled water to make ..... 1,000.0 ml

Dissolve the KH_2PO_4 in a small amount of water in a clean 1-liter volumetric flask. Add water to the 1 liter mark. Mix and store in a glass-stoppered bottle.

2. Solution B (M/15 Na_2HPO_4)

Disodium phosphate (Na_2HPO_4), anhydrous ..... 9.46 gm
Distilled water to make ..... 1,000.0 ml

Prepare as directed for Solution A.

Note: $Na_2HPO_4.12H_2O$ may be substituted for anhydrous Na_2HPO_4 using 23.88 gms of the crystalline compound per liter of solution.

3. Buffered Saline (0.5%, pH 7.4)

Sodium chloride (NaCl) ..... 5.0 gm
Solution A ..... 190.0 ml
Solution B ..... 810.0 ml

Combine solutions A and B and add the NaCl. Stir thoroughly to dissolve. Sterilize at 15 lbs pressure for 15 minutes. Store in the refrigerator.

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B. Preparation of Agar Slants

Disodium phosphate ($Na_2HPO_4.12H_2O$)	3.0 gm
Postassium phosphate (KH_2PO_4)	4.0 gm
Sodium citrate crystals	1.0 gm
Magnesium sulfate crystals	0.1 gm
Ferric ammonium citrate	0.1 gm
Bacto-asparagin (Difco)	2.0 gm
Bacto-tryptone (Difco)	5.0 gm
Glycerin (reagent grade)	10.0 ml
Distilled water	1,000.0 ml

Add the various ingredients, in the order listed, to the distilled water and heat to dissolve. Do not boil. Stir thoroughly. Then add, stirring thoroughly after each addition:

Bacto-agar (Difco)	10.0 gm
Norite A (Charcoal)[1]	10.0 gm
Cholesterol in Acetone, 1% solution[2] (0.25 gm cholesterol in 25 ml acetone)	25.0 ml

Note: Be sure to keep the flask away from flames when adding the cholesterol-acetone.

1. Heat the entire mixture to boiling to dissolve the agar.
2. Stir frequently to keep the charcoal in suspension.
3. Dispense the hot solution in 3-ml amounts in tubes and plug.
4. Autoclave for 15 minutes at 15 lbs pressure.
5. Resuspend the charcoal and allow the tubes to cool in a slanted position to form short butts or no butts.
6. Add 3 ml of sterile 0.5% buffered saline overlay to the slants.
7. Check the medium for sterility by placing it in the incubator overnight; then refrigerate.

Liquid Medium

Prepare as for the diphasic medium using the same ingredients and quantities, *except omit the agar and substitute 10 ml of distilled water.* The total amount of water will be 1,010 ml.

1. Bring the medium to a boil.
2. Add 1 liter of buffered 0.5% saline.

[1]From Pfanstiehl Laboratories, Inc., 1219 Glen Rock Ave. Waukegan, Ill. 60085
[2]Cholesterol, C.P., ash-free, for Kline Test, Pfanstiehl Laboratories, Inc.

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3. Dispense in 6.0-ml amounts in tubes and sterilize at 15 lbs pressure for 15 minutes.
4. Check for sterility by placing the tubes in the incubator overnight.
5. Store in refrigerator.

COMMERCIAL MEDIA

When only occasional cultures are desired, dehydrated commercial media are usually more practical and economical than laboratory prepared media. Bacto *Entamoeba* Medium (basically the Cleveland and Collier liver extract medium) can be purchased from the Digestive Ferments Co. (Difco), Detroit, Michigan 48201. Directions for preparing it are on the bottle. The charcoal agar may be purchased in dehydrated form as Hirsch Charcoal Agar Modified from Baltimore Biological Laboratory (BBL), Division of BioQuest, Cockeysville, Maryland 21030. The slant is prepared according to directions on the bottle and overlayed with sterile Locke's Solution rather than the simple buffered saline described here. Be sure the tubes are slanted so that a very short butt is formed.

•INOCULATION AND EXAMINATION TECHNIQUES

1. Just before use, add approximately 30 mgm of sterile rice starch to each tube of medium. This may be done with a sterile wire loop (use 1 loopful) or a dispensing funnel (fig. 23) by 1 or 2 sharp but gentle taps on top.
2. For each specimen to be cultured, warm 2 tubes of medium to approximately 37°C.
3. To 1 tube, add enough penicillin and streptomycin to give a final concentration of 200 to 250 units of each per ml of overlay (Spingarn and Edelman, 1952; Norman and Brooke, 1955a). This is approximately 1,000 units of each antibiotic per tube.
4. Inoculate as follows:
   a) Liquid or semi-solid material
   With a large-bore pipette, add 0.5 ml of specimen to each tube of medium. Add as little air as possible. Mix the inoculum with the overlay, being careful not to introduce air bubbles.

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b) **Formed feces**
With applicator sticks, transfer a portion of specimen the size of a small pea to the tube of medium. Mix with the overlay by carefully rubbing the material against the side of the tube. Portions for inoculating are preferably selected from any mucus, blood, or abnormal-appearing areas in the fecal material, if present.

5. Incubate all tubes at 37°C for 24 hours.
6. With a sterile, large-bore pipette, place a drop or two of the sediment on a slide and cover with a coverslip. Prepare an unstained mount and temporary stain preparation with Quensel's or buffered methylene blue solutions. The amebae grow at the bottom of the culture or on the surface of the slant near the bottom.
7. All cultures which show no trophozoites or an insufficient number for identification are transferred to fresh media. With a sterile large-bore pipette, transfer about half of the sediment to a tube of medium. Do not add antibotics to any of these transfers (secondary cultures).
8. Incubate both the original cultures and the transfers at 37°C for 48 hours and examine.
9. Sediment of cultures revealing organisms or questionable objects should be mixed in vials with PVA-fixative. Permanent stained films of the PVA-fixed sediments are prepared and carefully examined under the oil-immersion objective (Norman and Brooke, 1955b).

•SHIPPING MEDIUM

The media used for routine cultivation of protozoa in the laboratory are not suitable for transporting organisms from one location to another. Gleason *et al.* (1960) developed a special shipping medium in which trophozoites may survive for up to 4 days, thus permitting shipment of viable organisms to a reference laboratory for recovery and identification. It is Balamuth's egg extract medium enriched with dextrose, trypticase, and sodium thioglycollate.

MODIFIED BALAMUTH'S MEDIUM

Prepare as directed for Balamuth's Egg Yolk-Infusion Medium, but omit the liver extract solution.

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Enriched Locke's Solution

To 100 ml of modified Locke's Solution (formula on page 143), add the following:

Dextrose	110 mgm
Trypticase	2 mgm
Sodium thiogycollate	50 mgm

Preparation of Medium

Balamuth's Medium (without liver)	5 parts
Enriched Locke's solution	1 part

Dispense in 6-ml volumes in screw-cap tubes (approximately 15x125-mm). Sterilize at 15 lbs pressure for 15 minutes. Cool and store in the refrigerator. If kept refrigerated, the medium may be used up to 3 months after preparation.

Method of Use

Emulsify a small amount of feces about the size of a small pea in a tube of medium in the usual manner for fecal cultures. Add approximately 1,000 units each of streptomycin and penicillin to the tube (250 units of each per ml of overlay). Screw the cap on tightly and ship in a double mailing container (or suitable equivalent) to the laboratory.

When the specimen is received in the laboratory, replace the supernatant fluid with fresh medium (without adding antibiotics). Transfer about half the sediment to a second tube of medium. If the amount of sediment is very small (as may be true with sigmoidoscopic specimens), do not transfer. Incubate tubes at 37°C and examine daily.

In positive specimens, motile organisms usually can be detected in 24 hours if the storage or transit time was not more than 1 day. Cultures kept at room temperature for longer periods may need to be incubated longer before growth is apparent. For example, cultures left at room temperature for 3 days, may not show motile organisms for 3 to 5 days at 37°C.

Although organisms can be recovered after 4 days at room temperature, best results will be obtained with 1- to 2-day-old specimens.

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EXAMINATION AND CULTIVATION OF ASPIRATES FROM LIVER ABCESSES

Liver abscesses suspected of being caused by *Entamoeba histolytica* are often drained and the aspirate examined for the presence of the organisms. However, it is frequently difficult to demonstrate amebae in the thick pus removed from the abscesses because: (1) most of the organisms are in the peripheral area of the abscess and there are often relatively few free in the abscess contents; (2) the organisms may be partially immobilized with the white blood cells by coagulum; and (3) since the fluid is sterile, amebae will not grow in culture when the material is inoculated in the manner routinely used for stool cultures.

The Amoebiasis Research Unit, Durban, South Africa, has reported great success in using proteolytic enzymes in freeing the amebae from the aspirates of liver abscesses. The following procedure incorporates the recommendations of that group (Freedman *et al.*, 1958; Wilmot, 1962).

PROCEDURE

1. During aspiration a minimum of two portions of exudate is removed. These are kept separate. The first portion withdrawn, usually yellowish-white in color, seldom contains amebae and is not examined routinely. Later portions which are reddish in color are more likely to include organisms. The final portion containing most of the material from near the wall is most likely to be positive. (It is believed that collapse of the abscess and inflowing blood release amebae from the tissue.) Subsequent aspirations performed after appropriate rest periods may be more likely to reveal organisms.

2. The material may be examined directly or after centrifuging at 1,000 r/min (300 g) for 5 minutes. Prepare saline mount of the pus or of the centrifuged sediment and examine. If amebae are not seen, the aspirated pus should be treated with enzymes (streptodornase or streptokinase) to break up the coagulum and release organisms, if present. A combination of streptodornase and streptokinase (Varidase*) is commonly used.

3. Add 1 part of enzyme solution to 5 parts of aspirate material, mix, and incubate at 37°C for 1 hour. Shake at intervals. This process frees the amebae from the coagulum.

*Varidase, produced by Lederle Laboratories, is usually available as a lyophylized powder through pharmaceutical outlets.

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4. Centrifuge the mixture at 1,000 to 1,500 r/min (300-400g) for 5 minutes. If possible, use aseptic technique and keep the material sterile. The sediments may be microscopically examined in wet mounts. If amebae are not found, some of the sediment (if kept sterile) can be used to inoculate culture media, such as the egg slant medium overlayed with Locke's solution (Boeck and Drbohlav, 1925; Reardon and Rees, 1939); egg yolk-infusion medium (Balamuth, 1946); liver-infusion agar (Cleveland and Collier, 1930) or any medium that is satisfactory for isolation and growth of *E. histolytica.*
5. In culturing the material, condition the medium by inoculating with *Clostridium perfringens.* This can be done at the time of the inoculation with the aspirated material, though it is preferable to precondition the medium for 24 hours at 37°C, if possible. Sterile rice powder is added at the time of inoculation of the pus. Overlay with a 1:1 mixture of paraffin and petroleum jelly (Vaseline). The cultures are incubated at 37°C and examined for amebae after 24 to 48 hours. If organisms are not seen, continue to incubate and examine daily for 21 days before reporting as negative.

CULTIVATION OF *TRICHOMONAS VAGINALIS*

Several media are available for culturing *T. vaginalis.* Two of the most reliable are the Feinberg-Whittington medium (Feinberg and Whittington, 1957) and the modified Diamond's medium (Diamond, 1957; Fouts and Kraus, 1980).

•PREPARATION OF MEDIA

FEINBERG-WHITTINGTON MEDIUM

Panmede[1]	12.5 gm
NaCl	3.3 gm
Dextrose	2.5 gm
Horse serum (Inactivate at 57°C for 25 min)	40.0 ml
Distilled water	500.0 ml
Sodium penicillin G	500,000 units
Streptomycin sulfate	250 mgm

Combine ingredients. Adjust the pH to 6.5 with NaOH. Filter through a Seitz filter. Dispense into small 100x13-mm screw-cap tubes. Each tube should be about one-half full. Store in refrigerator.

[1] Proteolysed liver. Pabryn Laboratories, Greenford, Middlesex, England.

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MODIFIED DIAMOND'S MEDIUM

A. Broth

Trypticase (BBL)[1]	6.0 gm
Yeast extract (BBL)	3.0 gm
Maltose	1.5 gm
Cysteine HCl·H_2O	0.3 gm
Ascorbic acid	0.06 gm
Distilled water	225.0 ml

1. Dissolve the dry ingredients in the water. Stir thoroughly.
2. Check pH of the mixture and adjust to pH 6.0 with 4N NaOH or 1 HCl.
3. Dispense 7.5-ml portions of the mixture into 16x125-mm screwcap tubes.
4. Autoclave at 15 lbs pressure for 15 min.
5. Cool to room temperature.

B. Antibiotic Mixture

Distilled water, sterile	50 ml
Sodium penicillin G	1,000,000 units
Streptomycin sulfate	1 gm
Fungizone (Amphotericin B)	2 mgm

Add the antibiotic to the water and mix well.

C. To each cooled tube of broth medium add:

Sterile horse serum (inactivated at 56°C for 30 min)	1 ml
Antibiotic mixture	0.5 ml

D. Store tubes of completed medium in the refrigerator.

•TECHNIQUE

1. Either urine sediment or vaginal secretions obtained by swab can be used to inoculate the medium.
2. If swabs are used, squeeze the swab against the side of the tube.
3. Incubate at 37°C.
4. Examine at 24 and 48 hours. If the culture is not positive at 48 hours, it should be examined again at 72 hours.
5. Usually subculturing is not necessary, but if fungal growth (*Candida,* for example) is excessive, subculture at 48 hours. Examine subculture at 24,48, and 72 hours.

[1] Baltimore Biological Laboratory, Cockeysville, Maryland 21030

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HELMINTH LARVAE

In old fecal specimens, both hookworm and *Strongyloides* rhabditiform larvae may be present. If doubt as to species exists, the feces may be cultured to obtained filariform larvae. Hookworm requires 5 to 6 days to reach the filariform stage; *Strongyloides* requires only 2 to 3 days. Two methods of cultivation are given: the charcoal or sand culture and the test tube culture (Sasa *et al.*, 1958).

•CHARCOAL OR SAND CULTURE

PREPARATION

1. Mix a large portion of feces (about the size of a walnut) with an approximately equal quantity of fine granular wood or bone charcoal or sterilized sand. Powdered charcoal is not satisfactory.

2. Using 2 tongue depressors, "cut" the feces into the sand to form small "lumps" about the size of small peas or smaller.

3. Add water to moisten the material, but avoid getting it too wet.

4. Place the mixture on moistened filter paper in a petri dish. Waxed half-pint cartons, such as those recommended for specimen collection, can be used. The feces mixture tends to dry more rapidly in the carton, however, and daily "watering" may be necessary. To do this, sprinkle the surface of the culture until it appears visibly moist. Do not get the culture too wet. Cultures, whether in petri dishes or cartons, must be kept moist because drying will kill the larvae.

5. Keep the cultures at room temperature for 3 to 7 days. If petri dishes are used, larvae may be found in the drops of moisture on the lid. The lid or the surface of the culture may be examined with a hand lens or dissecting microscope to determine if larvae are present. Filariform larvae will migrate to the surface of the mixture or to the moisture drops.
   The culture should be checked for larvae on the 3rd day. If the species in question is *Strongyloides,* filariform larvae will probably be seen. If no larvae are visible, the culture should be checked again on the 5th and 7th days.

6. Species should be positively identified by microscopic examination of the larvae.

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RECOVERY OF LARVAE FROM CULTURES

Collect larvae as follows:

1. If present in the drops of moisture on the petri dish lid, wash the drops into a beaker, tube, or clean petri dish.
2. Use a Baermann apparatus to separate larvae from the culture material. A simple Baermann can be constructed from a large glass funnel with a short length of rubber tubing, fitted with a pinch clamp, attached to the bottom. A piece of wire screen is fitted into the top of the funnel and a layer of muslin (a piece of bed sheet is excellent) is placed over this (fig. 24). Gauze does not work as well as muslin, since it permits charcoal or sand to escape into the lower levels of water. The funnel is held upright by putting it into a ring stand.
   a) Fill the funnel with warm tap water (37°C).
   b) Place the top layers of the culture material, or the entire culture, if it is small, on the muslin-screen. The water should cover the bottom part of the material.
   c) Let stand for 30 minutes to 1 hour to allow the larvae to collect in the rubber tube and the lower part of the funnel.
   d) Draw off a few ml of the water into a breaker. If the larvae are few, the material can be centrifuged for 2 to 3 minutes at 1,500 r/min.
   e) Transfer a drop of the material (use the sediment, if centrifuged) to a slide and examine with the 10X objective.

•TEST-TUBE CULTURE (fig. 25)

1. Cut a strip of filter paper about 1 inch wide and 7 inches long.
2. Smear about 1/2 gram of feces (a quantity about the size of a small pea) over approximately 1/2 to 2/3 the length of the filter paper.
3. Place the strip in a large test tube with approximately 3 ml of tap water in the bottom. The lower edge of the filter paper should be in the water, but the feces do not have to be submerged (fig. 25).
4. Cover the tube – a cork stopper or cotton plug is satisfactory– keep at room temperature for 3 to 7 days. Filariform larvae will be found at the bottom of the tube.
5. Examine a drop after 3 days for *Strongyloides* larvae and after 5 to 7 days for hookworm larvae.

This method is simpler and easier than the charcoal or sand culture.

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Figure 24
Baermann Apparatus for Recovery of Larvae from Cultures or Soil Samples

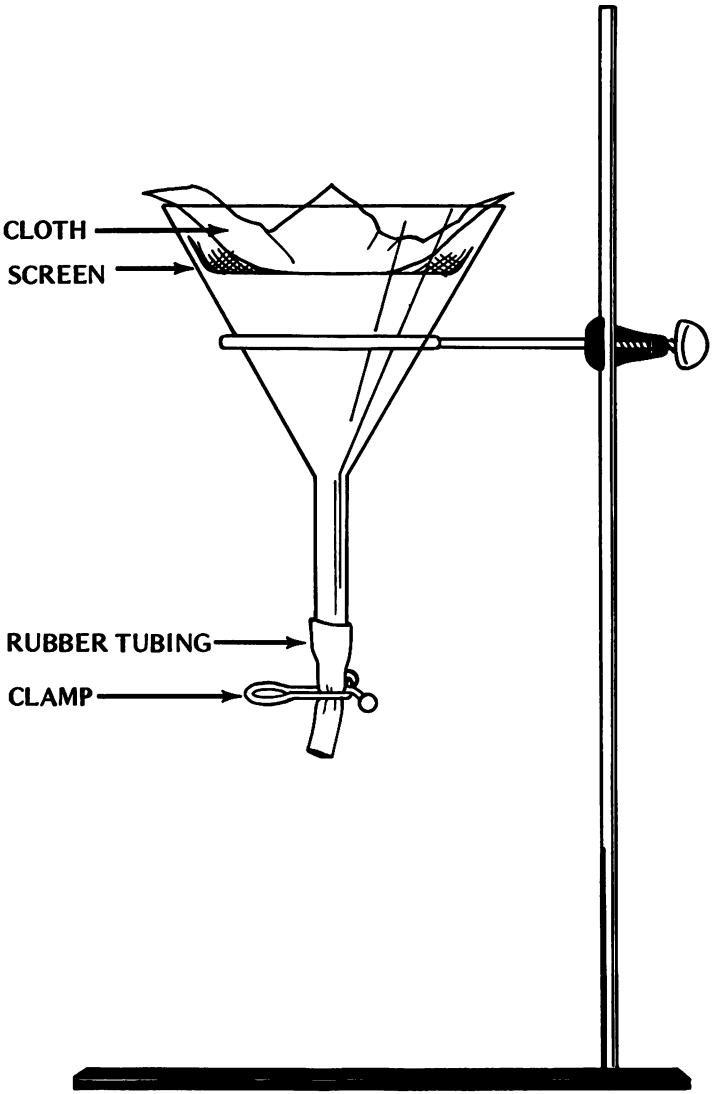

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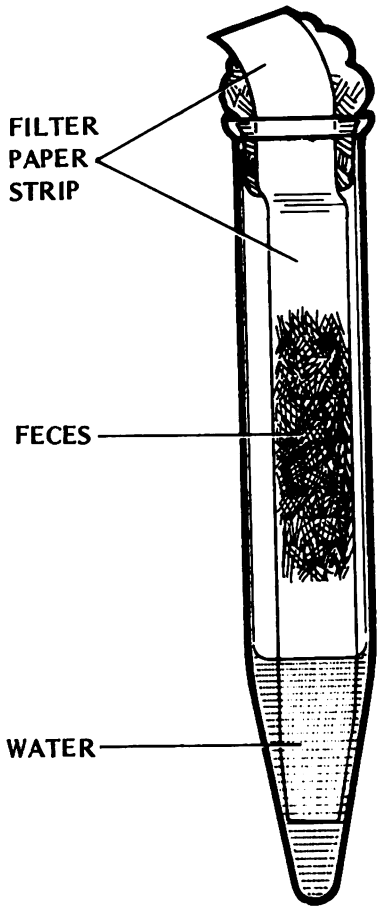
Figure 25
Test Tube (Filter Paper) Culture for Nematode Larvae



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•EXAMINATION

Examine unstained wet mounts for the presence of motile larvae, adjusting the light for optimum visibility. Rapidly moving larvae are difficult to observe. To kill the organisms, a drop of iodine or Formalin can be allowed to run under the coverslip.

Hookworm and *Strongyloides* filariform larvae can be differentiated by the length of the esophagus and the appearance of the tip of the tail. When cultures are examined at the end of 3 days, rhabditiform larvae of hookworm (alone or with *Strongyloides* filariform larvae) may be found. These are often larger than those seen in fecal specimens, but their characteristics are the same. Be careful to distinguish between rhabditiform and filariform larvae to avoid erroneous diagnoses. If fecal samples have been contaminated with soil, free-living larvae may have to be differentiated from parasitic larvae. The method for doing this is as follows (Shorb, 1937):

> Add 0.3 ml of concentrated hydrochloric acid per 10 ml of water containing larvae. A 1:30 concentration of hydrochloric acid is obtained. If less than 10 ml of suspension is available, use proportionate amounts of acid. Free-living nematodes are killed almost immediately; infective larvae of parasitic species live for about 24 hours. Larvae can be specifically identified by morphologic features.

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MISCELLANEOUS PROCEDURES

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MISCELLANEOUS PROCEDURES

TECHNIQUES FOR DIAGNOSING ENTEROBIASIS

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 for diagnosing enterobiasis. Two types of swabs, the cellulose-tape slide preparation (Graham, 1941) and the Vaseline-paraffin swab (Markey, 1950), are most commonly used for routine purposes. The procedure given below for handling cellulose-tape slides incorporates the modifications of Brooke *et al.* (1949) and Beaver (1949 b). The technique for Vaseline-paraffin swabs is that used at CDC; it is based on the original Markey (1950) method.

•CELLULOSE-TAPE SLIDE TECHNIQUE

PREPARATION OF SLIDES

Clear cellulose tape, ¾-inch wide, is best for this purpose. Cloudy or hazy tape interferes with examination. A piece of tape about 4 to 5 inches long is anchored on the underside of a 3x1-inch slide and smoothed across the top of the slide. A paper tab is affixed to the free end of the tape for labeling and identification. (See figure 26).

The unused tape preparations may be stored for several months in the refrigerator or in a cool place.

COLLECTION TECHNIQUE (See figure 26).

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. The perianal folds should be pressed open to insure access to eggs concealed in the crevices. To facilitate examination, use only the center portion of the tape, if possible.
3. Smooth the tape back into place with a piece of gauze or cotton. Be careful not to contaminate hands.

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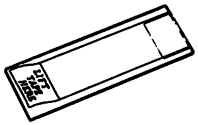
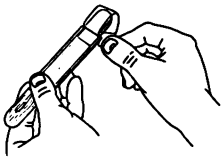
Figure 26.
Use of Cellulose-Tape Preparation for Diagnosis of Pinworm

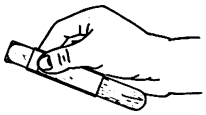


a. Cellulose-tape slide preparation

b. Hold slide against tongue depressor one inch from end and lift long portion of tape from slide

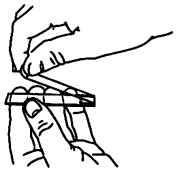


c. Loop tape over end of depressor to expose gummed surface

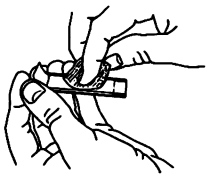


d. Hold tape and slide against tongue depressor

e. Press gummed surfaces against several areas of perianal region



f. Replace tape on slide



g. Smooth tape with cotton or gauze

Note: Specimens are best obtained a few hours after the person has retired, perhaps at 10 or 11P.M., or the first thing in the morning before a bowel movement or bath.

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EXAMINATION TECHNIQUE

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. Examine the center portion under the low power of the microscope. A 5X or 6X ocular will facilitate observations.
3. If eggs are not found in the center area, the rest of the slide should be scanned before reporting NPF (no parasites found).
4. If the preparations are to be stored before examination, the toluene should not be added until the time of examination, since prolonged contact with the toluene may cause the organism to degenerate more rapidly than it normally would.
5. Tape preparations may be stored in the refrigerator for several days or weeks before examination without extensive degeneration of the eggs.

•VASELINE-PARAFFIN SWAB TECHNIQUE

PREPARATION OF SWABS

1. Dip a cotton swab into a mixture of 4 parts of Vaseline to 1 part of parawax (paraffin) so that the swab is thoroughly coated. Let cool. If the mixture is too hot, the swab may have to be dipped twice to coat it thoroughly.
2. Put coated swab in a 100x13-mm tube and plug the tube with cotton. These swabs may be stored for long periods if the temperature is not so great that the Vaseline-paraffin on the swab melts. Refrigeration is preferable.

COLLECTION TECHNIQUE

1. Rub the swab gently over the perianal surface, spreading open the perianal folds. Insert the swab about one-fourth inch into the anal opening.
2. Replace swab in tube.

EXAMINATION TECHNIQUE

1. Fill the tube about one-third to one-half full of xylene, just covering the swab, and let stand for approximately 3 to 5 minutes.
2. Remove the swab and centrifuge for 1 minute at 2,000 r/min (500 g).

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3. With a pipette, carefully remove the supernatant; transfer the sediment to a 3x2-inch slide and examine for eggs. A coverslip is not necessary. To prevent the drop from spreading over a large area, draw a ring on the slide with a wax pencil. Some of the wax will dissolve, but the amount is usually not enough to interfere with the examination. A depression slide may also be used, but it necessitates more up and down focusing on different levels than the flat side.

KATO THICK-SMEAR TECHNIQUE FOR FECES

The Kato thick smear technique (Kato and Miura, 1954; Martin and Beaver, 1968) is satisfactory for detecting medium and large size helminth eggs in feces but is not good for small trematode eggs. Its chief advantage is that a larger quantity of feces can be put on the slide than in the usual direct wet mount. The basic steps are: (1) press a portion of the stool specimen between a glycerin-soaked cellophane strip or "coverslip" and a slide and (2) allow the smear to stand for a time to clear the fecal film. Hookworm eggs, however, may collapse and disappear shortly after clearing, and unless preparations are examined as soon as the fecal film has cleared, hookworm infections may be missed.

This technique is of little or no value in detecting protozoa infections.

A. MATERIALS NEEDED

1. 3x1-inch or 3x2-inch slides.
2. Cellophane strips - 22x30-mm (wettable cellophane about 40μm thick - no. 124 PD, E.I. DuPont de Nemours, Inc., Film Department, Wilimgton, Delaware)
3. Glycerin-malachite green solution. Prepare by mixing 100 ml glycerin, 100 ml water, and 1 ml 3% aqueous malachite green.
4. Applicator sticks.

B. PROCEDURE

1. Soak cellophane strips in the glycerin-malachite green solution for at least 24 hours. Note: Malachite green reduces glare and makes the eggs more clearly visible, but it can be omitted and just the glycerin-water mixture used.

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2. Place a portion of feces about the size of a small pea or bean (about 50 to 60 mgm) on the slide.

   Note: If the specimen contains a lot of coarse debris, it can be pressed through a steel mesh cloth. See the Kato thick smear quantitative procedure on page 175.

3. Cover the feces with glycerin-malachite green soaked cellophane "coverslips."

4. Invert the preparation and press it down on a flat absorbent surface (a sheet of thick soft paper or filter paper on a table surface is ideal) until the feces are spread over an area about 20 to 25 mm in diameter.

5. Allow the smear to stand for 1 hour at room temperature or 30 minutes in a 37°C incubator.

6. Examine the smear as soon as it is cleared. The fecal film will become almost transparent, leaving the eggs uncleared.

   Note: Since hookworm eggs and schistosome eggs tend to collapse and sometimes disappear if overcleared, the smear should be examined immediately after clearing.

ESTIMATING WORM BURDENS

Egg counting techniques are used to evaluate the degree or intensity of infection. These procedures are chiefly used to estimate worm burdens in a population group in relation to control or eradication programs or to determine the efficacy of anthelmintics. The methods do not give the exact number of worms present, but they do furnish an approximation that enables one to classify the infections as light, moderate, or heavy.

The procedures can also be used with individuals, but the results must be interpreted with caution, since counts are not reliable in determining an individual patient's worm burden. Many factors, such as egg output of individual worms, influence individual counts. Counts may be of use, however, to a physician in classifying the general level of a patient's infection and in evaluating the effectiveness of a drug regimen by obtaining counts before and after treatment.

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Several methods are available for making egg counts. The two commonly used for fecal specimens are the dilution egg-counting technique (Stoll, 1923) and the standard smear technique (Beaver, 1949a, 1950). The Kato thick-smear technique has also been adapted for quantitative determinations of helminth infections (Martin and Beaver, 1968). For estimating pinworm burdens, the coefficient of infection procedure is suggested (Sadun *et al.*, 1956).

•DILUTION EGG COUNTING TECHNIQUE

This is a dilution method for determining the number of eggs per gram of feces. Since weighing out a specific number of grams of feces is very time-consuming, the technique has been modified to obtain the specimen volumetrically by displacing an easily measurable quantity of liquid with feces.

This tends to yield counts approximately 5% too high, since 1 ml of soft feces has been found to weigh 1.03 to 1.04 grams, whereas the same amount of formed feces will weigh 1.05 to 1.07 grams. Such counts should be recorded as "eggs per ml" rather than "eggs per gram." These counts are sufficiently accurate, however, for routine purposes.

In some respects, the technique is similar to a blood count in that:

1. A measured quantity of feces is taken as the sample.
2. The sample is diluted in a constant volume of fluid.
3. A measured sample of homogeneous suspension is examined.

Decinormal sodium hydroxide is used as a diluent because it:

1. Saponifies fats.
2. Sets free eggs that are embedded in debris.
3. Makes the suspension clearer than water does.

APPARATUS:

Stoll dilution flasks - graduated at 56 ml and 60 ml* (fig. 27)
Stoll pipette (with rubber bulb) graduated at 0.075 ml and 0.15 ml
Small glass beads (6 mm)
4 rubber stopper
22x40-mm coverglasses (No. 2)
3x2-inch slides

*Stoll flasks and pipettes are available from Arthur H. Thomas Co., Philadelphia.

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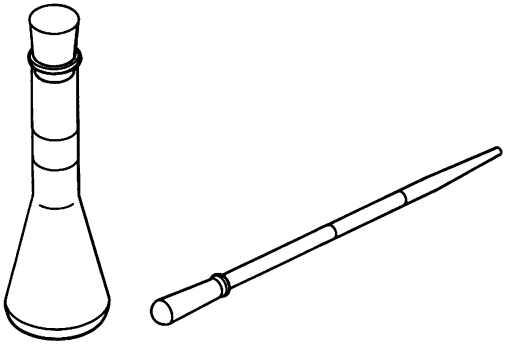
Figure 27.
Stoll Flask and Pipette



PREPARATION OF SOLUTION

Sodium hydroxide. . . . . .4 gm
Distilled water to make . . . . . .1,000 ml

Dissolve NaOH in a small amount of water in 1-liter volumetric flask, then add additional distilled water to make 1,000 ml. The approximate normality is 0.1N. The solution does not need to be standardized for egg-counting purposes.

PROCEDURE:

1. Fill the special Stoll flask with 0.1N sodium hydroxide to the bottom mark etched on the neck of the flask (56 ml).
2. Using two applicators, add sufficient feces to the fluid to raise the level to the upper mark etched on the neck (60 ml).
3. Add 6 to 8 glass beads and firmly insert a No. 4 rubber stopper.
4. If the feces are very hard, allow them to soak for a while.
5. Shake vigorously with a straight up and down motion for 1 minute to secure a homogeneous suspension. Some workers recommend shaking the flask in an inverted position. A circular motion must be avoided, since this motion may concentrate the eggs in a small area, defeating the purpose of the shaking.

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6. Routinely, it is best to run the sample to this point in the afternoon and then set it aside until the next morning. After standing overnight, an additional amount of eggs will be liberated from the fecal debris. Reshake the contents of the flask the following day. Complete and even mixing is essential for an accurate result.
7. The eggs and debris will begin to settle out immediately after shaking stops. Using a Stoll pipette immediately and quickly withdraw a sample of either 0.075 or 0.15 ml from the center of the suspension. The latter size is recommended since it provides a larger sample for counting. Inserting the tip of the pipette to the center of the flask decreases the error in counting due to settling of the eggs.
8. Expel the entire contents of the pipette on to a 3x2-inch slide.
9. Cover the drop with a 22x40-mm, No. 2 coverslip. Do not seal, since the paraffin may obscure some eggs.
10. Examine the mount systematically with the low power objective of the microscope. Cover the entire area of the coverslip.
11. Count all of the hookworm eggs present in the preparation, taking care not to count the same egg twice.
12. Multiply the number obtained by 100 if a 0.15 ml sample was used, or by 200 if a 0.075 ml was used. This will give the total number of eggs per ml of formed feces. With mushy stools, multiply this latter number by the correction factor 2, with diarrheic stools, by 4.

Calculations on which step 12 is based (with 0.15 ml sample):

A. 4 ml of feces to 56 ml of 0.1N NaOH =
4 ml of feces in 60 ml of suspension OR 1 ml of feces in 15 ml of suspension.

Therefore, in the 0.15 ml of suspension counted, 0.01 ml of feces is present, and the number of eggs counted should be multiplied by 100 to give the eggs per ml or eggs per gram.

B. Obviously a given volume of formed stool will contain more fecal material and eggs than an equal volume of liquid or diarrheic stool, which essentially represents a diluted specimen.

In a gram of formed stool, approximately 44 eggs have been found to represent one female *Necator americanus* worm. In a mushy stool, however, the egg count per gram has generally been found

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to be 22 and in a diarrheic stool, 12. This yields a ratio of 1:2:4 (formed:mushy:diarrheic). It is best to convert all counts to a "formed stool" basis by multiplying the count obtained from mushy stools by 2 and from liquid stools by 4.

INTERPRETATION OF RESULTS

Egg counts are applicable chiefly to infections with nematodes such as *Ascaris*, hookworm, and *Trichuris*, where the eggs are passed more or less continuously. Quantitative egg outputs have been used primarily in estimating hookworm burdens, particularly *N. americanus* burdens, and the procedure was originally developed for use in determining densities of infections with this worm in population groups. The interpretation of results, therefore, usually relates to hookworm, but it could be applied to other infections in which the egg output per gram of feces is known.

The number of *N. americanus* worms necessary to produce clinical symptoms varies from person to person and depends on several factors. As stated, a female *Necator* will shed an average of 44 eggs per gram of formed feces. Thus, a technique which can determine the number of eggs in a gram of feces in a given sample enables one to calculate the number of female worms present. Since males and females are usually found in almost equal numbers, doubling the original calculation yields the approximate total worm infection. Various workers consider that when there is a burden of 100 or more hookworms, clinical symptoms are likely to occur. This intensity level is represented by 2,600 or more eggs per ml of feces and is considered a moderate infection. In their hookworm surveys, Keller *et al.* (1934, 1940) used the following classification:

Intensity Grouping *(Eggs per ml of feces)*	Clinical Classification
100- 699	Very Light
700-22,599	Light
2,600-12,599	Moderate
12,600-25,099	Heavy
25,100 and over	Very Heavy

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For routine purposes, it is usually sufficient to indicate the clinical classification based on eggs per ml of feces. The number of worms present need not be calculated.

•STANDARD SMEAR EGG COUNTING TECHNIQUE

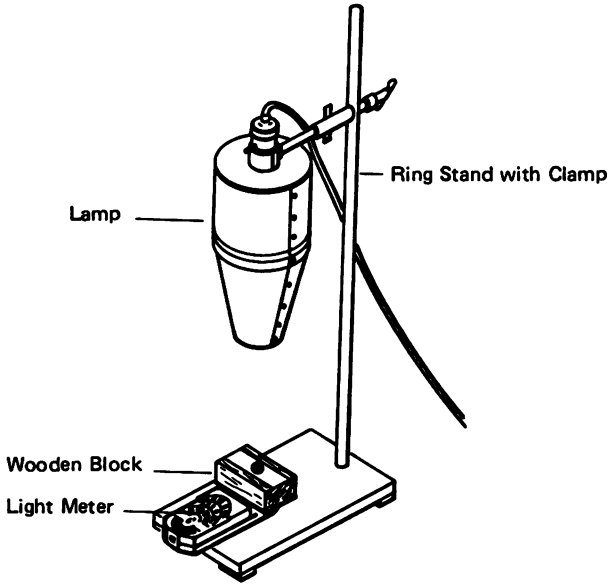
The direct smear technique for egg counting involves using a light meter or photo-electric foot-candle meter in preparing a fecal smear of a known standard density.

APPARATUS (fig. 28)

1. Light meter. The meter used should have the window on the same side as the scale.
2. A lamp, adjustable in height, suspended directly over the window of the meter. Either a 75- or 100-watt bulb may be used.
3. Block about 15-mm thick, 2 to 3 inches long, and with a central hole 16 mm in diameter. The inner surface of the hole may be covered with black ink to reduce light reflection and interference. The block is placed over the meter window to reduce the opening and to provide a uniform light area.

The apparatus can be standardized or calibrated by using barium sulfate suspensions as standards.

Figure 28.
Lamp and Light Meter Apparatus for Standard Smear Egg Count



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PREPARATION OF SOLUTIONS

Stock Solutions

Solutions of 2N sodium sulfate (Na_2SO_4) and 1 N barium chloride ($BaCl_2$) are prepared by analytical methods.

1. Na_2SO_4 (anhydrous) ..... 142.05 gm
   Distilled water to make ..... 1,000.0 ml

   In a 1-liter volumetric flask, dissolve the Na_2SO_4 in a part of the water, then add water to the 1-liter level.

2. $BaCl_2 \cdot H_2O$ ..... 122.16 gm
   Distilled water to make ..... 1000.0 ml

   Prepare as for Na_2SO_4 solution.

3. Prepare salt-glycerin mixtures by mixing (separately) 2 parts of each solution with 1 part of pure glycerin. The salt-glycerin mixtures are stored separately and mixed in varying proportions to give the desired concentration standard.

Standards

The barium chloride solution is added to the sodium sulfate solution slowly, drop by drop, with constant stirring. The proportions used for various standards are as follows:

Parts barium chloride mixture	Parts sodium sulfate mixture	Give barium sulfate suspension for standardizing smears containing feces in the amount of:
3	2	1/200 ml
2	3	1/300 ml
1	2	1/400 ml
1	3	1/500 ml (2 mgm)
1	6	1/1000 ml (1 mgm)

CALIBRATION OF LIGHT METER

1. Adjust the light to a predetermined reading on the scale (Beaver used 20 foot-candles as a setting).
2. Place a clean 3x1-inch slide over the opening of the block covering the light meter window.

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3. Using a pipette which delivers 0.05 ml per drop (a regular medicine dropper is usually satisfactory), place a drop of the thoroughly mixed barium sulfate suspension in the desired concentration on the slide over the block opening. The 1/1,000 (1 mgm) or 1/500 (2 mgm) standards are preferred for routine purposes.
4. With an applicator stick, carefully spread the suspension to exactly fit the opening. If the standard extends beyond this area, the slide should be discarded and a new one prepared.
5. Read the scale. Subsequent fecal preparations should be of sufficient density to cause the same light reduction.
6. With some meter scales, it may be easier to adjust the light so that a known, easily read scale reading is the end point. The main considerations are to determine the reduction in light and intensity with the desired standard concentration and to prepare fecal mounts of the same density.
7. After adjusting the light and calibrating the apparatus, the position should not be changed. Periodic checkings are necessary, however, to be sure that current fluctuations or jarring have not affected the adjustments. In some cases, a voltage regulator is desirable.

PREPARATION OF THE FECAL SMEAR

A 1-mgm or 2-mgm fecal suspension is usually easier to examine than thicker mounts, and the light meter should be calibrated with the corresponding standard (1/1,000 or 1/500).

1. Using a pipette which delivers a 0.05-ml drop (medicine dropper), place a drop of distilled water on a clean slide directly over the block opening.
2. With an applicator stick, emulsify feces in the water, spreading the film to fit the block opening exactly, until the reading on the scale is the same as that of the corresponding standard. If the preparation extends beyond this area or too much feces is added, it should be discarded and a new slide prepared.
3. Cover with a 22-mm square coverslip and, beginning with one corner, systematically count the number of eggs in the preparation. Do not seal the mount, since the paraffin may obscure some eggs.

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4. Use of a 5X ocular will decrease the examination time. Be careful not to overlap fields and recount eggs already counted.
5. To obtain eggs-per-gram count, multiply the number of eggs counted by 1,000 if a 1-mgm preparation is used or by 500 if a 2-mgm preparation is used. No correction factors for soft or loose stools, as required by the Stoll count, are necessary. If no eggs are found in the 2-mgm preparation, report "less than 500 eggs per gram." A 3-mgm (or heavier) preparation is too thick to count the eggs accurately.
6. If several counts are to be made, the meter should be checked with the standard at intervals to make sure the readings have not altered. This is especially important if the flow of electric current fluctuates. A voltage regulator may be used if the current fluctuation is pronounced.

USE OF NONSTANDARDIZED SMEAR FOR EGG COUNTS

If no light meter is available, a rough estimate of the worm burden can be made from ordinary direct wet mounts prepared for routine examination. Experienced technologists usually prepare smears containing 1.5- to 2-mgm of feces. For practical purposes, the number of eggs per gram can be estimated by using 750 for a multiplication factor in step 5. Some laboratories report simply "eggs-per-preparation" without attempting to convert the count to eggs-per-gram. This is satisfactory so long as the person receiving the information can interpret the results. For example, 1 or 2 eggs per smear may represent a very light infection while 15 or 20 may represent a heavy infection.

INTERPRETATION OF RESULTS

Egg counts are interpreted in the same manner as with the Stoll dilution count. In both cases, the counts are estimates and provide an *estimate* of the existing worm burden rather than a specific count.

•KATO THICK-SMEAR TECHNIQUE FOR QUANTITATIVE DETERMINATIONS

The Kato thick-smear technique is a simple quantitative method based on direct examination of feces (Martin and Beaver, 1968). The sample size is about 25 times greater than that used in the

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standardized smear technique. The procedure is the same as the usual Kato technique except a measured quantity of feces is used. The basic steps are: (1) press a standard quantity of feces between a glass slide and a strip of glycerin-soaked cellophane; (2) let the smear stand for a time to clear the fecal film; and (3) examine the entire smear microscopically and count all eggs seen. The technique is effective for most helminth eggs but hookworm and schistosome eggs often collapse shortly after clearing. Therefore, cleared preparations must be examined immediately.

MATERIALS NEEDED

1. 3x1-inch or 3x2-inch slides
2. Cellophane "coverslip" soaked in glycerin-malachite green solution (see Kato technique on page 166 for preparation).
3. Applicator sticks
4. 105-mesh stainless steel bolting cloth - 4-cm squares (W.S. Tyler Co., Cleveland, Ohio)

PROCEDURE

1. Press a portion of stool through the stainless steel mesh cloth.

   Note: If the specimen does not contain a lot of fibers, seeds or other large particles, the screening can be omitted.

2. Weigh out 50 mgm of the screened feces and put on the slide.
3. Cover the feces with glycerin-malachite green-soaked cellophane "coverslips."
4. Invert the preparation and press it down on a flat absorbent surface (a sheet of thick soft paper or filter paper on a table surface is ideal) until the feces are spread over an area about 20 to 25 mm in diameter.
5. Allow the smear to stand for 1 hour at room temperature or 30 minutes in a 37°C incubator.
6. Examine the smear as soon as it is cleared and count all eggs seen. The fecal film will become almost transparent, leaving the eggs uncleared.

   Note: Since hookworm eggs and schistosome eggs tend to collapse and sometimes disappear if overcleared, the smear should be examined immediately after clearing.

7. The number of eggs counted in the 50-mgm sample can be multiplied by 20 to give eggs-per-gram.

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Warren *et al.* (1974) have successfully used this procedure to determine severity of schistosome infections in population groups. They modified the procedure slightly by increasing the clearing time to 48 hours at room temperature. The embryo within the schistosome egg disappears, but the characteristic shape is visible.

•QUANTITATIVE METHOD FOR *SCHISTOSOMA HAEMATOBIUM* EGGS IN URINE

S. haematobium eggs in urine can be quantitated using a Nucleopore filter (Peters *et al.*, 1976a, 1976b). Eggs from a measured volume of urine are collected on a transparent Nucleopore filter, the filter placed on a slide, and the eggs counted. The procedure is rapid, simple and reliable; however, light infections with less than 1 egg per ml may not be detected.

MATERIALS

1. Nucleopore filters, 8-μm pore size, 13-mm diameter (Nucleopore Corporation, Pleasanton, California 94566).
2. Glass syringes.
3. Swinney adapter.
4. 3x2-inch slides.
5. Physiological saline.

PROCEDURE

1. Place the filter in the adapter and attach the syringe barrel.
2. Pour a 10-ml aliquot of the urine specimen into the syringe, insert the plunger, and pass the urine through the filter.
3. Remove the syringe, fill it with air and inject through the filter chamber.
4. Remove the filter and place face down on a slide. The dried filter will stick to the slide.
5. Moisten the filter with a drop of saline to make the eggs clearly visible.
6. With the low-power objective, count all of the eggs on the filter. No staining is necessary.

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7. Results are expressed as eggs/10 ml urine.
8. If the infection is heavy, the pores of the filter may clog. If this happens, use a 5-ml or 1-ml sample of urine.

•COEFFICIENT OF INFECTION FOR *ENTEROBIUS* INFECTIONS

The number of *Enterobius* eggs on a cellulose tape preparation does not reflect the number of worms the patient harbors, since many variables, such as time and method of collection, hygienic habits of the patient, and the egg-laying habits of the female worm, may affect the number of eggs picked up by the tape. Egg deposition apparently does not occur every day, and thus it can be assumed that the greater the number of worms present, the greater the chances that eggs will be found on the day of collection. With 6 consecutive daily collections, if all 6 swabs are positive, the worm burden can be assumed to be higher than if only 1 or 2 positives are found.

On the basis of these assumptions, a relative measure of the average intensity of infection can be determined by calculating the coefficient of infection (CI) (Sadun *et al.*, 1956) as follows:

1. Cellulose tape swabs are collected daily for 6 days from the patients concerned. The swabs should be collected at about the same time each day, preferably between 10 and 12 p.m. The perianal folds should be spread so that the sticky surface of the tape picks up eggs that are present.
2. Examine the tape preparations in the usual manner. Clearing the debris with toluene will facilitate the examination. (See page 165).
3. Record whether positive or negative. Only the presence or absence of eggs is significant; the number of eggs is not.
4. Calculate the CI of a group of patients by adding the products obtained by multiplying the percentage of individuals having positive specimens on no occasion, once, twice, and so on up to six times by the corresponding factors, 0, 1, 2, 3, 4, 5, or 6.

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Example: In a group of 100 patients

30% are positive 0 times = 0
20% are positive 1 times = 20
10% are positive 2 times = 20
20% are positive 3 times = 60
5% are positive 4 times = 20
10% are positive 5 times = 50
5% are positive 6 times = 30
CI 200

5. For individual patients, the intensity of infection can be determined by examining 6 consecutive daily swabs and assigning a value of 1 to a patient found positive 1 time out of the 6 daily specimens, a value of 2 to a patient positive on 2 occasions, and so on up to 6. A patient with a CI of 6 would be positive with every examination.

INTERPRETATION

It can be assumed that the higher the value of the CI, the heavier the infections. For example, if a group has a CI value of 200, the worm burdens can be assumed to be heavier than a group with a CI of 100, and lighter than a group with a value of 360. Likewise, an individual patient with a value of 6 has a heavier infection than a patient with a value of 1 or 2. Although the CI is a rough estimation of worm burden, it can be useful for clinical purposes and for evaluating the effect of therapy.

EGG-HATCHING FOR SCHISTOSOME MIRACIDIA

When feces containing mature living schistosome eggs are diluted with water, the eggs hatch within a few hours. The miracidia tend to collect in the upper layers of the water where they may be located with a magnifying glass such as a hand lens. This is an especially sensitive test for indirectly demonstrating small numbers of viable eggs present in fecal sample. McMullen and Beaver (1945) recommended a flask with a side arm (fig. 29) but for routine purposes an Erlenmeyer flask may be substituted.

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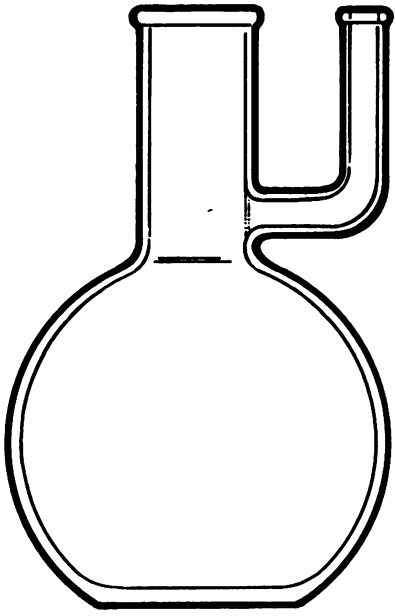
Figure 29.
Side-Arm Flask for Hatching Miracidia



TECHNIQUE

1. Place a sample of feces about the size of a large walnut (approximately 5 gm) into a 250-ml beaker and fill it one-fourth full with water. It is best to use chlorine-free water throughout this entire procedure, since tap water may contain a chlorine content sufficient to kill miracidia. Chlorine-free water may be obtained by boiling tap water for several minutes. The water should then be cooled to room temperature.
2. Comminute thoroughly using a tongue depressor.
3. Add sufficient water to fill the beaker three-fourths full.
4. Strain this suspension through 1 or 2 layers of wet cheesecloth into a sedimentation glass or cone-shaped graduate.
5. Allow the suspension to settle for 1 hour.
6. Carefully pour off supernatant fluid without allowing sediment to escape.

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7. Resuspend the sediment in a small quantity of water and pour it into a 500-ml Erlenmeyer flask or a side-arm flask (fig. 29).
8. Fill the flask almost to the lip with water.
9. Allow the flask to stand at room temperature several hours or overnight.
10. The eggs will hatch, liberating the miracidia, which will swim to the upper layers and collect in the neck or arm of the flask. Painting the lower part of the flask black, or covering it with paper, will improve collection of larvae in the upper layers. Thus the organisms are concentrated in a small area and are more easily visible.

    Caution: If the water is cloudy, the miracidia may be found in all layers of the fluid. This cloudiness may be prevented by washing the sediment once more after Step 6.
11. Against a dark background, the miracidia will be observed as minute, white organisms swimming rapidly in a straight course. They tend to collect around the margin of the flask (Chernin and Dunavan, 1962). A hand lens will aid in detecting the larvae.
12. Coprozoic, free-living ciliates, for example, *Colpoda steini,* may quickly appear in fecal-contaminated water and should not be confused with miracidia. To confirm identification, transfer a few organisms to a 3x2-inch slide and examine under the microscope. A drop of a weak iodine solution or dilute methylene blue will stop the motion of the miracidia.

PROCEDURES FOR MATURING COCCIDIA OOCYSTS

Immature oocysts of *Eimeria* species and of *Isospora* species are similar in appearance and may be difficult to distinguish. Man occasionally ingests oocysts of *Eimeria* accidentally or with animal tissue and subsequently passes them in the feces. Therefore, generic identification of oocysts found in a stool specimen may be important. Differentiation is based on the number of sporocysts present in the mature form and the number of sporozoites within each sporocyst.

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Mature oocysts can be obtained by mixing feces with saline or water, placing a small quantity in a petri dish, and allowing it to stand at room temperature for 2 to 3 days.

Better results can be obtained if bacterial growth is retarded by mixing feces with 2% potassium dichromate solution as follows:

PREPARATION OF POTASSIUM DICHROMATE SOLUTION

Potassium dichromate ($K_2 CR_2 O_7$) crystals..... 2 gm
Distilled water..... 100 ml

Dissolve the crystals in the water. Store in a glass-stoppered bottle.

TECHNIQUE

1. Emulsify a portion of the feces in about 2 volumes of potassium dichromate solution.
2. Pour a small amount into a petri dish to a depth of about one-fourth inch.
3. Cover and leave at room temperature for 2 to 4 days. Be sure the suspension does not dry up. Add a small amount of distilled water if necessary.
4. With a pipette, remove a drop of the sediment to a 3x2-inch slide and cover with a coverglass. Examine under low and high power for the presence of mature oocysts. Stains, such as iodine, are not necessary. The potassium dichromate, however, may stain the oocysts slightly.
5. If the oocysts are too few to be detected in this manner, the material can be concentrated by centrifugation. Transfer the entire contents of the petri dish to a 15-ml conical centrifuge tube and centrifuge at 2,000 r/min (500 g) for 1 to 2 minutes. Examine preparations from the sediment as described above. If the sediment is too dry or too thick, a drop of saline should be used in making the mount.

EXAMINATION OF RECTAL BIOPSY TISSUE

Rectal biopsies have been used with success in the diagnosis of schistosomiasis mansoni (Ottolina and Atencio, 1943) and amebiasis (Juniper *et al.*, 1958).

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•SCHISTOSOMIASIS

Generally, a small piece of tissue is removed from the level of the dorsal fold (Houston valve) with tracheal forceps. Ottolina (1951) demonstrated that the greatest concentration of eggs occurs at about 9 cm from the anus; thus the dorsal valve is a convenient biopsy area. No special preparation of the patient is necessary before biopsy; however, the bowel should be free of feces.

TECHNIQUE FOR EXAMINING

1. Rinse the tissue in saline to remove blood and debris.
2. Press between 2 slides and examine with the low-power magnification of the microscope.
3. Living mature eggs appear somewhat transparent, amber-colored, and contain a well-defined miracidium. Immature eggs, which are filled with a greyish granular mass, and calcified, dead eggs, which appear black, may also be seen (Maldonado, 1967).
4. Examination of the biopsied tissue may be facilitated by adding a drop or two of lacto-phenol (1:1) and letting the tissue stand a few minutes. This procedure clears the tissue, and the eggs may be more distinct than in untreated tissue.
5. Sectioning and staining biopsied tissue is less satisfactory than examining pressed tissue for eggs and is not recommended for diagnosis of *S. mansoni* infections.

• AMEBIASIS

Tissue is obtained by a punch biopsy technique. Juniper *et al.*, (1958) recommended using only lesions located below the middle valve of Houston. Minute mucosal lesions may be removed *in toto*, or if lesions vary in size, specimens should be obtained from both small and large ulcerations. Ulcers selected for biopsy should not be scraped. Prompt fixation and good histologic technique are necessary for positive identification of trophozoites.

TECHNIQUE FOR EXAMINING

1. Remove the biopsied material from the forceps and place on a piece of 3x5-inch card with the mucosal surface down. The specimen should be kept flat. All fragments of mucus and exudate should be retained with the tissue.

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2. Immediately place the card with the specimen face down in 10% Formalin for fixation.
3. Prepare a tissue block in the manner normally used for histologic specimens, being careful to include any tissue fragments or exudate.
4. Section and stain by the routine histologic procedures using a hematoxylin-eosin stain procedure. Prepare the hematoxylin stain as follows:

(a) **Stock hematoxylin stain**

1) Solution 1

Hematoxylin	5 gm
Absolute ethyl alcohol	50 ml

Dissolve the dye in the alcohol.

2) Solution 2

Aluminum ammonium sulfate	100 gm
Distilled water	1,000 ml
Ferric chloride	3 gm

Dissolve the ammonium alum in the water with the aid of heat. Add the ferric chloride.

3) Mix solutions 1 and 2 and bring to a boil.
4) Add 2.5 gm of mercuric oxide. The solution will turn dark purple.
5) Remove the solution from the heat and cool in cold water.

(b) **Stain solution for use**

Hematoxylin stock solution	180.0 ml
Distilled water	50.0 ml
Glacial acetic acid	2.4 ml

Add hematoxylin stock solution to the distilled water and mix. Add the acetic acid and mix again.

TECHNIQUES FOR TRICHINOSIS

Although trichinosis is more frequently diagnosed by serologic methods, it is occasionally desirable to examine biopsied tissue for

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the presence of larvae. In addition, diaphragms or other tissue obtained at human autopsy or from pigs may be examined. Several techniques are available, but the compression and digestion techniques are reliable (within the limits of tissue examination) and simple to perform.

•COMPRESSION TECHNIQUE

This is a simple and rapid method of demonstrating trichina larvae in tissue. Light infections may be missed, however, unless a large portion of tissue is examined. Such an examination would require several preparations. The compression technique is valuable in detecting long-standing infections in which the larvae are dead or calcified and in early infections in which the larvae are immature. In addition, no special equipment is needed. Gould (1945) advocated a simple slide compression as a routine procedure and a more elaborate method using a ring-shaped trichinoscope. The simple slide method is described here.

TECHNIQUE

1. Cut very thin slices from the tissue specimen and press between 2 slides.
2. Examine with low power for the presence of larvae.
3. A drop of eosin may be added to the tissue to slightly stain the larvae.

•DIGESTION TECHNIQUE

The digestion technique permits examination of a larger tissue sample than compression or sectioning and staining methods and may result in the detection of light cases that might otherwise be missed. It recovers living larvae very well, but it does not recover dead ones. Furthermore, the digestion technique may destroy immature larvae and thus miss early infections. Also, digestion will cause calcified cysts, usually present in long-standing infections, to disintegrate. Nolan and Bozicevich (1938) suggested the following procedure.

EQUIPMENT

1. Erlenmeyer flask 100-or 150-ml size. Larger sizes will be needed if large samples of tissue, such as whole diaphragms from pigs or human autopsies, are to be handled.

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2. Mechanical shaker or stirrer.
3. Baermann apparatus. For small tissue samples of approximately 1 gram, only a small funnel is needed. Larger samples will require a proportionately larger apparatus. (See figure 24, p. 159)
   (a) To the neck of a small funnel (2 to 3 inches wide) attach a short 2- to 3-inch length of rubber tubing fitted with a pinch clamp.
   (b) Place a piece of fine screen, about 80-mesh, in the top of the funnel. If a regular sieve with a metal edge is not available, use a circle of fine screen wire and turn the edges up about ½ inch or so to make it fit down inside the funnel.
   (c) Put the funnel apparatus in a ring holder on a ringstand to hold it upright.
4. Slides, 3x2-inch size.
5. Pipette with rubber bulb.

PREPARATION OF SOLUTION

Artificial Digestive Juice

Pepsin	5 gm
Hydrochloric acid, concentrated	7 ml
Distilled water	1,000 ml

Dissolve the pepsin in the water and add the acid. Mix thoroughly.

TECHNIQUE

1. Place 1 gm of finely ground tissue in 20 ml of digestive juice in a small Erlenmeyer flask.
2. Place flask in a 37°C incubator for 18 hours or longer.
3. Stir mixture thoroughly at half-hour intervals.
4. Add 2 to 3 volumes of water heated to 37° to 45°C to the digestate. The exact amount is unimportant.
5. Pour the diluted digestate into the Baermann funnel and add warm water sufficient to cover the screen.
6. Let stand 1 hour or longer. The larvae will collect in the lower part of the tube and in the neck of the funnel.
7. Draw off a few ml of fluid and place a drop on a 3x2-inch slide. Examine with the 10X objective for living larvae.

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8. If no larvae are found, the sample can be centrifuged at 2,000 r/min (500 g) for 1 to 2 minutes, and the sediment examined.
9. Large tissue specimens will require larger quantities of digestive fluid. For a sample of 50 gm or so, approximately 3 liters will be needed for proper digestion.

RECOVERY OF HELMINTHS FROM FECES

Occasionally, small helminths, proglottids and scoleces are passed in feces and must be recovered for identification. This may be done by comminuting a small amount of specimen with water and slowly straining the suspension in small portions through a piece of screen wire, a sieve, or a layer of wet gauze. Screens are more satisfactory than gauze, since small white objects such as scoleces often are trapped in the gauze fibers or are very difficult to detect against the white background. A small-mesh sieve (30-50 mesh) is especially useful for this purpose. The sediment remaining on the screen from each portion is washed by running a slow current of water over it. A large hand lens is helpful in examining the cleansed debris. Collecting the strained sediment in a shallow pan, the bottom of which has been painted black, or in a glass dish placed over a black surface will facilitate the examination by increasing the contrast of organisms against the background. Small worms and segments may be picked up with an applicator stick or a camel's hair brush, placed in a drop of saline on a slide and examined directly. If necessary, they may be cleared or stained.

An alternate method for recovery of scoleces, or similar small objects, is to pour small portions of a well-comminuted fecal suspension into a glass petri dish placed over a black background and to scan the thin layer with a hand lens. Discard each portion as it is scanned.

RECOVERY OF LARVAE AND EGGS FROM SOIL

Soil samples are sometimes examined for the presence of larvae. These samples can be cultured in the same fashion as feces and the larvae recovered with the Baermann apparatus. See remarks on helminth larvae in the section on Cultivation.

In some cases, culturing may not be necessary, and the soil sample can be placed directly in the Baermann apparatus. Larvae may mi-

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grate out of the soil more slowly than from the feces culture, and recovery may require several hours. Unfortunately, not all nematode species will migrate and be recovered by this method.

Larvae of free-living species must be separated from those of parasitic species. This can be done by the procedure described on p. 161. for helminth cultures. Eggs and larvae may possibly be recovered from soil, compost or sludge by the same methods used to concentrate feces: brine flotation, zinc sulfate centrifugal flotation, and Formalin-ether sedimentation. A modification of the Formalin-ether method using a nonionic detergent, sodium sulphonate, was developed by Steer *et al.* (1974) to recover eggs from compost.

Soil and sludge samples must be thoroughly mixed with water to provide a uniform suspension. If necessary, the mixture can be homogenized in a blender such as a Waring Blendor. The homogenized sample is then passed through a fine sieve. Aliquots (10 to 20 ml) are processed in the same manner as fecal specimens. If brine flotation is used, centrifuge the sample at 2,000 r/min (450-500g) for about 2 to 5 minutes and pour off the supernatant fluid. The sediment can be transferred to a flotation vial (2x1-inch) as described for the brine flotation method for feces. As an alternate method, brine can be added to the sediment in the centrifuge tube, mixed thoroughly, and the tube centrifuged for about 2 minutes at 2,000 r/min (450-500g). The surface film is examined in the same way as described for the zinc sulfate centrifugal flotation method for feces.

COLLECTING AND SHIPPING SPECIMENS FOR SEROLOGIC TESTS FOR PARASITES

Although this manual does not cover immunodiagnostic procedures, the collection of satisfactory specimens for these methods is included. Many laboratories submit blood or serum specimens to reference laboratories for serologic testing, and these specimens must be properly collected and shipped.

Serum specimens to be tested for parasitic disease are collected in the usual manner. Three to five ml of clean serum are usually adequate for testing. If the specimens are to be mailed, aqueous sodium azide (1:10,000) is added to prevent bacterial growth during shipment. It does not interfere with the tests.

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The following precautions should be exercised in securing and mailing specimens:

A. COLLECTION OF SERUM

1. Draw blood before the individual has had breakfast or at least 3 hours after the last meal in order to avoid chylous specimens.
2. Use only sterile, dry apparatus. All apparatus for collecting specimens should be washed free of alkali and acid before it is sterilized.
3. Use a dry needle and syringe.
4. Allow the blood to clot at room temperature (1 to 2 hours), then place it in the refrigerator to retract the clot. Draw off the serum with a sterile pipette.
5. The serum should not be inactivated (heated).

B. PREPARATION AND USE OF SODIUM AZIDE SOLUTION

1. Preparation of 1% Stock Sodium Azide Solution
   a. Dissolve 1 gm of powdered sodium azide in 100 ml of distilled water.
   b. Store in the refrigerator. The solution may be kept up to 30 days.
2. Use
   Add 0.01 ml of a 1% aqueous solution of sodium azide per ml of serum. This results in a 1:10,000 concentration of sodium azide.

C. PACKING AND MAILING SPECIMENS

1. Seal tubes containing serum for shipment with taped rubber stoppers or screw tops to prevent leakage. Specimens sent via airmail should have particularly well taped stoppers to prevent "blowing." Label properly for identification.
2. The tube should be wrapped in cotton, tissue, or some absorbent packing material and packed in the inner container or cylinder of a double mailing container, or in a specially designed styrofoam mailing container. Packing must be adequate to prevent breakage or leakage (which might soil the mail) and must conform to postal regulations for shipping such material.
3. Enclose a copy of the covering letter or report form with each serum specimen. If possible, include a brief statement of the patient's illness. Wrap the sheets around the outside of the inner

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cylinder of the mailing container or pack it in the styrofoam container.

4. Specimens that may be tested at CDC should be sent to the State Laboratory rather than directly to CDC. If the State Laboratory does not perform the test, it will forward the serum to CDC.
5. Whenever possible, ship specimens so that they do not arrive on the weekend. Be sure that the package is addressed properly and that the contents are identified (e.g., "Serum Specimen") for rapid handling in the reference laboratory.

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QUALITY CONTROL

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QUALITY CONTROL

Quality controls in parasitology are perhaps less well defined and less rigid than in other areas of microbiology. Nevertheless, certain checks and periodic monitoring should be undertaken to maintain a satisfactory level of diagnostic proficiency.

Reliable laboratory diagnosis of intestinal parasites is based on accurate identification of organisms and competent performance of the techniques for recovering and demonstrating these organisms. Proper performance requires appropriate equipment and periodic checks on both equipment and materials. Controls must apply in all areas.

TRAINING OF PERSONNEL

The person examining parasitologic specimens must be familiar with collection methods, the appropriate technical procedures to be used for the type of material and organisms being searched for, and the morphologic differentiation of parasites or their diagnostic stages. This capability is acquired through supervised study in a formal course or on-the-job training with a competent parasitologist. The amount of training required varies with the individual, but a general estimate of 150 hours of initial study, including both lectures and laboratory practice, may be considered adequate. Furthermore, unless specimens are being examined frequently, periodic refresher training is needed to maintain an acceptable level of proficiency.

Organized courses in laboratory methods in medical parasitology are presented at CDC. Applicants for these courses must have had 1 to 2 years' experience in a clinical or public health laboratory. For more details contact Centers for Disease Control, Laboratory Training and Consultation Division, Atlanta, Georgia 30333.

Self-instructional programs are available in some areas of parasitology and several atlases, slide sets, and other pictorial references have been produced by various sources.

The American Society of Clinical Pathologists (2100 West Harrison Street, Chicago, Illinois 60612) has three *Atlases of Diagnostic Parasitology* available as sets of 2x2-inch color slides or as microfiche. In addition, CDC has developed a training aid series entitled

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"Laboratory Update" that includes a number of parasitology programs. These consist of sets of 2x2-inch color slides with taped narrations. Some are self-instructional. The Laboratory Update programs are available on loan from most State Laboratories. A listing of programs available can be obtained from Parasitology Training Branch, Centers for Disease Control, Atlanta, Georgia 30333. Many of the programs can be purchased at a nominal cost from National Center for Continuing Education, P.O. Box 241, Batavia, Illinois 60510.

EQUIPMENT

Adequate working space with access to a sink is essential. Fortunately, however, parasitologic services require little special equipment. The basic items needed are a good binocular microscope, a centrifuge, a refrigerator, and a chemical exhaust or fume hood.

MICROSCOPE

Since the microscope is the most important single piece of equipment, it is essential that it meet the standards established for parasitology and that it be properly used and cared for. The criteria for the microscope and illuminator, their use and care are described in the section on Microscopy, p. 67.

CENTRIFUGE

Either a table or floor model centrifuge is satisfactory so long as it is equipped to handle 15-ml centrifuge tubes (for Formalin-ether) or the 100x13-mm tubes used for the zinc sulfate technique. Of the table model centrifuges, only the horizontal type should be used. Angle head centrifuges are unsatisfactory for parasitologic specimens.

The centrifuge should be calibrated with a tachometer and marked or labeled at the settings for the speeds routinely used.

The mechanism should receive periodic checks and maintenance.

REFRIGERATOR

Any ordinary household type of refrigerator is satisfactory. The temperature should be approximately 40°F or 5°C. Specimens should not be put in the freezer section since freezing and thawing may destroy organisms.

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OTHER EQUIPMENT

An exhaust or fume hood should be available for handling PVA-fixed specimens and similar materials. A 37°C incubator for drying slides is desirable but not essential. If cultures for protozoa are made, however, incubators are necessary.

In addition to major equipment, smaller items such as a hot plate for melting the paraffin-Vaseline mixture and a magnetic stirrer for preparing solutions such as PVA-fixative, which require extensive mixing, are desirable.

There are several expendable items, such as 3x2-inch slides, which are more or less peculiar to the parasitology laboratory. Suggested supplies and equipment for a laboratory doing parasitologic examinations are listed on pp. 199-201.

TECHNICAL PROCEDURES

The laboratory should establish a routine for collection and handling of parasitologic specimens that will insure reliable examinations.

Proper collection and handling of specimens and selection of techniques for clinical and public health laboratories are discussed in separate sections of this manual.

PERFORMANCE

The techniques should be performed carefully, with close attention to the steps where technical errors are most likely. For example:

Wet Mounts

1. Density of the preparation is important and each mount should be checked for proper density as it is prepared. Too thick or too thin preparations will reduce the accuracy and reliability of diagnosis.
2. To prevent contamination of the stock solutions, the drops of diluents should be put on the slide *before* the feces are added.

Concentrations

1. The amount of sediment obtained in both initial and final centrifugations is critical and should be controlled or regulated as suggested in the technique directions.
2. The gauze used for straining the specimen should be of the proper thickness and should be wet before using, to reduce loss of organisms.

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Staining

1. Timing of the various steps, especially staining and destaining, should be carefully regulated.
2. Transfer of solutions from one dish to another as the slides are moved should be kept to a minimum to maintain the solutions in good condition. Slides should be drained of excess solution between steps by touching the edges to a paper towel.
3. The stain dishes should be kept covered at all times except when slides are being transferred into or out of the dish. If left uncovered, dehydrating solutions will absorb moisture from the air and become ineffective.
4. The amount of mounting medium used should be regulated. Too much medium may interfere with focusing and proper examination of the smear. Too little material will result in equally poor preparations.
5. Stained smears should be examined with oil-immersion for accurate identification of organisms.

Solutions

In addition to proper performance of techniques, the solutions used in the various procedures must be carefully prepared, and many require periodic checking.

1. Saline solution. The solution in the dropping bottle should be checked occasionally for free-living organisms, particularly flagellates and ciliates. To do this, place a drop of solution on a slide, cover with a coverslip, and examine microscopically. Contamination may result from accidentally picking up feces on the end of the pipette when preparing wet mounts. Avoid this by putting the solution on the slide first.

   The solution in the dropping bottle should be replaced occasionally (approximately 2- to 3-month intervals) even though it does not appear contaminated.
2. Iodine solution. A 1% iodine solution, such as Dobell's or D'Antoni's, should be used and should be prepared fresh each 10 to 14 days (see figure 13). If Lugol's iodine is used, it should be diluted 1:5 with saline or distilled water. Stock Lugol's should be prepared fresh each 3 weeks; the dilution will need to be changed each 10 to 14 days.
3. Temporary stains: Buffered Methylene Blue and Quensel's. These are stable and may be used indefinitely. Contamination of the dropping bottle pipettes should be avoided.

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4. Zinc sulfate solution. The specific gravity should be checked with a hydrometer at the time of preparation and at 1- to 2-month intervals to insure the proper density (specific gravity) for floating organisms.

   A specific gravity of 1.18 should be used for fresh fecal specimens and 1.20 for formalinized specimens.
5. Formalin and ether do not need specific checks for controls, beyond safety precautions for storing and handling the ether. See section on Safety Precautions for recommended methods of storing and handling ether.
6. Staining solutions should be changed as needed. The exact time for changing will depend on the technique and frequency of use. New batches of stock stain should be checked for staining quality.
   a. Hematoxylin stain dilution (0.5%) is not stable and must be prepared fresh each day or each time used.
   b. Trichrome and chlorazol black E stains are stable but will need replacing when staining becomes poor. The criteria for good staining are described in the directions for these procedures.
   c. Iodine-alcohol should be a strong tea color and should be replaced when faded. This is approximately every 2 weeks. (See figure 20.)
   d. Iron-alum solutions should be prepared fresh each 2 to 3 days.
   e. Dehydrating and clearing solutions such as alcohols, carbol-xylene, and xylene must be kept free of moisture. Stock bottles should be sealed or tightly closed. Staining dishes of these solutions should be kept covered at all times except when slides are being transferred in or out. Coplin jars with screw-type lids or lids with ground rims should be used. A lubricant can be used with the latter type to insure a tight cover.

CHECKS

Ideally, positive control specimens should be run periodically to check on the reliability of the procedure. However, since positive materials are not always readily available, some compromise is necessary.

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If the criteria for satisfactory performance are observed, the technologist can assume that organisms present will be recovered or demonstrated. Stain preparations can be evaluated to some extend by the color and differentiation of the normal fecal elements. Smears of feces containing epithelial cells and pus cells are particularly useful for evaluating stains.

Positive Formalin specimens and unstained PVA-fixed fecal specimens can be purchased from certain commercial companies but may be too expensive for routine checks.

Positive animal specimens, such as monkey feces, can be substituted for human specimens, but they usually contain more coarse debris and may be more difficult to handle than human feces. The best sources of specimens for check purposes are those routinely received for diagnosis. Positive specimens should be preserved in Formalin or PVA-fixative for future use.

REFERENCE MATERIALS

Positive reference specimens should be available for comparison with unknown organisms. Textbook descriptions and pictures are usually inadequate, although diagrams may be used along with known specimens.

Ideally, a laboratory offering parasitologic services should have Formalin preserved specimens of all the common helminth eggs and larvae and protozoan cysts and stained fecal smears of protozoan trophozoites and cysts. A suggested list of reference species is on p. 198. Since parasites may be restricted in geographical distribution, in some laboratories certain of the species listed would probably be rarely, if ever, encountered. Therefore, the laboratory director or supervisor should determine which species should be available in a particular laboratory.

Laboratories should have positive specimens received for routine examination for use as reference material. Refer to the section on Preservation of Specimens, p. 59, for procedures for preparing Formalin suspensions. Stained reference slides are prepared in the same way as slides for diagnosis. Parasitologic material can also be purchased from commercial companies, but not all species are continuously or readily available.

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Color slides or transparencies may be substituted for actual specimens, but they are usually less useful for protozoa than for helminths. They can be used indefinitely, however, and provide a ready "picture" of the organism. Unfortunately, the level of focus cannot be changed, and a change is often necessary for accurate identification. Color slides may be purchased from several commercial sources. The CDC Laboratory Update series 2x2-inch color slides could also serve as reference material.

A current list of commercial companies offering specimens and/or color slides can be obtained on request from CDC. (See address on p. 192.)

The laboratory should also have reference manuals on hand. These may include textbooks on clinical parasitology and technical manuals. Separate manuals of procedures used routinely in the laboratory should be kept at the bench where the work is performed. Changes in the procedures and adoption of new procedures should be initialed and dated by the director.

In addition to the present manual, CDC has produced four other manuals dealing with intestinal parasites. These are currently available from National Technical Information Service, 5285 Port Royal Road, Springfield, Virginia 22161. The NTIS number is listed for each publication; contact NTIS directly for prices.

1. *Laboratory Procedures for the Diagnosis of Intestinal Parasites,* third edition, 1982. NTIS#: PB297958.
2. *Morphology of Diagnostic Stages of Intestinal Parasites of Man* - Diagrams of the various diagnostic stages drawn to scale. (Revised 1978): NTIS#: PB297962.
3. *Common Intestinal Helminths of Man - Life Cycle Charts* (1964) NTIS#: PB80-186976.
4. *Common Intestinal Protozoa of Man - Life Cycle Charts* (1964) NTIS#: PB80-186994
5. *Common Intestinal Protozoa of Man - Life Cycle Charts* and *Common Intestinal Helminths of Man - Life Cycle Charts* - combined NTIS#: PB80-186960.
6. *Amebiasis: Laboratory Diagnosis, a Self-Instructional Lesson* (Revised 1976)

I.	*Life Cycle of Entamoeba histolytica*	NTIS#: PB80-145592
II.	*Identification of Intestinal Amebae*	NTIS#: PB80-145600
III.	*Laboratory Procedures*	NTIS#: PB80-145618
Complete set: Parts I, II, and III		NTIS#: PB80-145584

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PARASITE REFERENCE COLLECTION

INTESTINAL AND UROGENITAL PARASITES

A. Protozoa

Entamoeba histolytica trophozoites and cysts
Entamoeba hartmanni trophozoites and cysts
Entamoeba coli trophozoites and cysts
Endolimax nana trophozoites and cysts
Iodamoeba bütschlii trophozoites and cysts
Dientamoeba fragilis trophozoites
Trichomonas hominis trophozoites
Trichomonas vaginalis trophozoites
Giardia lambia trophozoites and cysts
Chilomastix mesnili trophozoites and cysts

B. Helminths

Enterobius vermicularis eggs
Trichuris trichiura eggs
Ascaris lumbricoides eggs
Hookworm eggs
Strongyloides stercoralis larvae
Taenia spp. eggs
Hymenolepis nana eggs

C. Optional Helminths

These species may be important in some areas but not in others.

Diphyllobothrium latum eggs
Schistosoma mansoni eggs
Schistosoma japonicum eggs
Schistosoma haematobium eggs
Clonorchis sinensis eggs
Paragonimus westermani eggs
Fasciolopsis buski eggs

BLOOD PARASITES

A. Malaria

Plasmodium vivax
Plasmodium falciparum

B. Hemoflagellates (Optional)

Trypanosoma cruzi
Trypanosoma brucei gambiense or *rhodesiense*

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EQUIPMENT AND MATERIALS FOR LABORATORY DIAGNOSTIC SERVICES IN PARASITOLOGY

For routine diagnostic services in parasitology, both blood and intestinal parasites, a laboratory should have the following in addition to the usual glassware and chemicals ordinarily available. Certain items listed are used in specific procedures and if the laboratory does not perform the procedure, these items can be omitted.

NONEXPENDABLE

1. Microscope(s) with adequate illumination.
2. Calibrated ocular micrometer.
3. Centrifuge —either table model or floor model. The centrifuge should not be an angle-head model.
4. Incubator, 37°C. Optional; however, an incubator is essential if fecal cultures are done.
5. Refrigerator—ordinary household model.
6. Exhaust hood.

EXPENDABLE

1. Applicator sticks, wooden.
2. Blood lancets, disposable.
3. Bottle of immersion oil, low viscosity.
4. Cotton swabs.
5. Coverslips, 22-mm square, #1 thickness.
6. Dispensing or squeeze bottles, 250 ml.
7. Dropping bottles:
   - Physiological saline.
   - Dobell's or D'Antoni's iodine (or Lugol's diluted 1:5).
   - Buffered methylene blue or Quensel's
   - Methyl alcohol (for blood staining).
8. Facial tissue.
9. Forceps.
10. Funnels, small or 4-oz conical paper cups with tips cut off.
11. Gauze squares—approximately 6-inch squares for straining fecal specimens. (If mesh is narrow or fine, 1 thickness is sufficient; if mesh is wide, 2 thicknesses are necessary).
12. Gauze squares, 2x2-inch, sterile (for cleaning fingers).

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13. Hand lens.
14. Hot plate.
15. Labeling pens or markers.
16. Lens paper.
17. Microscope slides - 3x1-inch, plain.
    3x1-inch, frosted ends.
    3x2-inch.
18. Pipettes, capillary (Pasteur), 5¾ inches long, disposable.
19. Pipettes, serologic – 1 ml, 5 ml, 10 ml.
20. Rubber bulbs for capillary pipettes.
21. Scotch tape, clear ¾-inch width (for pinworm preparations).
22. Staining dishes, Coplin 50-ml capacity, with lids.
23. Slide boxes–50 or 100 capacity.
24. Slide folders - to hold 20 3x1-inch slides; to hold 10 3x2-inch slides.
25. Slide labels.
26. Timer.
27. Tongue blades.
28. Vaseline-paraffin (in beaker).
29. For Formalin-ether or acid-ether techniques:
    a. Centrifuge tubes, 15-ml, pointed, preferably graduated.
    b. 4-oz flat-bottom paper cups (or small beakers).
    c. Rubber stoppers, #0 to fit tubes.
    d. Rack to hold 15-ml tubes.
30. Zinc sulfate technique:
    a. 100x13-mm tubes.
    b. Rack to hold tubes
    c. Wire loops.

SOLUTIONS AND CHEMICALS

The individual chemicals or dyes needed to make the various solutions are not listed; however, certain compounds which must be readily available are listed. Some of the items are peculiar to certain procedures, and every laboratory may not need them.

1. Alcohols:
    a. Ethyl alcohol–70%, 95%, and 100%.
    b. Methyl alcohol, absolute.
    c. Acid alcohol–for trichrome stain.
2. Buffers, stock M/15 solutions (for blood staining):
    a. Sodium dihydrogen phosphate.
    b. Disodium hydrogen phosphate.
3. Carbol-xylene.

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4. Distilled water.
5. Ether—anesthesia or technical grade—or - Ethyl acetate.
6. Ferric alum crystals—if hematoxylin stains are used. (Store in refrigerator.)
7. Formalin—5% and 10%.
8. Glacial acetic acid.
9. Glycerin.
10. Immersion oil, low vicosity.
11. Iodine crystals.
    Note: To prepare Dobell's, D'Antoni's, or Lugol's iodine, the crystals are added to KI at the time of use. However, a strong iodine-alcohol solution (for staining) can be prepared, stored in a brown bottle (for several weeks) and diluted with 70% alcohol to the proper color at the time of use.
12. Physiological saline.
13. Potassium iodide - 2% (5% if Lugol's iodine solution is used).
14. PVA-fixative solution.
15. Phosphotungstic acid, 2%—if used in the staining procedure employed.
16. Permount or similar mounting medium.
17. Schaudinn's fixative.
18. Stains:
    a. Buffered methylene blue or Quensel's.
    b. Giemsa.
    c. Trichrome or stock hematoxylin or chlorazol black E.
19. Xylene.
20. Zinc sulfate, sp. grav. 1.18 - if technique is used.

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SAFETY PRECAUTIONS

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SAFETY PRECAUTIONS

Because of the moderate risks involved, safety precautions as applied to parasitology are less elaborate than those required for other areas of microbiology but are nonetheless important. The major factors are personal hygiene and health safeguards; disposal of specimens containing viable organisms and handling of dangerous chemicals; storage and disposal of flammable solvents and other reagents; and equipment safety and maintenance. Most of the material presented here has been taken directly or adapted from the CDC Manual, *Laboratory Safety at the Centers for Disease Control,* and is intended to suggest safety practices in clinical laboratories performing parasitologic examinations. The material does not include all of the safety precautions that should be employed by clinical laboratories nor does it cover regulations specified by the various regulatory agencies.

PERSONAL HYGIENE AND HEALTH PRACTICES

The most effective health measure in parasitology is *good personal hygiene.*

Most intestinal parasitic infections are acquired by ingesting the infective stage. Among the helminths, with a few exceptions, the diagnostic stages are not infective and thus present no real hazard; however, protozoan cysts, which may be present in the feces, are infective and precautions are needed. Follow these rules:

a. Do not eat, drink or smoke in the laboratory.
b. Wash hands frequently with soap and hot water.
c. Wear a protective garment (lab coat or apron) in the laboratory; but do *not* wear this garment outside of the laboratory area.
d. Clean up spills with paper towels or sponges and wash this area with disinfectant followed by hot soapy water. Soiled sponges should be cleaned by rinsing them several times in disinfectant and then in hot water. Soiled paper towels can be discarded along with similar laboratory wastes, or in lieu of this, can be soaked in disinfectant for 10 to 15 minutes or longer, and then discarded in the waste basket. Hands should be washed thoroughly with hot soapy water when you finish cleaning up.

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e. Eye wash fountains or sprays should be readily available for emergency use.

Although in the average clinical laboratory the blood and tissue parasites pose fewer problems with regard to personal hygiene, certain blood parasites can be acquired through contamination of lesions or cuts on the hands and tissue parasites, such as trichinosis, can be acquired by ingestion of infected material. Following the rules listed here, plus wearing rubber gloves if you have cuts or lesions on your hands, should reduce the possibility of infection. Additional information on handling infectious organisms will be found in the section dealing with viable organisms.

IMMUNIZATIONS

It is suggested that persons working with fecal specimens that might contain viable organisms be immunized against polio and possibly other organisms that might be passed in feces, if recommended by your hospital or institution. The immunizations should be given in accordance with the current recommendations of the PHS Advisory Committee on Immunization Practices.

Unfortunately, at the present time, there are no effective immunizations for parasitic infections.

DISPOSAL OF SPECIMENS AND HANDLING OF SPECIFIC ORGANISMS

A. SPECIMENS

Specimens containing viable organisms may include feces, blood or other fluid or tissue. In some cases, these may be potential sources of infection and must not only be *handled* with care but *disposed* of with care. The disinfectants used for bacterial agents are generally effective against parasites and covering or immersing the specimen in such a solution for several minutes will kill them. In the absence of other solutions, 10% Formalin, 70% ethyl alcohol, isopropyl alcohol, or undiluted laundry bleaches (50,000 ppm available chlorine) can be used. If fecal specimens are collected in paper containers, the entire carton and contents can be incinerated.

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Incineration is a recommended method of disposing of parasitologic specimens; however, this may not be feasible or practical. If specimens cannot be incinerated, they can be treated with disinfectants as described in the preceding paragraph and then flushed down the toilet or disposed of in some other suitable manner. Most laboratories already have methods of disposing of such material.

Autoclaving is also effective in destroying parasites but is not recommended for fecal specimens because of the extremely obnoxious odors produced.

Fresh fecal specimens may also contain pathogenic bacteria and viruses. Thus, for personal safety, these specimens should be treated with the precautions usual for such agents.

B. SPECIFIC ORGANISMS

Relatively few of the parasite species would be considered hazardous to the average clinical laboratory worker engaged only in parasitologic diagnoses and not involved in research studies. The organisms that should be given special consideration are *Leishmania* species, *Trypanosoma* species, *Naegleria* or *Acanthamoeba* species, *Toxoplasma gondii*, and malaria species. These organisms can gain access to the body by inhalation of aerosols, through accidental inoculation of viable material, or through cuts or lesions on the skin. Material containing, or suspected to contain, these organisms in a viable state should be handled with rubber gloves and under a biological safety cabinet. Workers should wear gowns and surgical masks. If pipetting is necessary, automatic pipettes or pipettes fitted with rubber bulbs are mandatory; pipetting by mouth should *NOT* be done. (High-speed dispensing pipettes are not recommended for pipetting highly virulent organisms because if the line ruptures, a widespread aerosol is created which may be dangerous to the user.) Work surfaces should be cleaned thoroughly with a disinfectant when tasks are finished. In general, the precautions normally employed for other highly infectious agents should apply to these species. Dried blood films or other tissue smears containing these parasites are not infectious, of course, and may be handled as other similar preparations.

Cysts of *Entamoeba histolytica, Giardia lamblia* and other intestinal protozoa are also infective to man, but good personal hygiene should eliminate or reduce the likelihood of infection

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with these agents since they are acquired by ingestion of contaminated material.

Helminth infections are less likely to be acquired in the laboratory than are the protozoan infections, since the diagnostic stages found in feces or other material are usually not infective. There are, however, two exceptions which might be encountered in clinical laboratories: *Enterobius vermicularis* (pinworm) and *Hymenolepis nana* (dwarf tapeworm). The eggs of each of these worms are infective immediately or shortly after passage and the microscopist making the examinations could possibly be exposed to infections. Fortunately, neither of these species causes serious or life-threatening conditions and both are generally self-limiting in the absence of reinfection. Nevertheless, few people relish the idea of harboring a worm parasite no matter how benign it might be and precautions to avoid infection should be exercised. Since, like most intestinal parasitoses, they are acquired by ingestion (in this case, ingestion of the embryonated eggs), good personal hygiene should reduce or eliminate the possibilities of infection.

One other helminth might possibly be considered: *Trichinella spiralis*. Because the *T. spiralis* larvae in tissue are infective, accidental ingestion of material containing these organisms could possibly result in infection. Again, good personal hygiene and care in handling the specimen should prevent infection.

HANDLING OF DANGEROUS CHEMICALS

A. CLASSIFICATION

Dangerous chemicals include the following classifications commonly found in parasitology laboratories.

1. **Caustic or corrosive.** Examples: Acids or bases which may burn or otherwise damage the skin and other human tissue. Consideration also must be given to corrosion of equipment.
2. **Poison.** In this category are substances which are so poisonous that inhalation or ingestion of relatively small amounts will produce death or other serious effects. These may be solid, liquid or gas. Containers should be clearly marked as POISON.
3. **Flammable.** (Liquids and solids.) Such materials that will easily ignite, burn, and serve as fuel for a fire.

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4. **Explosive.** Although many explosive materials are also flammable, these substances will explode under special conditions, e.g., when subjected to shock or excess heat. Such materials must receive handling designed to eliminate exposure to or attainment of those conditions.
5. Other classes of chemicals less often used in parasitology laboratories include compressed gases, oxidizers and peroxides, and radioactive chemicals.

Special care should be taken when handling, storing or disposing of these chemicals. Several "dangerous chemicals" are used in parasitology including phenol crystals and picric acid (explosive), mercuric chloride (poison and corrosive), sodium hydroxide and various acids (caustic), among others.

Polyvinyl alcohol (PVA)-fixative and Schaudinn's fixative solutions are poisonous because they contain large amounts of mercuric chloride and should be clearly marked "poison." If vials of PVA-fixative are given to patients for collecting fecal specimens, be sure the vials are clearly labelled POISON and the patient is instructed not to swallow any of the solution.

B. DISPOSAL OF DANGEROUS CHEMICALS

Disposal of acids, caustic and corrosive substances should be done with care. Most laboratories have procedures for doing this. If not, procedures are outlined in the manual, *Guide for Safety in the Clinical Laboratory* produced by Manufacturing Chemists Association. (See p. 212 for source.) All laboratories should have access to a copy of this manual.

One of the chief problems in parasitology is disposal of fixative solutions containing mercuric chloride, commonly, Schaudinn's fixative. Mercury compounds *cannot* be poured down the sink or discarded on or in the soil because of subsequent environmental contamination. Most states as well as the Environmental Protection Agency (EPA) have regulations prohibiting disposal of mercury compounds into the environment. At present, there are no really good recommendations for getting rid of this solution. Regulations for feasible and proper disposal are being developed by EPA, but until these are available, the solutions should be disposed of in a currently approved manner. Consult the manual referred to in the previous paragraph for methods of handling mercuric compounds.

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STORAGE AND DISPOSAL OF FLAMMABLE SOLVENTS

A. DEFINITION

Flammable solvents are defined as liquid substances having a flash point below 140°F (60°C) and having a vapor pressure not exceeding 40 p.s.i. at 100°F (38°C). These include several compounds used in parasitology: acetone, ether, xylene, toluene, benzene and alcohols: ethyl, methyl, and isopropyl.

B. STORAGE

1. Refrigerators

Kitchen type refrigerators are occasionally used in the laboratory. Some of these may have been modified to make them safe for flammable solvents; others have not. Nonmodified refrigerators must *NOT* be used for storing flammable solvents. It is suggested that each refrigerator be properly identified by a sign on the door, for example:

CAUTION. THIS BOX IS NOT EXPLOSION PROOF, BUT INTERNAL WIRING HAS BEEN MODIFIED TO PERMIT STORAGE OF WELL-STOPPERED FLAMMABLE OR EXPLOSIVE MATERIALS

or

DO NOT STORE ETHER, ACETONE, OR OTHER VOLATILE OR FLAMMABLE MATERIALS IN THIS LOCATION

2. Open Shelf

Considerable confusion exists about using refrigerators to store flammable solvents. *In many cases it is better to store them on well-ventilated open shelves.*

Flammable solvents generally should be stored in refrigerators *only* if chilled solvent is required in a laboratory procedure. Solvents with very low flash points, for example, ethyl ether (F.P. −49°F), should *NOT* be stored in refrigerators since considerable evaporation can occur at low temperatures. Opening the door can release these vapors, and, if an ignition source exists in the room, an explosion may result.

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There are other disadvantages to refrigerator storage. Cold solvents pick up atmospheric moisture much faster when opened than those kept at ambient temperature. Also, cold does not retard the formation of peroxides in ethers. In addition, containers can leak or spill in the refrigerator. When this occurs, the fluid evaporates but the fumes are not carried away. Because the fumes remain in the box, it is very important that no ignition source be present when the door is opened.

Therefore, *CDC does not recommend storing ether or other solvents with flash points below refrigeration temperature in refrigerators, even in modified refrigerators.* Small quantities may be stored on open shelves; larger quantities should be stored in approved flammable solvent storage cabinets. The quantities vary with the standards prescribed by various organizations (for example, the Occupational Safety and Health Administration (OSHA) and the Joint Commission on Accreditation of Hospitals (JCAH), but the practice at CDC is to store no more than 2 gallons per room on the open shelf.

The smallest size containers of ether or other flammable solvents, should be purchased unless you use large quantities routinely. It is recommended that ether be purchased in ¼-lb containers, and, ideally, the entire amount in the container should be used on the day it is opened. This may not be practical in every case, but if only a small quantity of ether is left in the can, this should be disposed of rather than kept. (See Section C for suggested disposal methods.) If opened cans are kept, however, they should be securely stoppered or closed to prevent leakage. Cork stoppers are preferable to plastic caps which may pop off. Well-stoppered cans may be left on the open shelf.

3. Storage Cabinet

The following should be stored in National Fire Protection Association approved-type solvent storage cabinets:

a. Containers of flammable solvents larger than one-half gallon.
b. *All* flammable solvents supplies, if the total quantity exceeds 2 gallons.

At CDC, all unopened flammable solvents are stored in solvent storage cabinets. Only opened bottles or cans are kept on the open shelf.

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Laboratory supervisors should determine their storage needs and obtain appropriate size cabinets either from commercial suppliers, or from the hospital or institution workshop, or a local cabinet maker. Cabinets can be constructed to fit available space and funds. Plans for a wooden storage cabinet that meets National Fire Protection Association standards are on page 214 (fig. 30).

4. **Recommendations**

   a. Use the smallest containers of flammable solvents compatible with the work.
   b. Keep only the minimum supply on hand.
   c. Anticipate spills and other accidents by removing all ignition sources when using flammable solvents, i.e., turn off burners, disconnect motors, heaters, etc.

C. DISPOSAL OF WASTE SOLVENTS

Follow specific directions provided by your institution's safety office or those in the *Guide for Safety in the Chemical Laboratory* referred to on page 207. Flushing small quantities of water-miscible waste solvents down the drain, using copious quantities of water, is common practice, but is not without hazards. A better solution is to pour the waste solvent into a waste solvent can and have it disposed of in an approved manner. Waste solvent cans are available commercially.

An alternate method is to evaporate small quantities of solvents such as ether under a fume hood. Even this method is not entirely free of hazards, however, especially if the fume hood is not functioning properly.

EQUIPMENT SAFETY

In parasitology, the microscope and the centrifuge are the most commonly used equipment items.

A. MICROSCOPE

The chief safety hazard associated with the microscope is contamination of the stage from "sloppy" preparations. If this happens, the stage should be wiped off with a small amount of disinfectant, alcohol or hot soapy water.

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To prevent or reduce the chances of specimen material spilling on the microscope stage, wet-mount preparations of feces, blood, urine, or other body material should be prepared on 3x2-inch slides and sealed with melted paraffin-Vaseline (1:1) mixture. The wider slides can also be handled with less danger of contaminating hands.

B. CENTRIFUGE

Each operator should be instructed on proper operating procedures before being allowed to use the instrument. Instructions should include balancing loads, using the proper head, and using accessory equipment.

The person using a centrifuge should be responsible for the condition of the machine at the end of the procedure. This includes turning off the power and cleaning up spills or broken glass.

1. Operation

The lid should be closed when the centrifuge is in operation. If an unusual condition (such as noise or vibration) begins, the centrifuge should be stopped immediately. An unbalanced load may cause the instrument to vibrate. The operator should check to be sure that heads are symmetrically loaded, tube caps are correctly seated, and swinging buckets are symmetrically placed. Particular attention should be given to verify that swing-out cups are supported correctly.

a. Tubes

Plastic and glass centrifuge tubes should be carefully inspected before each centrifuge cycle. Only tubes free from defects should be used. After tubes have been through several cycles, the pressure cometimes causes them to break. Tubes likely to fail can often be identified by stress lines which appear in the area of junction of the sides and the bottom. Such tubes should be discarded.

b. Carrier Cups and Rotors

Accidents, such as centrifuge tubes being broken, have a potential for producing large volumes of very finely divided aerosols. The inside of the cup should be clean and smooth, and thus, carrier cups need to be cleaned on a scheduled basis. They should be washed in warm, soapy water and scrubbed with a nylon brush. In some instances, fine steel wool should be used to remove stubborn deposits. Cleanliness

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is the best preventive for rotor corrosion. If solid deposits occur, a mild detergent solution should be used with a stiff test tube brush. Then the detergent should be rinsed away with warm tap water and distilled water. Caustic solutions are particularly damaging and washing should be prompt.

RESPONSIBILITY FOR LABORATORY SAFETY

Responsibility for safety procedures rests primarily with the workers and supervisors in the laboratory. Everyone who works in a laboratory should be trained in safe laboratory procedures, since a worker who is aware of risks is less likely to be injured or become infected.

The success of a safety program depends on each participant having the necessary knowledge to protect himself and those with whom he comes in contact and to deal with laboratory emergencies such as exposure to dangerous agents or contamination of the physical environment.

The laboratory director or supervisor should have information on safety precautions and safety manuals readily available to the laboratory personnel. Some suggested manuals are listed below. The CDC course "Laboratory Safety Management" is also recommended. For more information on this program, contact the Laboratory Training and Consultation Division, Centers for Disease Control, Atlanta, Georgia 30333.

PUBLICATIONS ON SAFETY

1. *Guide for Safety in the Chemical Laboratory*, 2nd Ed. 1972. Manufacturing Chemists Association. Available from: International Thomson Education Publishing, Inc., 7625 Empire Drive, Florence, Kentucky 41042.
2. *Handbook of Laboratory Safety*, 2nd Ed. 1971. The Chemical Rubber Company, 18901 Cranwood Parkway, Cleveland, Ohio 44128.
3. *The Care, Handling, and Disposal of Dangerous Chemicals.* 1970. P.J. Gaston, Author. William F. Heinman, 1966 Broadway, New York, New York 10023.

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4. *Lab Safety at the Center for Disease Control*, 1977. Office of Biosafety, Centers for Disease Control, Atlanta, Ga. 30333.

5. *Dangerous Properties of Industrial Materials*, 3rd Ed. 1975. Irving Sax. Reinhold Publishing Company, New York.

6. *Safety in the Chemical Laboratory*. Edited by Norman Steere. American Chemical Society, Easton, Pa. 18042.

7. *The Merck Index*, 9th Ed. 1976. Merck and Company, Rahway, New Jersey.

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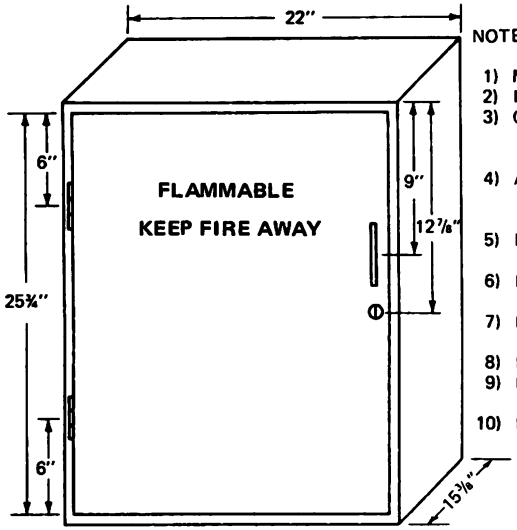
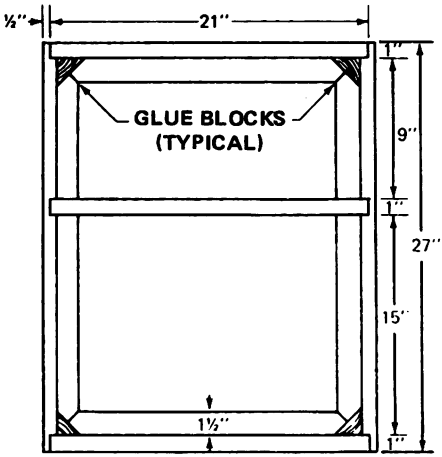
Figure 30.
Storage Cabinet for Flammable and Combustible Liquids

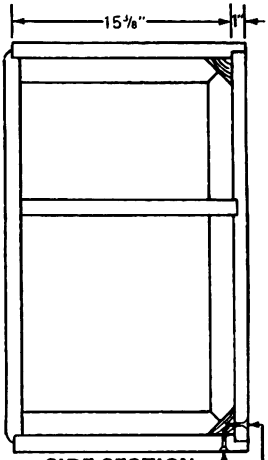


NOTES:

1) Material – 1" thick fir plywood
2) Inside finish – seal and varnish
3) Outside finish – yellow enamel with red lettering, "FLAMMABLE – KEEP FIRE AWAY"
4) All joints to be glued and secured with 1½" glue blocks and #10x1½" wood screws
5) Hinges to be secured with machine screws
6) Door hinges: Self-closing, Sears 9K6057 or equivalent
7) Door lock: McMasters desk lock #1681 D3 or equivalent (lock is optional)
8) Door handle: standard lab furniture
9) Door catch, magnetic: Sears 9K6136 or equivalent
10) Dimensions (w, l, & h) may be varied to fit existing space



FRONT SECTION



SIDE SECTION

#10 x 1½" LONG WOODSCREWS (TYPICAL)

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Storage Cabinet for Flammable and Combustible Liquids

The plywood cabinet depicted on the opposite page was designed by the Centers for Disease Control to meet the requirements of the Occupational Safety and Health Act of 1970 (OSHA). The OSHA standards were taken from National Fire Protection Association (NFPA) Code 30-1969.

Generally, the requirements for wooden storage cabinets are:

1) Maximum capacity of no more than 60 gallons of Class I or II liquids nor more than 120 gallons of Class III.
2) Construction to be of an approved grade of at least 1-inch thick plywood.
3) During a 10-minute fire test as specified in NFPA Code 251-1969:
   a. hinges must not lose holding effectiveness,
   b. joints and seams must remain intact and the door must remain closed, and
   c. internal temperature must not exceed 325°F.
4) All joints shall be rabbetted and fastened in two directions with flathead wood screws.
5) When more than one door is used, there shall be a rabbetted overlap of at least one inch.
6) Cabinets must be labeled in conspicuous lettering – "FLAMMABLE – KEEP FIRE AWAY."

Reference: Title 29 CFR, Chapter XVIII, Part 1910, Occupational Safety and Health Standards.

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REFERENCES

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